AN ULTRASTRUCTURAL AND CYTOCHEMICAL STUDY OF MINK (MUSTELA VISON) SPERMATOZOA

by

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We accept this thesis as conforming to the required standard

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This study was undertaken to investigate the ultrastructure, cytochemistry and maturation changes of mink spermatozoa which are important in biological research and in their relevance to artificial insemination.

Mature standard dark mink were used in this study. Spermatozoa were released from the testis and epididymis (caput, corpus and cauda) of the male mink, and were also collected from the vagina of female mink immediately after mating. Conventionally prepared thin sections were observed under a transmission electron microscope. Enzymes were cytochemically localized in spermatozoa.

The mink spermatozoon head showed six swellings on the dorsoventral aspects: two connected hump-like structures at the anterior border of the equatorial segment of the acrosome, and one at the postacrosomal sheath on each side. These swellings, which show a strong acid phosphatase activity, appeared to be a species-specific structural feature which might be necessary for the recognition of the ovum or for sperm-ovum attachment in fertilization. The occurrence of the postacrosomal swelling in spermatozoa was significantly increased (p < 0.01) during the passage of spermatozoa through the reproductive tract.

Although the total length of the head did not change significantly during the passage of spermatozoa down the reproductive tract, the anterior acrosomal length was significantly decreased (p < 0.001), while the postacrosomal length was significantly increased (p < 0.05).
The cell membrane on the peripheral part of the acrosome, with the exception of the tip of the acrosome, was significantly separated ($p < 0.05$) during the passage of spermatozoa through the reproductive tract.

The neck appeared to show dorsoventrally continuous but laterally separated capitulum which was followed by two major and five minor columns, forming at first a striated ring and then joining with the dense fibers of the axial fiber bundle. Some axoneme remnants were found in the interior of the column bundle.

The shape of the annulus was triangular in longitudinal sections. The occurrence of the cytoplasmic droplet was significantly decreased ($p < 0.001$) during the passage of spermatozoa through the testis and epididymis. The motility of spermatozoa was significantly increased ($p < 0.05$) as spermatozoa passed the successive parts of the reproductive tract.

The activities of acid and alkaline phosphatases, ADPase, ATPase and DOPA oxidase were found to be distributed in the head, middle and principal pieces of epididymal spermatozoa. Glucose-6-phosphatase, 5-nucleotidase, non-specific esterase, malate, succinate, lactate and isocitrate dehydrogenases, and NADH diaphorase activities were seen to be confined to the middle piece, while the esterase and malate dehydrogenase activities extended to the head base. The activity of 6-phosphogluconic dehydrogenase was not detected.

Although most enzyme activities of spermatozoa were enhanced during the passage of spermatozoa through the
reproductive tract, several enzyme activities (acid and alkaline phosphatases, ADPase, ATPase, and malate dehydroge- nase) were distinctly reduced in spermatozoa from ejaculated semen recovered from female mink following mating. The presence of enzyme inhibiting factors in the seminal plasma or female reproductive tract was discussed.
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GENERAL INTRODUCTION

Following the invention of the microscope, fundamental studies of spermatozoa have been made by many investigators. However, the application of the basic knowledge in this field for practical purposes began when Ivanov (1907) succeeded with the artificial insemination (AI) of domestic animals.

It is well known that several physiological, morphological and chemical aspects of spermatozoa correlate with fertility in mammals. For example, motility, swimming pattern, live-dead ratio, abnormal spermatozoan ratio, the form of the acrosome, the occurrence of dynein arms in an ultrastructural level, methylene blue or resazurin color reduction time, and enzyme activity are utilized as indicators to predict fertility.

Although there are numerous reports on the spermatozoa of other domestic animals, comparatively few reports are available on mink spermatozoa (Enders, 1952; Ishikawa et al., 1965; Onstad, 1967).

The distribution of the mink is confined to the northern parts of the Northern Hemisphere; the mink produces neither food nor wool, but a luxurious fur. Furthermore, the mink has a short breeding season which lasts about one month of the year, and only a small amount of semen can be acquired per ejaculation. These geographical, economical, and physiological factors tend to hinder elaborate studies of mink spermatozoa.

However, the application of AI in the mink industry is imminent. As mink farms are becoming larger and more professionally organized, much labor is required during the
short breeding season. Especially notable are the facts that about 10 per cent of the male mink suffer from anomalies of the genitalia, copulation problems and lack of libido (Ishikawa et al., 1965). Also, a considerable number of the mink are injured due to fighting while pairing. Pairing occurs several times with different partners apparently to increase fertility.

Application of AI to the mink industry not only eliminates these particular problems in the mink but also ensures the general advantages of AI: genetic improvement, decreasing the number of males required for breeding purposes and control of venereal diseases. Although AI in mink has been sporadically attempted for 30 years, little success has been achieved due to insufficient basic knowledge (Adams, 1975). On the other hand, because of the ease of recovering spermatozoa from the excised epididymis and the possible usage of the preserved spermatozoa of pelted male mink at the end of the breeding season, epididymal spermatozoa have been used in AI of mink (Pomytko et al., 1972; Adams, 1975; Ahmad et al., 1975b).

In order to obtain basic knowledge of mink spermatozoa, the present investigation has studied the ultrastructural and cytochemical aspects of epididymal spermatozoa, as well as the maturation of mink spermatozoa.
Chapter I

AN ULTRASTRUCTURAL STUDY OF MINK SPERMATOZOA

A. INTRODUCTION

Since Ham first observed spermatozoa almost 300 years ago, shortly after the invention of the light microscope (Kircher, 1646), numerous studies in the basic knowledge of the structure and physiology of spermatozoa have been recorded. Detailed appreciation of the spermatozoan structure has been accomplished first with the improvement of the light microscope and more recently with the advent of the electron microscope (Knoll and Ruska, 1932). The improvement in the transmission and scanning electron microscope, and in fixation, embedding, thin sectioning and freeze cleaving techniques have enriched the understanding of the internal and external structure of spermatozoa. The physiological aspect of spermatozoa have also been elucidated by the application of histochemical techniques.

Spermatozoa from several mammalian species have been extensively studied using the electron microscope. Although all mammalian spermatozoa have the same basic structure, there is a variation in the size, shape and fine structure among the species. Although there are numerous studies on the ultrastructure of mammalian spermatozoa, there is no record of a study describing mink spermatozoa. The present investigation was undertaken to describe the fine structure of mink spermatozoa and to discuss the significance of this structure to the reproductive physiology of the mink.
B. REVIEW OF RELATED LITERATURE

1. Early Studies of Spermatozoa

Spermatozoa were first observed by Ham in 1677 (Meyer, 1938) soon after the invention of the light microscope. Almost 150 years later, Prévost and Dumas (1824c) showed that spermatozoa were produced by the sexually potent male. Eventually, the testis was proven as the site of spermatozoan production (Peltier, 1835). The egg (Gegenbauer, 1861) and spermatozoon (Schweigger-Seidel, 1865) were recognized as single cells and equal in their contribution to the new individual (van Beneden, 1875). van Beneden (1883) demonstrated the joining of pronuclei of the spermatozoon with that of the egg, showing that each contained half the number of somatic cell chromosomes.

During this early period, the microscope and techniques employed were primitive and interpretations of observations were influenced by preconceived notions of what should be present in a germ cell. Even the presence of alimentary tracts and micro-embryos in spermatozoa was reported (Hartsoeker, 1694). As optics improved throughout the 19th century, descriptions of spermatozoon morphology became more accurate (Donné, 1845; Schweigger-Seidel, 1865; Leydig, 1883; Jensen, 1887). Retzius (1902) described a spermatozoon as an organism consisting of a head, a neck and a tail which was divided into three parts consisting of a connecting piece, main piece, and end piece.
Through the development of new kinds of microscopes (polarising, ultraviolet, electron, phase-contrast, interference-contrast, and fluorescence), of histochemistry and of physical and chemical analytical techniques during the 20th century, the knowledge of the spermatozoon has grown rapidly. Although the early electron microscope observations with the "whole cell" technique added only limited new information (Seymour and Benmosche, 1941; Schnall, 1952; Wu and McKenzie, 1954, 1955; Bradfield, 1955), the introduction of the thin-sectioning technique and the improvement of embedding, fixation, and post-staining techniques combined with the achievement of increased resolution in the electron microscope, have made possible a more detailed ultrastructural study of spermatozoa (Afzelius, 1955, 1959; Anberg, 1957; Hancock, 1957a,b, 1966; Fawcett, 1958, 1962, 1970, 1975; Blom and Birch-Andersen, 1960, 1961, 1965; Saacke and Almquist, 1964a, b; Pedersen, 1970a,b, 1972a,b,c).

2. General Morphology of Mink Spermatozoa

Mature mink spermatozoon has the basic morphological features described by Retzius (1902) for the spermatozoon in general, namely the head, neck, middle piece, principal piece, and end piece.

The overall length of mink spermatozoon is about 70 μm. The head appears almost round when seen from the flat side; the breadth is approximately the same as the length (about 7.5 μm). It is flat and about 1 μm thick (Onstad, 1967).
3. The Spermatozoan Head

The spermatozoan head consists mainly of the nucleus which is covered at the anterior by the acrosome and at the posterior by the postacrosomal sheath. The cell membrane envelopes the entire cell over the nuclear coverings.

a. The Nucleus. The greater part of the head is occupied by the nucleus filled with densely packed chromatin which is homogeneously distributed in the nucleus with slightly increasing concentration posteriorly (Friend, 1936). The caudal surface of the nucleus has a recess, the implantation fossa, for the attachment of the capitulum of the connecting piece. The shape of the implantation fossa is variable according to species (Fawcett, 1970).

The nucleus often contains vacuoles (Retzius, 1902; Schnall, 1952), which are not limited by a membrane and may represent an accumulation of gaseous end-products of metabolism (Schnall, 1952). Fawcett (1958) suggested that they appeared due to a condensation process of chromatin during spermiogenesis. The nucleus contains a small amount of non-basic protein (Bishop and Walton, 1966a) and polysaccharide (Mann, 1964).

b. The Acrosome. A secretion of the Golgi apparatus forms the acrosome (Burgos and Fawcett, 1955; Ånberg, 1957). Although Hancock (1957a) postulated a double acrosomeal covering, the acrosome is a membrane-limited cap-like structure closely applied to the tapering anterior portion of the
nucleus (Fawcett, 1958). The space between the membranes is occupied by a layer of homogeneous substance of moderate electron density (Fawcett, 1958).

In mature spermatozoa, the size and shape of the acrosome differs among species (Bishop and Walton, 1966a; Fawcett and Phillips, 1970). The acrosome can be distinguished in appearance by its three segments: an apical segment projecting beyond the anterior margin of the nucleus; a main segment extending back over the anterior half of the nucleus; and a differentiated equatorial segment comprising the caudal portion of the acrosome (Hancock, 1957a). The shape of the apical segment of the acrosome is a characteristic of the species (Fawcett, 1965, 1970; Bishop and Walton, 1966a); the equatorial segment varies in its length among species (Nicander and Bane, 1966) and the acrosome-postacrosomal sheath junction has a species-specific structural feature (Koehler, 1969).

The galea capitis or head cap which seems to be a separate structure overlying the acrosome is an artifactual loosening and elevation of the anterior portion of the cell membrane (Fawcett, 1975).

The acrosome is composed of carbohydrate, lipid, and protein (Clermont et al., 1955b; Hartree and Srivastava, 1965). Moreover, the acrosome is a lysosome or lysosome-like structure (Teichman and Bernstein, 1969; Allison and Hartree, 1970). The lytic action of the acrosomal enzyme enables spermatozoa to penetrate the egg envelopes (McRorie and Williams, 1974).
Because the spermatozoon depends upon the acrosome to enter the ovum, an acrosomal defect might impair fertilization. Spermatozoa having knobbed acrosomes are unable to penetrate or attach to the zona pellucida of the ovum (Buttle and Hancock, 1965); acrosome retention after incubation of semen at 37°C post-thawing is closely related to fertility (Saacke and White, 1972). Furthermore, an abnormality of the spermatozoan acrosome is an inherited cause of sterility in the bull (Donald and Hancock, 1953; Hancock, 1953).

c. The Perforatorium. The perforatorium lies between the acrosome and the anterior edge of the nucleus. It is distinct from the acrosome in origin, site and cytological characteristics (Bishop and Walton, 1966a; Hancock, 1966). The perforatorium originates from a layer of cytoplasm (Hadek, 1963a) and appears to be a modification of the inner membrane of the acrosome rather than of the nuclear membrane (Clermont et al., 1955a; Austin and Bishop, 1958). The perforatorium has been described as a pyramidal structure in the spermatozoa of murine rodents (Bishop and Walton, 1966a; Hancock, 1966; Fawcett, 1970), while that of the domestic ungulates appears as a cone-like projection (Hancock, 1966). Conversely, Fawcett and Phillips (1969a) argued that there was no resistant fibrous structure which might play the mechanical role implied by the term "perforatorium" in mammalian spermatozoa. Therefore, they proposed the term "subacrosomal space" instead of "perforatorium" in mammalian spermatozoa.
d. **The Postacrosomal Sheath.** The presence of the postacrosomal sheath has been established by the use of thin-sectioning techniques with electron microscopy (Blom and Birch-Andersen, 1961). The postacrosomal specialization covers the posterior portion of the acrosome. It is a cell membrane underlaid by a thin dense layer which is called the postacrosomal dense lamina or the postacrosomal sheath (Fawcett and Ito, 1965). It used to be called the "postnuclear cap" by several workers (Gatenby and Beams, 1935; Blom and Birch-Andersen, 1965; Hancock, 1966). However, Fawcett (1958) argued that the term "postnuclear cap" was inexact because the sheath was found to be confined to the lateral aspects of the nucleus except the base of the nucleus. Although Rahlmann (1961) claimed in his description that the postacrosomal sheath extended under the acrosome for a short distance, several workers (Hancock, 1952; Hancock and Trevan, 1956; Bishop and Walton, 1966a) reported the presence of the nuclear ring, which corresponded with the equatorial segment, located on the boundary of the acrosome with the postacrosomal sheath.

The chemical nature of the postacrosomal sheath is still obscure. However, it is much more resistant to distortion than the acrosome and seldom shows separations from the nucleus (Bishop and Walton, 1966a).

e. **The Cell Membrane.** The cell membrane of the spermatozoon originates from that of the spermatid. It envelops the entire spermatozoon as a continuous membrane. The composition of the cell membrane differs between the head and the tail parts
with respect to agglutinating properties (Bishop and Walton, 1966a). The cell membrane is composed of a lipid-protein complex and forms a triple layered unit membrane (Blom and Birch-Andersen, 1965).

The cell membrane closely apposes the apical part at its lateral and anterior edges. It may become loose from the subjacent structure in the rest of the apical part and the main segment of the acroosome (Bedford, 1963c, 1965b; Blom and Birch-Andersen, 1965; Fawcett and Phillips, 1969a; Fawcett, 1970) and disappears for a considerable part of the main segment of the acroosome owing to the vulnerable structure of the membrane (Blom and Birch-Andersen, 1965). Posterior to the acroosome, on the other hand, the membrane is closely associated with the postacrosomal sheath, and at the lateral edges of the posterior ring it is anchored firmly, with the postacrosomal sheath, to the nuclear membrane (Blom and Birch-Andersen, 1965; Pedersen, 1972c). The nuclear envelope forms a scroll that extends for a variable distance back into the neck region (Nicander and Bane, 1962a; Blom and Birch-Andersen, 1965).

The cell membrane is loosely attached to the mitochondrial sheath and anchors at the lateral surface of the Jensen's annulus (Saacke and Almquist, 1964b).

4. The Spermatozoan Neck

The neck of mammalian spermatozoa is the short anterior end of the tail that connects the tail with the head. It originates from a basal body that is partly embedded in the base of the head (Bishop and Walton, 1966a). The structure of
this region is a very complex portion of the entire spermatozoon and is poorly understood.

The major component of the neck is the connecting piece which is attached anteriorly to the basal plate that covers the outer surface of the implantation fossa and posteriorly to the dense outer fibers of the flagellum (Fawcett, 1970). The connecting piece has the capitulum, a convex articular region which conforms to the concavity of the basal plate and posteriorly connected with the columns. The capitulum is supported on either side by two main columns that are funnel-shaped, transverse striated columns. On its dorsal and ventral aspects the main stems are joined by five minor columns. Further in the posterior, each of the two main columns divides into two. Thus, altogether nine columns join the nine dense fibers (Kojima, 1962; Hancock, 1966). Although some workers (Hancock, 1957a; Rahlmann, 1961) found three granule-like bodies at the base of the head, other investigators (Anberg, 1957; Fawcett, 1958, 1962; Blom and Birch-Andersen, 1960) could not verify the occurrence of the granules. In longitudinal sections, the connecting pieces are composed of approximately 13 to 14 dense segments altering with narrower light bands (Blom and Birch-Andersen, 1960; Saacke and Almquist, 1964b; Fawcett, 1965; Wu and Newstead, 1966; Zamboni and Stefanini, 1971), and each of the two bands has fine transverse striations (Fawcett, 1975).

Although the chemical nature of the cross-banded columns has not been established, the columns are believed to be analogous to the cross-striated rootlets associated with the
basal bodies of epithelial cilia (Fawcett, 1975). The alternate contraction and expansion of two laminated columns in the neck may initiate the flagella wave (Saacke and Almquist, 1964b).

Immediately beneath the capitulum, there is the proximal centriole in the interior of the connecting piece (Burgos and Fawcett, 1955; Ånberg, 1957; Blom and Birch-Andersen, 1960). The centriole is a typical cylindrical structure with dense walls and a less dense center, with nine tubular triplets set in the walls (Fawcett, 1958). The distal centriole is absent in mature spermatozoa (Fawcett, 1965; Fawcett and Ito, 1965), although the remnants might occasionally be found in the inner aspect of the nine columns (Fawcett, 1975). Therefore, the distal centriole is neither the kinetic center nor the site of the origin of the flagella wave (Woolley and Fawcett, 1973). The longitudinal filaments of the flagellum originate from near the proximal centriole (Burgos and Fawcett, 1955). Zamboni and Stefanini (1971) suggested that the proximal centriole was possible the basal body of the flagellum and the center of the spermatozoan motility.

A few flattened mitochondria projecting from the mitochondrial sheath of the middle piece and a scroll of redundant nuclear envelope are often located in the neck region. Because the mitochondria send small projections between the longitudinal columns into the interior of the connecting piece, Fawcett (1970) suggested that the connecting piece had energy-requiring functions. The functional significance of the scroll, however, remains obscure.
5. The Spermatozoan Tail

The tail of mammalian spermatozoa is differentiated into three parts, i.e. the middle piece, principal piece, and end piece. Ballowitz (1888) observed by means of the light microscope that the tail occasionally frayed into a tuft of exceedingly fine fibrils. A complex structure consisting of several filaments of the tail has been confirmed by Reed and Reed (1948), Schnall (1952) and Wu and McKenzie (1955) using the electron microscope.

a. The Axial Fiber Bundle. The axial fiber bundle of mammalian spermatozoa consists of 20 fibers arranged concentrically in two rows of nine each around a central pair and is known as the 9+9+2 fiber pattern (Bradfield, 1955; Änberg, 1957; Fawcett, 1958; Blom and Birch-Anderson, 1960). The outer nine fibers are considerably thicker and more dense than the inner nine fibers and central pair.

The inner nine fibers and central pair (9+2) are called the axoneme or axial filament complex. This pattern in cilia and flagella is the basic pattern of all motile cells which occurs throughout the plant and animal kingdoms (Manton et al., 1952; Fawcett and Porter, 1954). The axoneme begins in the neck region and extends to the end piece but it does not retain the 9+2 pattern somewhere in the end piece (Änberg, 1957).

The inner nine fibers are double tubes known as the doublets or double microtubules; the central pair consists of two single microtubules (Bradfield, 1955; Afzelius, 1959; Blom and Birch-Anderson, 1960; Telkka et al., 1961; Hancock, 1966). The
doublets consist of two subunits: a subunit A, a complete microtubule and a subunit B which is C-shaped in cross section with its ends attached to the wall of the subunit A. Subunit A is closer to the center of the tail than subunit B (Rahlmann, 1961) and it is solid rather than a hollow structure (Gibbons and Grimstone, 1960; Telkka et al., 1961). Viewed from the flagella base looking toward the tip, subunit A has two short diverging arms that project clockwise from its wall toward the next doublet in the row (Afzelius, 1959; Gibbons and Grimstone, 1960; Telkka et al., 1961). The outermost arm has a flag-like appendage projecting at the end of the arm towards the inner arm. Each subunit A has two slender nexin links that connect it to the adjacent doublets (Stephens, 1970) and a radical spoke that joins it to a helical sheath around the central pair (Anberg, 1957; Afzelius, 1959; Rahlmann, 1961; Telkka et al., 1961). The cylindrical wall of subunit A is made up of 13 tubulins, protein protofilaments, and subunit B is composed of about 10 tubulins (Warner, 1970; Tilney et al., 1973; Amos and Klug, 1974). The arms consist of dynein, a protein with adenosine triphosphatase (ATPase) activity (Gibbons, 1965; Gibbons and Rowe, 1965; Gibbons and Fronk, 1972). Each microtubule of the central pair is composed of 13 tubulins and is enclosed in a helical sheath (Pedersen, 1972c). Although the mechanism of the flagellar movement is still obscure, the dynein seems to convert the chemical energy of adenosine triphosphate (ATP) into mechanical energy and induce the sliding of neighboring doublets (Satir, 1965; Gibbons, 1965, 1975; Summers and Gibbons, 1971). It has been reported that immotile spermatozoa
have no dynein arm and cause sterility (Afzelius et al., 1975; Pedersen and Rebbe, 1975).

