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## A COMPARISON OF ACROSOMAL PROTEINASE ACTIVITY IN THE MALE AND FEMALE REPRODUCTIVE TRACTS OF THE GUINEA PIG

Thomas Joseph Morris

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A COMPARISON OF ACROSOMAL PROTEINASE ACTIVITY  
IN THE MALE AND FEMALE REPRODUCTIVE TRACTS OF  
THE GUINEA PIG

by

Thomas Joseph Morris

BS, Virginia Military Institute, 1969

THESIS

Submitted in partial fulfillment of the requirements for the  
Degree of Master of Science in the Department of Anatomy  
at the Medical College of Virginia

Richmond, Virginia

April, 1975

TO MY FAMILY  
and  
REV. JOSEPH C. GREGOREK

Read not to contradict and confuse,  
nor to believe and take for granted  
but to weigh and consider.

--Francis Bacon

**This** thesis by Thomas Joseph Morris is accepted in its  
**present form** as satisfying the thesis requirement for the degree  
**of Master of Science**

Date:

APPROVED

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Adviser, Chairman of Graduate Committee

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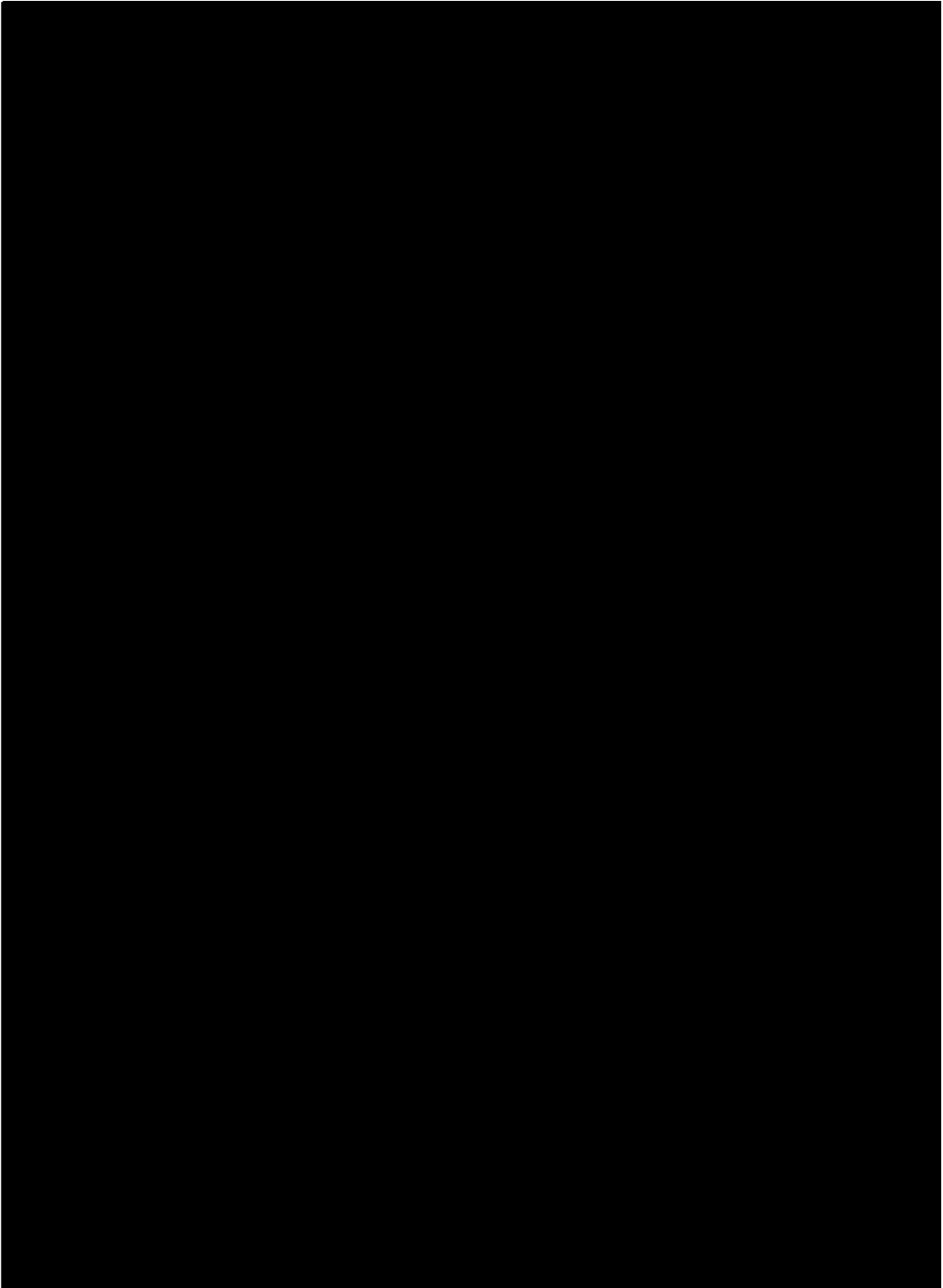
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APPROVED:..

Dean of the School of Basic Sciences

## CURRICULUM VITAE





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## I. INTRODUCTION AND LITERATURE REVIEW

### Anatomy of Guinea Pig Sperm

The spermatozoon has been the subject of numerous investigations because of its remarkable specialization for locomotion and fertilization. Although the spermatozoon of the guinea pig is relatively large when compared with other mammalian spermatozoa, its small size has hidden many of the details of its structure from the light microscopist. It was not until the invention of the electron microscope that a thorough study of spermatozoon structure could be accomplished. The following anatomical description is based on the works of Fawcett and Hollenberg (1963), Fawcett (1965), and Fawcett (1970). The principle parts of the guinea pig spermatozoon are the head and the tail with the tail being subdivided into the neck, middle-piece, principal-piece, and the end-piece. These and other anatomical structures which will be discussed are depicted in figures I and II which were taken from the work by Fawcett (1965).

#### 1. Head

The head consists of two major components, the nucleus and the acrosomal cap. The nucleus is flattened. Its broad oval outline is rounded anteriorly and truncated posteriorly. On the caudal surface of the nucleus there is an eccentrically placed shallow concavity for the reception of the convex articular surface of the tail.

D. W. FAWCETT:

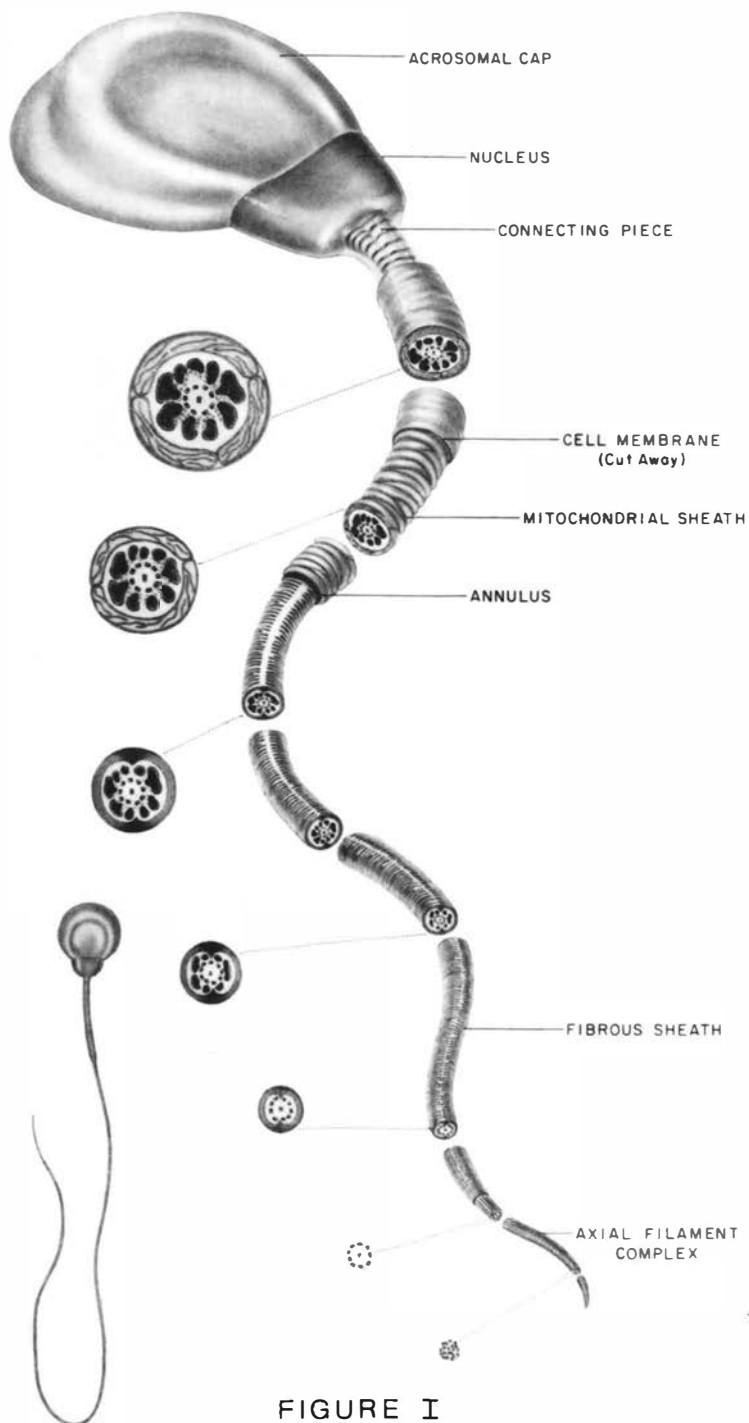


FIGURE I

—F. Diagrammatic representation of various regions of the guinea pig spermatozoon. A Sagittal section of the anterior portion of the spermatozoon showing the relative proportions of the head and middle-piece; the flexion of the head with respect to the long axis of the tail; and the inclination of the acrosome with respect to the long axis of the nucleus. B The base of the head, the neck, and beginning of the middle-piece as seen in a longitudinal section parallel to the broad surface of the flattened nucleus. The overlapping junction of the longitudinal columns of the connecting piece with the tapering proximal ends of the outer dense fibers is illustrated; the attachment of the connecting piece to the basal plate of the nucleus; and the centriole set obliquely within a niche in one of the major segmented columns of the connecting piece. C A parasagittal section showing the convergence and fusion of the minor columns of the connecting piece with its articular surface. The centriole immediately below, is seen in oblique section. The basal plate is also shown, extending well beyond the margin of the implantation fossa on the ventral aspect of the head. D, E, and F Representations of the cross sectional appearance of the tail at the levels indicated by the lead lines. In D, are depicted the two major and five minor columns of the connecting piece in cross section, and the penetration of processes of the mitochondria into its interior. The irregular shape of the longitudinal elements of the neck in the region of overlap of the columns of the connecting piece and the outer dense fibers is shown in E. Cross section D and E can be compared with the appearance of a typical cross section of the middle piece in F