Peripheral to the axoneme of mammalian spermatozoa, there are nine petal-like dense fibers but these are present only in the middle piece and the major part of the principal piece with decreasing dimension (Blom and Birch-Andersen, 1960; Rahlmann, 1961). In cross sections, each of the nine dense fibers are aligned on the same radius as the corresponding doublet (Saacke and Almquist, 1964b). Although Fawcett (1958) proposed that these fibers had originated from the distal centriole, Fawcett and Phillips (1969b) suggested later that the dense fibers arose as outgrowths from the wall of each doublet. The nine dense fibers differ from one another in the cross sectional area and shape. In many species, fiber number 1, 5, and 6, as designated by Bradfield (1955), are distinctly larger than the others, but in a few species number 9, 1, 5, and 6 are larger (Blom and Birch-Andersen, 1960). There are also marked interspecific differences in the cross sectional configuration, initial size and longitudinal extent of the dense fibers (Fawcett, 1970).

The dense fibers are round at the outer contour and taper toward the corresponding doublets (Fawcett, 1970). The fibers have a thick medulla and a thin cortical layer. Oblique striations on the surface replicas of the cortex of the dense fiber suggest globular subunit compositions (Phillips and Olson, 1975). The medulla also has a very fine periodicity (Pedersen, 1972c). Although several investigators reported a high content of cysteine and the absence of ATPase activity in both the medulla
and cortex of the dense fibers (Baccetti, et al., 1973a; Pihlaja and Roth, 1973; Price, 1973). Nelson (1958) and Baccetti et al. (1975), however, demonstrated ATPase activity in the cortex. ATPase activity between the doublets and around the central tubules was also detected (Baccetti et al., 1973b; Burton, 1973). Therefore, a contraction of dense fibers might be involved in the spermatozoan motility.

Satellite fibers are present in limited numbers between the dense fibers through the anterior third of the flagellum (Telkka et al., 1961; Fawcett and Ito, 1965). They arise from the cortex of the dense fibers (Fawcett and Phillips, 1969b), and they might be accessory tensile elements of the motor apparatus (Fawcett and Phillips, 1970). Their forms and numbers are markedly different among species (Fawcett, 1970).

b. The Mitochondrial Sheath. The middle piece is characterized by the presence of a mitochondrial sheath which surrounds the axial fiber bundle. Because of the mitochondrial sheath, the middle piece is thicker than the other portion of the tail (Wu and McKenzie, 1955).

Jensen (1887) first observed the sheath and traced its origin to the mitochondria. Several investigators (Hodge, 1949; Wu and Newstead, 1966) have described a double helix. However, Blom and Birch-Andersen (1960) reported a single chain which doubled upon itself to form a loop with its two free ends terminating near the head. Saacke and Almquist (1964b) also suggested that the helix was composed of one or more strands and some strands failed to reach the end of the middle piece.
In general, the mitochondria of the middle piece are elongate and are arranged end to end in a single helix around the flagellum (Ånberg, 1957; Fawcett, 1958, 1965, 1975).

The mitochondria are not fused but retain their individuality (Fawcett, 1958) and there are species variations in the form and arrangement of the elements (Fawcett, 1962; Fawcett and Ito, 1965). The length of the middle piece, which is proportional to the number of mitochondrial gyres, is remarkably different among species (Fawcett, 1970). During spermiogenesis, the mitochondria of spermatids undergo extensive internal reorganization (André, 1962). In the early spermatid mitochondria, the two membranes of foliate cristae tend to separate to form the expanded intracristal space (pseudomatrix) and condensed intercristal matrix (true matrix). However, some species do not show conspicuous pseudomatrix but show normal intracristal space in the mitochondria sheath of spermatozoa (Fawcett, 1970). Although the functional implication of those differences among species is obscure, it might be related to the species-specific energy requirement of spermatozoa to achieve fertilization.

c. The Annulus (Jensen's ring, ring centriole). The annulus surrounds the axial fiber bundle at the junction of the middle piece and principal piece of mammalian spermatozoa (Jensen, 1887; Blom and Birch-Andersen, 1960). It is composed of closely packed filamentous subunits oriented circumferentially. The form of the longitudinal sections of the annulus varies among the species. It might be triangular in some species
with the base adhering to the cell membrane and the apex projecting inward toward the dense fibers in contact with the mitochondrial helix and fibrous sheath laterally. In other species it might be semicircular (Fawcett, 1970). The cell membrane adheres closely to the annulus without interruption. It might traverse and shed the cytoplasmic droplet at the caudal end of the middle piece (Saacke and Almquist, 1964b). The annulus appears to prevent caudal displacement of the mitochondria during the tail movement (Fawcett, 1970).

d. The Cytoplasmic Droplet. The cytoplasmic droplet, described first by Retzius (1909), borders on the neck and middle piece of spermatozoa. The internal structure of the droplet consists of derivatives of the Golgi material: fine, curved tubules, many small and some larger vesicles, and a ground cytoplasm, all bounded by a distinct membrane (Gatenby and Woodger, 1921; Burgos and Fawcett, 1955; Bloom and Nicander, 1961). It contains some lipid, carbohydrate (Mann, 1964), lysosomal enzymes (Dott and Dingle, 1968), glycolytic enzymes (Harrison and White, 1974) and phosphatases (Moniem and Glover, 1972).

Although the role of the cytoplasmic droplet is obscure, the droplet normally moves down the middle piece and is sloughed off during the passage of spermatozoa through the epididymis, though some ejaculated spermatozoa retain it. There are conflicting reports about the relationship between the occurrence of the droplet and the degree of maturity, but the presence and location of the droplet are taken as an
approximate index of spermatozoan maturity. On the other hand, White and Wales (1961) reported that the resistance of epididymal spermatozoa to cold shock was correlated with the attachment of the cytoplasmic droplet.

e. **The Fibrous Sheath.** The principal piece of mammalian spermatozoa is surrounded by the fibrous sheath. The sheath contains two longitudinal elements which travel down on both sides of the principal piece (Bradfield, 1955; Fawcett, 1958, 1962, 1965; Telkka et al., 1961; Saacke and Almquist, 1964b; Pedersen, 1970b). The fibrous sheath is made up of a series of closely spaced and occasionally interconnected semi-circular ribs that attach at either end to two longitudinal columns (Telkka et al., 1961). The sheath becomes thinner and closer to the axial filament as it moves posteriorly along the axoneme. The sheath then ends abruptly 2 to 3 μm from the tip of the flagellum in the bull (Saacke and Almquist, 1964b).

The attachment of the dorsal and ventral columns to fibers 3 and 8 in the proximal part of the principal piece might prevent caudal displacement of the sheath from the axoneme (Fawcett and Phillips, 1970). The continuous longitudinal columns and interspaced periodic ribs possibly endow the sheath with elastic properties which induce flagellar bending by resisting the sliding movement propagated along the axoneme (Fawcett, 1970, 1975).

f. **The End Piece (terminal piece).** The end piece is extended beyond the termination of the fibrous sheath; therefore, the axoneme is bound only by the cell membrane. The length of the end piece is quite variable depending on the species (Fawcett, 1970).
C. MATERIALS AND METHODS

Standard dark male mink of not more than three years of age were used for the ultrastructural study of spermatozoa. During the period February 26 to March 19, 1975, reproductive tracts were removed from five male mink under sodium pentobarbital anaesthesia. Small pieces of tissue were sliced from the cauda epididymis and immediately immersed in 1 ml of Krebs-Henseleit-Ringer solution (Jones, 1971) where the spermatozoa were released by squeezing the tissue. The suspended spermatozoa were concentrated by centrifugation (700G, 15 min), fixed in glutaraldehyde-cacodylate (100 mM glutaraldehyde and 150 mM sodium cacodylate), washed three times, postfixed in osmium tetroxide, and rewashed (Jones, 1971). The samples were again concentrated (1500G, 15 min), supported in 3% agar, dehydrated, and embedded in Epon 812 (Luft, 1961). Sections were cut with glass knives using an LKB ultramicrotome, double stained by 5% uranyl acetate in 1% acetic acid and lead citrate (Venable and Coggeshall, 1965) and examined with an AEI-Corinth 275 transmission electron microscope.
D. RESULTS

1. The Spermatozoan Head

The head was found to be dorsoventrally flattened and ovate in outline, with an average length of 5.83 \( \mu m \) and a breadth of 0.35 \( \mu m \) in lateral view at the equatorial segment (Figs. 1, 2 and Table 3). In sagittal sections, where the nucleus occupied a major part of the head, the posterior part of the nucleus was thicker than the anterior part (Fig. 1). The nuclear material consisted of an electron dense mass with a number of vacuoles of approximately 20 nm diameter (Fig. 1). The vacuoles, varying in size, were scattered throughout the nucleus with an increased number in the posterior portion (Fig. 1). In the anterior two-thirds of the head, the nucleus was covered with the acrosome containing a less dense amorphous matrix (Figs. 1, 3). The posterior one-third of the head was covered with the post-acrosomal sheath. Depending upon acrosomal volume around the nucleus, the acrosome appeared in three parts: the apical segment, 0.68 \( \mu m \) long, occupied the largest area and extended a little beyond the tip of the nucleus; the main segment, 1.61 \( \mu m \) long, followed the apical segment; and the equatorial segment, 1.55 \( \mu m \) long, occupied the smallest area (Figs. 1, 3, 9 and Table 3). The perforatorium, composed of an amorphous matrix, was triangular and 0.34 \( \mu m \) long in longitudinal sections (Figs. 3, 4). At the proximal border of the equatorial segment, in sagittal sections, the acrosome appeared to be separated from the nucleus, producing a bilateral swelling (acrosomal swelling, Fig. 1) formed as a connected hump-like
structure in frontal sections (Fig. 2). Immediately posterior to the equatorial segment, the postacrosomal sheath also formed a bilateral swelling. A total of six swellings are present in the head. Some disintegrating membranous or amorphous material was observed in the empty space of the swellings (Fig. 1). Although the contents of the apical and the main parts of the acrosome were dislodged, the perforatorium, acrosomal swelling, and the other part of the head remained intact (Figs. 1,4). The postacrosomal sheath was 1.99 μm long and a 6 nm layer of dense material was identifiable on the inner aspect of the cell membrane (Fig. 1 and Table 3). The caudal surface of the nucleus formed the implantation fossa to accommodate the capitulum of the connecting piece (Figs. 5,6,7).

The inner nuclear membrane was so closely opposed to the nucleus that it was difficult to identify (Fig. 3). However, both the inner and outer nuclear membranes became distinguishable at the caudal end of the nucleus where they appeared to be 4 nm apart (Fig. 5).

The acrosome was bounded by distinct outer and inner acrosomal membranes (Fig. 3). The cell membrane was usually separated from the cell in the acrosomal part of the head except for the apical portion and the postacrosomal sheath (Fig. 1). At the distal end of the postacrosomal sheath, the cell membrane was anchored firmly on the nuclear membrane forming the posterior ring (Fig. 7). All spermatozoan membranes were unit membranes (Figs. 1,3,4).
2. **The Spermatozoan Neck**

The spermatozoan neck consisted of the capitulum, cross striated longitudinal columns, proximal centriole, a few mitochondria, and scrolls of membrane (Figs. 5, 7, 8). At the base of the head, the outer nuclear membrane was supported posteriorly by a 48 nm thick basal plate confined in the implantation fossa (Fig. 5). The basal plate was associated posteriorly with the capitulum by means of a diffused electron dense material. The capitulum, an articular structure, lay immediately between the basal plate above and the cross striated columns below. Because the capitulum was continuous in the dorsal and ventral aspects but separated on both sides it appeared that the capitulum consisted of a dorsal and ventral plates (Figs. 5, 6). The cross striated column was 0.7 μm long and consisted of 10 to 13 striations (Fig. 5). The cross striations were composed of 43 nm thick dense bands which alternate with 6.6 nm light bands. Individual fibers of the column were widest at the proximal end, adjacent to the capitulum, and tapered distally (Figs. 5, 6). Because these fibers were also arranged parallel to each other, they appeared to form a "connected ring" of cross striations (Figs. 5, 9). In addition, the circular aspect of the connected ring of fibers appeared discontinued by a dorsal, a ventral and two lateral vertical clefts (Figs. 5, 6). Thus, four cross striated columns were followed further distally in the neck by two major and five minor columns (Figs. 5, 6, 9, 11). Below the neck region, it appeared
that each major column split into two, thus forming with the five minor columns a total of nine columns connected to the nine dense fibers (Figs. 5,12,13).

Immediately beneath the inner surface of the capitulum, the proximal centriole was situated in the center of the connected ring (Figs. 5,7). The longitudinal axis of the centriole lay at about 70° to the main axis (Fig. 5). The nine tubular triplets were obliquely inserted at a regular spacing in the wall of the centriole giving a whirl-like appearance in cross sections (Fig. 7).

The central pair of microtubules of the axoneme was also found in the interior of the bundle formed by the columns (Fig. 11). At a lower level of the neck region, cross sections revealed the presence of double microtubules of the axoneme (Figs. 12,13). In the neck region, there was usually one or two longitudinally oriented mitochondria which marked the anterior margin of the mitochondria helix (Figs. 7,10). Enclosed by the cell membrane on the sides of the neck, there were redundant membranes which appeared as scrolls (Figs. 7,8).

3. The Spermatozoan Tail

The transverse section of the middle piece showed a 9 + 9 + 2 arrangement of the axial filament bundle (Fig. 14). The central pair consisted of two single microtubules of 28 nm diameter which were connected by the central sheath and surrounded by a cylindrical bundle of nine uniformly spaced double microtubules. Each double microtubule consisted of subfiber A and B sharing a sector of their wall (Figs. 14-17).
Subfiber A was a complete circular tubule (29 nm diameter) with nearly homogeneous density. It had inner and outer arms which were directed toward the subfiber B of the neighboring doublet. The distal end of the outer arm projected inward by a distinct appendage (Fig. 14). Subfiber B was 27 nm in diameter and appeared to be hollow in the micrographs. Subfiber A was slightly tilted toward the center of the axoneme. Each subfiber A was connected with a radial spoke directed centrally (Figs. 14 - 17). At the midpoint of each of these spokes, there was a slight thickening (Fig. 14). Slight electron dense material was found between the fibers of the axial filament complex (Fig. 14).

In the middle and principal piece regions, peripheral to the axoneme (9 + 2), there were 9 outer dense fibers. The outer border of dense fiber appeared smooth in cross sections, while the inner border appeared serrated or irregular (Fig. 14). The shape and size of the dense fibers were different: number 9, 1, 5, and 6 were elliptical forms and larger in diameter than the other fibers which were bonnet forms. The dense fibers were large at the outset, then gradually diminished along the length of the principal piece (Figs. 14 - 16). Dense fibers 3 and 8 ended before the rest of the fibers and were replaced by the inward extension of the longitudinal elements of the fibrous sheath (Fig. 15). Further distally, each dense fiber terminated close to corresponding doublets at different levels of the terminal part of the principal piece (Figs. 15, 16). Small round or polygonal satellite fibrils were also found between the dense fibers and around the inner border of the...
fibers (Fig. 14).

In the middle piece region, the dense fibers were surrounded by a single layer of helically coiled mitochondria sheath (Figs. 10–14, 21, 22). The middle piece was 10 μm long with about 53–57 gyres of mitochondria (Fig. 10). Mitochondria varied in size (Figs. 10, 21, 22) and some appeared pseudomatrix (Fig. 21). The annulus was composed of non-homogeneous electron dense material (Fig. 23). In longitudinal sections, it appeared as a triangle with the base juxtaposed to the cell membrane and projected downward to face the outer coarse fibers at its apex. Cytoplasmic droplets were observed on the distal end of the middle piece in some spermatozoa (Fig. 23).

In the principal piece, the axonemal complex was surrounded by a fibrous sheath which was composed of circumferentially oriented ribs extending halfway around the columns (Figs. 19–21). The ribs were joined at their ends to form two longitudinal elements that ran along the dorsal and ventral aspects of the principal piece (Figs. 15–17). The sheath ended abruptly at a distance of 1.5 μm from the tip of the flagellum (Fig. 20) and marked the junction of the principal piece and the end piece.

In the end piece, an axial filament complex (9 + 2 pattern of the fibers) was surrounded only by the cell membrane (Figs. 18, 20).
Figures 1 to 4. Ultrastructure of the epididymal spermatozoan head.

Figure 1. Sagittal sections of the head showing bilateral swellings on the proximal border of the equatorial segment (acrosomal swelling, AS) and on the postacrosomal sheath (PS). Disintegrating membranous materials (MM) and amorphous materials are observed inside the postacrosomal swellings. At the lower left, the acrosomal swellings and perforatorium (P) remain intact even though the content of the acrosome (AO) appears damaged. The nucleus (N) contains vacuoles (V). The cell membrane (CM) is separated from part of the acrosome (AC), but it coheres to the apical end of the acrosome (AE) and postacrosomal sheath. 30,000X.

Figure 2. Frontal section showing connected hump-like swellings (AS) and the postacrosomal swelling (PS) of a testicular spermatozoon. 18,000X.

Figure 3. Sagittal section of the apical part of head showing triangular perforatorium (P). The acrosome is filled with amorphous content surrounded by acrosomal membrane (AM). The cell membrane (CM), acrosomal membrane, and nuclear membrane (NM) are double membranes. 80,000X.

Figure 4. Sagittal section of the apical part of the head showing perforatorium containing nonhomogeneous matrix and a vacuole (V), all enclosed by the clear membrane (PM). 120,000X.
Figures 5 to 8. Ultrastructure of the spermatozoan neck.

Figure 5. Frontal section of the neck region. The basal plate (BAP) is confined to the implantation fossa (area between two arrows). It is associated anteriorly with the nuclear membrane (NM) and posteriorly with the capitulum (C) by diffused electron material. The striated columns (SC) consist of dark and light striations and are connected with the dense fibers (DF) distally. Note that the capitulum (C) is continuous, but a cleft divides the connected ring (CR). The proximal centriole is located in the center of the connected ring. A unit nuclear membrane (NM) is seen at the caudal end of the nucleus. 125,000X.

Figure 6. Lateral section showing a cleft dividing the capitulum (C) and also the connected ring of the striated columns (SC). 180,000X.

Figure 7. Sagittal section showing a proximal centriole (PC) sectioned transversely and a scroll (S). The nine triplets (T) are regularly arranged as a while. Inserted white lines show the orientation of triplets. The posterior ring is distinctive (arrow). A longitudinally oriented mitochondrion (M) is seen. 50,000X.

Figure 8. Oblique section showing scrolls (S) situated bilaterally. 37,500X.
Figures 9 to 13. Ultrastructure of the neck and middle piece in spermatozoa.

Figure 9. Sagittal section of the neck region showing the connected ring (CR) of striated columns. 80,000X.

Figure 10. Axial sections of the entire middle piece (MP) showing approximately 53 gyres of mitochondria (M). Note that the shape and size of the mitochondria (arrow) close to the nucleus (N) is different; a helix of mitochondria (MH) covers the surface of the middle piece. The principal piece (PP) has no mitochondrial helix. 12,000X.

Figure 11. Cross section at an upper level of the neck region showing two major (MC) and five minor columns and a central pair of the axial filament complex (CP) in the center. Mitochondria (M) surround the bundle of columns. 50,000X.

Figure 12. Cross section at a lower level of the neck region showing two double microtubules (DM) of the axial filament complex inside the bundle of columns (CO) which is surrounded by mitochondria (M). 80,000X.

Figure 13. Oblique section at a lower level of the neck region showing a bundle of nine columns (CO) and a double microtubule (DM). 50,000X.
Figures 14 to 23. Ultrastructure of the spermatozoan tail.

Figure 14. Cross section of the middle piece showing components of the axial fiber bundle. The dense fibers, number 9, 1, 5 and 6, are larger than the rest. Subfiber A (a) appears to be larger than subfiber B (b). The outer arms shows an appendage (ap) projecting centrally. Many satellite fibrils (sf) are seen. The electron dense matrix is also scattered between the fibers of the axoneme. The bundle of fibers is enclosed by the mitochondrial helix (MH) and cell membrane (CM). 80,000X.