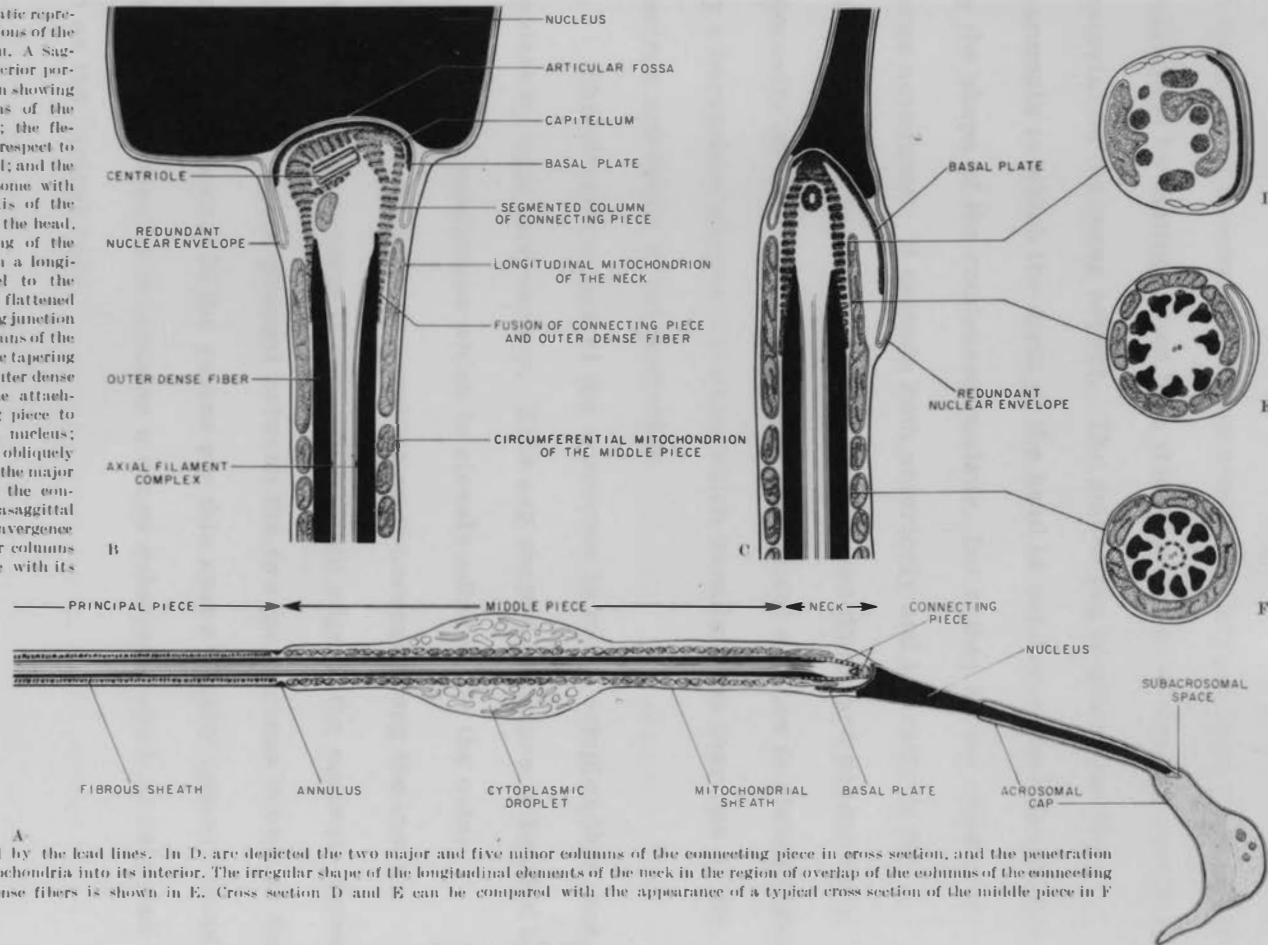


FIGURE II

This concavity, the implantation fossa, is lined by the two membranes of the nuclear envelope. The outermost membrane forms a basal plate which may be distinguished by a thickened layer of dense amorphous material on the outer aspect. The guinea pig is one of the few mammals in which the form of the head is not determined primarily by the shape of the condensed nucleus, but rather by the unusually large acrosome. It extends both anteriorly and laterally to the margins of the nucleus. The border curves downward producing a concavity on the ventral surface. The dorsal surface is distinguished by a horseshoe-shaped elevation which parallels the margins of the nucleus and opens to the posterior.

The ultrastructure of the acrosome is more complex than was evident by light microscopy. This cap shaped structure is limited by a continuous membrane which is closely adherent to the outer membrane of the nuclear envelope as it courses along the inner surface of the acrosomal cap. At the thin edge of the nucleus, there is a narrow recess present between the two membranes known as the acrosomal space. In the guinea pig this space usually appears empty, but in other species it contains a dense substance which is referred to as the "apical body."

## 2. Tail

The tail is subdivided into four regions: the neck, middle-piece, principal-piece, and end-piece.

The neck is the region between the nucleus and the first gyrus

of the mitochondrial helix of the middle-piece. This region also contained mitochondria, but they are irregular in shape and are oriented longitudinally. The connecting piece is the principle structural component of the neck. It is formed by the convergence and fusion of nine longitudinal segmented columns. Four of the segmented columns fuse cephalad to form two major columns and five minor columns. Of the two major columns, one is expanded anteriorly to form a capitellum which fuses with the basal plate and thus attaches the head to the tail. The remaining columns fuse with the capitellum to support it. Enclosed within this structure, immediately beneath its articular surface, is a centriole. It is situated at  $45^{\circ}$  to the long axis of the tail and one end opposes the inner surface of the capitellum while the other contacts the inner aspect of the second major column.

An eccentrically placed implantation fossa is present on the caudal surface of the nucleus and it is here that the base of the tail and the head come together. The articular surface is lined by two membranes of the nuclear envelope. The outermost of these is thickened on its outer aspect to form a basal plate.

Of interest is the observation that a redundant portion of the nuclear envelope is reflected away from the condensed chromatin and extends back over the neck region.

The middle-piece region of the tail is composed primarily of

mitochondria arranged in helical fashion to form approximately forty-two gyri of two mitochondria each. A flagellar membrane is closely adapted to these structures except in the region of the cytoplasmic droplet, about midway along its length. This structure has a cytoplasmic matrix in which numerous smooth-surfaced membranes may be found. These are believed to be remnants of endoplasmic reticulum and the Golgi complex.

A dense ring-like structure, the annulus, may be found distal to the last gyrus of the mitochondrial sheath, and it is this structure that defines the posterior border of the middle piece.

The principal piece is characterized by a fibrous sheath, a series of closely-spaced fibrous elements arranged circumferentially around the axial bundle of longitudinal fibrils. These fibrils are divided into two groups, an inner group of eleven and an outer group of nine thicker dense fibrils. The inner group or axial filament complex is further subdivided into two central single fibrils surrounded by nine evenly spaced double fibrils. Each doublet is divided into a subfibril A and a subfibril B. Subfibril A has a dense interior and it bears short divergent arms that project toward the next doublet. Subfibril B is the larger member of the pair. It has a clear center and bears no arms.

The radial symmetry of the outer fibers changes in the principal piece. This is because all the fibers taper from base to

tip. Fibers 3 and 8 terminate in the anterior part of the principal piece while fibers 1, 5, and 6 appear to continue.

The end piece consists of the remaining axial filament complex which is enclosed by the flagellar membrane. In the guinea pig, the nine doublets divide to form eighteen single units which end at various levels.

### Acrosome Formation

The development of the acrosome is an integral part of the four phases of spermiogenesis, the developmental events by which spermatids are transformed into mature sperm. The following description is based on the works of Bloom and Fawcett, (1969); Vilar, (1973); and Zamboni, (1971).

The first Golgi phase is characterized by the appearance of several small proacrosomal granules within the juxtannuclear Golgi apparatus of the spermatid. As development progresses, the proacrosomal granules coalesce to form a single, dense, PAS positive acrosomal granule within an acrosomal vesicle which adheres to the outer aspect of the nuclear envelope. The Golgi apparatus continues to form these proacrosomal granules which coalesce with the acrosomal vesicle contributing to its enlargement.

The second stage is the cap stage. During this time, the acrosomal vesicle increases in its area of adherence to the nuclear envelope and thus forms what appears to be a head cap.

In the acrosomal phase there is a redistribution of the acrosomal substance into the fold of membrane comprising the head cap. Once this is complete, the acrosomal granule and head cap are coextensive and therefore, form the acrosome.

The maturation phase of spermiogenesis in the guinea pig is characterized by further alterations in the acrosome. It is here that the acrosome assumes its characteristic shape.

### Role of the Acrosome and Enzyme Studies

Many possible roles have been proposed for the acrosome. Perhaps the most popular has been the theory that the acrosome is a specialized lysosome which evolved to facilitate fertilization in multicellular organisms, (Allison and Hartree, 1970). According to Zamboni (1971), "the Golgi-mediated formation of the acrosome, as well as the PAS positivity and enzymatic activity of this organelle, make the acrosome comparable to the lysosomes of somatic cells."

In 1970, Allison and Hartree demonstrated these lysosomal properties by vitally staining the acrosomes of guinea pig, hamster, and several rodents with aminoacridines. In the developmental stages of the spermatid they observed red-fluorescing granules around the Golgi zone which condensed to form the red-fluorescent proacrosome. In the mature spermatozoon, the acrosome fluoresced a brilliant orange-red which is typical of lysosomes.

If the acrosome is indeed a specialized lysosome, then it

becomes important to study not only the enzymatic content, but also the function of these enzymes. Probably one of the most widely accepted concepts of fertilization is the sequential release of enzymes from the acrosome which was proposed by Bedford (1968). He demonstrated an acrosomal reaction characterized by the fusion of the plasma membrane and the outer acrosomal membrane to form vesicles as the spermatozoon penetrates the follicular cells of ovulated rabbit eggs. He postulated that hyaluronidase, which is to a large extent located on the plasma membrane and more readily released from acrosomes than is protease, is available at this time to digest the intercellular substance between the cells of the cumulus oophorus. This enzyme, which appears to initiate the start of sperm penetration of the egg investments, affects neither the corona radiata nor the zona pellucida (Allison and Hartree, 1969; Enders, 1970). According to Zaneveld and Williams (1970), the second phase of fertilization, as demonstrated in the rabbit and the hamster, is effected by a corona penetrating enzyme (CPE), which is capable of digesting the cementing substance between the corona cells. Now, the acrosomal reaction is considered to be complete; the proteolytic enzymes which are released from the surface of the inner acrosomal membrane, are available to digest the zona pellucida. It has also been reported that these proteases facilitate penetration of the corona radiata (Allison and Hartree, 1970). This should not be misinterpreted as

evidence that CPE is a protease. There is no indication at this point that CPE might be a proteolytic enzyme. Such a possibility is unlikely in light of the fact that soybean trypsin inhibitor did not affect the activity of CPE (McRorie and Williams, 1974).

There are other enzymes in the acrosome which have been the subject of much investigation. A comparative study of the acrosomal enzymes of rabbit, rhesus monkey, and human spermatozoa was conducted by Stambaugh and Buckley (1970). They were able to isolate subcellular fractions of the various spermatozoa by using sucrose-density gradients. All three species were found to have hyaluronidase and a specific "trypsin-like" enzyme in the head fraction, but it was only in rabbit and rhesus monkey that both enzymes specifically were localized in the acrosome. To avoid any confusion on the part of the reader, acrosin is now the official term used to designate a trypsin-like acrosomal proteinase (McRorie and Williams, 1974).

A "chymotrypsin-like" enzyme was found in ejaculated but not in epididymal sperm. This they hypothesized was absorbed from the seminal plasma. Allison and Hartree (1970) confirmed the presence of several lysosomal enzymes in the ram from acrosomal preparations and buffer washings. They include the following: acid phosphatase, aryl sulphatase,  $\beta$ -*N*-acetyl-glucosaminidase, phospholipase A, and proteinases. They reported that the protease activity was greatest at pH 7.5. They also reported the presence of

several acid hydrolases: acid phosphatase, 5 bromo - indoxylacetate esterase, and 5 - bromo - 4 - chloroindoxylacetate in guinea pig acrosomes.

Perhaps the most significant enzymatic study in view of the present investigation is that of Owers (1970b) and Owers (1971). This researcher identified many of the proteolytic enzymes in the guinea pig acrosome by fluorometric assay procedures. They include the following exopeptidases: dipeptidylaryl-amidases (DAP), including DAP I, DAP II, DAP III, and DAP IV. The endopeptidases observed were "trypsin," cathepsin B' and cathepsin D.

#### Apical Body and Cytoplasmic Droplet

Other structures of the spermatozoon either have been implicated in fertilization or are closely related to the acrosome.

Gordon (1969) studied the apical body in both guinea pig and human spermatozoa with phosphotungstic acid. He hypothesized that this structure, which is located between the inner acrosome membrane and the nucleus, may be important for the movement of the sperm through the zona pellucida of the egg after vesiculation of the outer membrane of the acrosome. He was able to localize the apical body utilizing ethanolic phosphotungstic acid (E-PTA), and because E-PTA bonds to proteins, he suggested that this proteinaceous structure might possess enzymatic properties which promote the passage of the sperm through the zona pellucida of the ovum.

The cytoplasmic droplet is important in this study of the acrosome because it may be derived from degenerating Golgi apparatus and endoplasmic reticulum as seen by Bloom and Nicander (1961). Therefore, this structure may facilitate a greater insight into the enzymatic makeup of the acrosome. Garbers, Wakabayashi, and Reed (1970) examined bovine cytoplasmic droplets and found them to be rich in hydrolase enzymes which operate over a wide pH range. These droplets contained appreciable acid phosphatase,  $\beta$ -glucuronidase, acid protease, and RNase activity. Other enzymes of a lysosomal nature which were found include 5'-nucleotidase,  $\beta$ -galactosidase, and low levels of  $\alpha$ -mannosidase,  $\alpha$ -D-glucosidase,  $\beta$ -D-glucosidase and a neutral esterase. These authors did not suggest a role for these enzymes other than to say that they were of a lysosomal nature. But they did state that although these droplets contained low activities of some enzymes linked to intermediary carbohydrate metabolism they appeared to be metabolically inert.

Dingle and Dott (1969), however, suggested that droplet lysis could contribute enzymatic activity to seminal plasma.

### Gelatin Membrane Studies

A gelatin membrane technique for the detection of proteases released in vitro was first developed by Owers (1970a), and later modified by Denker (1974). It utilizes a glutaraldehyde fixed gelatin film which is susceptible to digestion by proteinases. The

significant feature of this technique is that protease produced and released as a result of metabolism and function, or those released due to cell death or injury can be detected for a single cell. However, it must be noted that this technique is specific for endopeptidases. Only after the initial cleavage of the protein fiber by enzymes can the exopeptidases exert their action.