Figure 15. Cross section of the principal piece devoid of the mitochondrial sheath. Note that the dense fibers, number 3 and 8, are replaced by longitudinal columns of the fibrous sheath (FS). 60,000X.

Figure 16. Cross section of the middle of the principal piece. The remnants of the dense fibers, number 9, 1, 5 and 6, are still present near the corresponding double microtubules. 75,000X.

Figure 17. Cross section of the lower part of the principal piece showing no dense fibers. 60,000X.

Figure 18. Cross section of the end piece showing no fibrous sheath. 60,000X.

Figure 19. Longitudinal section through a broken principal piece showing microtubules (MT). The hexial structure of the fibrous sheath (FS) is obvious. 37,500X.
Figure 20. Longitudinal section through the end piece (area between two arrows) showing no fibrous sheath (FS). 37,500X.

Figure 21. Longitudinal section through the middle (MP) and principal piece (PP). Some mitochondria are pseudomatrix (PX). In the principal piece (left), the axial filament complex is surrounded by the hexial fibrous sheath (FS). 50,000X.

Figure 22. Longitudinal section of the mitochondria helix showing the arrangement of mitochondria (M). 50,000X.

Figure 23. Longitudinal section at the junction of the middle piece (MP) and the principal piece (PP). The cell membrane (CM) anchors at a triangular annulus (AN). The cytoplasmic droplet (CD) is sometimes found on the distal end of the middle piece. 37,500X.
E. DISCUSSION

1. The spermatozoan Head

Except for small irregular vacuoles which were scattered throughout the nucleus, the chromatin appeared nearly homogeneous. The vacuoles were numerous in the posterior portion of the head in mink spermatozoa as reported for the bull (Saacke and Almquist, 1964a; Blom and Birch-Andersen, 1965). Because of the irregularities in the number, shape, and orientation of vacuoles, they were considered as artifacts (Saacke and Almquist, 1964a) or results of accidents in chromatin condensation during spermiogenesis (Fawcett, 1958). The size of the vacuole in mink spermatozoa was smaller than that in human spermatozoa (Bishop and Walton, 1966a).

The acrosome of mink spermatozoa was relatively small and its apical segment extended little beyond the nucleus as in the spermatozoa from man, monkey, bull, boar, rabbit, hare, ram, dog, horse, and cat (Nicander and Bane, 1966; Fawcett, 1970). On the other hand, the spermatozoa from chinchilla, guinea pig, and ground squirrel have much larger acrosomes (Fawcett, 1970).

The elongated cone shape of the perforatorium and its tendency to remain intact even when the content of the acrosome had disappeared suggest that this structure in mink spermatozoa may serve a useful function in fertilization. Although some workers reported no corresponding structure in human (Fawcett, 1958; Bedford, 1967a; Pedersen, 1970b), guinea pig (Fawcett, 1965) and bat (Fawcett and Ito, 1965) spermatozoa, the occurrence of the perforatorium has been clearly demon-
strated in spermatozoan heads from the horse, bull, ram, boar, dog, cat, rabbit, hare, and guinea pig (Bane and Nicander, 1963; Nicander and Bane, 1966).

The special feature of mink spermatozoa is the occurrence of swellings anterior and posterior to the equatorial segment of the head. Strong acid phosphatase activity was evident on the anterior and posterior borders of the equatorial segment (Fig. 25) where the swellings were observed in the present ultrastructural study. Moreover, the surface of this portion showed a swollen appearance, according to a scanning electron microscopic observation of mink spermatozoa (Ahmad, unpublished). Similar structures have also been reported for rabbit (Nicander and Bane, 1966; Teichman and Bernstein, 1969, 1971; Phillips, 1972; Bernstein and Teichman, 1972, Flechon and Bustos-Obregon, 1974) and hare spermatozoan heads (Nicander and Bane, 1966). However, the swellings of the rabbit and hare are confined to the anterior border of the equatorial segment, while the caudal part of the postacrosomal region is lightly swollen in rabbit spermatozoa. Although Yanagimachi and Noda (1970a) showed the presence of the subacrosomal ring in conjunction with the anterior equatorial border of hamster spermatozoa, it was not conspicuously large compared to that found in rabbit, hare, or mink spermatozoa.

In mammals, the initial contact between the plasma membrane of a spermatozoon and an ovum occurs at the postacrosomal region (Pikó and Tyler, 1964; Yanagimachi and Noda, 1970b,c) while Barros and Franklin (1968) reported it as occurring at the equatorial region. The mammalian spermatozoa have a
characteristic structure at the equatorial segment (Nicander and Bane, 1966). Moreover, spermatozoan heads have species-specific structural features of the equatorial segment-post-acrosomal junction which might be essential for recognition or attachment mechanism during fertilization and subsequent egg activation (Koehler, 1969). Special structural modification of the equatorial region and the high content of acid phosphatase activity in this region in mink spermatozoa might be useful in ensuring fertilization during a short breeding season in the mink.

Although the acrosomal content was dispersed during processing for electron microscopy, the acrosomal swellings located on the anterior end of the equatorial segment remained intact. The stability of the equatorial segment compared to the anterior part of the acrosome has been reported in several mammals (Bedford, 1964; Saacke and Almquist, 1964a; Blom and Birch-Andersen, 1965; Nicander and Bane, 1966; Pedersen, 1970b). Furthermore, the equatorial region of mammalian spermatozoa remains intact during the initial stage of spermatozoan degeneration (Nicander and Bane, 1966; Yanagimachi and Noda, 1970a) and remains visible even after spermatozoa penetrate the corona cells (Bedford, 1967c; Barros et al., 1967). This stable structure might be useful in the recognition of or attachment to an ovum in the initial stage of fertilization in the mink.

The presence of postacrosomal sheath in mink spermatozoa, a distinct electron dense material in close apposition to the cell membrane of the postacrosomal region, conforms to the
findings of Wu and Newstead (1966) in the bovine and Bedford (1967a) and Pedersen (1970b) in the human and monkey spermatozoa. However, Ånberg (1957) could not identify a post-acrosomal sheath in human spermatozoa.

The cell membrane of the mink spermatozoan head was found to adhere firmly only to the tip of the acrosome and to the postacrosomal sheath while it appeared to fit loosely around the rest of the acrosome. Although Hadek (1963b) considered it as a fixation artifact, several investigators (Saacke and Almquist, 1964a; Bedford, 1965b; Fawcett and Phillips, 1969a) confirmed it as a normal feature in mammalian spermatozoa. The double nuclear membrane was distinctive only at the caudal end of the nucleus and this is similar to that of human spermatozoa (Pedersen, 1972c). Although Saacke and Almquist (1964a) reported the presence of pores in the nuclear membrane in bovine spermatozoa, no definite nuclear pores were observed in this study of mink spermatozoa or in the study of bull spermatozoa by Blom and Birch-Andersen (1965).

2. The Spermatozoan Neck

The neck of mink spermatozoa is structurally as complicated as in the spermatozoa of other mammals (Fawcett, 1970). Immediately below the head, the basal plate lies within the boundary of the implantation fossa as shown in the bull (Saacke and Almquist, 1964b). In this the mink spermatozoa differs from the case of the guinea pig spermatozoa where an arcuate prolongation of the basal plate has been observed (Fawcett, 1965). The space between the basal plate and the capitulum was bridged by electron dense material as reported by Saacke
and Almquist (1964a) for the bull, but no fine filaments were observed as reported for Chinese hamster spermatozoa (Fawcett and Phillips, 1969b). A dorsal and ventral plate of the capitulum was found articulated with the basal plate above and joined with the cross striated columns of connecting piece fibers below. Although the presence of the capitulum in mature spermatozoa was denied by Zamboni and Stefanini (1971), the capitulum has been well documented in mammalian spermatozoa (Fawcett, 1975). Whether or not the specific fibers contribute to the formation of capitulum could not be determined either in this study or in that of bull spermatozoa (Saacke and Almquist, 1964b).

Following the capitulum, at least four cross striated columns were arranged parallel to each other along their long axes. However, being widest proximally, they formed a connected ring-like structure with four vertical clefts. Distally, they were divided into two major and five minor columns. Finally they became nine columns as each major column was split into two. Thereafter, the cross striations of the columns were connected to solid and homogeneously dense matrix material which had no striation similar to those demonstrated by Fawcett (1965) and Fawcett and Ito (1965) in the guinea pig and bat.

Triplets of the proximal centriole appeared in varied forms (doublets, tetralets, and pentalets) in the sagittal sections of the neck region. The cutting angle of the sectioned material made it difficult to verify if these forms were artifacts or the normal structural variation in mink spermatozoa. On the other hand, Fawcett (1958) mentioned that
the proximal centriole of mammalian spermatozoon was not
different from the typical centriole of a somatic cell. The
occurrence of the central pair and double microtubules, which
seem to be remnants of the centriole triplets or the extension
of the axoneme in the interior of the connecting piece, suggests
that the mink is probably one of the special species which have
microtubules in the neck region of matured spermatozoa. Fawcett
and Phillips (1969b) stated that some species had the central
pair in the connecting piece of matured spermatozoa. The scroll
in the neck region of mink spermatozoa is similar in structure
to those of the bat, dormouse, Russian hamster (Fawcett, 1970)
and boar (Nicander and Bane, 1962b).

3. The Spermatozoan Tail

Unlike the head, the tail of the spermatozoon shows con-
siderably less species variation. The axial fiber bundle of
mink spermatozoan tail follows the usual 9 + 9 + 2 pattern
which is commonly observed in other mammals (Telkka et al.,
1961; Fawcett and Ito, 1965; Fawcett, 1970). In the axial
filament complex of microtubules, the diameter of subfiber A
appeared slightly larger than B, which agrees with earlier
observation of sea urchin spermatozoa (Afzelius, 1959). How-
ever, Fawcett (1970, 1975) with regard to mammalian, and
Warner and Satir (1973) in the case of fresh water mussel
spermatozoa reported that subfiber B was larger than A. The
outer arm of subfiber A which bore a flag-like projection
distally, appeared to be structurally different from the inner
arm, and this agrees with the findings of Warner and Satir
(1973). In general, the structure of microtubules, radial
spokes and central sheath of mink spermatozoa appear similar to that of mammalian spermatozoa (Hancock, 1966).

In the middle piece region of mink spermatozoa, the nine doublets resemble those of other mammals, but the nine outer dense fibers appear to be smaller when compared to those of the lower vertebrates (Fawcett, 1970). Furthermore, four of the dense fibers (number 9, 1, 5, and 6) were larger in diameter than the rest of the dense fibers. This is consonant with the finding of Blom and Birch-Andersen (1960) in the bull, but differs from the result of Bradfield (1955) who described, as a usual phenomenon, three thicker (number 1, 5, and 6) and six thinner dense fibers in bull spermatozoa. Saacke and Almquist (1964b) suggested that the comparative size difference between each of the dense fibers varied not only with species but also with breeds of the same species. On the other hand, Wu and Newstead (1966) reported that three or sometimes four dense fibers were larger than the others in the bull. Therefore, four larger dense fibers seem to be normal in mammalian spermatozoa. Considering the length of the fibers, the long axes of two fibers (number 3 and 8) appeared to end in the distal part of the tail sooner than the rest of the dense fibers.

Mink spermatozoa had a comparatively long mitochondrial sheath which seems to be a single helix. The number of mitochondrial gyres in mink spermatozoa is greater than those found in spermatozoa of human, bull, dog, and chinchilla, but it is less than in the dormouse, suni, Russian hamster, mouse, bat, and rat spermatozoa (Fawcett, 1970). Because mink spermatozoa need more energy for their longer period of existence in the
uterine tract of the female (Enders, 1952) as compared with that of other domestic animals, they might require a larger number of mitochondria. The cytoplasmic droplets were found on the middle piece in some mink spermatozoa as in other ejaculated or epididymal mammalian spermatozoa (Hancock, 1957a; Bishop and Walton, 1966a; Orgebin-Crist, 1967a).

The triangular annulus was present in mink spermatozoa as it is in the bat, dormouse, Chinese hamster, suni antelope, guinea pig (Fawcett, 1965, 1970), blue fox (Andersen, 1974), bull (Saacke and Almquist, 1964b), and man (Pedersen, 1970b). On the other hand, the cell membrane appeared to anchor itself on the annulus, which is closely consonant with the findings of Saacke and Almquist (1964b) in bull spermatozoa.

An earlier investigator denied the existence of longitudinal columns in the fibrous sheath (Anberg, 1957). However, mink spermatozoa had longitudinal columns even though they are not very conspicuous. This finding is in accordance with those with regard to several mammals (Fawcett, 1970). The basic pattern of the axoneme (9 + 2) was maintained in the end piece, while Anberg (1957) reported a possible loss of 9 + 2 pattern somewhere in the end piece in human spermatozoa.

The present ultrastructural study of mink spermatozoa reveals no fundamental difference in structure from those of other mammals. However, the occurrence of swellings anterior and posterior to the equatorial segment of the head and the arrangement of the connecting piece, the modified capitulum and the grouping of the striated columns, are some of the important features characteristic of mink spermatozoa.
F. SUMMARY

The subcellular structure of epididymal spermatozoa obtained from standard dark mink was studied by means of electron microscopy, using thin-sectioning techniques.

The spermatozoan head, composed mostly of an electron dense nucleus with a number of vacuoles, was seen to be dorso-ventrally flattened and ovate in outline. The anterior two-thirds of the nucleus was covered with the acrosome and the posterior one-third with the postacrosomal sheath. The acrosome may be divided into the apical, main, and equatorial segments according to the acrosomal content around the nucleus. On the tip of the nucleus, the perforatorium was visible and remained intact even when the content of the acrosome was dislodged. On the dorsal and ventral aspects of the head, six swellings were observed; two of these were located at each side of the proximal border of the equatorial segment, and one was associated with the postacrosomal region on each side. The postacrosomal sheath was a dense layer deposited on the inner aspect of the cell membrane. The equatorial and postacrosomal segments were more stable than the anterior part. The cell membrane adhered firmly to the tip of the acrosome and to the postacrosomal sheath, but was found to be usually separated from the rest of the acrosome. The caudal surface of the nucleus accommodated the capitulum of the neck by forming the implantation fossa.

The articular structure of the neck appeared to show separate dorsal and ventral plates of the capitulum which
were followed by a ring of striated columns of the connecting piece. These striated columns were followed by two major and five minor columns which appeared to continue with the nine dense fibers of the axial fiber bundles. Beneath the inner surface of the capitulum, the proximal centriole was found in the center of the connected ring. The remnants of the axoneme were occasionally visible in the interior of the column bundle. A few mitochondria and scrolls were also found in the neck region.

The spermatozoan tail displayed a 9 + 9 + 2 pattern in the organization of the axial fiber bundle consisting of the dense fibers, double microtubules, and a central pair. The dense fibers number 9, 1, 5, and 6 were larger in diameter than the rest of the dense fibers. In the axonemal complex, the diameter of subfiber A was larger than the central fiber, while that of subfiber B was the smallest. The middle piece was of medium length compared with other mammalian spermatozoa. The annulus was triangular in longitudinal sections and contained non-homogeneously distributed electron dense material. In some spermatozoa, cytoplasmic droplets were found on the caudal end of the middle piece. The fibrous sheath had longitudinal columns. Although the end piece maintained the 9 + 2 pattern, it did not have the fibrous sheath.
Chapter II

CYTOCHEMICAL OBSERVATIONS OF MINK SPERMATOZOA

A. INTRODUCTION

A basic problem in reproductive physiology is the transport of spermatozoa to the egg with subsequent penetration of cumulus cells and membranes of the egg. Enzymes play an essential role in the motility and in the lytic action of spermatozoa.

ATP is the energy source of spermatozoan motility. The energy of fructose in seminal plasma, phospholipid in spermatozoa, and other substrates in semen are transformed to ATP by either glycolysis or respiration and ATP is stored in the spermatozoa. ATPase in the flagella of spermatozoa, known as the dynein arms of the axonemal doublets, hydrolyses ATP and releases energy for motility. Several coenzymes, dehydrogenases, and other enzymes are involved in the Embden-Meyerhof pathway of glycolysis and aerobic tricarboxylic acid cycle. Related enzymes are mostly localized in the mitochondrial sheath of the middle piece and the principal piece of spermatozoa.

The egg is surrounded by several protective coats. To form the zygote, the spermatozoon must penetrate the investment of the ovum by the lytic action of the acrosomal enzyme. Hyaluronidase and acid phosphatase are important enzymes among many acrosomal enzymes. Some phosphatases are involved in the transport of phosphate groups between seminal plasma and spermatozoa.
Although several investigators have studied the relation between the activity of some enzymes and the semen quality, convincing results are few. Moreover, the enzyme activity of semen differs among species. Enzyme localization of spermatozoa in different species might be helpful to elucidate the nature of spermatozoan metabolism and fertilization process, and it might also be used as an index to predict fertility.

Although there have been several studies regarding the enzyme localization of mammalian spermatozoa, there is no report on the enzyme in mink spermatozoa. The purpose of the present study was to identify the localization sites of 14 enzymes (phosphatases, esterase, oxidase, and dehydrogenases) in mink spermatozoa and the implication of these enzymes in regards to the reproductive physiology of the mink.
B. REVIEW OF RELATED LITERATURE

1. Early Studies of Spermatozoan Cytochemistry

Although early investigators did not entirely neglect the seminal plasma as a vehicle (Leeuwenhoek, 1677), observations on smell and taste of semen were first reported by Hunter in 1786, one century after the discovery of spermatozoa. Later, Miescher (1897) gave much information concerning spermatozoa and seminal plasma. However, until Ivanov (1907) succeeded in semen collection from domestic animals for artificial insemination, most early workers used to deal with invertebrate spermatozoa and semen.

In 1919 Lillie stated that spermatozoa could not receive nutrients from seminal plasma after being fully differentiated. Afterwards, Walton et al. (1928) found that mammalian spermatozoa could survive under anaerobic conditions, and McCarthy et al. (1927) demonstrated glycolysis in human semen. Later Redenz (1933) showed that mammalian spermatozoa could utilize extra-cellular substrates occurring in the seminal plasma, i.e. fructose and glucose, through glycolytic breakdown under both aerobic and anaerobic conditions. Under aerobic conditions, the lactic acid produced by glycolysis is further degraded to carbon dioxide and water.

A powerful solvent action on the egg membrane of the toad and mussel is provided by the spermatozoan acrosome (Parat, 1933; Wada et al., 1956). Hoffman and Duran-Reynals (1931) and McClean (1931) discovered the existence of a "spreading" or "diffusing" factor in mammalian testes. The depolymeriza-
tion and hydrolysis of hyaluronic acid by hyaluronidase were reported by Meyer et al. (1937), McClean and Rowlands (1942) and Weissmann (1955). Spermatozoan suspensions or extracts from rabbit spermatozoa disperse the follicle cell of the cumulus oophorus of the rabbit ova (Yamane, 1930, 1935b).

2. The Role of Enzymes in Spermatozoan Motility

The immediate energy source of spermatozoan motility is ATP in spermatozoa (Lardy and Phillips, 1945; Mann, 1945a; Ivanov et al. 1946). Mammalian spermatozoa lose their motility when ATP reserves are depleted (Lardy and Phillips, 1945; Mann, 1945a,b,c). However, the isolated spermatozoan tail model initiates movement when ATP is added (Bishop, 1958). While the decrease of ATP is rapid in the absence of a substrate, the loss of ATP is decreased by the addition of glucose or fructose (Lardy and Phillips, 1945; Mann, 1945a), and by phospholipid (Lardy and Phillips, 1945). Energy-rich phosphate of ATP is furnished by glycolysis or oxidative metabolism (Mann, 1964; Bishop and Walton, 1966a). The energy is released from ATP by the action of ATPase. Actually, the arms of the axonemal doublets of the tail are composed of ATPase (Gibbons and Rowe, 1965). Although there are species differences in energy acquiring systems, the major energy source for ATP generation in mammalian spermatozoa is fructose-rich seminal plasma for anaerobic glycolysis (Gonse, 1962; Mann, 1964); the secondary energy source is endogenous phospholipid (Lardy and Phillips, 1941a,b, 1945; Lardy et al., 1945a; Hartree and Mann, 1961). Because spermatozoa use glycolytic phosphorylation preferen-
tially, even under aerobic conditions (Salisbury and Lodge, 1962), the metabolic pattern of spermatozoa is unusual and it can be regarded as a metabolic adaptation to the special requirements for survival with maintenance of motility. The glycolytic degradation of hexose in mammalian spermatozoa is accomplished through the Embden-Meyerhof scheme (Mann, 1945b, c; Flipse, 1954, 1956a; Flipse and Almquist, 1955, Wu et al., 1959; Turner and Johnson, 1973a; Storey and Kayne, 1975). Several enzymes and coenzymes are involved in this pathway and are found in intact spermatozoa (Mann, 1945b, 1964). Finally, the substrate is hydrated and dephosphorylated to produce pyruvic acid, which is converted to lactic acid in the absence of oxygen.