The application of this technique has been very diversified including the study of spermatozoa (Gaddum and Blandau, 1970; Owers, 1970b; Denker, 1974), the blastocyst (Owers and Blandau, 1971) and certain bacteria, fungi, and mammalian cells (Owers, 1970a).

Particular emphasis has been given to the study of testicular, epididymal, and ejaculated spermatozoa of various species, Owers (1970b) examined the acrosomes of epididymal sperm from guinea pig, rat, dog, monkey, quail, and roosters and found maximal activity with an optimum pH in the alkaline range and reduced activity in the acidic range. He hypothesized that this reduced activity in the acidic range was due to the presence of cathepsin D and B'. A similar study by Gaddum and Blandau (1970) studied the reactivity of sperm acrosomes from the ejaculated spermatozoa from humans and the epididymal sperm from guinea pigs, rats, mice, and hamsters. These authors stated that the guinea pig spermatozoa display large reaction circles in four to six hours from pH 7.5 to pH 9.5, while only slight to moderate reactions were observed below

pH 7.5. Rabbit ejaculate has also been studied and zones of reactivity reported (Denker, 1974).

### Purpose

It has been well established that a certain amount of enzymatic activity exists in the mammalian acrosome of the spermatozoa from the male testis, epididymis, and ejaculate. Both histochemical and biochemical studies have been utilized to identify the nature of these enzymes, and gelatin membrane studies have demonstrated the presence and relative quantities of the proteolytic enzymes which are present. However, no extensive studies have been performed to study the proteolytic enzymes of the acrosome once it is within the female reproductive tract.

The purpose of the present investigation is to conduct an in-depth gelatin membrane study of the proteinase activity of the guinea pig sperm acrosome in both the male and female reproductive tracts and in the ejaculate of the male. Specifically, this research will attempt to answer the following questions:

1. Does proteolytic enzyme activity persist in the acrosome while the sperm travels through the female reproductive tract (vagina, uterus, and oviduct)?
2. Does this enzymatic activity vary according to the location of the sperm in the male and female

reproductive tracts ?

3. Does enzymatic activity vary for the pH value (4.5, 7.0, or 8.4) of the veronal acetate buffer used in vitro?
4. And finally, does proteolytic enzyme activity differ at any of the locations in the male and female reproductive tracts for each of the pH values used in the veronal acetate buffer ?

## METHODS AND MATERIALS

### Gelatin Membrane Technique

#### A. Preparation of Gelatin Membrane

1. A mixture consisting of 408mg of dry gelatin granules in 6 ml of distilled water was heated in a water bath at 40°C until the gelatin dissolved.
2. 0.33 ml of India ink (Higgins 4465) was added to 6 ml distilled water. This mixture was also warmed to 40°C in a water bath.
3. 6 ml of gelatin solution as given in Step 1 and 6 ml of the ink solution as given in Step 2 were mixed and warmed to 50°C (Fig. 2, p. 29).
4. A 1 cc plastic tuberculin syringe was used to apply 0.1 ml of the warmed mixture to a clean, dry glass microscope slide, covering an area 45 x 25 mm, and allowed to air dry for 18 hours (Fig. 3, p. 29).

#### B. Fixation of Gelatin Membrane

1. The slides were placed with the dry gelatin membranes in a solution of 0.05% glutaraldehyde in veronal acetate buffer pH  $7.0 \pm 0.05$  for 2 minutes, then transferred to each of two changes of buffer only for 10 seconds each. Excess buffer was shaken off, and the slides were allowed

to air dry for at least one hour.

2. The slides were then rinsed in three changes of distilled water for 10 seconds each. Excess water was shaken off and the slides were allowed to dry for at least one hour (Figs, 4 and 5, p. 31).

#### C. Test for Membrane Sensitivity

One slide from each batch of twenty prepared, was subjected to quality control, as follows. Trypsin, 1mg/ml in veronal acetate buffer pH 8.0, was prepared and 10 lambda drops of this enzyme were placed on 2 separate portions of the gelatin membrane. The slides were then incubated at 37°C for one hour. The appearance of a clear zone in the membrane signified the completion of digestion. The veronal acetate buffers were also used in control preparations where 10 lambda drops of the buffer (without trypsin) were added to each of these slides. No digestion was observed for the buffer only, at pH 4.5, 7.0, or 8.4. This completed the quality control test for the gelatin membrane (Fig. 6, p. 33).

#### Experimental and Operative Procedures

Young, sexually mature (Fox and Laird, 1970) male and female English short hair albino guinea pigs, Cavia porcellus, weighing approximately 500gm were used in this investigation. All

animals were housed in hanging cages and received food and water ad libitum. A supplemental dose of Vitamin C, 30mg/0.5ml Mead-Johnson Tri-Vi-Sol, was administered intraorally to each guinea pig once daily (Lane-Petter and Porter, 1968). The animal room was maintained at a constant temperature of 21°C and on a light-dark cycle of 14:10 hours. Prior to surgery a final body weight was taken.

The animals were divided into two experimental groups. The first group consisted only of males from which sperm was obtained directly by dissection or by electroejaculation (1). The second group consisted of both male and female animals, and sperm was obtained from the female reproductive tracts after mating (2).

#### 1. Collection of Testicular, Epididymal, and Ejaculated Sperm.

The male animals were anesthetized with 50mg/cc of sodium pentobarbital, intraperitoneally (0.074cc/100gm body weight) (Valenstein, 1961; Phoenix, 1970). The testes and epididymides (Fig. III, p. 19) were then removed from the male guinea pigs after an incision was made into the lower abdominal cavity. These two components were then separated and each macerated on the inner surface of a glass funnel with a pair of iris scissors. The tissue was then washed into a beaker with a nutrient solution (0.9% NaCl, 0.5% glucose) which had been pre-warmed to 37°C. This sperm-containing solution was then placed in an incubator for 15 minutes to

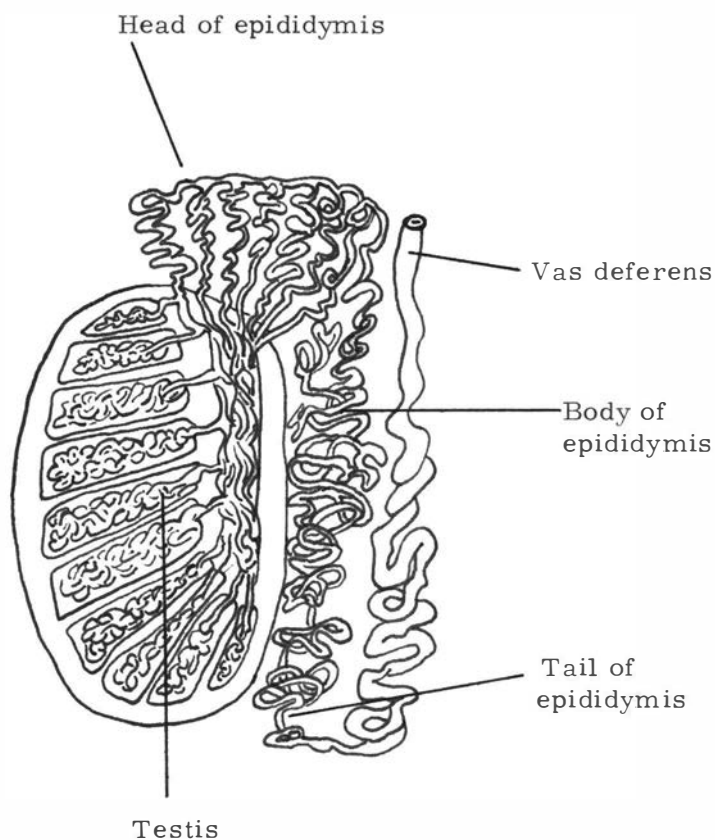


FIGURE III. Diagrammatic Representation Showing the Tubular Arrangement of the Testis, Epididymis, and Vas Deferens (Adapted from Crafts, 1966).

allow for separation of the sperm and macerated tissue. Three samples of 0.5ml each were removed from this sperm suspension and added to three separate centrifuge tubes. The samples were then spun down to form a pellet and the supernatant was discarded. To each of these centrifuge tubes a different veronal acetate buffer solution (pH 4.5, 7.0, or 8.4) was added. These tubes were then agitated to produce a homogenous buffered sperm suspension. A micropipette was used to deposit 20  $\mu$ l of each suspension onto a gelatin membrane (pre-incubated to 37°C), and the drop was smeared over the membrane surface using a Fisher Beve-L-Edge microscope slide in much the same manner as a blood smear is made. While the membrane was still moist, one or two drops of mineral oil were added to the area to be studied and the slides were incubated for 12 hours at 37°C to allow for maximal reactivity of the acrosomal proteinases.

The males in the electroejaculation group, while unanesthetized (Scott and Oziuk, 1959), were subjected to an intermittent square wave of 25 volts rms (root mean square) at 1,000 cycles with an automatic 3 second "on period" and a 12 second "off period." The electro-ejaculation apparatus assembled by the author (Fig. 7, p. 35) was duplicated after that used by Freund (1958 and 1969), in his experiments on guinea pig semen. The hair was clipped from the lumbar region of the animal and electrode jelly was applied to the clipped area. The guinea pig was then strapped on its back to the animal board (Fig. 8, p. 35) and held by the pelvic crest so that it contacted the lumbar

electrode (round brass disc). The anal electrode, a smooth brass tube, was inserted 4-6 cm into the rectum.

The ejaculate was collected and prepared for study in two different ways. First, a sample was collected in its natural physiological state. A smear was made on the gelatin membrane and mineral oil was added before incubation. The second method involved collecting the ejaculate in 2ml of 4% sodium citrate (Scott and Oziuk, 1959). The ejaculate was then transferred to a graduate test tube containing 7ml of 4% sodium citrate and the sperm was suspended by vibration. This suspension was divided into three equal portions each of which was spun down to form a pellet, and then the supernatant discarded.

To the first pellet 2ml of veronal acetate buffer, pH 4.5, was added; to the second 2ml of pH 7.0; and to the third 2ml of pH 8.4; and the pellets were resuspended. A 20 lambda sample was removed from each centrifuge tube; and a smear was made on the gelatin membrane, covered with mineral oil and incubated for 12 hours.

## 2. Collection of Sperm From the Female Reproductive Tract

Two females were placed in a cage with a male guinea pig. The females were examined at 10 hour intervals for evidence of mating. Two criteria were used to determine the occurrence of coitus (Phoenix, 1970). First, the animals were examined for the presence of an ejaculation plug. If this was not visibly present, then a cotton tip

applicator, which had been moistened with physiological saline, was inserted into the vagina, and the specimen obtained was used to make a smear on a glass slide for examination under the light microscope. If the animal was found to have either an ejaculation plug or a smear, positive for sperm, then it was considered to have mated. These females were then placed into one of two groups by random selection; those to be operated upon immediately and those which would be operated upon after ten additional hours had elapsed. The original intent for this division was to examine spermatozoa for differences in the amount of proteinase activity from 0-10 hours and from 10-20 hours post coitus. No difference between these two groups was noticed and therefore, the protocol was changed to include a period of 0-20 hours post coitus. This time frame coincides closely with the limit of time, 22 hours, during which fertilizing capacity is retained by guinea pig spermatozoa introduced into the genital tracts of females by artificial insemination prior to the beginning of heat (Soderwall and Young, 1940).

Just prior to the operative procedure, the animals were sacrificed by a sharp blow to the back of the head followed immediately by decapitation. In the operative procedure, the abdominal cavity of the female was opened and silk ligatures were placed around each of the uterine horns at the origin of their bifurcation and at the junction of the uterine horns to the oviducts (Fig. IV, p. 23). The vagina was then lavaged with veronal acetate

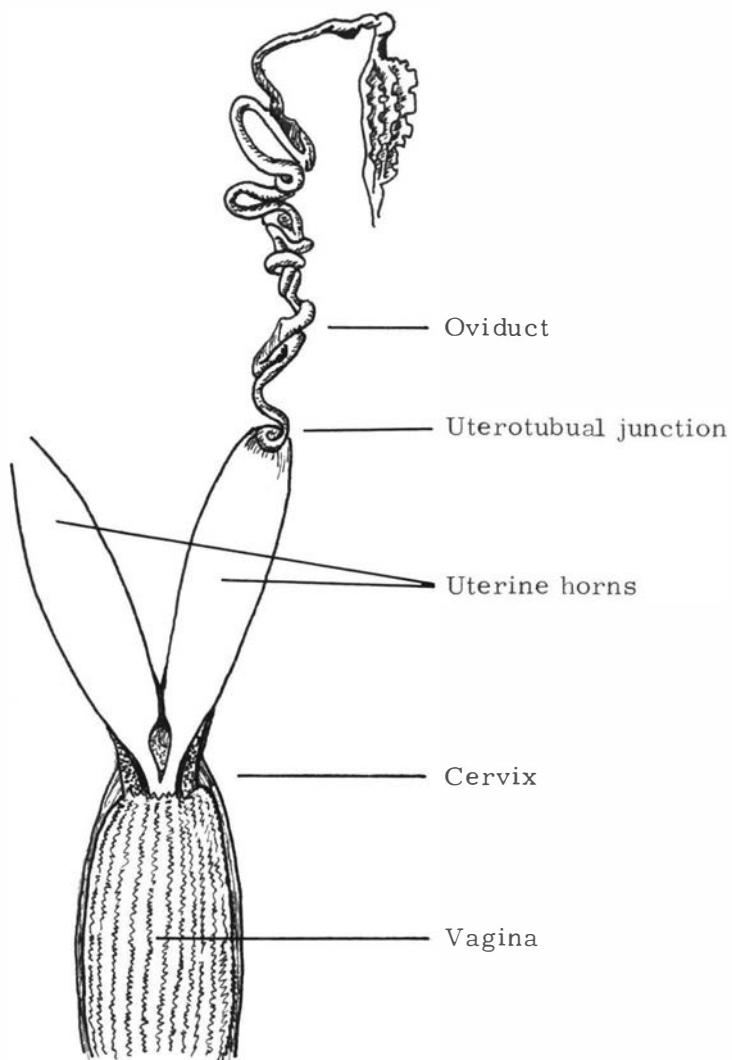


FIGURE IV. Diagrammatic Representation of the Reproductive Tract of the Female Guinea Pig (Adapted from Hafez, 1970).

buffer of a predetermined pH and a smear made from this sample on the gelatin membrane. The remaining portion of the female reproductive tract was dissected free and placed in a culture dish containing Hank's solution at 37°C. Extraneous tissue was then removed under the dissecting microscope and the individual uterine horns and oviducts were flushed by a syringe containing 0.1 cc of a veronal acetate buffer (either pH 4.5, or 7.0, or 8.4) onto a gelatin membrane. Smears were made, mineral oil added, and the slides incubated for 12 hours at 37°C.

#### Observational Procedure and Method of Data Collection and Analysis

An inverted phase contrast microscope (Fig. 1, p. 27) was used to observe the gelatin membranes for reactive spermatozoa. The slides were scanned at 100x to determine the area of reactivity which was most representative. This determination was made at the discretion of the researcher and included those areas in which there were both maximal activity and the highest percentage of reactive versus non-reactive sperm (Figs. 10 and 11). From this field, 50 reactive spermatozoa randomly were selected by making straight, uniform scans back and forth across the field, counting only those sperm which were intact and which fell within the confines of a net micrometer reticle. If less than 50 reactive sperm were observed on the entire slide, then all reactive sperm were counted and measured. The only exception to this was one female in which counts taken from the uterus and oviducts were in excess of 1000.

The diameter of the zone of lysis for each of the reactive sperm was measured to the closest one-half of a unit on a linear micrometer reticle where each unit was equal to 0.01 mm at 100x. For area the conversion factor is  $10 \times 10^{-5} \text{mm}^2$  per square reticle unit, and this appears in the equation below. The diameters then were averaged for each portion of the male and female reproductive tract observed at each specific pH value, and the average diameter (d) was used in the following equation to compute the average area (A) of the zone of lysis.

$$A = \pi \left(\frac{d}{2}\right)^2 (10) (10^{-5}) \text{mm}^2$$

A statistical analysis was run on the data to determine their significance. Because of the many variables involved in this experiment, an analysis of variance was performed.

FIG. 1. Inverted phase and brightfield camera microscope used to make observations on the gelatin membrane.

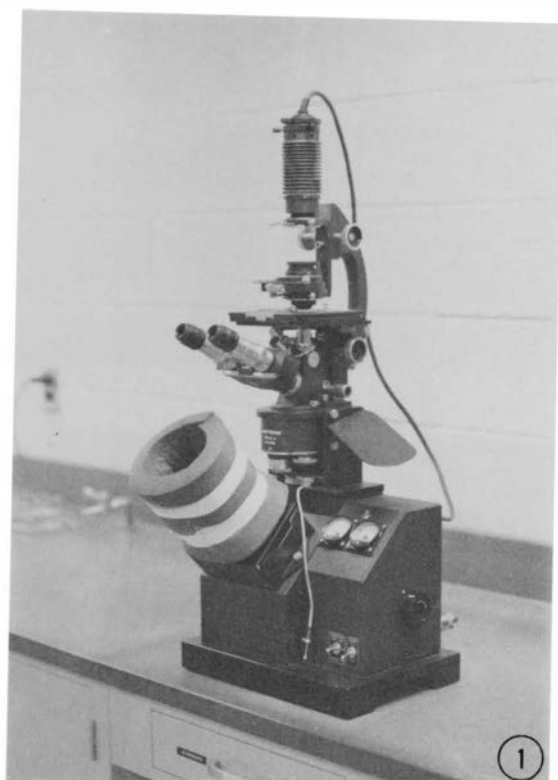


FIG. 2. Water bath containing separate gelatin and India ink solutions at  $40^{\circ}\text{C}$ . These later will be mixed and warmed to  $50^{\circ}\text{C}$ .

FIG. 3. India ink impregnated gelatin solution being applied to glass microscope slides. The slides are then allowed to air dry on a level surface.

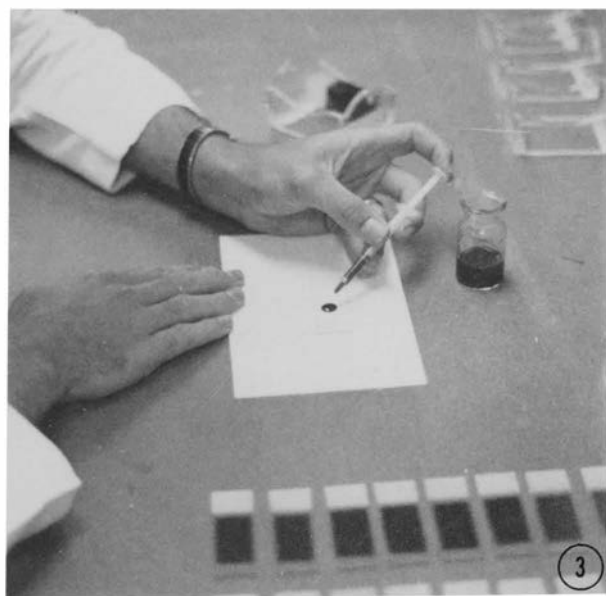
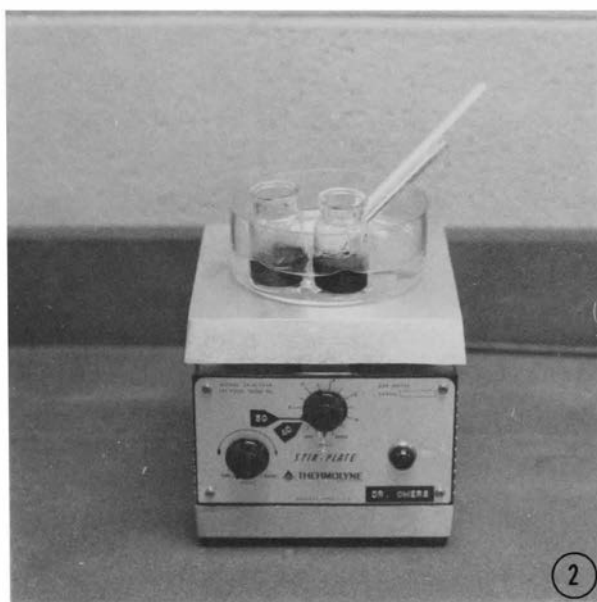


FIG. 4. Fixation of the gelatin membranes with a solution of 0.05 percent glutaraldehyde in veronal acetate buffer of pH  $7.0 \pm 0.05$ . The slides are then rinsed twice in buffer, allowed to air dry, and finally washed three times in distilled water.

FIG. 5. Excess water is shaken off and the slides are allowed to air dry for at least one hour.

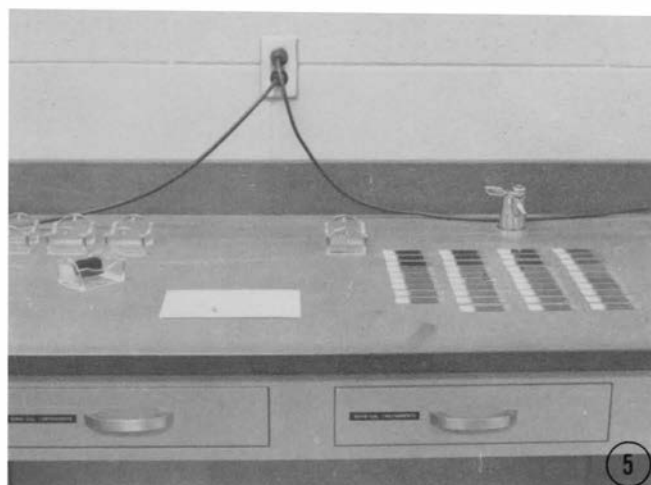


FIG. 6. A gelatin membrane slide used in the test for membrane sensitivity. The two circles were the result of membrane digestion by trypsin in veronal acetate buffer. Buffer alone did not digest the membrane. 1 1/2 X.

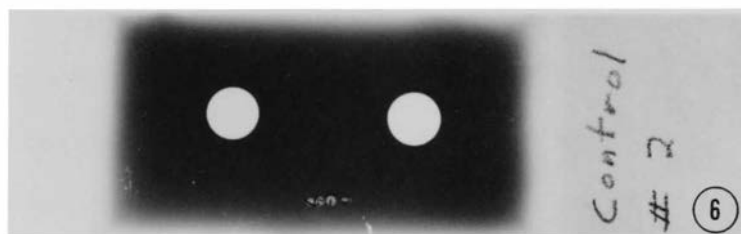
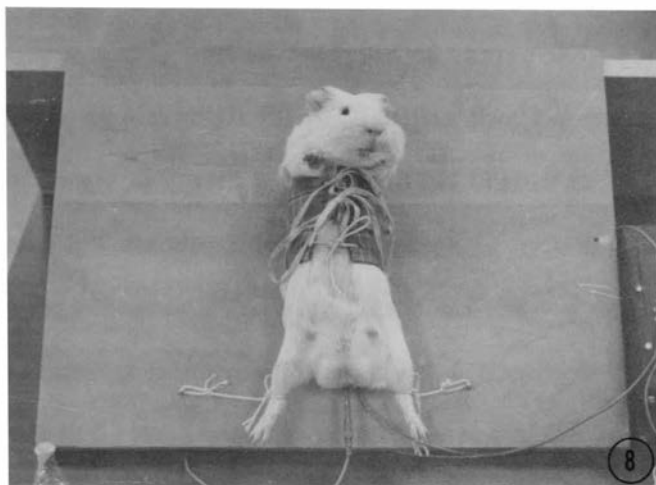
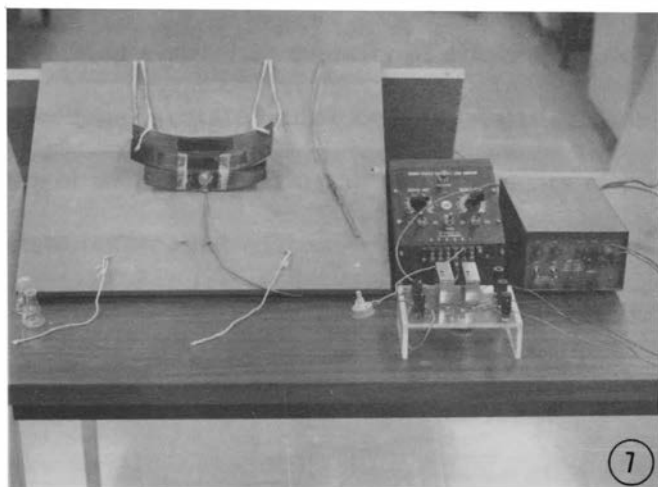


FIG. 7. Animal board and electroejaculation apparatus built by the author for the collection of the ejaculate from the guinea pig. Note the lumbar electrode in the harness, center of animal board, and the anal electrode on the board to its right.

FIG. 8. Guinea pig strapped to the animal board with the lumbar and anal electrodes of the electroejaculation apparatus in place.



### III. RESULTS AND CONCLUSIONS

#### Control

A qualitative analysis of gelatin membrane sensitivity was performed utilizing a specific quantity of a known proteinase dissolved in veronal acetate buffer on the gelatin membrane. A negative control was run utilizing an equal quantity of the same veronal acetate buffer, but without a proteinase. The details for the control were given previously in the section on materials and methods found on page 17.