In the presence of oxygen, mammalian spermatozoa show considerable respiratory activity consuming glycolysable sugars, lactate, pyruvate, acetate, propionate, butyrate, and oxaloacetate (Lardy and Phillips, 1945; Mann and Lutwak-Mann, 1948; Melrose and Terner, 1953; Turner and Johnson, 1973b). The respiratory activity depends on the Krebs tricarboxylic acid cycle and catalytic activity of the cytochrome system. Mammalian spermatozoa can also metabolize glycerol (Mann and White, 1957; White, 1957), lipid (Lardy and Phillips, 1941a,b, 1945) and amino acids (Flipse, 1956b; Flipse and Benson, 1957) by the Krebs cycle. The enzyme system involved in the respiration of spermatozoa includes specific coenzymes and several enzymes: ATP (Lardy et al., 1945a; Mann, 1945a,c; Ivanov et al., 1946), cytochromes (Mann, 1945a,c, 1951), ATPase (Nelson, 1954, 1958; Mann, 1964; Uesugi and Yamazoe,

The metabolic enzymes appear to be distributed through the spermatozoan tail while the respiratory enzymes seem to be confined to the mitochondria of the middle piece (Bishop and Walton, 1966b). On the other hand, some metabolic enzymes exist in the seminal plasma (Mann, 1967). The effects of glycolysis inhibitors (Lardy and Phillips, 1941c), oxidative processes (Lardy and Phillips, 1941a,c) and various substrates (Lardy and Phillips, 1941a,b; Scott et al., 1962) are different among species. Therefore, the presence and distribution of enzymes in spermatozoa might also differ among species.

3. The Role of Enzymes in Fertilization

Prior to fertilization spermatozoa must acquire the ability to penetrate the ovum through capacitation in the female reproductive tract (Austin, 1951; Chang, 1951). The timing of the acrosome reaction is influenced by the types of energy sources in the environment (Rogers and Yanagimachi, 1975). Through the capacitation, labilized plasma and acrosomal membranes enable the release of hyaluronidase, which digests the hyaluronic acid between the cells of the cumulus oophorus (McClean and Rowlands, 1942; Talbot and Franklin, 1974). The cementing substance between the corona cells of the ovum is digested by corona-penetrating enzymes (Zanerveld et al., 1969a; Zanerveld and Williams, 1970) which have been
recently classified as esterases (Bradford et al., 1976a,b). Proteolytic enzymes on the inner surface of the acrosomal membrane are either gradually released or activated in a bound state (Srivastava et al., 1974). A limited fissure of the ovum is digested through the zona pellucida by an array of proteolytic enzymes (Srivastava et al., 1965; Zaneveld et al., 1973). The crude extract of the acrosome frequently digest the vitelline membrane, the last barrier to spermatozoan penetration (Srivastava et al., 1965). During fertilization, therefore, the acrosome plays an essential role to provide lytic agents.

Owing to the improvement of physical and chemical treatments to remove the acrosome of mammalian spermatozoa, numerous lytic enzymes have been localized in the acrosome. These are: hyaluronidase (Rowlands, 1944; Srivastava et al., 1965; Bernstein and Teichman, 1973), proteolytic enzymes that resemble plasmin and trypsin (Yamane, 1935a,b; Srivastava et al., 1965; Zaneveld et al., 1969b, 1973; Bernstein and Teichman, 1973; Stambaugh and Smith, 1973), acid phosphatase (Wislocki, 1950; Bernstein and Teichman, 1973), β-glucuronidase (Bernstein and Teichman, 1973), β-N-acetylglucosaminidase (Conchie and Mann, 1957; Allison and Hartree, 1970), d-mannosidase (Conchie and Mann, 1957), non-specific esterase (Bryan and Unnithan, 1972, 1973; Bradford et al., 1976a,b), 5-nucleotidase (Garbers et al., 1970; Multamäki et al., 1975), neuramidinidase, and others (McRorie and Williams, 1974; Multamäki et al., 1975).
Some phosphatase activities are detected in spermatozoa (Melampy et al., 1952; Teichman and Bernstein, 1969; Allison and Hartree, 1970; Wislocki, 1950; Uesugi and Yamazoe, 1966; Quinn and White, 1968; Mathur, 1971), and powerful phosphatase activity of the semen exists in the seminal plasma (Mann, 1964). Acid phosphatase and alkaline phosphatase are the most active dephosphorylating enzymes in the semen. 5-nucleotidase, pyrophosphatase, and several enzymes which split ATP are also contained in the seminal plasma (Mann, 1964).

4. Epididymal Spermatozoa as a Model for the Enzyme Study

Although most investigators consider epididymal spermatozoa as immature (Salisbury and Lodge, 1962), the spermatozoa collected from the cauda epididymis appear to be mature and fertile not only in the mink (Pomytko et al., 1972; Ahmad et al., 1975b) but also in other mammals (Young, 1931; Orgegin-Crist, 1967a, 1968; Horan and Bedford, 1972). The difference of metabolic pattern between epididymal and ejaculated spermatozoa has been reported by several investigators. Epididymal spermatozoa have a lower endogenous respiratory exchange compared to ejaculated spermatozoa (Henle and Zittle, 1942; Lardy et al., 1945b). However, the addition of glucose to epididymal spermatozoa stimulates oxygen uptake (Henle and Zittle, 1942). Moreover, on addition of sugar, epididymal spermatozoa produce lactic acid more rapidly under anaerobic than under aerobic conditions. In ejaculated spermatozoa the glycolysis rate is not much higher in the presence than in the absence of oxygen (Mann, 1964). Washed ejaculated
spermatozoa exhibit only a feeble Pasteur effect, i.e. the extent of glycolysis inhibition by oxygen, compared to fresh epididymal spermatozoa (Lardy and Phillips, 1941a,b,c, 1943a, b). On the contrary, White and Wales (1961), Voglmayr et al. (1966), and Murdoch and White (1968) reported no difference in Pasteur effect between epididymal and ejaculated spermatozoa. The basic mechanism of glucose utilization in cauda epididymal spermatozoa is via the Embden-Meyerhof pathway (Wu et al., 1959; Storey and Kayne, 1975). The major energy source of ejaculated spermatozoa is also via anaerobic glycolysis (Mann, 1964; Gonse, 1962). Moreover, testicular spermatozoa stored in testicular fluid change their metabolic pattern toward that of ejaculated spermatozoa (Lardy et al., 1945b; Voglmayr et al., 1967). Consequently, epididymal spermatozoa have been used as a convenient model for the demonstration of the metabolism of spermatozoa which might be applied for ejaculated samples.

Furthermore, a proteinase inhibitor is absorbed by spermatozoa from seminal plasma during ejaculation (Zaneveld et al., 1973), and is later lost after capacitation in utero (Zaneveld et al., 1971, 1973). L-tartrate inhibits the acid phosphatase activity of prostatic origin (Sivaram and Bami, 1971). Acrosin activity, acrosomal proteinase digesting a passway for spermatozoa through the zona pellucida, is significantly higher in epididymal and capacitated spermatozoa than in ejaculated rabbit spermatozoa (Zaneveld et al., 1969b). If such inhibiting factors of other enzymes exist in the seminal plasma and inhibit the enzyme activity of ejaculated
spermatozoa, epididymal spermatozoa might be a good model for studying the real distribution of enzymes.
C. MATERIALS AND METHODS

For the cytochemical investigation small pieces of the cauda epididymis were immediately immersed in 1 ml of 0.85% saline solution where spermatozoa were released by squeezing the tissue. Motile spermatozoa thus released into the suspension were used for preparation of microscope slides and stained for enzyme characterization as indicated in Table 1.

All the substrates were obtained from Sigma Chemical Company. The stained sperm preparations were mounted in Farrant's medium and examined by a light microscope. The presence of the enzymes was confirmed by comparison with the staining characteristics of appropriate control slides prepared by the deletion of substrate or by the addition of inhibitors.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Method</th>
<th>Incubation</th>
<th>Control</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>Gomori lead phosphate method</td>
<td>37°C, 2 h</td>
<td>Addition of 0.01M sodium fluoride</td>
<td>Chayen et al. (1973) pp. 112-114</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Gomori-Takamatsu procedure</td>
<td>37°C, 1 h</td>
<td>Deletion of the substrate</td>
<td>Chayen et al. (1973) pp. 108-109</td>
</tr>
<tr>
<td>5-nucleotidase</td>
<td>Lead method</td>
<td>37°C, 20 min</td>
<td>The substrate was replaced by sodium-B-glycerophosphate</td>
<td>Chayen et al. (1973) p. 118</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>Lead method</td>
<td>37°C, 20 min</td>
<td>- ditto -</td>
<td>Chayen et al. (1973) pp. 120-121</td>
</tr>
<tr>
<td>ADPase</td>
<td>Lead method</td>
<td>37°C, 1 h</td>
<td>Deletion of the substrate</td>
<td>Wachstein and Meisel (1957)</td>
</tr>
<tr>
<td>ATPase</td>
<td>Calcium-activated method</td>
<td>37°C, 20 min</td>
<td>The substrate was replaced by sodium-B-glycerophosphate</td>
<td>Chayen et al. (1973) p. 124</td>
</tr>
<tr>
<td>Non-specific esterase</td>
<td>Indoxyl acetate method</td>
<td>37°C, 30 min</td>
<td>Deletion of the substrate</td>
<td>Chayen et al. (1973) pp. 137-139</td>
</tr>
<tr>
<td>DOPA oxidase</td>
<td>Diengdoh method</td>
<td>37°C, 2 h</td>
<td>Addition of potassium cyanide</td>
<td>Chayen et al. (1973) pp. 170-171</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>NAD and MTT</td>
<td>Room temp, 1 h</td>
<td>Deletion of the substrate</td>
<td>Pearse (1972) pp. 1343-1344</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>Nitro BT</td>
<td>37°C, 1 h</td>
<td>- ditto -</td>
<td>Nachlas et al. (1957)</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>NAD and MTT</td>
<td>Room temp, 1 h</td>
<td>- ditto -</td>
<td>Pearse (1972) pp. 1343-1344</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>NAD and MTT</td>
<td>Room temp, 1 h</td>
<td>Addition of sodium cyanide</td>
<td>- ditto -</td>
</tr>
<tr>
<td>6-phosphogluconic dehydrogenase</td>
<td>NADP and MTT</td>
<td>Room temp, 1 h</td>
<td>- ditto -</td>
<td>Nachlas et al. (1958)</td>
</tr>
<tr>
<td>NADH diaphorase</td>
<td>NAD and Nitro BT</td>
<td>Room temp, 30 min</td>
<td>Deletion of the substrate</td>
<td></td>
</tr>
</tbody>
</table>
D. RESULTS

1. Phosphatases

Acid phosphatase (EC 3.1.3.2) activity was most strongly localized among the examined enzymes (Fig. 25). The equatorial region of the head and tail (middle piece and principal piece) were deeply stained. It was especially noticed that in the head there were two deeply stained prominances appearing as two connected humps on the anterior border of the equatorial segment. The galea capitis, acrosome, and postacrosomal sheath were also stained. Alkaline phosphatase (EC 3.1.3.1) activity was strongly and evenly distributed in the galea capitis, acrosome, postacrosomal sheath and tail (Fig. 26). The activity of 5-nucleotidase (EC 3.1.3.5) was weakly localized in the middle piece (Fig. 27). Very strong glucose-6-phosphatase (G-6-Pase, EC 3.1.3.9) activity was confined to the middle piece (Fig. 28). Adenosine diphosphatase (ADPase, EC 3.6.1.5) activity was distributed strongly in the tail while weak activity was found in the acrosome and postacrosomal sheath (Fig. 29). Adenosine triphosphatase (ATPase, EC 3.6.1.3) activity was distributed strongly in the head base and tail while weak activity was detected in the galea capitis, acrosome and postacrosomal sheath (Fig. 30). All the localized phosphatase complexes determined by the present technique were stained brown in colour.

2. Esterase and Oxidase

Non-specific esterase activity was weakly localized in the base of the head and the middle piece (Fig. 31).
3,4-dihydroxyphenylalanine (DOPA) oxidase (histochemically demonstrated as catechol oxidase, EC 1.14.18.1) activity was faintly present in the acrosome, postacrosomal sheath, and tail (Fig. 32). The esterase and oxidase complexes were stained light-grey and intense-grey respectively.

3. Dehydrogenases

Most of the dehydrogenases examined were confined only to the middle piece, and their reaction products were stained grey. The reaction of succinate dehydrogenase (SDH, EC 1.3.99.1) and reduced nicotinamide adenine dinucleotide (NADH) diaphorase was strong (Figs. 34,37), and that of lactate dehydrogenase (LDH, EC 1.1.1.27) and isocitrate dehydrogenase (IDH, EC 1.1.1.41) was comparatively weak (Figs. 35,36). The activity of malate dehydrogenase (MDH, EC 1.1.1.37) was detected as being strong in the middle piece and weak in the base of the head and principal piece (Fig. 33) while 6-phosphogluconic dehydrogenase (PDH, EC 1.1.1.43) activity was undetectable.
Figures 24 to 37. Enzyme localization in epididymal spermatozoa. 1,640X.

Figure 24. Control for alkaline phosphatase activity.

Figure 25. Very strong acid phosphatase activity is localized in the head and tail. The connected hump-like localization on the anterior border of the equatorial segment is especially prominent. The galea capitis, acrosome and postacrosomal sheath are also stained.

Figure 26. Strong alkaline phosphatase activity can be seen in the galea capitis, acrosome, postacrosomal sheath and tail.

Figure 27. 5-nucleotidase activity is weakly localized in the middle piece.

Figure 28. Very strong glucose-6-phosphatase activity is confined to the middle piece.

Figure 29. Adenosine diphosphatase activity is distributed strongly in the tail and weakly in the acrosome and postacrosomal sheath.

Figure 30. Adenosine triphosphatase activity is localized strongly in the head base and tail, and weakly in the galea capitis, acrosome and postacrosomal sheath.

Figure 31. Non-specific esterase activity can be seen in the head base and middle piece.

Figure 32. DOPA oxidase activity is localized in the acrosome, postacrosomal sheath, and tail.
Figure 33. Malate dehydrogenase activity is localized strongly in the middle piece and head base and weakly in the principal piece.

Figure 34. Strong succinate dehydrogenase activity is confined to the middle piece.

Figure 35. Lactate dehydrogenase activity can be seen in the middle piece.

Figure 36. Very weak isocitrate dehydrogenase activity is localized in the middle piece.

Figure 37. Strong NADH diaphorase activity is confined to the middle piece.
E. DISCUSSION

1. Phosphatases

Very strong acid phosphatase activity was localized in the postacrosomal sheath, tail and especially in the equatorial region where it produced a connected hump-like formation. Although several investigators have reported the presence of acid phosphatase in spermatozoa of the human (Wislocki, 1950; Teichman and Bernstein, 1969; Allison and Hartree, 1970), bull (Melampy et al., 1952; Roussel and Stallcup, 1966; Teichman and Bernstein, 1969, 1971; Allison and Hartree, 1970; Guraya and Sidhu, 1975), rabbit (Teichman and Bernstein, 1969, 1971), mouse (Poirier, 1975), ram, boar, guinea pig, hamster and rodent (Allison and Hartree, 1970), some investigators have reported very little or no acid phosphatase activity in the rabbit (Stambaugh and Buckley, 1969) and mouse spermatozoa (Mathur, 1971; Bryan and Unnithan, 1973). The difference in the results obtained for rabbit and mouse spermatozoa appears to be dependent on the different substrates (Chayen et al., 1973), fixations (Bryan and Unnithan, 1973), incubation times (Poirier, 1975) or to be due to an association of the enzyme with stable membrane (Allison and Hartree, 1970; Teichman and Bernstein, 1971). In the present study, however, the same substrate and a method similar to that applied by Melampy et al. (1952), Teichman and Bernstein (1971), Mathur (1971), and Guraya and Sidhu (1975) were used and a strong acid phosphatase activity in mink spermatozoa was demonstrated.
The acid phosphatase complex was found at the distinct hump-like structure in the head region of mink spermatozoa which resembles the complex illustrated for rabbit spermatozoa (Teichman and Bernstein, 1969, 1971). This pattern of acid phosphatase localization appears to be related to the structural modification (swellings) in the head region of mink spermatozoa (Fig. 2). A strong acid phosphatase activity in this region of mink spermatozoa might account for the lysosomal action of releasing acrosomal enzyme during the initial contact of a spermatozoon and an ovum. Acid phosphatase, a major component of the acrosome, is a lysosomal enzyme.

Mann (1964) suggested that semen owes its powerful phosphatase activity mainly to the seminal plasma derived from the male accessory organs of reproduction. The acid phosphatase activity was located in both the seminal plasma and spermatozoa of the human (Wislocki, 1950) and bull (Melampy et al., 1952). Roussel and Staccup (1966) have shown that acid phosphatase activity was three times higher in the seminal plasma than in spermatozoa of the bull. They also demonstrated that slight amounts of this enzyme was present in the caput epididymis with increasingly larger amounts in the corpus and cauda epididymis. MacLeod and Summerston (1946) reported that human spermatozoa washed free of seminal plasma could not split the phosphate group except ATP. Jones and Glover (1973) also showed that acid phosphatase is an extracellular enzyme. However, Stambaugh and Smith (1973) reported trace acid phosphatase activity in the extract of subcellular fractionation of rabbit spermatozoa. Recently, Poirier (1975)
ultrastructurally demonstrated acid phosphatase activity between the plasmalemma and the outer acrosomal membrane of the head of mice spermatozoa collected from the cauda epididymis. Teichman and Bernstein (1969) suggested that rabbit and bull acid phosphatase appeared to be membrane bound and subject to species-specific distribution. The present study demonstrated a strong activity of acid phosphatase in the membrane of mink spermatozoa collected from the cauda epididymis.

Acid phosphatase activity in the acrosome, postacrosomal sheath and tail of mink spermatozoa appears similar to that localized in human and bull spermatozoa (Wislocki, 1950; Melampy et al., 1952). This suggests that acid phosphatase is involved not only in the lytic action of the acrosome, but also in the dephosphorylation and transport of phosphate groups between seminal plasma and spermatozoa as demonstrated by Mann (1964). Changes in the distribution of the enzyme in mice spermatozoan heads during maturation (Poirier, 1975), and the migration of the cytoplasmic droplet which contains acid phosphatase as spermatozoa pass through the epididymis (Dott and Dingle, 1968; Moniem and Glover, 1972; Bedford, 1973) appear to show the involvement of acid phosphatase in spermatozoan maturation.

The acid phosphatase activity of bull semen has a positive correlation with spermatozoan concentration (Reid et al., 1948; Haq and Mullen, 1949; Roussel and Stallcup, 1966), and with the percentage of motile spermatozoa (Reid et al., 1948; Roussel
and Stallcup, 1966) but a negative correlation with the percentage of abnormal spermatozoa (Roussel and Stallcup, 1966). Feeding of additional vitamins and minerals increases the activity of acid and alkaline phosphatases in bull semen (Reid et al., 1948). However, Stallcup (1965) failed to show any convincing correlation between fertility and acid phosphatase activity in spermatozoa from the bull, and Back et al. (1975) in the rat and rabbit.

Acid phosphatase has phenotype variability: three isozymes appear in the seminal plasma of bulls and five in that of rams (Georgiev and Yossifov, 1973). L-tartrate inhibits acid phosphatase activity in human semen (Sivaram and Bami, 1971). Such inhibition might be a reason for failures in demonstrating the enzyme reaction in ejaculated spermatozoa of the rabbit, human (Gordon, 1973) and mink in this study (Chapter III, Table 5).

Alkaline phosphatase activity was distributed strongly and evenly in the galea capitis, acrosome, postacrosomal sheath and tail. Although several investigators (Bavdek and Glover, 1970; Moniem and Glover, 1972) found the enzyme activity restricted to the cytoplasmic droplet in the rabbit and ram, alkaline phosphatase activity was found in the head and tail of spermatozoa in human (Wislocki, 1950), bulls (Melampy et al., 1952; Guraya and Sidhu, 1975) and rams (Friedlaender and Fraser, 1952). On the other hand, the reaction of alkaline phosphatase was not detected in spermatozoa from the hamster, rat (Momiem and Glover, 1972), and mouse (Mathur, 1971).