#### Effect of In Vitro pH

If the mean areas of the zones of lysis for all four locations analysed (epididymis, vagina, uterus, and oviduct) are averaged for each of the pH values (4.5, 7.0, and 8.4) of the veronal acetate buffer which was used, and an analysis of variance is run on these data, then there is a significant probability that the mean area of the zone of lysis does vary with a change in pH (Table II, p. 42). This is true despite the standard deviation which is included in this analysis for significance. No significant difference was noted between the zones of lysis produced at pH 4.5 and pH 7.0 or between pH 7.0 and pH 8.4. The significant difference occurred between the areas produced by acrosomes subjected to the veronal acetate buffer at pH 4.5 and pH 8.4 with the greatest mean area being produced at the alkaline pH.

### Effect of Location of Sperm Removal

The probability that the site from which spermatozoa are extracted has an effect on the area of the zone of lysis is 0.0615 where significance is determined by  $P = 0.05$ . The closeness of this value to the range of significance suggests that there may be a difference in the quantity of active proteolytic enzymes present in the acrosome at different locations in the male and female reproductive tracts. Working under this assumption, the greatest quantity of active proteinases appears to be found in the acrosomes of sperm removed from the epididymis and from the uterus with those from the uterus exhibiting the largest mean area for the zone of lysis. Sperm removed from the vagina and the oviduct produced the smallest mean areas (Table I, p. 42).

However, the null hypothesis that no change in area does occur has not been disproven; and therefore a difference in enzyme activity according to location cannot be demonstrated under the conditions of this study.

### Effects of In Vitro pH and Location of Sperm Removal

The probability that the mean area of the zone of lysis will vary for sperm collected from each of the four different locations analysed when the mean area for each location is determined for each of the three pH variables used, a total of twelve mean areas in all, is insignificant (Tables III and IV, pp. 43-44). However, because the

probability is being determined for a statical interaction between both pH and location, the P value is considered to be close enough to the region of significance to lend some support to these data. With this understanding, the data will be analysed; but it must be remembered that the null hypothesis, that no change in the mean areas does occur with a change in both location and in vitro pH, has not been disproven.

The apparent change in mean area can best be understood if the mean areas for all the locations studied are compared for only one of the three pH values used in the experiment at a time (Table III, Fig. V, p. 43). In addition, a comparison may be made of the mean areas for all three pH values at each individual location (Table IV, Fig. V, p. 44).

pH 4.5. At pH 4.5 there appears to be no relative change in the mean area of the zone of lysis for any of the locations from which sperm were removed (Fig. V, and plate figs. 16, 23, 28, 29, 32, and 35).

pH 7.0. The mean areas at pH 7.0 appear to increase as the sperm progress through the male and female reproductive tracts until the oviduct is reached. The mean area apparently decreases in the oviduct after reaching its peak in the uterus (Fig. V, and plate figs. 17, 25, 26, 30, and 33).

pH 8.4. The changes in mean area at pH 8.4 are the most variable. In the epididymis, the mean area for the zone of lysis is

relatively greater than that observed at either pH 4.5 or pH 7.0.

There is then a pronounced decline in the vagina, followed by a peak in the uterus, and finally by a second decline in the oviduct (Fig. V, and plate figs. 18, 24, 27, 31, and 34).

Epididymis. In the epididymis, the mean areas appear to differ considerably depending upon the pH value of the veronal acetate buffer which was used to test the sperm. The greatest mean area was found to occur at pH 8.4 and the smallest at pH 4.5 (Fig. V, and plate figs. 16-18).

Vagina. The range of the mean areas decreases in the vagina with the areas for all three pH values being closely grouped together. At this location, the greatest mean area appears to occur at pH 7.0, but there is very little difference between this area and the area that results at pH 8.4 (Fig. V and plate figs. 23-27).

Uterus. The uterus appears to be the location at which the greatest range in mean areas for the zones of lysis occurs. It is at this location that the mean areas for both pH 7.0 and pH 8.4 reach their peak. Again, as in the epididymis, the largest mean area for the zones of lysis appears to coincide with pH 8.4 and the smallest with pH 4.5 (Fig. V, and plate figs. 28-31).

Oviduct. In the oviduct, the observations are very similar to those seen in the vagina. The range for the mean areas is relatively small, and at this location there appears to be a generalized

decrease for the mean areas at both pH 7.0 and pH 8.4 with little or no change at pH 4.5. It should be noted that the greatest mean area was observed at pH 7.0 and the least at pH 8.4 (Figs. 32-35).

#### Acrosomal Proteinases in Testicular and Ejaculated Sperm

Testis. A trial was conducted on one male guinea pig to determine if there was proteinase activity in the acrosome of testicular spermatozoa. Only sperm of normal morphology were included in the observations, but no attempt was made to determine their stage of maturity. As in the previous experiments, veronal acetate buffers of pH 4.5, 7.0, and 8.4 were used; and proteolytic enzyme activity was found to be present at all three of these pH values. The greatest mean area for the zone of lysis was computed for pH 8.4 (Table V, and plate Figs. 12-15).

Ejaculate. The ejaculated spermatozoa from three male guinea pigs was examined in either its natural physiological state or in veronal acetate buffer of pH 4.5, 7.0, or 8.4 on the gelatin membrane (Table V). Proteolytic enzyme activity was found to be present in each of the above cases (Figs. 19-22). When the sperm was tested in its natural physiological state, the mean area of the zone of lysis was found to be relatively small compared to those areas produced under the influence of the veronal acetate buffer. The greatest mean area was found to occur at pH 8.4 and the smallest at pH 4.5.

### Changes in Tail Morphology

On several occasions, the tail of the guinea pig sperm was observed to undergo a morphological change. This change which occurred only in sperm removed from the uterus and the oviduct, consisted of a segmentation of the tail in the region of the principal piece (Figs. 30, 34, and 36). It is of interest to note that, in each of the examples which are pictured in this text, the tail is divided into two major divisions with three or four smaller segments between them.

TABLE I

EFFECT OF THE LOCATION OF SPERM REMOVAL ON THE AREA OF THE ZONE OF LYSIS FOR SPERMATOZOA EXTRACTED FROM THE MALE AND FEMALE REPRODUCTIVE TRACTS OF THE GUINEA PIG AT pH 4.5, 7.0, AND 8.4 (P = 0.0615)

<u>LOCATION</u>	<u>NUMBER OF ANIMALS</u>	<u>MEAN AREA <math>\pm</math> STANDARD DEVIATION (<math>\times 10^{-5}</math> mm<sup>2</sup>)</u>	<u>RANGE OF MEAN AREAS (<math>\times 10^{-5}</math> mm<sup>2</sup>)</u>
EPIDIDYMIS	18	25.07 $\pm$ 15.13	10.75 - 61.98
VAGINA	11	24.10 $\pm$ 5.97	17.66 - 34.62
UTERUS	24	33.54 $\pm$ 23.55	7.85 - 88.10
OVIDUCT	16	20.10 $\pm$ 9.08	7.85 - 49.06

TABLE II

EFFECT OF IN VITRO pH ON THE AREA OF THE ZONE OF LYSIS FOR SPERMATOZOA EXTRACTED FROM THE EPIDIDYMIS, VAGINA, UTERUS, AND OVIDUCT OF THE GUINEA PIG (P = 0.0149)

<u>pH</u>	<u>NUMBER OF ANIMALS</u>	<u>MEAN AREA <math>\pm</math> STANDARD DEVIATION (<math>\times 10^{-5}</math> mm<sup>2</sup>)</u>	<u>RANGE OF MEAN AREAS (<math>\times 10^{-5}</math> mm<sup>2</sup>)</u>
4.5	20	18.54 $\pm$ 5.66	7.85 - 31.40
7.0	24	27.18 $\pm$ 14.79	14.10 - 70.65
8.4	25	32.80 $\pm$ 22.62	7.85 - 88.10

TABLE III

EFFECTS OF IN VITRO pH AND LOCATION OF SPERM REMOVAL ON THE AREA OF THE ZONE OF LYSIS FOR SPERMATOZOA EXTRACTED FROM THE MALE AND FEMALE REPRODUCTIVE TRACTS OF THE GUINEA PIG (P = 0.0945)

<u>pH</u>	<u>LOCATION</u>	<u>NUMBER OF ANIMALS</u>	<u>MEAN AREA <math>\pm</math> STANDARD DEVIATION (<math>\times 10^{-5}</math> mm<sup>2</sup>)</u>	<u>RANGE OF MEAN AREAS (<math>\times 10^{-5}</math> mm<sup>2</sup>)</u>
4.5	EPIDIDYMIS	6	17.53 $\pm$ 4.07	10.75 - 22.69
	VAGINA	3	20.63 $\pm$ 5.15	17.66 - 26.58
	UTERUS	6	18.65 $\pm$ 7.81	7.85 - 31.40
	OVIDUCT	5	18.34 $\pm$ 6.10	7.85 - 22.69
7.0	EPIDIDYMIS	6	21.25 $\pm$ 8.06	14.95 - 35.62
	VAGINA	3	26.25 $\pm$ 7.51	20.10 - 34.62
	UTERUS	9	32.68 $\pm$ 20.51	14.10 - 70.65
	OVIDUCT	6	25.34 $\pm$ 12.02	16.96 - 49.06
8.4	EPIDIDYMIS	6	36.43 $\pm$ 21.35	14.73 - 61.98
	VAGINA	5	24.88 $\pm$ 5.86	17.66 - 31.40
	UTERUS	9	44.33 $\pm$ 28.95	13.27 - 88.10
	OVIDUCT	5	15.59 $\pm$ 4.57	7.85 - 19.60

TABLE IV

EFFECTS OF LOCATION OF SPERM REMOVAL AND IN VITRO pH ON THE AREA OF THE ZONE OF LYSIS FOR SPERMATOOA EXTRACTED FROM THE MALE AND FEMALE REPRODUCTIVE TRACTS OF THE GUINEA PIG (P = 0.0945)

<u>LOCATION</u>	<u>pH</u>	<u>NUMBER OF ANIMALS</u>	<u>MEAN AREA <math>\pm</math> STANDARD DEVIATION (<math>\times 10^{-5}</math> mm<sup>2</sup>)</u>	<u>RANGE OF MEAN AREAS (<math>\times 10^{-5}</math> mm<sup>2</sup>)</u>
EPIDIDYMIS	4.5	6	17.53 $\pm$ 4.07	10.75 - 22.69
	7.0	6	21.25 $\pm$ 8.06	14.95 - 35.62
	8.4	6	36.43 $\pm$ 21.35	14.73 - 61.98
VAGINA	4.5	3	20.63 $\pm$ 5.15	17.66 - 26.58
	7.0	3	26.25 $\pm$ 7.51	20.10 - 34.62
	8.4	5	24.88 $\pm$ 5.86	17.66 - 31.40
UTERUS	4.5	6	18.65 $\pm$ 7.81	7.85 - 31.40
	7.0	9	32.68 $\pm$ 20.51	14.10 - 70.65
	8.4	9	44.33 $\pm$ 28.95	13.27 - 88.10
OVIDUCT	4.5	5	18.34 $\pm$ 6.10	7.85 - 22.69
	7.0	6	25.34 $\pm$ 12.02	16.96 - 49.06
	8.4	5	15.59 $\pm$ 4.57	7.85 - 19.60

TABLE V

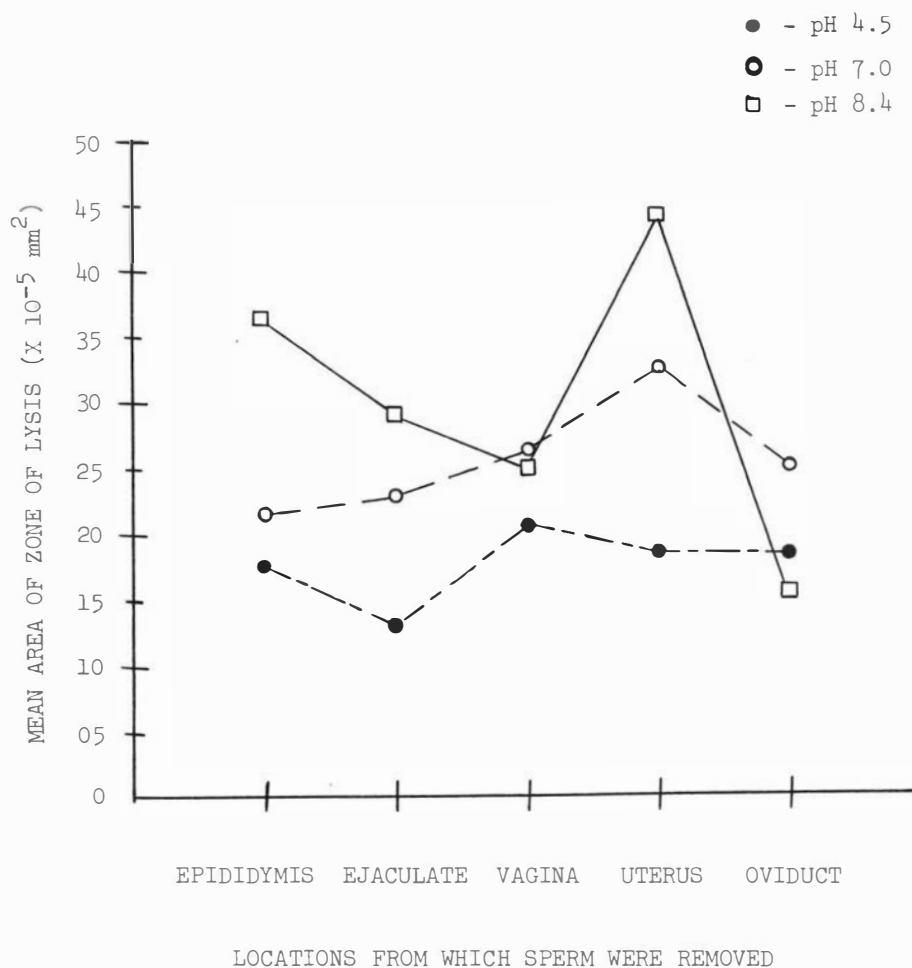
EFFECT OF IN VITRO pH ON THE AREA OF THE ZONE OF LYSIS FOR SPERM  
EXTRACTED FROM THE TESTIS AND EJACULATE OF THE MALE GUINEA PIG

<u>pH</u>	<u>LOCATION</u>	<u>NUMBER OF ANIMALS</u>	<u>MEAN AREA (<math>\times 10^{-5}</math> mm<sup>2</sup>)</u>	<u>RANGE (<math>\times 10^{-5}</math> mm<sup>2</sup>)</u>
4.5	TESTIS	1	15.90	7.85 - 31.40
	EJACULATE	1	12.98	7.85 - 17.66
7.0	TESTIS	1	10.68	7.85 - 17.66
	EJACULATE	1	23.13	7.85 - 70.65
8.4	TESTIS	1	25.59	7.85 - 49.06
	EJACULATE	1	29.25	7.85 - 70.65
NONE*	EJACULATE	2	15.52	7.85 - 31.40

\* The ejaculate was tested on the gelatin membrane in its natural state without the addition of buffer.

FIGURE V

EFFECTS OF IN VITRO pH AND LOCATION OF SPERM REMOVAL ON THE AREA OF THE ZONE OF LYSIS FOR SPERMATOZOA EXTRACTED FROM THE MALE AND FEMALE REPRODUCTIVE TRACTS OF THE GUINEA PIG



#### IV. DISCUSSION

This investigation has attempted to answer several questions concerning the proteolytic enzyme activity of the guinea pig acrosome. First, is acrosomal proteinase activity present in spermatozoa recovered from different locations of the male and female reproductive tracts?

Second, does proteinase activity differ at different pH levels which represent the acid, neutral, and basic ranges?

Third, does the location of the spermatozoa in the male or female reproductive tract have an affect on proteinase activity of the acrosome?

And finally, can both location and pH be shown to have a combined affect on proteolytic enzyme activity on the gelatin membrane?

In all segments of the male and female reproductive tracts and in the ejaculate, proteolytic enzyme activity was shown to be present in the acrosome (Figs. 12-37). In each case, both reactive and non-reactive spermatozoa were observed within the same microscope field on the gelatin membrane (Figs. 10, 11, and 25); and the proteinase activity or area of the zone of lysis for the reactive spermatozoa was found to vary (Figs. 16 and 17). These findings support the works of Owers (1971) and Gaddum and Blandau (1970) who first reported that epididymal spermatozoa of the guinea pig exhibit proteolytic activity on the gelatin membrane. It also lends

support to the work of Denker (1974) who used a modification of the gelatin membrane technique, which he called the proteinase substrate film test, to show proteinase activity in the acrosome of the rabbit ejaculate.

It is important to note that this is the first report that acrosomes of individual spermatozoa exhibit proteolytic enzyme activity in the testis, vagina, uterus, and oviduct. This finding supports the view that the proteolytic enzymes in the acrosome are intrinsic to the sperm and develop by differentiation at the cellular level.

#### Acrosomal Proteinase Activity and pH

If the sum total of proteinase activity for all four locations from which sperm were extracted (epididymis, vagina, uterus, and oviduct) is compared for each individual pH variable, a significant difference in proteolytic enzyme activity is noted between pH 4.5 and pH 8.4. The greatest mean area is observed at the more alkaline pH. This supports an observation made by Gaddum and Blandau (1970). This difference in activity on the gelatin membrane according to pH may very well mean that there is a greater quantity of proteinases in the guinea pig acrosome which have their pH optima in the alkaline range. This finding may have even greater significance in that proteolytic enzymes with pH optima in the acidic, neutral, and alkaline ranges have been discovered in the guinea pig by Owers (1971) and McDonald, Zeitman and Owers (unpublished). It is possible that

some of these enzymes may be activated at a particular pH, and therefore, the variable pH range of the female reproductive tract, acidic to slightly alkaline, might be one of the factors involved in the process of capacitation, a physiological change that the sperm must undergo before it is capable of penetrating the egg (Austin, 1952; Seitz, Brackett, and Mastroianni, 1973).

#### Acrosomal Proteinase Activity and Location

The author was unable to disprove the null hypothesis which states that no significant change in acrosomal proteinase activity occurs on the gelatin membrane for spermatozoa removed from different locations in the male and female reproductive tracts. This observation was based on the mean area of all three pH variables for each one of the four locations which were studied.

This observation has two possible implications. First, the probability that a change does exist is very close to the region of significance and thus, under different experimental conditions, a difference might very well be demonstrated. Due to combining all the pH variables together, any slight differences which might appear at different locations might be assumed to result from the presence or absence of enzymatic inhibitors which are unaffected by pH.

The second possibility is that no change does take place in the acrosomal proteinase activity according to location. This is the null hypothesis, and it may indicate that the enzymatic content of the

acrosomal package remains intact with no significant loss of enzymes as the mature spermatozoon passes through the male and female reproductive tracts.

#### Effect of pH and Location on Acrosomal Proteinase Activity

It is improbable that both pH and location have a combined effect on the acrosomal proteinase activity. The observed variability in proteolytic enzyme activity for any one of the three pH values used over the four locations studied is statistically insignificant. However, the pH value, because it is based on a statistical interaction, is considered to be close to the region of significance. Therefore, this researcher would like to give his impression or hypothesis as to what may be occurring in the hope that it may help future researchers.

In interpreting the data, an assumption is made that the mean area created by the proteinases is probably proportional to the quantity of enzymes at any particular locus.

Epididymis. Those proteolytic enzymes which have their optimal reactivity in an acidic environment (Barrett and Dingle, 1971) are probably the most stable because of the little variability in activity at different locations as demonstrated in Figure V. It has been reported by Cannon (1969) that human spermatozoa which are stored in the ampullae are inactive metabolically because of the acidic environment and diminished oxygen supply.

Those enzymes which have maximal reactivity in a neutral or alkaline range (Barrett and Dingle, 1971) are probably unaffected by the acidic environment of the epididymis and hence show greater proteinase activity when exposed to their respective pH optima.

Ejaculate. The seminal plasma of the ejaculate has remarkable effects upon the spermatozoa. To interpret the changes in acrosomal proteinase activity, it is necessary to take note of the various components of the seminal plasma and their effect.

The pH of the freshly ejaculated semen varies with the species. Hamner (1970) reported a range of anywhere from pH 6.1 - 7.0 in the dog and pH 7.0 - 7.2 in the cock, while Cannon (1969) stated that human semen was slightly alkaline at about pH 7.7. Because the pH of guinea pig semen has not been reported, it would be impossible to make any correlation according to pH.

Zaneveld, Srivastava and Williams (1970) demonstrated in the rabbit that trypsin-like enzyme, corona penetrating enzyme, and neuraminidase activities are high in epididymal sperm extracts and are decreased by contact with seminal plasma. They attributed this finding to the presence in the semen of a trypsin inhibitor and a decapacitation factor (DF), a substance which functionally reverses the capacitation process. The DF inhibits corona penetrating enzyme and sperm neuramidinase while the trypsin inhibitor inhibits the trypsin-like enzyme. This observation is supported by an earlier work (Chang, 1957) in which seminal plasma was found to be

detrimental to both motility and fertilizing capacity in the rabbit after capacitation in the female tract. This observation is important because a trypsin-like enzyme is one of the proteinases found in the guinea pig acrosome and according to Fritz, Trautschold, Haendle, and Werle (1968) there is a heat-stable polypeptide trypsin inhibitor in the seminal vesicles of guinea pigs. This is supported by another observation that a trypsin-like activity is high in epididymal spermatozoa, absent in ejaculated spermatozoa and again present in capacitated spermatozoa in the rabbits (Zaneveld, Srivastava, and Williams, 1969). Because this particular enzyme has been shown to have its optimal activity at pH 8.0 in both the guinea pig (McDonald, Zeitman, and Owers, unpublished) and in the rabbit (McRorie and Williams, 1974), this may very well explain the apparent drop in reactivity of the basic proteinases in the ejaculate and the vagina (Fig. V). The neutral proteolytic enzymes appear to be unaffected by inhibitors and their activity increases in both the ejaculate and vagina in a steady fashion. Both phenomena may be pH variable. Those enzymes in the acidic range appear to show a slight decrease in activity in the ejaculate with a light increase in the vagina. These changes appear to be within the limits of normal variability.

Vagina. Spermatozoa were found in the vagina even though it has been reported that the guinea pig ejaculates small amounts of gelled semen direct into the uterus (Vickery and Bennett, 1968;

Hamner, 1970; Hafez, 1973). This researcher finds this difficult to support because he has seen both spermatozoa and an ejaculation plug in the vagina. But this finding may be due either to reflux of spermatozoa or a miss by the animal. However, the acrosomal proteinase activity at all three pH values in this hostile environment is very closely grouped together and the changes seen with respect to the epididymis may merely be an extension of the effects of the seminal plasma.

Uterus. The uterus has long been considered as one of the sites of capacitation (Hamner, 1970). Dukelow and Chernoff (1969) reiterated a theory that the presence of DF in the seminal plasma of a species is construed as an indication of the need for capacitation of that species. Because it was hypothesised that DF was a glycoprotein coat surrounding the sperm head and that this must be removed during capacitation, he interpreted the ability of various laboratory animal uteri to remove tetracycline fluorescence from rhesus and human sperm as an indication of capacitation. The removal of this protective coat of glycoprotein and therefore, any inhibitors associated with it might very well account for the sudden upward surge in acrosomal proteinase activity in the uterus at pH 8.4 and 7.0. Again, those enzymes with their pH optima in the acidic range appear to be stable and relatively unaffected by the uterine environment.

Hamner and Sojka (1968) reported that rabbit uterine and oviductal secretions do not contain all the factors necessary for capacitation. They concluded that the factors needed to be bound to the uterine and oviduct epithelium, requiring the sperm to have intimate contact with the epithelium. Thus, the generalized peak in acrosomal proteinase activity in the uterus may be an indication of the first step in capacitation.

Oviduct. The oviduct plays many roles in the reproductive process. It has been reported, like the uterus, to be another site of capacitation (Hamner and Sojka, 1968; Brackett, 1969), and of course, the site of fertilization. Other authors feel that the principal means of capacitation here is via the follicular fluid of the egg (Edwards and Fowler, 1970; Yanagimachi, 1969; Barros and Austin, 1967). Bavister (1969) however, felt that tubal or follicular fluids may not capacitate spermatozoa directly, but simply create suitable environmental conditions for the spontaneous occurrence of this process. He also reported that fertilization could be achieved in the hamster over a pH range of 7.2 to 7.8 with the highest rate of fertilization at pH 7.6 to 7.8. Hamner and Fox (1969) reported that the oviduct of the rabbit had an alkaline pH while that of the human was neutral or slightly alkaline. Therefore, in the oviduct, we most probably have an alkaline environment (Bishop, 1969) in which capacitation is completed and fertilization occurs. How then can the sudden change in proteinase activity between the

uterus and oviduct be explained? It is very possible that the environmental pH plays its major role in the oviduct. After capacitation, the acrosomal proteinases should be free of inhibitors, pH labile, and capable of affecting fertilization. The changes in acrosomal proteinase activity seen at this location indicate an actual utilization or loss of these enzymes. Again, those enzymes with a pH optima at an acidic pH are the most stable and appear to be unaffected by the alkalinity of the oviduct. However, as pH increases, enzymatic reactivity appears to decrease with the most marked decrease being observed among alkaline groups of proteinases.

### Changes in Tail Morphology

The segmentation of the tail of the guinea pig sperm was observed only in those sperm removed from the uterus and oviduct. It is not known whether this is due to an in vitro effect or whether it is part of the normal physiological aging process for sperm in the female reproductive tract. It is, however, suspected that this may indeed be linked to the deterioration of sperm which have failed to fertilize the female's egg.