Alkaline phosphatase activity of bull spermatozoa is
positively correlated with the percentage of motile spermatozoa and the percentage of live spermatozoa, while the activity in seminal plasma is negatively correlated with the percentage of abnormal spermatozoa (Roussel and Stallcup, 1966). High alkaline phosphatase activity in bull semen is also correlated with high nonreturn per cent (Stallcup, 1965). Alkaline phosphatase is associated with the dephosphorylation and transport of phosphate groups across membranes (Bern, 1949; Mann, 1964). In addition to this, Bavdek and Glover (1970) suggested an involvement of this enzyme in the glycogen utilization within the cytoplasmic droplet. The activity of alkaline phosphatase in the cytoplasmic droplet of rabbit spermatozoa is reduced during epididymal transit and the enzyme might therefore be involved in the early maturation of epididymal spermatozoa (Moniem and Glover, 1972).

The activity of 5-nucleotidase was found to be confined to the middle piece of mink spermatozoa. Guraya and Sidhu (1975) showed the enzyme activity in the postacrosomal sheath and middle piece of buffalo spermatozoa. Mathur (1971) indicated its absence in epididymal spermatozoa of mice. It has been suggested that the presence of this enzyme in seminal plasma (Mann, 1964) could contaminate spermatozoa and produce a false positive result. However, in the present study, mink spermatozoa were collected from the cauda epididymis where there is very little chance of contamination due to seminal plasma.

Mann (1964) summarized the role of 5-nucleotidase in the seminal plasma as dephosphorylyzing ribose-5-phosphate, adenylc acid, and other nucleotides. Guraya and Sidhu (1975)
assumed its role in permeability and transport process. Moreover, the presence of enzyme activity in the postacrosomal sheath (Guraya and Sidhu, 1975), acrosome and cytoplasmic droplet (Garbers et al., 1970; Multamäki et al., 1975) suggests its lysosomal action and involvement in fertilization. However, 5-nucleotidase in mink spermatozoa might not display such function because this enzyme activity has not been found in the acrosome or in the postacrosomal sheath.

The strong activity of G-6-Pase was confined to the middle piece of mink spermatozoa. Although Gordon (1973) could not localize the enzyme in spermatozoa of the rabbit and human, Mathur (1971) found its activity in the acrosomal tip, head base, and tail of mice spermatozoa. Glucose-6-phosphate plays an essential role in glycogen metabolism because it is a common intermediate in glycogenesis, glycogenolysis, and glycolysis (Krebs and Fisher, 1962). Consequently, G-6-Pase activity, which hydrolyses glucose-6-phosphate, influences the disposition of glucose-6-phosphate and the regulation of glycogen metabolism (Anderson and Personne, 1970). Although Mann (1964) stated that the glycogen and glucose content in spermatozoa and seminal plasma of mammals was low, the strong activity of G-6-Pase in mink spermatozoa raises the question whether glucose and glycogen content of spermatozoa and/or semen is relatively high compared to other mammals.

Although ADPase activity was not found in rabbit and human spermatozoa (Gordon, 1973), this enzyme appeared to be distributed strongly in the tail and weakly in
the acrosome and postacrosomal sheath of mink spermatozoa. With regard to ATPase, a weak activity was detected in the galea capitis, acrosome, and postacrosomal sheath and strong activity in the tail of mink spermatozoa. This result is in agreement with previous reports in that ATPase activity was confined almost exclusively to the tail with lowest activity in the head of other mammalian spermatozoa (Nelson, 1954, Engelgardt and Burnasheva, 1957, Quinn and White, 1968, Guraya and Sidhu, 1975, in bulls; Quinn and White, 1968, Voglmayr et al., 1969, in rams; Uesugi and Yamazoe, 1966, in boars; Durr et al., 1972, in human; Nelson, 1958, Gordon and Barrnett, 1967, in rats; Gordon and Barrnett, 1967, in guinea pigs; Mathur, 1971, in mice). Ultrastructurally, ATPase activity is localized in the axial filament complex of the tail (Nelson, 1958; Gordon and Barrnett, 1967), dense fibers (Nelson, 1958; Baccetti et al., 1973) and around the doublets and central tubules (Baccetti et al., 1973a). Particularly, it is to be noted that the arms, located on the outer doublets of the axoneme, are constituted of ATPase (Gibbons, 1965, 1966; Gibbons and Rowe, 1965; Gibbons and Fronk, 1972). ATPase activity is also present in semen (Mann, 1945a, Heppel and Hilmoe, 1953) and in cytoplasmic droplets (Garbers et al., 1970).

ATPase and ADPase bring about the hydrolysis of ATP to adenosine diphosphate and successively to adenosine monophosphate with the release of energy for spermatozoan motility (Mann, 1964). The active transport of alkali metal ions between spermatozoa and seminal plasma involves a sodium-
potassium activated ATPase (Uesugi and Yamazoe, 1966; Quinn and White, 1967b, 1968). Gordon and Barnett (1967) suggested the involvement of ATPase in spermatozoan capacitation. These are differentiated ATPase activities in spermatozoa based on pH optimum, effects of fixation, activation ions, substrate specificity, and sensitivity to inhibitors (Uesugi and Yamazoe, 1966; Gordon and Barnett, 1967; Voglmayr et al., 1969; Gordon, 1973). Therefore, Gordon and Barnett (1967) suggested that these ATPases, which cytochemically and morphologically constitute at least two distinct units, might play separate roles in spermatozoan motility.

2. Esterase and Oxidase

Non-specific esterase activity in mink spermatozoa was located in the base of the head and middle piece. Meizel (1970) and Meizel et al. (1971) reported esterase activity in the head and tail of bull spermatozoa and Mathur (1971) showed its activity in the middle piece of mice spermatozoa. On the other hand, esterase activities were found to be confined only in the acrosome of bull spermatozoa (Bryan and Unnithan, 1972) and mice and hamster spermatozoa (Bryan and Unnithan, 1973). Niemi and Kormano (1965), however, could not detect the esterase activity in the acrosome of rat spermatozoa. The esterase reaction also occurred in the seminal plasma of the bull (Meizel, 1970; Meizel et al., 1971) and rabbit (Bradford et al., 1976a) and the cytoplasmic droplet (Meizel et al., 1971) of the bull. By an electrophoretic study, Meizel et al. (1971) demonstrated that a certain esterase activity of spermatozoa was due to absorbed
seminal plasma esterases.

Recently Bradford et al. (1976a,b) reported that the esterolytic activity of the acrosome dispersed the intracellular cement material of the corona radiata which contained an essential ester linkage, while hyaluronidase dispersed the cumulus layer. They also proposed that the reported esterase might be classified as EC 3.1.1 and probably as EC 3.1.1.2, arylesterases. Several isozymes of esterase were demonstrated in spermatozoa and seminal plasma of the human (Beckman and Kjessler, 1968) and bull (Meizel, 1970).

The DOPA oxidase activity was weakly spread in the acrosome, postacrosomal sheath and tail of mink spermatozoa. Mathur (1971) showed its activity in the tail of spermatozoa and he reported a difference in the degree of activity between strains. The activity of this enzyme has also been found in spermatozoa of human (Ackerman, 1972), bulls (Mukherjee, 1964; Pant and Mukherjee, 1971), goats, rams (Mukherjee, 1964), mice (Mathur and Beatty, 1974), and rabbits (Beatty, 1956). Although the DOPA oxidase is present in seminal plasma (Beatty, 1970), the DOPA activity of spermatozoa is not dependent on the enzyme in seminal plasma because washed spermatozoa also shows strong DOPA reaction (Beatty, 1956).

The DOPA oxidase reaction, that is the melanizing activity of spermatozoa, is associated with somatic pigmentation of the rabbit (Beatty, 1956) and human (Ackerman, 1972). However, Mukherjee (1964) and Pant and Mukherjee (1971) could not find a definite correlation between coat colours and melanizing activities. Afterwards, Beatty (1970) also admitted that the
melanizing activity and pelt colour could not be directly related. On the other hand, the melanizing activity of spermatozoa is negatively correlated with resistance to temperature shock, the proportion of live (unstained in eosin-nigrosin) spermatozoa, and fructolysis rates of the bull, goat, and ram spermatozoa (Mukherjee, 1964; Pant and Mukherjee, 1971). The DOPA activity is correlated with the degree of the natural colour of the semen (Beatty, 1970). Although Mukherjee (1964) reported the difference of melanizing activity between animals and between ejaculates from the same animal, Mathur and Beatty (1974) demonstrated a variation in the melanizing activity affected by either age or strain, or by both.

3. Dehydrogenases

The activity of MDH appeared strongly in the middle piece and weakly in the principal piece, and head base of mink spermatozoa. A comparatively strong reaction of the enzyme is confined to the middle piece of spermatozoa from the mouse (Balogh and Cohen, 1964; Mathur, 1971), human, rat, guinea pig, cat, and dog (Balogh and Cohen, 1964). However, Mohri et al. (1965) demonstrated the distribution of the enzyme mostly in the tail and small portion of the head in bull spermatozoa which is in close agreement with the result of this study.

MDH is involved in the oxidation of malate in the tricarboxylic acid cycle. Human spermatozoa contain two electrophoretically distinct MDH of which MDH₁ has more activity (Goldberg, 1963). The activity of MDH in bovine spermatozoa is stimulated by egg yolk but inhibited by cholesterol (Smith
et al., 1956).

The activity of SDH was found in this study to be confined to the middle piece of mink spermatozoa. This is in agreement with the findings of other workers who studied spermatozoa from the human (Balogh and Cohen, 1964; Hrudka, 1965), bull (Hrudka, 1965), rabbit (Edwards and Valentine, 1963; Hrudka, 1965), mouse (Mathur, 1971; Balogh and Cohen, 1964), rat, guinea pig, cat, and dog (Balogh and Cohen, 1964). However, Nelson (1955) reported that the SDH activity in bull spermatozoa was concentrated in the principal and middle piece region in a ratio of 2 : 1. He subsequently (1959) demonstrated the presence of this enzyme in the nine outer dense fibers of the rat spermatozoa flagellum. Furthermore, there is no SDH activity in spider spermatozoa which lack mitochondrial complement (Gupta and Kamboj, 1962).

At the initial stage in the electron transport system, SDH plays a key role in sperm metabolism (Nelson, 1959). Mammalian spermatozoa can utilize the tricarboxylic acid cycle as a major source of energy (Bishop, 1962) and succinate stimulates oxygen uptake of spermatozoa (Lardy et al., 1945a,b). However, ejaculated bull spermatozoa cannot utilize the four-carbon dicarboxylic acids, succinate, fumarate, and malate (Lardy and Phillips, 1945) because the spermatozoa cell membrane is impermeable to these intermediates of the tricarboxylic acid cycle (Koefoed-Johnsen and Mann, 1954; Lardy and Phillips, 1945). However, Nelson (1954, 1955) demonstrated that both SDH and ATPase activities were concentrated in the spermatozoan flagellar fibers and suggested (Nelson, 1959) that ATPase was
synthesized within the flagellum and assured a continuous supply of ATP for its motility. Although the role of SDH in spermatozoa metabolism has not been fully elucidated, the importance of dehydrogenases to spermatozoa has been confirmed by the relationships between reduction time, motility, longevity and nonreturn rate (Smith et al., 1956). While cholesterol and carotene of egg yolk stimulate SDH activity, yolk lecithin fails to stimulate or inhibit SDH activity of bovine spermatozoa (Smith et al., 1956). The activity of SDH in spermatozoa withstands freezing (-196°C) and subsequent drying (Keilin and Hartree, 1949) and washing (Jones and Holt, 1974).

The LDH activity of mink spermatozoa is weakly localized in the middle piece. This agrees with those found in the mouse (Balogh and Cohen, 1964; Mathur, 1971), human (Balogh and Cohen, 1964; Churg et al., 1974), rabbit (Churg et al., 1974; Storey and Kayne, 1975), rat (Balogh and Cohen, 1964; Anderson and Personne, 1970), guinea pig, cat, and dog (Balogh and Cohen, 1964). The LDH activity is primarily localized in the matrix of mitochondria (Anderson and Personne, 1970). On the other hand, Mohri et al. (1965) reported that most of the LDH activity was located in the principal piece rather than in the middle piece or head region of bull spermatozoa. The LDH activity in mice spermatozoa differs according to age or strain, or by both (Mathur, 1971; Mathur and Beatty, 1974).

Most mammalian tissues contain five LDH isozymes which are composed of H and M subunits (Wilkinson, 1965). In addition to these isozymes, testes and spermatozoa contain
a large amount of a sixth isozyme, LDH-X (Blanco and Zinkham, 1963; Goldberg, 1963, 1975a; Schatz and Segal, 1969). LDH-X is a sperm-specific isozyme. Semen samples from oligospermic and vasectomized males have no LDH-X (Gregoire and Moran, 1972, 1973) and the activity of LDH-X in the seminal plasma correlates with the spermatozoa density (Eliasson, 1968). Immunization of female rabbits and mice with LDH-X significantly reduces the fertility of these animals through blockage of fertilization and increase of the embryo mortality (Goldberg, 1973, 1975b). Furthermore, an extra LDH band (between the second and third LDH bands) relates to the motility factor of low spermatozoan count semen (Prasad et al., 1976). The activity of LDH$_5$ increases in malignant tissue as in prostate cancer (Eliasson, 1968).

The parent form of five isozymes are differently distributed. The middle piece, site of aerobic glycolysis in the mitochondria, contains the X and H subunits which are best suited for aerobic glycolysis. The tail fibers contain the M subunit which is best suited for anaerobic glycolysis (Stambaugh and Post, 1967; Stambaugh and Buckley, 1969).

The LDH regulate the balance of NADH$_2$-NAD under low oxygen tension in the spermatozoan metabolism (Anderson and Personne, 1970).

In spite of repeated attempts, no reaction product could be identified to show the presence of PDH activity in mink spermatozoa. This result differs from that of Mathur (1971) who reported that this enzyme was localized in the middle piece of mice spermatozoa. As PDH is involved in the hexose
monophosphate shunt pathway, it appears that this pathway is not utilized by epididymal spermatozoa in the mink. Bishop and Walton (1966b) and Voglmayr et al. (1970) also concluded that there was no hexose monophosphate shunt activity in either testicular or ejaculated spermatozoa of bulls.

The middle piece of mink spermatozoa showed faintly localized IDH activity. It is consonant with the results in spermatozoa from the mouse (Balogh and Cohen, 1964; Mathur, 1971), rat, guinea pig, cat, and dog (Balogh and Cohen, 1964). The IDH is involved in the oxidation of isocitrate in the tricarboxylic acid cycle. In all tested samples, dehydrogenase activity was confined to the middle piece of mink spermatozoa with the exception of MDH (distributed in the middle piece, head base and principal piece) and PGD (negative reaction). Dehydrogenase reaction in the mitochondrial sheath of the middle piece is related to the ability of spermatozoa to make use of anaerobic and aerobic metabolic processes as a source of energy for their motility (Hrudka, 1965).

Strong NADH diaphorase activity was confined to the middle piece of mink spermatozoa. This closely agrees with the result in the mouse (Balogh and Cohen, 1964; Mathur, 1971), guinea pig (Birns and Masek, 1961; Balogh and Cohen, 1964), rat, cat, dog, and human (Balogh and Cohen, 1964).

Several dehydrogenases involved in the Embden-Meyerhof pathway and tricarboxylic acid cycle depend for their oxidative action on coenzyme nicotinamide adenine dinucleotide (NAD) forming reduced NAD (NADH) through the transfer of hydrogen from substrates to the coenzyme. The reduced
coenzyme (NADH) is oxidized back to NAD by NADH diaphorase (Chayen et al., 1973). Therefore, NADH diaphorase plays a key role in both glycolytic and respiratory metabolism.

Although the enzymes reported in the spermatozoa of other mammals have also been detected in mink epididymal spermatozoa (with the exception that PDH was not localized) there is some variation in the sites of localization. The results of this study suggest that mink spermatozoa utilize the tricarboxylic acid cycle and Embden-Meyerhof pathway for various oxidative, reductive and other biochemical processes. The long survival of mink spermatozoa in the uterus for more than 19 days post-coitus (Enders, 1952) might be accounted for by the strong enzyme activities.
F. SUMMARY

Epididymal spermatozoa of mink were used to study the cellular localization of several enzymes.

Very strong acid phosphatase activity was found in the equatorial region of the head and tail (middle and principal pieces) of spermatozoa while strong activity was found in the galea capitis, acrosome, and postacrosomal sheath. Deeply stained connected hump-like prominences on the anterior border of the equatorial segment suggest a structural modification in the head region of mink spermatozoa. Strong alkaline phosphatase activity was detected in the galea capitis, acrosome, post-acrosomal sheath and tail. The activity of 5-nucleotidase and glucose-6-phosphatase were confined to the middle piece. The reaction of 5-nucleotidase appeared weak while that of glucose-6-phosphatase was very strong. The activity of ADPase and ATPase was distributed strongly in the tail and it was localized weakly in the acrosome and postacrosomal sheath. The galea capitis was weakly stained by ATPase. Most phosphatases were localized in the acrosome, postacrosomal sheath, and tail, with the exception of 5-nucleotidase and glucose-6-phosphatase which were confined to the middle piece.

Weak non-specific esterase activity was located in the base of the head and middle piece. The activity of DOPA oxidase was weakly spread in the acrosome, postacrosomal sheath, and tail. Although the activity of malate dehydrogenase was distributed in the base of the head
and tail, the rest of the dehydrogenase activity examined (succinate, lactate, and isocitrate dehydrogenases except 6-phosphogluconic dehydrogenase) was confined to the middle piece. The activity of succinate dehydrogenase was the strongest among the examined dehydrogenases. The activity of NADH diaphorase was strongly confined to the middle piece. The metabolic enzymes (glucose-6-phosphatase, dehydrogenases, and NADH diaphorase) were confined to the middle piece, while the activity of malate dehydrogenase was found to be extended to the head base and principal piece. The lytic enzymes, acid phosphatase and esterase, were localized in the acrosomal portion.

Although there was some variation in the site of localization, all examined enzymes in this study, with the exception of 6-phosphogluconic dehydrogenase, were localized in mink spermatozoa as found in spermatozoa of other mammals.
Chapter III

ULTRASTRUCTURAL AND CYTOCHEMICAL CHANGES OF MINK SPERMATOZOA COLLECTED FROM THE REPRODUCTIVE TRACT AND SEMEN

A. INTRODUCTION

Spermatozoa are produced in the seminiferous tubules of the testis and undergo further maturation in the epididymis where they are stored until ejaculation takes place. During spermatogenesis, a number of morphological, chemical and physiological changes occur in spermatozoa to acquire fertilizing ability. Most morphological changes take place in the testis with minor alterations in the epididymis.

On the other hand, most physiological changes take place in the epididymis. Mammalian spermatozoa are not physiologically mature when they enter the epididymis. During passage through the epididymis, spermatozoa attain physiological maturity, as measured by the fertilization rate and the per cent of motile spermatozoa. Variations in response to certain staining, temperature shock, alkalinity or acidity, differences in negative surface charge, agglutinating property, contact ability, chemical composition, enzyme distribution, metabolic pattern, and specific gravity have been noted not only between epididymal and ejaculated spermatozoa but also between spermatozoa collected from the proximal and distal segments of the epididymis.

The introduction and improvement of electron microscopy, the development of continuous spermatozoan collecting
techniques from the testis and epididymis, and the improvement of histochemical methods have facilitated the elucidation of maturation mechanisms of spermatogenic cells in several mammals. However, there is no report on mink spermatozoan maturation. The aim of the present study was therefore the investigation of the maturation changes in mink spermatozoa.
B. REVIEW OF RELATED LITERATURE

1. Early Studies of the Reproductive Tract

Aristotle (300 B.C.) studied the physiology of animal reproduction by introducing the classic technique of gonadal extirpation. After a long interval, the first microscopic examination of spermatozoa was conducted by Ham in 1677 (Meyer, 1938). Prévost and Dumas (1824a,b) reported the presence of spermatozoa in the testes of all examined species. They then characterized the differences between spermatozoa taken from various parts of the reproductive tract. In 1841 von Kölliker discovered that spermatozoa developed from cells residing in the testis, and this discovery was followed by microscopic description of the interstitial cell (Leydig, 1850) and Sertoli's cells (Sertoli, 1865). The various germinal epithelium cells were morphologically classified by von LaVallete (1876); Regaud (1901) established the concept of the spermatogenic cycle.

The histology of the epididymis was described in the 19th century by Becker (1857). Hammar (1897) and Aiger (1900) reported the regional differences in the epididymal duct. The secretory activity of the epididymal epithelium (Hammer, 1897; Aiger, 1900) and the absorbing ability of the epididymis were reported by von Möllendorff (1920) and Young (1933). In 1924, von Lanz emphasized that the function of the epididymis extended beyond the transportation of spermatozoa from the testis to the vas deferens. Furthermore, the epididymis concentrates the dilute spermatozoan suspension, retains
spermatozoa for sufficient time to allow maturation, and stores matured spermatozoa until ejaculation. The spermatozoa collected from successive levels of the reproductive tract show an increasing capacity for motility and fertility (Redenz, 1926; Young, 1929a,b, 1931; Yochem, 1930). Finally, Young (1931) demonstrated that mammalian spermatozoa undergo maturation during epididymal transit by showing a two fold increase of fertility in the distal end compared to the proximal end of the epididymis.