This observation may be similar to that reported by Joshi, Yaron and Lindner (1970). They found that the luminal fluid of the uterus of proestrous rats contained a non-dialyzable factor which on incubation with epididymal spermatozoa of rat or mice brought about separation of the head from the midpiece - tail segment; it did not,

however, decapitate epididymal spermatozoa of the bull, ram, or guinea pig. It was also discovered that sperm decapitation was inhibited by the secretions of the coagulating gland or the seminal vesicles and that ejaculated spermatozoa remained intact in the uterine lumen for at least 12 hours after mating. They concluded that the proteolytic activity of the uterine fluid was probably responsible for the sperm breakage effect.

### Significance of Results

If it can be presumed that the enzyme package of the acrosome has its function in the process of fertilization and therefore, needs to be preserved from the time of its origin until the time it reaches the egg, then no significant changes can be expected to occur from origin to destination. The large p values in the determination of data significance may be due to the influence of the following factors:

1. The passage of sperm through the male and female reproductive tracts is a biological system subject to the same degree of variability as any other biological system. Variability exists not only among the individual animals but also among the individual spermatozoa (Simeone and Young, 1931; Young, 1931; Laurence and Carpuk, 1963). There is no guarantee that all the animals, both male and female, are fertile or that all spermatozoa would contain the same enzymatic make up in terms of quantity of

enzymes, and the relative proportion of one enzyme to another.

2. There may be multiple as yet unknown enzyme inhibitors and activators which are present at various unknown sites; and even when modifying factors are known to be present, they are difficult to control. For example, Hamner (1970) recognized that the capacitating ability of the female reproductive tract is influenced by hormones: estrogen enhances it and progesterone inhibits it.
3. There is also the variable of time. It is possible that changes may occur in the enzymatic make up of the acrosome as the sperm ages.
4. There is also a distinct possibility that additional proteinase, not seen in the epididymis, may have been absorbed from seminal plasma and incorporated into or on the acrosome (Lundquist, 1953).
5. Finally, the gelatin membrane technique is sensitive enough for determining the presence of proteinase activity in a single cell but does not indicate the number of proteinases present or their relative concentrations.

## SUMMARY

The gelatin membrane technique was utilized to study proteinase activity in the acrosome of guinea pig spermatozoa. It was shown that the individual spermatozoa exhibit proteolytic enzymatic activity in the region of the acrosome when extracted from the testis, epididymis, ejaculate, vagina, uterus, and oviduct. This activity was found to vary with pH for these spermatozoa recovered from the epididymis, vagina, uterus, and oviduct. The greatest reactivity was found to be at pH 8.4 and the least at pH 4.5. There was no significant difference in reactivity between the different locations from which spermatozoa were removed and there also appeared to be no significant difference in reactivity for the different locations at different pH values from which sperm were removed.

It seems apparent that the proteinase package in the acrosome has no particular function during the course of its passage through the male and female reproductive tract but only at the time of encounter with the egg.

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## KEY TO TERMS

Ac - acrosome

cd - cellular debris

gmd - gelatin membrane defect

N - nucleus

t - tail

ZL - zone of lysis

## PLATES

FIG. 9. Guinea pig spermatozoon extracted from the oviduct of the female and tested on the gelatin membrane. Note the acrosome, nucleus, and tail. A zone of lysis may be observed surrounding the head region. (pH 8.4) 800X.

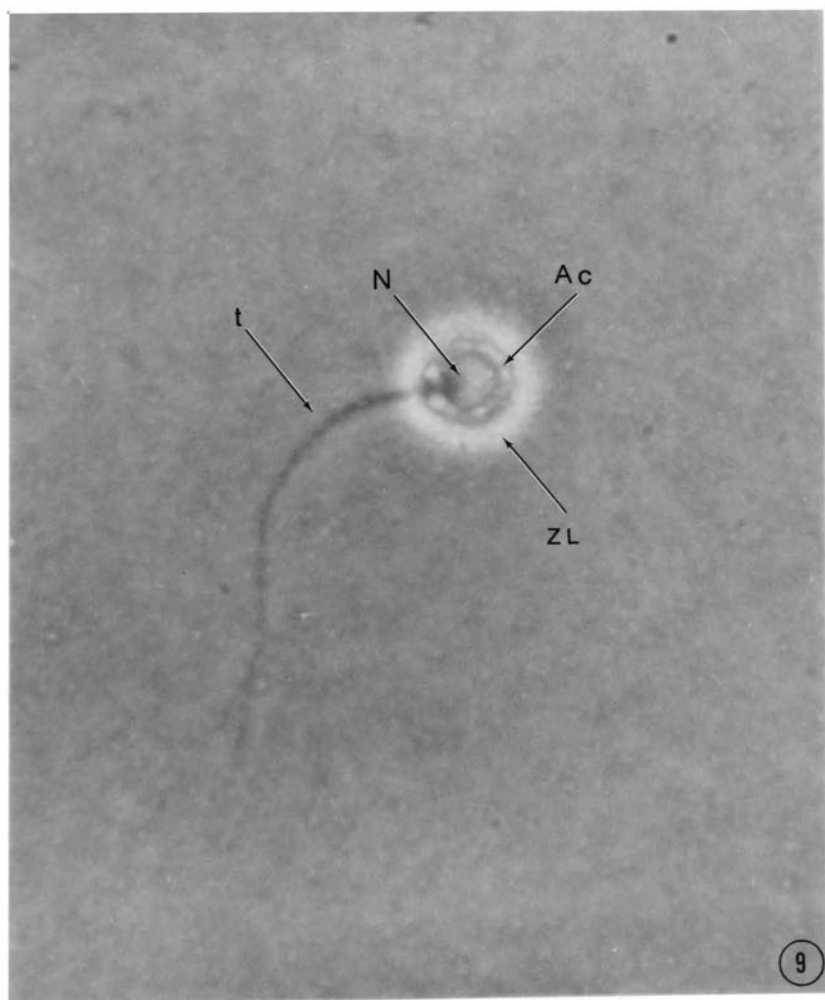


FIG. 10. Example of an enzymatically reactive spermatozoon, as demonstrated by the zone of lysis. 800X.

FIG. 11. Example of a spermatozoon showing no proteinase activity. 800X.

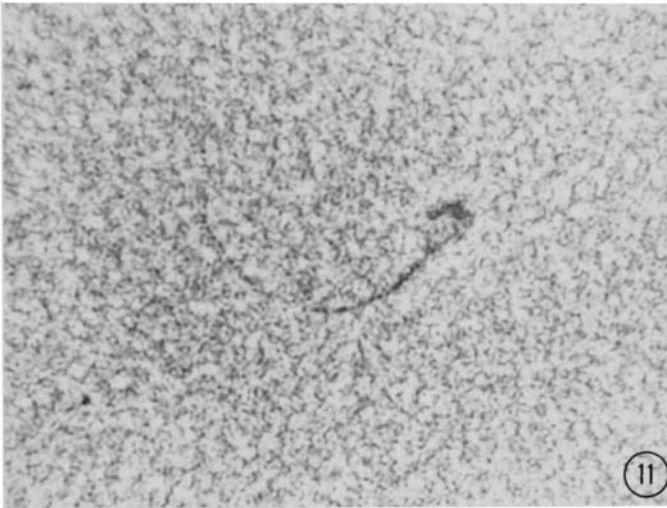
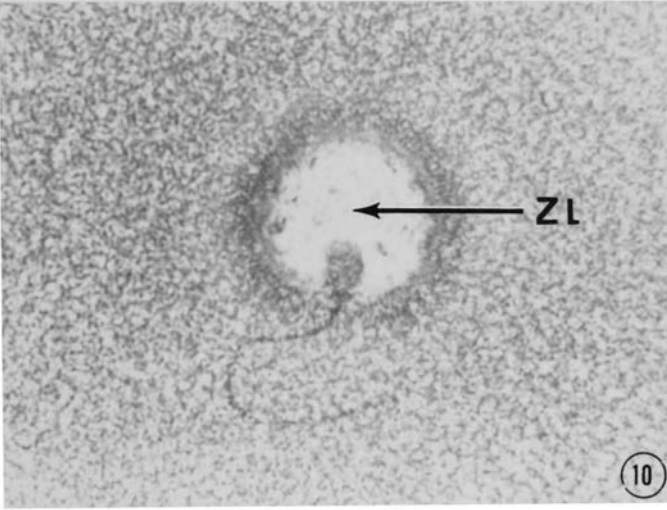


FIG. 12. A weekly reactive sperm (arrow) from the testis of the male guinea pig. (pH 4.5) 400X.

FIG. 13. The zone of lysis is well delineated for this spermatozoon removed from the testis. (pH 4.5) 400X.

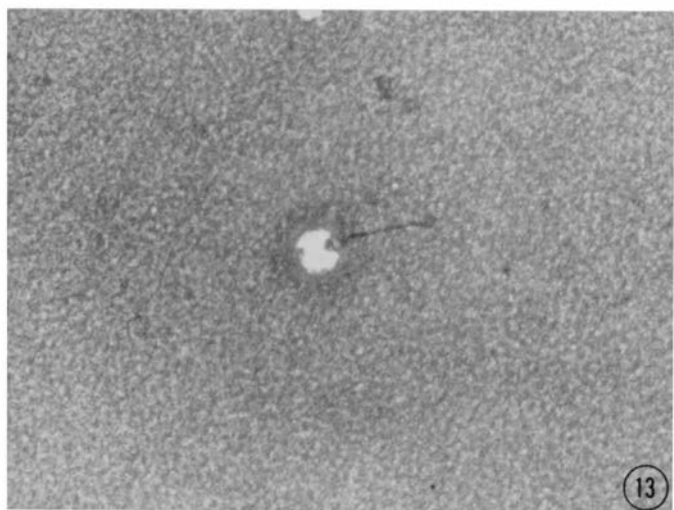
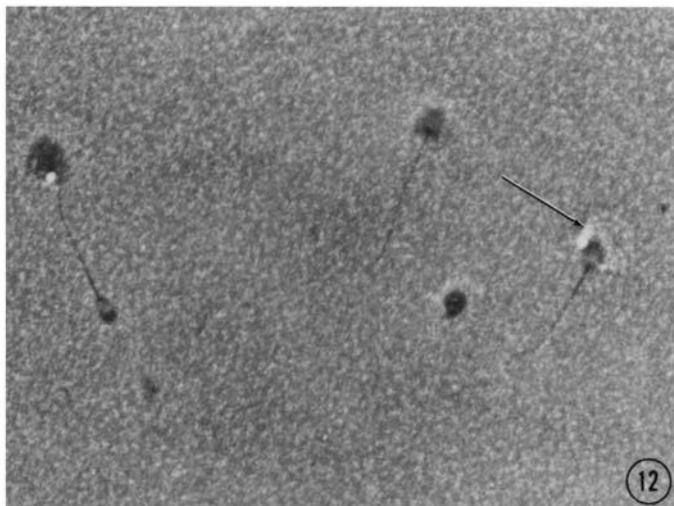


FIG. 14. These spermatozoa were removed from the testis and subjected to a neutral environment on the gelatin membrane. Although the activity appears to be minimal, the zone of lysis in the upper right hand corner is representative of the greatest activity observed at this pH for the testis. (pH 7.0) 400X.

FIG. 15. Testicular sperm tested at an alkaline pH exhibited the greatest overall reactivity. This observation is demonstrated by the zone of lysis for this spermatozoon. (pH 8.4) 400X.

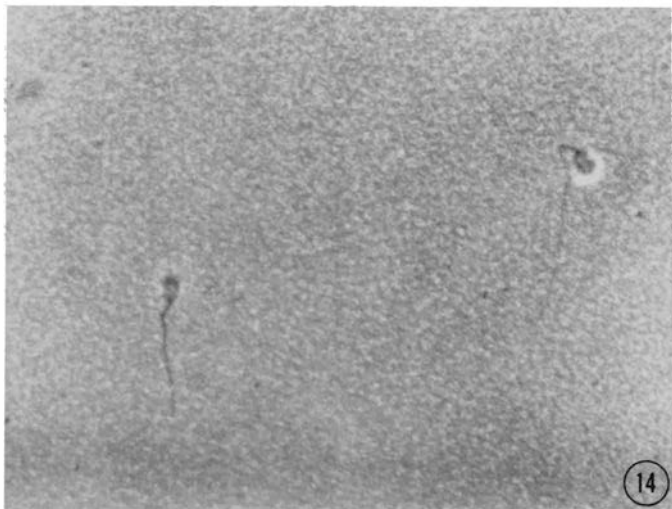


FIG. 16. Guinea pig spermatozoa extracted from the epididymis. Varying degrees of reactivity are present here. Whenever a zone of lysis was found to be the result of several sperm in combination, it was not included in the observations (arrows). (pH 4.5) 400X.