2. Morphological Changes

a. The Cytoplasmic Droplet. The most obvious morphological change in spermatozoa along the passage through the reproductive tract is noticed in the presence and position of the cytoplasmic droplet. Redenz (1924) reported that the droplet moved down the middle piece and finally sloughed off as spermatozoa reached the cauda epididymis. The cytoplasmic droplet is usually found at the neck region or distal end of the middle piece. The relative proportions of these types of spermatozoa vary with the level of the reproductive tract from which they are removed (Lagerlöf, 1934; Selivanova, 1937; Branton and Salisbury, 1947; Hancock, 1957a; Bedford, 1963a). The internal structure of the distal droplet is considerably different from that of the proximal droplet (Bloom and Nicander, 1961). The tubular and vesicular components of the droplet bounded by a distinct membrane become smaller as the droplet reaches the distal end of the middle piece (Rao and Hart, 1948; Bloom and Nicander, 1961;
Bedford, 1965b). While Selivanova (1937) and Rao and Hart (1948) suggested that the droplet slid off at the middle piece, Kojima and Ishikawa (1963) reported the resorption of the droplet by spermatozoa.

The secretion of the cauda epididymis, prostate gland (Selivanova, 1937), seminal vesicle (Selivanova, 1937; Bialy and Smith, 1958), and physiological saline solution (Rao and Hart, 1948) induce droplet regression.

Although several investigators (Rao and Hart, 1948; Mann, 1964) considered that the droplet was a nutritive substance required for spermatozoan maturation, the role of the cytoplasmic droplet still remains obscure. Orgebin-Crist (1967a) maintained that the absence of the droplet could not give an accurate index of spermatozoan maturation in terms of fertilizing ability. Bedford (1967b) also suggested that the movement and loss of the droplet could not be of importance in the development of motility and maturity of spermatozoa. Nevertheless, it is generally accepted that the presence and location of the cytoplasmic droplet are indications of the relative maturity of spermatozoa (Bedford, 1963a).

b. The Acrosome. Acrosomal changes in maturing bovine spermatozoa were first observed by Mukherjee and Bhattacharya (1949) who reported that the dimension of the head of spermatozoa from the caput epididymis was significantly greater than that of ejaculated spermatozoa.

Subsequent light and electron microscopic studies have shown structural modifications, including a reduction in the
acrosomal dimensions of spermatozoa in several species: the guinea pig (Fawcett and Hollenberg, 1963; Fawcett and Phillips, 1969a), chinchilla (Fawcett and Phillips, 1969a), rabbit (Bedford, 1963a, 1965b; Gaddum and Glover, 1965; Fulka and Koefoed-Johnsen, 1966; Paüfler and Foote, 1968; Bedford and Nicander, 1971), monkey (Bedford and Nicander, 1971), bull (Dickey, 1965), boar (Jones, 1971), stallion, ram, dog, hare and white-tailed rat (Bedford, 1975). However, no significant modification was observed in the acrosomal structure of spermatozoa as they pass through the epididymal duct in the human (Bedford et al., 1973), rat and mouse (Bedford, 1975). Because a marked reduction in the acrosomal size measured by a light microscope occurred only after the cytoplasmic droplet left the base of the head, Bedford (1963a) related these changes to the development of fertilizing ability. However, an ultrastructural discovery of the presence of the droplet on the neck region in matured spermatozoa (Bedford, 1965b) implies that there is perhaps no physiological relationship between the acrosome maturation and the state of the droplet. On the other hand, Fawcett and Hollenberg (1963) suspected either the increase in fertilizing capacity of spermatozoa was the result of matura­tion of the acrosome or a consequence of accompanying changes of the head shape which might affect the swimming pattern of spermatozoa. However, the functional meaning of these acrosomal differences is still unknown.

c. The Cell Membrane. The cell membrane around the
acrosome of caput spermatozoa becomes swollen as spermatozoa move down the epididymis and are ejaculated. Bedford (1965b) related this change to the osmotic status of the sperm head during maturation. Fawcett and Phillips (1969a) also observed the same change in the chinchilla and guinea pig. They felt that the increasing separation of the membrane might be caused by a progressive decrease in the volume of the underlying structure, or an increase in membrane amount, or an alternation in membrane permeability. However, Zamboni and Stefanini (1968) and Zamboni et al. (1968) did not find any difference in cell membrane configuration of caput and cauda spermatozoa of the rabbit. Also Jones's (1971) findings on the ultrastructure of the head membrane of the boar spermatozoa suggested that the changes in the membrane structure were not due to the sources of spermatozoa (caput or cauda epididymis) but due to the fixatives used for electron microscopy.

Modification of the staining properties (Glover, 1962a), electrophoretic properties (Bedford, 1963b) and surface-adhesive properties (Bedford, 1965a; Martan and Hruban, 1970) of the cell membrane as spermatozoa move through the epididymis may account for the surface change in the membrane. Since fertilization is followed by (i) capacitation of spermatozoa, (ii) penetration of the egg membrane, and (iii) fusion of the cell membrane of the egg and spermatozoon (Saunders, 1970), it is possible that changes in membrane property of spermatozoa is normal for their maturation.
d. The Middle Piece. This becomes thicker and straighter as the cytoplasmic droplet moves distally (Rao and Hart, 1948). Ånberg (1957) observed some changes in the arrangement and structural organization of mitochondria during epididymal transit in human spermatozoa.

e. Abnormal Spermatozoa. Amann and Almquist (1962b) observed the decrease of abnormal heads but the increase of bent or broken tails as spermatozoa passed through the epididymis. Paüfler and Foote (1968) and ElWishy (1975) reported that the number of abnormal spermatozoa decreased during epididymal transit. However, Lagerlöf (1934), Branton and Salisbury (1947) and Igboeli and Foote (1968) did not find such a difference between spermatozoa from the caput and cauda epididymis. In addition, Branton and Salisbury (1947) and Bedford et al. (1973) concluded that the original source of morphologically abnormal spermatozoa was the testis.

f. Specific Gravity. Specific gravity of spermatogenic cells was increased during the process of ripening (Lindahl and Kihlström, 1952; Lindahl and Thunqvist, 1965; Lavon et al., 1966). The suggested reason for this change was the loss of the residual cytoplasmic droplet, provided that the latter has a comparatively low density (Lindahl and Kihlström, 1952). However, Lindahl and Thunqvist (1965) later reported an increase of the specific gravity of the head was not caused by the loss of the droplet. An increase in the per cent of dry matter was also reported by Lavon et al. (1970).
g. Reflecting Capacity to Light. As the water content in the spermatozoan head decreases with ripening, the reflecting capacity to light increases (Lindahl et al., 1952).

3. Chemical Changes

a. Chemical Composition. Although the structural appearance of the normal spermatozoan nucleus remains constant from the time of spermiation until fertilization, significant changes in the deoxyribonucleoprotein (DNP) complex take place during epididymal passage. As the nucleus of the spermatid condenses, the composition of the basic nuclear protein changes qualitatively (Gledhill et al., 1966). Subsequently there is a continuous reduction in Feulgen positive material (DNA) (Gledhill, 1966, 1971; Gledhill et al., 1966; Setchell et al., 1969; Orgebin-Crist, 1969). Because there is no quantitative change in total deoxyribonucleic acid (DNA) content determined by the light absorption (Lavon et al., 1971), this has been interpreted as a qualitative alteration in the composition and binding of the DNP complex to DNA during epididymal maturation (Gledhill, 1966). Furthermore, a progressive decrease of binding sites for actinomycin D (Brachet and Hulin, 1969; Gledhill, 1971) and a considerable increment of disulfide bonds in the nuclear chromatin of eutherian mammals (Calvin and Bedford, 1971; Calvin et al., 1973; Marushige and Marushige, 1975) suggest condensation and stabilization of the nuclear chromatin during the passage of spermatozoa through the epididymis (Meistrich et al., 1976). It has been observed that
disulfide cross links are also formed in the tail structure and perinuclear material during epididymal transit (Bedford, 1975). The total content of lipid and protein of spermatozoa was estimated to decrease during maturation (Lavon et al., 1970, 1971; Terner et al., 1975). While phospholipid and choline plasmalogen decreased during epididymal transit in the boar and ram (Quinn and White, 1967a; Scott et al., 1967; Lavon et al., 1970), choline plasmalogen was accumulated by rat and rabbit spermatozoa (Dawson and Scott, 1964). A decrease of sodium and chloride contents in spermatozoa during passage through the reproductive tract has also been reported (Quinn, 1967).

b. Enzyme Distribution. Variations in enzyme activity in spermatozoa during passage through the reproductive tract have been reported by several investigators. Acid phosphatase activity of spermatozoa decreases during transit from the caput to cauda epididymis in the rat (Terner et al., 1975) and mouse (Poirier, 1975). However, there are conflicting reports on changes in alkaline phosphatase activity in the cytoplasmic droplet of rabbit spermatozoa (Bavdek and Glover, 1970; Moniem and Glover, 1972). Indophenol oxidase activity decreases while spermatozoa pass through the epididymis (Ostwald, 1907). On the other hand, phosphatases activities in ejaculated spermatozoa could not be elicited, while they were detected in cauda spermatozoa (Gordon, 1973).

c. Metabolism. The metabolic aspects of epididymal and ejaculated spermatozoa have been mentioned already in Chapter II
(pp. 53-55). In this section, therefore, the emphasis is on the metabolism of testicular spermatozoa.

Wu et al. (1959) suggested that bovine testicular spermatozoa might metabolize glucose via the pentose shunt pathway, whereas epididymal spermatozoa utilized glucose via the Embden-Meyerhof scheme of glycolysis. Nevertheless, Voglmayr et al. (1967, 1970) denied the occurrence of the pentosose cycle in either testicular or ejaculated spermatozoa.

Ram testicular spermatozoa, but not ejaculated spermatozoa, convert appreciable amounts of glucose to amino acids (Murdoch and White, 1968; Voglmayr et al., 1966, 1967). Moreover, inositol synthesis was demonstrated only in testicular spermatozoa (Voglmayr and White, 1971).

The rate of incorporation of exogenous carbon into phospholipids by testicular spermatozoa was 3 to 4 times greater than ejaculated spermatozoa (Scott et al., 1967). On the other hand, testicular spermatozoa incorporated much less $^{14}$C from glucose into volatile fatty acids and differed from ejaculated spermatozoa in that the incorporated $^{14}$C appeared largely as formate rather than as the acetate formed by ejaculated spermatozoa (Scott et al., 1967). Ram testicular spermatozoa failed to hydrolyze choline or ethanolamine phosphoglycerides, but both were metabolized by ejaculated spermatozoa (Scott and Dawson, 1968).

4. **Physiological Changes**

a. **Fertility.** The most important functional change of spermatozoa that takes place in the epididymis is the
acquisition of fertilizing ability during epididymal transit. It was shown in several species: the guinea pig (Young, 1931), rat (Blandau and Rumery, 1961), mouse (Hoppe, 1975), rabbit (Nishikawa and Waida, 1952; Bedford, 1966a,b, 1967b; Fulka and Koefoed-Johnsen, 1966; Orgebin-Crist, 1967a,b; Paüfler and Foote, 1968; Cummins and Orgebin-Crist, 1971), hamster (Horan and Bedford, 1972), bull (Amann and Griel, 1974) and boar (Holtz and Smidt, 1976; Hunter et al., 1976). The incidence of polyploid ova (Bedford, 1966a; Orgebin-Crist, 1967a), the striking delay in the cleavage rate (Orgebin-Crist, 1968; Cummins and Orgebin-Crist, 1971), and embryonic mortality (Holtz and Smidt, 1976) were observed after insemination with epididymal spermatozoa. On the contrary, Bedford (1975) reported that, once implantation had occurred, there was no statistical difference in mortality between embryos conceived with ejaculated or with epididymal spermatozoa.

The controversy, whether maturation changes are brought about by the intrinsic quality in the spermatozoon alone (van der Stricht, 1893; Young, 1931; Glover, 1962b; Gaddum and Glover, 1965) or by the epididymal influence (Tournade, 1913; Redenz, 1924; von Lanz, 1924; Fulka and Koefoed-Johnsen, 1966; Bedford, 1975; Orgebin-Crist et al., 1975) has been debated for many years. However, observations on the dynamic aspects of the epididymal epithelium (Hammar, 1897; Nicander, 1957a,b; Basrur and Ramos, 1973; Hamilton, 1975), on androgenic support of the epithelium (Marshall and Jolly, 1905; Young, 1929a; Dyson, 1973; Orgebin-Crist et al.,
1975), on different chemical compositions of fluids obtained from the testis and epididymis (White, 1973b), on the formation of disulfide cross-linking in the nucleus (Calvin and Bedford, 1971) and on spermatozoan membrane changes during the passage of spermatozoa through the epididymis (Bedford, 1967b; Cooper and Bedford, 1971), indicate that the epididymis is an essential site of spermatozoan maturation. Nevertheless, the concluding remark of Paüfler and Foote (1968), that both intrinsic and extrinsic factors were required for rabbit spermatozoa to become fully fertile in the epididymis, appears to hold good for several species of animals.

The maturation of mammalian spermatozoa takes place in different parts of the epididymis and probably require quite different environmental conditions (Glover and Nicander, 1971). Fertile spermatozoa first appear in the lower half of the corpus epididymis of the rabbit (Bedford, 1966a,b, 1967b; Fulka and Koefoed-Johnsen, 1966; Orgebin-Crist, 1967a; Cummins and Orgebin-Crist, 1971) or in the cauda epididymis of the mouse (Hoppe, 1975), rat (Dyson, 1973), hamster (Horan and Bedford, 1972), and pig (Holtz and Smidt, 1976; Hunter et al., 1976). Species differences in the anatomy and physiology of the epididymis (Glover and Nicander, 1971) might account for diverse sites of maturation changes.

b. Motility. Among several maturation changes, motility might have the most direct functional connection with fertility (Mann, 1964). Although the correlation between the per cent of progressively motile spermatozoa and fertility
was estimated to be very low (Kelly and Hurst, 1963),
Linford et al. (1976) recently reported that the nonreturn
values were correlated with motility. Spermatozoa may lose
their fertilizing capacity prior to cessation of motility
(Young, 1929a; Asdell and Salisbury, 1941).

In several species, spermatozoan motility increases as
spermatozoa pass through the epididymis. This has been found
in the dog (Hammar, 1897; Tournade, 1913), rat (Tournade,
1913; Young, 1929a; Yochem, 1930), guinea pig (Tournade, 1913;
Young, 1929a), rabbit (Tournade, 1913; Bedford, 1966a, 1967b;
Orgebin-Crist, 1967a), bull (Young, 1929a, Mukherjee and
Bhattacharya, 1949; Amann and Almquist, 1962a), ram (Young,
1929a; Mukherjee and Bhattacharya, 1949), and human (Bedford,
1973).

Recently, the changing pattern of spermatozoan motility
in the epididymis has been much emphasized. Spermatozoa
released from the testis and caput epididymis show weak
vibratory or circular movement with no forward progress.
Those released from the corpus epididymis develop forward
progression, and most spermatozoa released from the cauda
epididymis show progressive forward movement with longitudinal
rotation (Tournade, 1913; Young, 1929a; Nishikawa and Waida,
1952; Blandau and Rumery, 1961; Fulka and Koefoed-Johnsen,
1966; Gaddum, 1968; Igboeli and Foote, 1968; Bedford, 1973;
Bedford et al., 1973; Amann and Griel, 1974). The per cent
of progressive movement of ejaculated mink spermatozoa is
40-80% (Onstad, 1967). In accordance with maturation, the
neck region and various tail organelles seem to become less
flexible and are stabilized by the establishment of disulphide bonds, and thus mature spermatozoa develop a more rigid type of movement (Bedford et al., 1973; Bedford, 1975).

c. Eosinophilic Property. Lasley et al. (1942) introduced a vital stain using eosin as a quality test for ejaculated spermatozoa. Because of increased membrane permeability, dead spermatozoa stain readily, whereas active or potentially active spermatozoa do not stain (Lasley et al., 1942; Amann and Almquist, 1962b).

Brochart and Debaténe (1953) reported a fourfold increase in unstained spermatozoa from the caput to cauda epididymis of ruminants. However, other workers found no such difference between spermatozoa collected from the caput and cauda and marked a decrease in unstained spermatozoa from the corpus (Branton and Salisbury, 1947; Amann and Almquist, 1962b; Igboeli and Foote, 1968). Therefore, Amann and Almquist (1962b) suspected that these conflicting results might have been due to different staining techniques or sampling differences.

d. Temperature Shock. Spermatozoa from the cauda epididymis are more resistant to high temperature than those from the testis or ductus deferens (Yochem, 1930; Young, 1931). On the contrary, resistance of spermatozoa to cold decreases from the testis to cauda epididymis and ductus deferens (Yochem, 1930; Lasley et al., 1942; Bialy and Smith, 1959; White and Wales, 1961; Voglmayer et al., 1967; Setchell et al., 1969).
e. Resistance to Alkalinity and Acidity. The resistance of spermatozoa to alkalinity, acidity (Yochem, 1930) and harmful alkaloids (Metalnikov, 1911) increases from the testis to cauda epididymis, then decreases in the ductus deferens.

f. Net Negative Surface Charge. The net negative surface charge of spermatozoa increases during maturation in the epididymis of the rabbit (Bedford, 1963b; Cooper and Bedford, 1971), monkey (Cooper and Bedford, 1971), and human (Cooper and Bedford, 1971; Bedford, 1973; Bedford et al., 1973). This appears to reflect a modification in the distribution of charges or the nature of ionized groups over the cell membrane during epididymal passage. This may lead to head-to-head autoagglutination. However, the role of the negative charge in spermatozoan maturation, capacitation and fertilization is obscure.

g. Agglutinating Property. Individual and randomly moving testicular spermatozoa aggregate into large rouleaux-like head-to-head bundles during epididymal transit in the guinea pig (Fawcett and Hollenberg, 1963) and rabbit (Nicander, 1957a). This head-to-head agglutination was also reported in ejaculated mink spermatozoa (Onstat, 1967).

This adhesiveness is increased as changes in net charges and in antigen distribution in specific regions of the spermatozoan surface. It is enhanced in the presence of divalent cations or small amounts of normal serum (Bedford, 1975). Furthermore, Lindahl (1968) reported that ATP increased head-to-head agglutination, while ADP lowered the
frequency. However, anthropoid monkey and human spermatozoa failed to show head-to-head autoagglutination in normal serum (Bedford et al., 1973). The autoagglutination of human spermatozoa seems to be related to sterility (Wilson, 1956).

h. Contact Ability. Spermatozoa collected from the proximal region of the rabbit epididymis fail to fertilize not only because of lack of motility, but also because they are unable to establish contact with the ovum surface (Bedford, 1966a). Perhaps during passage through the epididymis, the cell membrane of spermatozoa develops their ability to initiate and maintain contact with the ovum. Although this phenomenon was also confirmed in the bull (Amann and Griel, 1974) and Syrian hamster (Horan and Bedford, 1972), the nature of this contact remains unknown.
C. MATERIALS AND METHODS

1. The Preparation of Material from the Reproductive Tract

The reproductive tracts used for this part of the investigation were the same as for the ultrastructure study. Small pieces of tissue were sliced from the testis and three parts of the epididymis (caput, corpus and cauda) of five minks (Fig. 38). The spermatozoa were obtained by immersing these tissues either in 1 ml of Krebs-Henseleit-Ringer solution for electron microscopic study or in 1 ml of 0.9% sodium chloride solution for enzyme localization.

2. The Preparation of Ejaculated Semen and Viability Test

For the ejaculated spermatozoan study, semen was collected from vaginae after mating by a 1 ml syringe fitted with a plastic tube (Ahmad et al., 1975a). Since the mink ejaculates such a small amount of semen, the semen was collected individually from 8-12 different minks and pooled as a test sample. Five test samples were used in this study.

The percentage of motile spermatozoa were estimated by a light microscope at room temperature. For viability test, a drop of semen was mixed with the same amount of stain (3% Eosin B in 2.9% sodium citrate solution, Ahmad - unpublished) on a slide and dried immediately. Using a light microscope, a total count of 300 cells for each sample was classified as stained and unstained spermatozoa.

A portion of the ejaculated semen was washed twice with Krebs-Henseleit-Ringer solution for electron microscopic study.
Fig. 38. Schematic drawing of a mink reproductive tract to show the position of the various parts (shaded regions)
or with sperm-Ringer solution (Mann, 1946) for enzyme localization.

3. The Procedure for Electron Microscopy

The suspensions of spermatozoa obtained from described locations of the reproductive tract and washed ejaculated spermatozoa were prepared for electron microscopy (p. 20) and cytochemical study (pp. 56-57).

Approximately 50 electron micrographs of sagittal sections of spermatozoan heads for each sample (5 locations, i.e. testis, caput, corpus, cauda epididymis and ejaculated semen X 5 replicates) were randomly selected for the measurement of several parts of the head and for the estimation of other fine morphological changes. For each section, the length of total head, anterior acrosome (the sum total of the apical and main segments of the acrosome), equatorial segment, postacrosomal sheath, apical segment, nucleus, and acrosomal thickness were measured as outlined in Fig. 39. The coherence of the cell membrane, the occurrence of swellings on the head and the cytoplasmic droplet on the middle piece were also calculated. The significance of the difference in motility, staining property, the means of the spermatozoan head parameters measured and other morphological changes were determined statistically by the analysis of variance and Newman-Keul's test (Armitage, 1971).
Fig. 39. The positions of the head measured
D. RESULTS

1. The Viability of Spermatozoa

The per cent of motile spermatozoa and unstained spermatozoa removed from the testis and epididymis and from the ejaculated semen are shown in Table 2. The mean values of ejaculated spermatozoa in this study were excluded from statistical comparisons because ejaculated spermatozoa were collected from animals not used for the reproductive tract study. The number of motile spermatozoa was significantly increased \((p < 0.05)\) from the testis to each part of the epididymis as spermatozoa passed through the reproductive tract. No activity was detected in spermatozoa collected from the testis. On the other hand, the motility of spermatozoa obtained from the cauda epididymis was higher than that of ejaculated spermatozoa.

Testicular and cauda spermatozoa showed a significantly higher \((p < 0.05)\) rate of unstained (viable) spermatozoa than spermatozoa from the caput epididymis. Although there was no significant difference between spermatozoa from the caput and corpus epididymis, the mean values of unstained spermatozoa increased during passage through the epididymis. Similar to the motility, the per cent of unstained spermatozoa in ejaculated sample was low as compared to that of the cauda epididymis.

2. Changes in the Spermatozoan Head Length

The mean values of several portions of spermatozoan head
Table 2. The per cent of motile spermatozoa and unstained spermatozoa collected from successive parts of the reproductive tract and ejaculated semen

<table>
<thead>
<tr>
<th>Part</th>
<th>Testis</th>
<th>Epididymis</th>
<th>Ejaculated</th>
<th>Semen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Caput</td>
<td>Corpus</td>
<td>Cauda</td>
</tr>
<tr>
<td>Motile spermatozoa % (Mean ± SE)</td>
<td>a*</td>
<td>b</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>7.2 ± 1.0</td>
<td>20.0 ± 2.4</td>
<td>84.0 ± 3.6</td>
</tr>
<tr>
<td>Unstained spermatozoa % (Mean ± SE)</td>
<td>a,b</td>
<td>c</td>
<td>b,c</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>52.0 ± 3.0</td>
<td>31.2 ± 2.7</td>
<td>39.8 ± 2.9</td>
<td>56.8 ± 5.5</td>
</tr>
</tbody>
</table>

*Means on the same line with the same superscript letter are not different but with different letters are significantly different, p < 0.05.
lengths from four parts of the reproductive tract and ejaculated semen are shown in Table 3. There was no significant difference in the means of total head length between spermatozoa collected from different parts of the reproductive tract. However, the total head length of ejaculated spermatozoa was smaller than that of spermatozoa collected from the reproductive tract.

The length of the anterior acrosome of testicular spermatozoa was significantly greater \( (p < 0.001) \) than those of spermatozoa from the various part of the epididymis. In the analysis of variance, however, there were significant differences between animals, and in the interaction of animals and different parts of the reproductive tract. The anterior acrosomal lengths of spermatozoa from the reproductive tract were greater than that of ejaculated spermatozoa.

Although there was no significant difference in equatorial length between testicular and corpus spermatozoa, the mean values of equatorial length in spermatozoa from each part of the epididymis appeared to be greater than that in spermatozoa from the testis. The equatorial length of ejaculated spermatozoa was smaller than those of spermatozoa from the reproductive tract.

The length of postacrosomal region of epididymal spermatozoa were significantly greater \( (p < 0.05) \) than that of testicular spermatozoa. Even though there was no statistical difference, the mean values of postacrosomal length of spermatozoa appeared to increase during passage through each part of the epididymis. The value of ejaculated
Table 3. Dimensional changes at different portions of the spermatozoan head

<table>
<thead>
<tr>
<th>Part</th>
<th>Testis</th>
<th>Epididymis</th>
<th>Ejaculated Semen</th>
<th>Significance Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Caput</td>
<td>Corpus</td>
<td>Cauda</td>
</tr>
<tr>
<td>Total head length, µm (Mean ± SE)</td>
<td>a*</td>
<td>5.80 ± 0.06</td>
<td>5.71 ± 0.05</td>
<td>5.83 ± 0.05</td>
</tr>
<tr>
<td>Anterior acrosomal length, µm (Mean ± SE)</td>
<td>a</td>
<td>2.34 ± 0.05</td>
<td>2.25 ± 0.04</td>
<td>2.29 ± 0.04</td>
</tr>
<tr>
<td>Equatorial length, µm (Mean ± SE)</td>
<td>a</td>
<td>1.56 ± 0.03</td>
<td>1.53 ± 0.02</td>
<td>1.55 ± 0.02</td>
</tr>
<tr>
<td>Postacrosomal length, µm (Mean ± SE)</td>
<td>a</td>
<td>1.89 ± 0.03</td>
<td>1.93 ± 0.02</td>
<td>1.99 ± 0.02</td>
</tr>
<tr>
<td>Apical length, µm (Mean ± SE)</td>
<td>a</td>
<td>0.88 ± 0.03</td>
<td>0.67 ± 0.01</td>
<td>0.68 ± 0.01</td>
</tr>
<tr>
<td>Nuclear length, µm (Mean ± SE)</td>
<td>a</td>
<td>4.89 ± 0.06</td>
<td>5.04 ± 0.04</td>
<td>5.15 ± 0.04</td>
</tr>
<tr>
<td>Acrosomal thickness, µm (Mean ± SE)</td>
<td>a</td>
<td>0.52 ± 0.01</td>
<td>0.50 ± 0.01</td>
<td>0.52 ± 0.01</td>
</tr>
</tbody>
</table>

*Means on the same line with the same superscript letter are not different but with different letters are significantly different as indicated.
spermatozoa was smaller than those of epididymal spermatozoa.

Because the total head length of spermatozoa was divided in this study into the anterior acrosomal, equatorial, and postacrosomal length, a significant decrease of the anterior acrosomal length compensated for a significant increase of the postacrosomal length during the transit of spermatozoa from the testis to the epididymis. Thus, the total lengths of spermatozoa remained, statistically, of similar values.

The mean values of the apical length of spermatozoa of the testis and caput epididymis were significantly larger (p < 0.001) than those of the corpus and cauda epididymis. The apical length of ejaculated spermatozoa was similar to that of cauda spermatozoa. On the other hand, the nuclear length of spermatozoa did not change significantly during passage through the reproductive tract. The nuclear length of ejaculated spermatozoa was less than those of spermatozoa from the reproductive tract.

The acrosomal thickness of spermatozoa from the testis was significantly greater (p < 0.001) than those of spermatozoa from the epididymis. Ejaculated spermatozoa had greater acrosomal thickness than epididymidal spermatozoa.

In brief, the major changes in the dimensions of spermatozoan head during their transit through the reproductive tract were the decrease of the anterior acrosomal length and acrosomal thickness and the increase of the postacrosomal length without alteration of the total head length.
3. Changes in the Morphology of the Spermatozoan Head

Because only very fine morphological changes take place in spermatozoa during their passage through the reproductive tract, spermatozoa collected from testis, epididymis and semen, did not differ in general microscopic appearance.

a. The acrosome. The acrosomal dimension decreased successively as spermatozoa passed through various parts of the epididymis and were eventually ejaculated (Table 3). A similar sequence of qualitative change in other aspects of acrosome was noted (Figs. 40–44). In this case the acrosomal matrix became more electron dense (Figs. 40–44). The number and size of nuclear vacuoles significantly decreased in accordance with the above sequence of the spermatozoan transit, i.e. from the testis (Figs. 49, 52) to the cauda epididymis and ejaculated semen (Figs. 43, 44).

In sagittal sections, the oval form of the acrosome in spermatozoa from the testis and caput epididymis (Figs. 40, 41) changed to a slender form in corpus, cauda, and ejaculated spermatozoa (Figs. 42–44). Several bizarre forms of acrosomes such as a pyriform shape (Fig. 50) and an S-shape (Fig. 52) were observed in testicular spermatozoa and spermatozoa from the caput epididymis (Figs. 51, 53). There was more space between the nucleus and the acrosomal region in testicular spermatozoa (Figs. 40, 49, 54) than in corpus (Fig. 42), cauda (Fig. 43) and ejaculated spermatozoa (Fig. 44).
b. The cell membrane. The extent of separation of acrosomal membrane was usually more pronounced in ejaculated spermatozoa (Fig. 44) than in testicular or caput spermatozoa (Figs. 41, 49, 50, 54, and Table 4).

The ratio of the apposed cell membrane of testicular spermatozoa was significantly greater ($p < 0.01$) than those of epididymal spermatozoa. Although there was no significant difference between parts of the epididymis, the mean values of the apposed membrane ratio of spermatozoa in each part of the epididymis were decreased as spermatozoa moved through the epididymis. Ejaculated spermatozoa had less apposed membrane than cauda spermatozoa. There was a significant increase ($p < 0.05$) of separated membrane in spermatozoa collected from the corpus and cauda epididymis compared to that from the testis. Ejaculated spermatozoa had still more separated membrane than cauda spermatozoa.

There was no significant difference in the incidence of damaged membrane during the passage of spermatozoa through the reproductive tract. Ejaculated spermatozoa had more damaged membrane than those from the cauda epididymis. Cell membrane may remain to be connected with the edge of the acrosome and postacrosomal sheath even though the membrane along other parts of spermatozoa was separated or damaged (Figs. 41, 43, 44, 55).

c. Spermatism. It is well known that the process of spermatocytogenesis and spermiogenesis occur in the testis. As shown in the electron micrographs, the microstructure of spermatids was clearly demonstrated (Figs. 45-48). Occasionally, immature sperm cells were observed in the caput epididymis.
Table 4. Changes in the cell membrane configuration and in the occurrence of swellings and the cytoplasmic droplet

<table>
<thead>
<tr>
<th>Part</th>
<th>Testis</th>
<th>Epididymis</th>
<th>Ejaculated Semen</th>
<th>Significance Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Caput</td>
<td>Corpus</td>
<td>Cauda</td>
</tr>
<tr>
<td>Cell membrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>configuration:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apposed acrosomal membrane %</td>
<td>98.0 ± 1.3</td>
<td>56.8 ± 19.3</td>
<td>33.8 ± 9.1</td>
<td>26.4 ± 5.5</td>
</tr>
<tr>
<td>% (Mean ± SE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Separated acrosomal membrane %</td>
<td>1.0 ± 0.6</td>
<td>19.6 ± 11.3</td>
<td>41.7 ± 11.5</td>
<td>48.8 ± 7.5</td>
</tr>
<tr>
<td>% (Mean ± SE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Damaged acrosomal membrane %</td>
<td>1.0 ± 0.6</td>
<td>23.6 ± 18.3</td>
<td>24.5 ± 7.2</td>
<td>24.8 ± 9.4</td>
</tr>
<tr>
<td>% (Mean ± SE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence of swellings:</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Acrosomal swellings %</td>
<td>44.7 ± 21.2</td>
<td>24.5 ± 13.3</td>
<td>86.1 ± 5.5</td>
<td>87.6 ± 4.6</td>
</tr>
<tr>
<td>% (Mean ± SE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postacrosomal swellings %</td>
<td>18.8 ± 15.9</td>
<td>30.9 ± 16.4</td>
<td>92.5 ± 3.9</td>
<td>90.0 ± 4.7</td>
</tr>
<tr>
<td>% (Mean ± SE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence of cytoplasmic droplets</td>
<td>62.2 ± 5.8</td>
<td>21.6 ± 10.3</td>
<td>0.8 ± 0.8</td>
<td>0.8 ± 0.8</td>
</tr>
<tr>
<td>% (Mean ± SE)</td>
<td></td>
<td></td>
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</tbody>
</table>

*Means on the same line with the same superscript letter are not different but with different letters are significantly different as indicated.
(Figs. 51, 53). However, spermiogenesis has not been observed in spermatozoa from regions posterior to the caput epididymis.

d. The swelling. Testicular spermatozoan heads had more acrosomal swellings than those of caput spermatozoa (Table 4). However, during the transit through the epididymis, the incidence of acrosomal swellings was significantly increased \((p < 0.05)\) from caput spermatozoa to corpus and cauda spermatozoa. On the other hand, the occurrence of postacrosomal swellings was found significantly increased \((p < 0.01)\) from testicular and caput to corpus and cauda spermatozoa. Almost all ejaculated spermatozoa had both types of swellings.

There was no definite trend in the occurrence and length of perforatorium in the spermatozoan head as spermatozoa passed through the reproductive tract and subsequent ejaculation. Through all parts of the reproductive tract, the perforatorium remained intact even in cases where the entire acrosomal content was separated or damaged (Figs. 14, 57).

4. Changes in the Morphology of the Spermatozoan Tail

Owing to the difficulty of getting longitudinal sections of the spermatozoan tail, detailed morphological changes of the tail could not be observed. The only obvious change was the movement of the cytoplasmic droplet from the neck portion to the distal end of the middle piece (Fig. 23, 58 and Table 4). There was a significant difference \((p < 0.001)\) between spermatozoa collected from the testis and from the epididymis. The cytoplasmic droplet was found virtually to disappear by the time of ejaculation.
The scroll was observed in cauda and ejaculated spermatozoa (Figs. 7, 8). The form and number of satellite fibrils were almost similar between ejaculated spermatozoa and spermatozoa from the reproductive tract (Figs. 14, 56).

5. Changes in the Enzyme Activity

The pattern of enzyme distribution in mink spermatozoa from the reproductive tract was, as described in Chapter II, similar to that in spermatozoa from the cauda epididymis. However, the intensity of the enzyme activity in testicular spermatozoa was weaker than that in epididymal spermatozoa with the exception of DOPA oxidase. Some enzyme activities (acid and alkaline phosphatases, G-6-Pase, ATPase, MDH, SDH, LDH, NADH diaphorase) appeared to be increased during the spermatozoan passage through the epididymis, while the other enzyme activities (5-nucleotidase, ADPase, esterase, DOPA oxidase, IDH) remained unchanged. Although some enzymes (5-nucleotidase, DOPA oxidase, LDH, IDH, NADH diaphorase) showed similar activity between spermatozoa from the reproductive tract and those in ejaculated semen, other enzymes activities (G-6-Pase, esterase, SDH and particularly acid and alkaline phosphatases, ADPase, ATPase, MDH) decreased following ejaculation.
Table 5. Changes in enzyme activities in spermatozoa from testis, epididymis and ejaculated semen

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Testis</th>
<th>Epididymis</th>
<th>Ejaculated Semen</th>
<th>Location</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Caput</td>
<td>Corpus</td>
<td>Cauda</td>
</tr>
<tr>
<td>Phosphatases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+*</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>±±</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>5-nucleotidase</td>
<td>±±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G-6-Pase</td>
<td>±±</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>ADPase</td>
<td>±±</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ATPase</td>
<td>±±</td>
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<td>++</td>
<td>+</td>
</tr>
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<td>Non-specific Esterase</td>
<td>±±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DOPA oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dehydrogenases</td>
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<tr>
<td>Malate</td>
<td>±±</td>
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<td>++</td>
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<td>Succinate</td>
<td>+±</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Lactate</td>
<td>+±</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>±</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6-phosphogluconic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NADH diaphorase</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

*Key to abbreviations: ± weak reaction; + moderate reaction; ++ strong reaction; +++ very strong reaction; - negative reaction.
Figures 40 to 44. Acrosomal changes of spermatozoan heads during passage through the reproductive tract and subsequent ejaculation (sagittal sections).

Figure 40. The testicular spermatozoan head showing large oval acrosome (AC) composed of granular electron less dense matrix. The perforatorium (P) is prominent. There is comparatively wide space between the acrosome and nucleus (N) which has several vacuoles (arrows). The acrosomal (AS) and postacrosomal swellings (PS) are obvious. 30,000X.

Figure 41. The caput spermatozoan head showing erected oval-shaped acrosome. The nucleus and acrosome contain electron less dense material and the cell membrane (CM) is separated. 27,000X.

Figure 42. The corpus spermatozoan head showing rounded cone-shaped dense acrosome. The space between the nucleus and acrosome is narrow and swellings are prominent. 27,000X.

Figure 43. The cauda spermatozoan heads showing rounded cone-shaped dense acrosomes. The cell membrane shows different configurations between the cells. The nucleus becomes dense and has few vacuoles (arrows). The postacrosomal swellings have disintegrating membraneous material (MM). 27,000X.

Figure 44. The ejaculated spermatozoan head showing long rounded cone-shaped acrosome. Although the
cell membrane (CM) is separated from the peri-acrosomal surface, the apical end (AE) of the acrosome and postacrosomal sheath (PS) adhere to the membrane. The electron dense nucleus has small vacuoles. 25,000X.
Figures 45 to 49. Sagittal sections of the spermatids and spermatozoa in the testis.

Figure 45. The spermatid in cap phase showing football-shaped acrosomal granule (AG) and fine granular chromatin (GC). The chromatin has been condensed at the peripheral portions of the nucleus. 9,000X.

Figure 46. The elongated spermatid in acrosomal phase showing redistribution of the acrosomal substances, evenly distributed chromatin (GC) and formation of the manchette (MA). 9,000X.

Figure 47. The middle piece of a spermatid showing mitochondria (M) arranged around the axial fiber bundle (AF). 30,000X.

Figure 48. The spermatid in maturation phase showing inversed half-moon form acrosome. It has the nuclear ring (NR), manchette (MA) and the dense granules (DG) which have increased in size. 12,000X.

Figure 49. The spermatozoon showing elongated nucleus (N) composed of a condensed homogenous electron dense material which contains numerous vacuoles (V). The cell membrane (CM) appose to the acrosome and the nuclear ring (NR) is clearly visible. 30,000X.
Figures 50 to 54. Bizarre forms of the acrosome and the separation of the acrosome from the nucleus of the head in testicular and caput spermatozoa (sagittal sections).

Figure 50. Pyriform-shaped acrosome (AC) showing the different distribution of acrosomal content in a testicular spermatozoon. The cell membrane (CM) is mostly apposed to the acrosomal membrane. 45,000X.

Figure 51. Hook-shaped acrosome in a caput spermatozoon. The nuclear material has a granular appearance. 30,000X.

Figure 52. S-shaped acrosome in a testicular spermatozoon. The nucleus has numerous vacuoles (V). 18,000X.

Figure 53. Knob-shaped acrosome in a caput spermatozoon. 30,000X.

Figure 54. The testicular spermatozoon showing distinct space between the acrosome (AC) and the nucleus (N). However, the cell membrane (CM) is closely apposed to the acrosome. 25,000X.
Figures 55 to 58. The movement of the cytoplasmic droplet, the adhesiveness of the cell membrane at the tip of the acrosome, and the occurrence of the satellite fibrils.

Figure 55. Damaged and separated cell membrane (CM) except the tip of the acrosome (AE) is seen in a caput spermatozoon (sagittal section). 15,000X.

Figure 56. Cross section of the middle piece of a caput spermatozoon showing many satellite fibrils (SF, arrows). 80,000X.

Figure 57. Sagittal section of a caput spermatozoon showing intact perforatorium (P), acrosomal (AS) and postacrosomal swellings (PS) even though the acrosomal content has been dislodged. The cytoplasmic droplet (CD) remains on the neck region. 24,000X.

Figure 58. Axial section of the tail of a cauda spermatozoon showing the cytoplasmic droplet (CD) on the distal end of the middle piece (MP). On the lower right side, a cross section of the cytoplasmic droplet and the middle piece is shown. 12,000X.
Spermatozoa produced in the seminiferous tubules are transported from the testis via the epididymis and vas deferens before ejaculation. During their passage through the reproductive tract, the spermatozoa become mature and acquire the ability to fertilize eggs.

As seen from the electron micrographs in this study, immature spermatozoa were found in all parts of the epididymis. Bedford (1965b) reported that there was no absolute correlation of different stages of maturation with any particular segment of the epididymal duct in the rabbit. On examining the cauda epididymis of the rabbit, Orgebin-Crist (1967a) also found the protoplasmic droplet in the spermatozoan neck. On the contrary, Fawcett and Hollenberg (1963) indicated that the acrosomal changes were closely correlated with particular regions of the epididymal duct of the guinea pig. The occurrence of immature spermatozoa in all parts of the epididymis as observed in this study suggests that spermatozoan mixing takes place within the epididymis in the mink as reported for the rabbit and pig. Holtz and Smidt (1976) reported that considerable mixing of spermatozoa could occur within the epididymis of the pig.

1. The Viability of Spermatozoa

The motility of epididymal spermatozoa released from the epididymal duct was significantly increased from the caput,
via the corpus, to cauda epididymis. However, testicular spermatozoa were not motile and the motility of ejaculated spermatozoa was less than that of cauda spermatozoa. Slight motility was observed in testicular spermatozoa of the rabbit (Gaddum, 1968), ram and bull (Setchell et al., 1969). However, no motility, or only weak movement at best, was detected in the testicular spermatozoa of the ram (Voglmayr et al., 1966, 1967; Murdoch and White, 1968) and bull (Amann and Griel, 1974), and also in the caput spermatozoa of the bull (Igboeli and Foote, 1968; Amann and Almquist, 1962b), stallion, ass (ElWishy, 1975), and human (Bedford et al., 1973).

The absence of motility of testicular spermatozoa, low motility of caput spermatozoa, and increased motility of cauda spermatozoa as observed in this study are observations in agreement with reports on spermatozoa from the rabbit (Gaddum and Glover, 1965; Paüfler and Foote, 1968; Gaddum, 1968; Orgebin-Crist, 1967a), mouse (Hoppe, 1975), ass, stallion (ElWishy, 1975), bull (Amann and Almquist, 1962b; Amann and Griel, 1974), and human (Bedford et al., 1973).

The increase of spermatozoan motility during passage through the epididymis of the mink was more rapid than that reported for the stallion, ass (ElWishy, 1975) or rabbit (Paüfler and Foote, 1968). However, a rapid increase in spermatozoan motility was reported in the bull (Amann and Almquist, 1962b). The motility of cauda spermatozoa in the mink (84.0% in this study and 81% by Ahmad et al., 1975b) was higher than that of the bull (32.1%; Amann and Almquist, 1962b), stallion (10 - 40%; ElWishy, 1975), ass (10 - 50%; ElWishy, 1975)
and rabbit (61%; Pañfler and Foote, 1968). Although the estimation of motility is somewhat subjective, the value for mink spermatozoa is definitely higher than for other species. The striking increase of spermatozoan motility during passage through the reproductive tract of the mink implies that mink spermatozoa acquire high motility within a short time period, which might be related to the short breeding season of the mink.

The motility of ejaculated spermatozoa was lower compared to that of cauda spermatozoa. Perhaps the time between the ejaculation and motility estimation or the influence of the secretion of the male and/or female reproductive tract are responsible factors.

In spite of increasing spermatozoan motility during the spermatozoan transit through the reproductive tract, the mean percentage of unstained spermatozoa did not show any specific trend. This agrees with the results from studies in the rabbit (Glover, 1962a; Gaddum and Glover, 1965) and bull (Amann and Almquist, 1962b; Igboeli and Foote, 1968). A decrease of unstained spermatozoa in the caput and corpus epididymis compared to the testis where the motility of spermatozoa was absent, cannot be interpreted as an increase of dead spermatozoa (Lasley et al., 1942; Glover, 1962b). However, this change in the staining behavior of spermatozoa might be a result of a temporary increase in membrane permeability as suggested by Amann and Almquist (1962b).
2. Changes in the Spermatozoan Head Length

The total head lengths of the spermatozoa taken from different parts of the reproductive tract were not statistically different. However, the anterior acrosomal length and acrosomal thickness of testicular spermatozoa were significantly greater than those of spermatozoa from three parts of the epididymis. This decrease in the anterior acrosomal length and acrosomal thickness of mink spermatozoa as they pass along the reproductive tract is in agreement with observations in the cases of the guinea pig, chinchilla (Fawcett and Phillips, 1969a), rabbit, monkey (Bedford and Nicander, 1971), bull (Dickey, 1965), boar (Jones, 1971), stallion, ram, dog, hare, and white tailed rat (Bedford, 1975). However, no modification of the acrosome was reported in human (Bedford, 1973), rats and mice (Bedford, 1975).

The acrosome is a secreted product of the Golgi apparatus and varies greatly in size and shape as spermatozoa pass through the reproductive tract. Whether there is an actual reduction in volume, or just a redistribution of acrosomal contents has not been determined (Jones, 1971).

The decrease in the number and size of the nuclear vacuoles and in the space between the nucleus and the acrosome, coupled with the increased electron density of the acrosome revealed by the micrographs of spermatozoa passing down the epididymis may indicate an increase in the specific gravity of spermatozoa due to the dehydration of spermatozoa as suggested by Lindahl and Kihlström (1952).
While the equatorial length did not show any definite change, the postacrosomal length of spermatozoa increased during the spermatozoan passage through the reproductive tract. Since the postacrosomal sheath is the first site of contact with the ovum, this increased postacrosomal length might enhance fertilization.

Similar to the total head length, the nuclear length of spermatozoa did not change significantly during passage through the reproductive tract. However, the apical length significantly decreased during the passage of spermatozoa from the testis and caput epididymis to the corpus and cauda epididymis, in accordance with the decrease of the anterior acrosomal length since the former is a component of the latter.

The head of ejaculated spermatozoa were shorter than those of the spermatozoa collected from the reproductive tract. Although the reason for this decrease is not clear, it may be caused by an artifact of the process of preparing samples or by an influence of the secretion of the male and/or female reproductive tract.

3. Changes in the Morphology of the Spermatozoan Head

More abnormal acrosome of mink spermatozoa were observed in the testis and caput epididymis than in the corpus and cauda epididymis. This is in agreement with results obtained in the rabbit (Paúfler and Foote, 1968), bull (Amann and Almquist, 1962b), ass and stallion (ElWishy, 1975). The ratio of abnormal spermatozoa decreased during the spermatozoan passage through the epididymis.
The cell membrane of spermatozoa tends to separate from the acrosome as spermatozoa pass down the reproductive tract. However, there was no significant difference in the damaged membrane between spermatozoa taken from successive parts of the reproductive tract. Bedford (1964) and Hadek (1963b) found loosely fitted cell membrane around the acrosome in ejaculated rabbit spermatozoa and suggested that this might be due partially to the fixation procedure. Furthermore, Zamboni and Stefanini (1968), Zamboni et al. (1968) compared several kinds of fixatives used for rabbit and mouse epididymal spermatozoa and human and rabbit ejaculated spermatozoa. They concluded that the abnormal configuration of the cell membrane was the result of poor cell preservation. Nevertheless, their report showed different configurations of peri-acrosomal cell membranes between caput and cauda spermatozoa of the rabbit. In boar spermatozoa, Jones (1971) compared glutaraldehyde fixatives buffered with 100 mM and 150 mM sodium cacodylate. She also concluded that spermatozoa fixed in 150 mM solution remained in close apposition with the acrosome membrane while that fixed in 100 mM solution got separated from the subjacent structure. However, in this study, the cell membrane was separating from the acrosome during the spermatozoan passage through the reproductive tract even though 150 mM sodium cacodylate buffered fixative was used. Therefore, the separating of the cell membrane from the acrosome as spermatozoa pass down the epididymis might be a species-specific phenomenon.

As shown in electron micrographs, microstructure of spermatids has been observed in sperm samples taken from the testis. Spermatids have also been observed in samples taken from the caput epididymis. Although the epididymis is a
spermatozoan transport, maturation and storage organ, the caput epididymis might have spermatids because the epididymis is directly connected with ductuli efferentes of the testis (Clermont and Huckins, 1961).

Testicular spermatozoa had more acrosomal swellings than caput spermatozoa. This is difficult to explain because caput spermatozoa had an inordinately low acrosomal swelling ratio compared to the spermatozoa removed from the other parts of the reproductive tract. Except for this case, generally, the occurrence of the swellings on the equatorial and postacrosomal segments of the mink spermatozoa head was seen to increase as spermatozoa moved through the reproductive tract. This change might be related to the maturity and fertility of spermatozoa because the site of initial contact between the entering spermatozoon and the mammalian ovum is the equatorial segment or post-acrosomal region.

4. Movement of the Cytoplasmic Droplet

A significant decrease in the cytoplasmic droplet occurred during spermatozoan transit from the testis to caput and from the caput to corpus epididymis. Therefore, the low incidence of the cytoplasmic droplet was recorded in spermatozoa from the corpus, cauda epididymis and ejaculated semen. Although Branton and Salisbury (1947) reported a dramatic decrease of the proximal cytoplasmic droplet from the corpus to cauda epididymis of the bull, many investigators indicated that such a decrease occurred as spermatozoa passed
from the caput to the corpus epididymis in the bull (Amann and Almquist, 1962b), ass, stallion (ElWishy, 1975), and rabbit (Bedford, 1965b; Orgebin-Crist, 1967a; Paüßler and Foote, 1968). Nicander (1957b) also reported that the movement of the droplet away from the neck always occurred in the proximal end of the corpus epididymis. Orgebin-Crist (1967a) concluded that, for most spermatozoa, the cytoplasmic droplet migration occurred from the distal caput to corpus epididymis. This finding agrees with the results of the present study.

It is interesting to note that while the ratio of the cytoplasmic droplet was found to be strikingly reduced from caput to corpus in mink spermatozoa, the motility of these spermatozoa was greatly increased from the corpus to the cauda epididymis (Tables 2 and 4). This may indicate that the detachment of protoplasmic droplets from spermatozoa is simply associated with spermatozoan maturation. Bedford (1967b) concluded, for instance, that the motility of caput spermatozoa was not influenced by the presence of cytoplasmic droplets in the rabbit.

5. Changes in the Enzyme Activity

During passage through the reproductive tract, most enzyme activities of spermatozoa were enhanced, while those activities were found dramatically reduced in ejaculated spermatozoa when compared with those from the cauda epididymis. The chemical composition (White, 1973a) and enzyme distribution (Roussel and Stallcup, 1966; Einarsson et al., 1976) of the testicular and epididymal fluid are different. Therefore,
the difference in the enzyme reaction might be due to the environmental change encountered by spermatozoa as they pass along the reproductive tract (White, 1973b) or to an increase in the amount of enzyme, or an unmasking of the enzyme (Voglmayr, 1970).

On the other hand, Terner et al. (1975) reported that the activity of acid phosphatase in rat spermatozoa decreased during transit from the caput to cauda epididymis. Poirier (1975) also demonstrated acid phosphatase activity in the head membrane of mice spermatozoa from the testis, caput, and to a smaller extent from the cauda epididymis, but it was absent from the ductus deferens. The increased enzyme activity in mink spermatozoa during the spermatozoan transit through the reproductive tract may indicate, in addition to the occurrence of swellings, another special feature in mink spermatozoa.

Alkaline phosphatase activity in mink spermatozoa also increased as spermatozoa moved through the reproductive tract. This agrees with increasing alkaline phosphatase activity in the cytoplasmic droplet of rabbit spermatozoa (Bavdek and Glover, 1970). Einarsson et al. (1976) reported the increase of alkaline phosphatase activity from the testicular fluid to the cauda epididymal fluid in the boar. However, the activity of the enzyme in mouse spermatozoa decreased during their transit through the epididymis (Terner et al., 1975) and in the cytoplasmic droplet of rabbit spermatozoa (Moniem and Glover, 1972). Moniem and Glover (1972) suggested the difficulty of histochemical methods and the subjectivity of some interpretations as possible reasons for the contradictory results.
Voglmayr et al. (1969) reported more ATPase activity in ejaculated spermatozoa than in testicular spermatozoa of the ram. However, Gordon (1973) recorded ATPase activity in the periacrososomal portion of the plasmalemma of rabbit and human spermatozoa from the cauda epididymis, while ejaculated spermatozoa showed no reaction. The absence of ATPase activity in ejaculated mink spermatozoa is in agreement with that found in the rabbit and human. In the rat, lactate dehydrogenase activity was slightly higher in spermatozoa from the cauda than from the caput epididymis (Terner et al., 1975), which agrees with the results of this study in mink spermatozoa.

Strong activity of acid and alkaline phosphatases, ADPase, ATPase and MDH in mink spermatozoa of the cauda epididymis were found dramatically decreased to weak or negative reactions in ejaculated spermatozoa. Since the ejaculated spermatozoa in this study were collected from females after mating, several factors might have inhibited some enzyme activities of ejaculated spermatozoa in this study. It has been reported that L-tartrate inhibits acid phosphatase activity in the semen (Sivaram and Bami, 1971) and low concentration of iodoacetic acid completely inhibits succinate dehydrogenase and alkaline phosphatase in spermatozoa (Lardy and Phillips, 1941c). Spermatozoa also absorb a proteinase inhibitor from seminal plasma during ejaculation (Zaneveld, et al., 1973). Gordon (1973) reported that there was no phosphatase activity (ATPase, ADPase, adenosin 5'-monophosphatase, G-6-Pase and others) in the plasmalemma of either washed or unwashed ejaculated rabbit and human spermatozoa. In this study unwashed
ejaculated spermatozoa were examined for enzyme activities but here also the results obtained were negative.

A significant decrease of acrosin activity from epididymal to ejaculated spermatozoa was demonstrated in several mammals (Zaneveld et al., 1973). Such inhibition would prevent premature spermatozoan changes during transport in the female reproductive tract. Afterwards, the proteinase activity increased in capacitated spermatozoa (Zaneveld et al., 1969b). Capacitation \textit{in utero} may remove any inhibitor involved in the spermatozoan head (Zaneveld et al., 1972). Considering these successive events, it is assumed that the strong activity of acid and alkaline phosphatases, ADPase and ATPase, which might be important in fertilization, would have been blocked or inhibited during transportation from the epididymis to the uterus.

The present study indicated that most morphological changes of spermatozoa took place in two steps: first, during their passage from the testis to the caput epididymis, and second, from the caput to the corpus epididymis. There was no significant difference in the morphological aspect of spermatozoa from the corpus to the cauda epididymis.
F. SUMMARY

The maturation changes in viability, morphology, and enzyme activity were studied in the present investigation with spermatozoa collected from the testis, three successive parts of the epididymis and from ejaculated semen in the mink.

The motility of spermatozoa was found to be significantly increased ($p < 0.05$) during the spermatozoan passage through successive parts of the reproductive system. Ejaculated spermatozoa collected from females after mating was less active compared to that of cauda spermatozoa. While testicular spermatozoa was non-motile, a comparatively high per cent of motility was acquired by spermatozoa during their transit in the epididymis. Although the total head length of spermatozoa changed very little, the anterior acrosomal length was significantly decreased ($p < 0.001$) while the postacrosomal length increased significantly ($p < 0.05$) during transit from the testis to the epididymis. The equatorial length of testicular spermatozoa was smaller than that of epididymal spermatozoa. Even though the apical length significantly decreased ($p < 0.001$) when spermatozoa moved from the testis and caput epididymis to the corpus and cauda epididymis, the nuclear length maintained almost similar values during passage through the reproductive tract. The acrosomal thickness of spermatozoa significantly decreased ($p < 0.001$) from the testis to the epididymis. The head length of ejaculated spermatozoa were smaller than those of spermatozoa obtained from the reproductive tract.
During transit in the reproductive tract, the acrosomal matrix appeared to become more electron dense; the number and size of nuclear vacuoles decreased. Many abnormal spermatozoa were found in the testis and caput epididymis. There was an increase of separation of the cell membrane from the acrosome \((p < 0.05)\) as spermatozoa passed down the reproductive tract. Such cell membrane separation from the acrosome was more pronounced in ejaculated spermatozoa. There was no statistical difference between spermatozoa removed from different parts of the reproductive tract in the damage of the acrosomal membrane. The occurrence of the postacrosomal swelling significantly increased \((p < 0.01)\) from testicular and caput spermatozoa to corpus and cauda spermatozoa. Almost all ejaculated spermatozoa had swellings.

The incidence of the cytoplasmic droplet significantly \((p < 0.001)\) diminished from testicular spermatozoa to epididymal spermatozoa. A few ejaculated spermatozoa had the droplet.

The activities of several enzymes, except DOPA oxidase, were increased during the spermatozoan transit through the reproductive tract. However, lowered enzyme activities (acid and alkaline phosphatases, ADPase, ATPase and malate dehydrogenase) were found in ejaculated spermatozoa recovered from female mink after mating.
The head of mink spermatozoa was dorsoventrally flattened and ovate in outline. The total head length did not change significantly during the passage of spermatozoa through the testis, and epididymis (caput, corpus and cauda). However, the head of ejaculated spermatozoa were smaller than spermatozoa collected from the reproductive tract.

The anterior two-third of the nucleus was covered with the acrosome which can be divided into three segments (apical, main, and equatorial) according to the acrosomal content. The posterior one-third was covered with the postacrosomal sheath. During the spermatozoan transit through the reproductive tract, the anterior acrosomal length, which was composed of the apical and main segments of the acrosome, was significantly decreased ($p < 0.001$), while the postacrosomal length was significantly increased ($p < 0.05$). The apical length also significantly decreased ($p < 0.001$) during transit of spermatozoa from the testis and caput epididymis to the corpus and cauda epididymis; the acrosomal thickness significantly decreased ($p < 0.001$) during transit from the testis to the epididymis. In accordance with the changes in dimensions, the acrosomal matrix appeared to be more electron dense and the number and size of vacuoles in the nucleus decreased as spermatozoa passed down the reproductive tract.

The perforatorium was triangular in longitudinal sections and remained intact even though the acrosomal content disappeared. On the dorsal and ventral aspects of the head,
two swellings as hump-like structures were found at the anterior border of the equatorial segment. One similar swelling was also found on each side of the postacrosomal sheath. A strong acid phosphatase activity was located at these swellings in epididymal spermatozoa. The occurrence of postacrosomal swellings was significantly increased (p < 0.01) during the passage of spermatozoa from the testis and caput epididymis to the corpus and cauda epididymis. Almost all cauda and ejaculated spermatozoa showed the acrosomal and postacrosomal swellings. The postacrosomal sheath was composed of a dense layer deposited on the cell membrane.

In the acrosomal region the cell membrane appeared to be separating from the acrosomal proper, except at the tip, as spermatozoa passed down the reproductive tract (p < 0.05). However, the cell membrane covering the postacrosomal sheath remained intact.

A strong acid phosphatase activity was localized not only in the acrosomal and postacrosomal swellings but also in the galea capitis and tail of epididymal spermatozoa. Non-specific esterase was localized in the postacrosomal sheath. Besides these, the activities of alkaline phosphatase, ADPase, ATPase, DOPA oxidase and malate dehydrogenase were also localized in the head of epididymal spermatozoa.

The neck was composed of the dorsoventrally continuous but laterally separated capitulum connected proximally with a ring of striated columns which was followed by two major and five minor columns, the proximal centriole, a few mitochondria and scrolls. The remnants of the axoneme were found in the
interior of the column bundle.

The tail consisted of nine dense fibers, nine double microtubules, and two central fibers. The diameters of dense fibers number 9, 1, 5 and 6 were larger than the rest of the dense fibers. In the doublets, the diameter of subfiber A was larger than the subfiber B. In the region of the middle piece, the axial fiber bundle was surrounded by 53 to 57 helically coiled gyres of mitochondria. The shape of the annulus was triangular in longitudinal sections. The fibrous sheath, which covered the principal piece of the tail, had two longitudinal columns along the dorsoventral aspects. The end piece retained the 9 + 2 pattern. The occurrence of the cytoplasmic droplet was significantly decreased (p < 0.001) from testicular to epididymal spermatozoa. Within the epididymis, the occurrence of the droplet was found markedly reduced from caput to corpus spermatozoa. Therefore, a few corpus, cauda and ejaculated spermatozoa had the droplet on the caudal end of the middle piece.

The motility of spermatozoa was significantly increased (p < 0.05) from no motile spermatozoa in the testis to 84% motile spermatozoa in the cauda epididymis. The motility of ejaculated spermatozoa collected from female after coitus was 65%.

A number of enzyme activities, with the exception of 6-phosphogluconic dehydrogenase, were localized in the tail of epididymal spermatozoa. Glucose-6-phosphatase, succinate, lactate and isocitrate dehydrogenases, and NADH diaphorase were confined to the middle piece, while malate dehydrogenase
activity extended to the head base and the principal piece of
the tail. The enzyme 5-nucleotidase was also confined to the
middle piece.

The activities of these enzymes in mink spermatozoa,
with the exception of DOPA oxidase, were found to be enhanced
during passage through the reproductive tract. However, the
activities of acid and alkaline phosphatases, ADPase, ATPase,
and malate dehydrogenase were significantly lowered in
ejaculated spermatozoa which were recovered from female minks
immediately following mating.
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