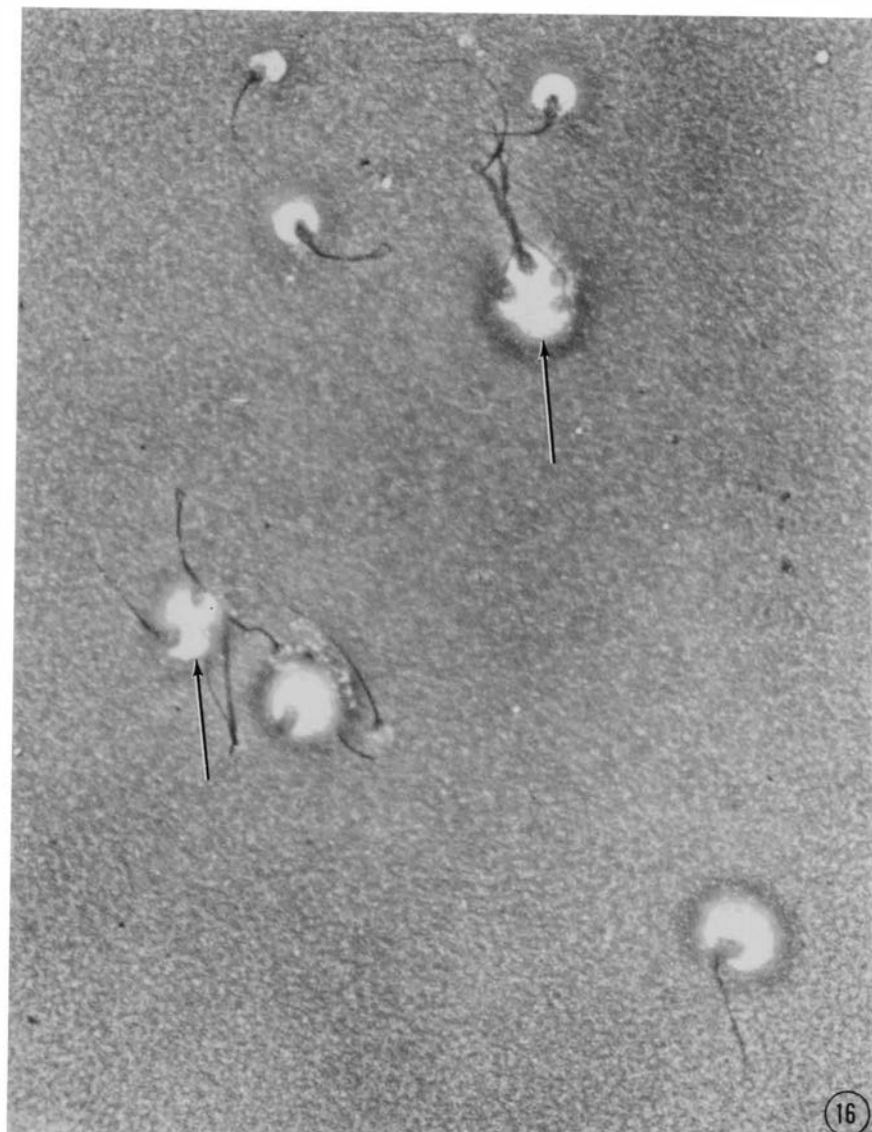


FIG. 17. The zones of lysis for these spermatozoa which were removed from the epididymis and tested at a neutral pH represent the spectrum of proteinase reactivity from the least reactive, far left, to the most reactive, far right. This type of wide distribution in reactivity was typical of most of the observations made in this thesis. (pH 7.0) 400X.

FIG. 18. The reactivity of epididymal spermatozoa was greatest when subjected to a basic veronal acetate buffer. (pH 8.4) 400X.

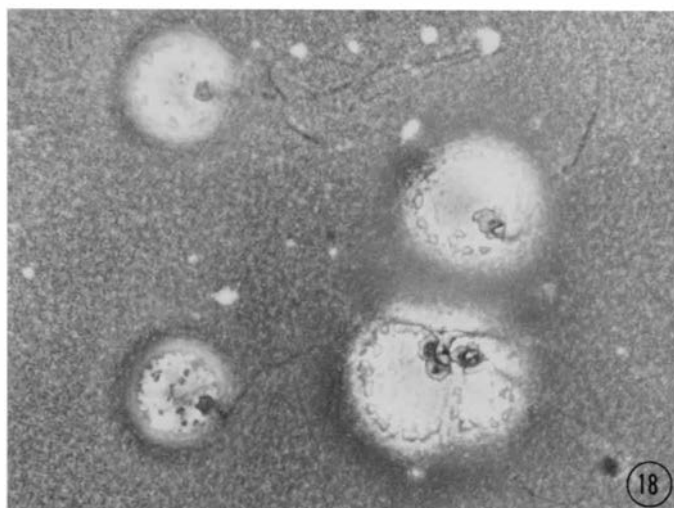
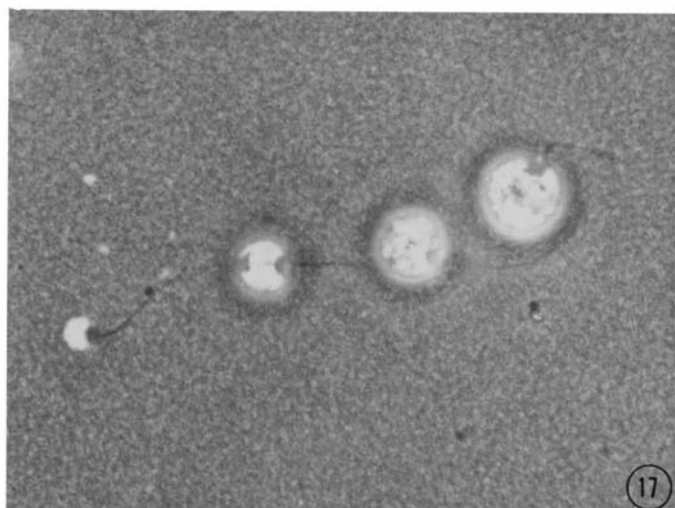


FIG. 19. Guinea pig spermatozoon from the ejaculate.

This amount of reactivity was typical for specimens tested at an acidic pH. (pH 4.5) 400X.

FIG. 20. The ejaculate was tested without the addition of pH adjusted buffers. The sperm pictured here are lysing the gelatin membrane even though they have been exposed only to the seminal plasma of the male guinea pig. 400X.

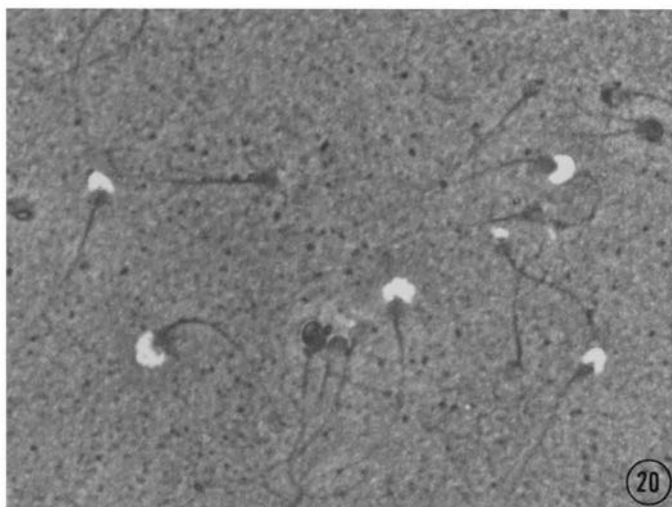
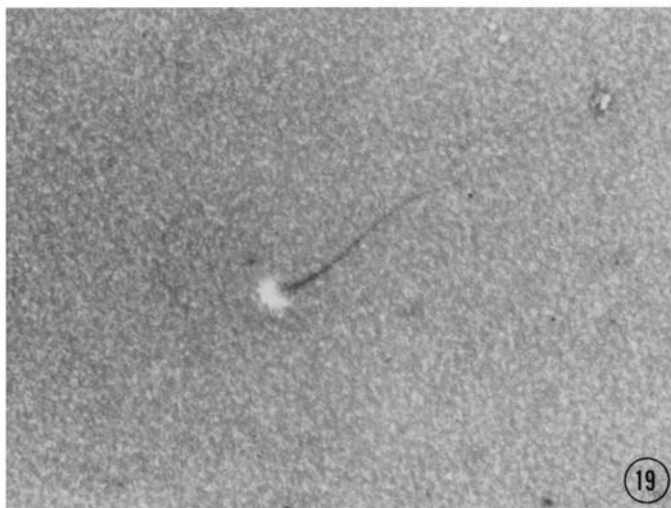


FIG. 21. Sperm from the ejaculate, when tested with neutral pH veronal acetate buffer, showed greater reactivity than ejaculated sperm to which no buffer was added. Note the gelatin membrane defect which is contiguous with a zone of lysis, to the right of the photograph. (pH 7.0) 400X.

FIG. 22. Guinea pig spermatozoon from the ejaculate of the male. (pH 8.4) 400X.

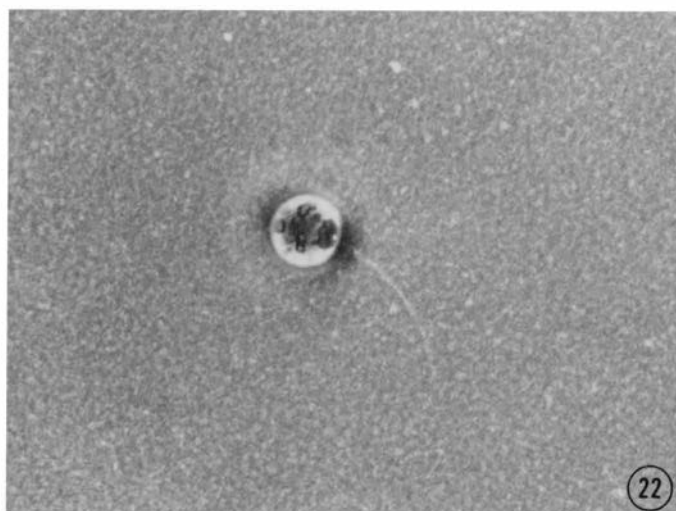
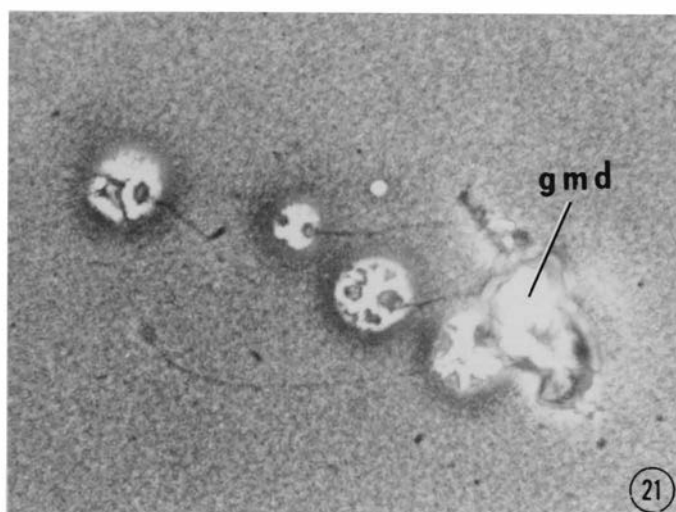


FIG. 23. These sperm were recovered from the vagina within 20 hours after mating. Note the multiple tails associated with the spermatozoon in the upper left hand corner. This may be due to stacking of the guinea pig sperm, and therefore was discounted as an observation. (pH 4.5) 400X.

FIG. 24. Guinea pig spermatozoon removed from the vagina and tested at a basic pH. (pH 8.4) 400X.

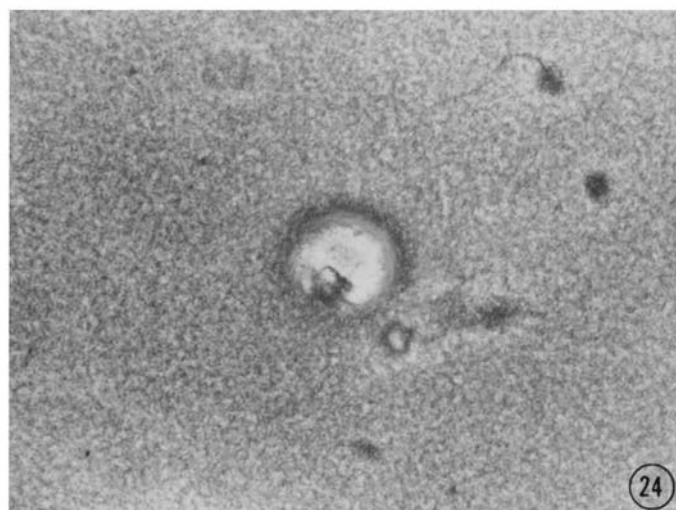
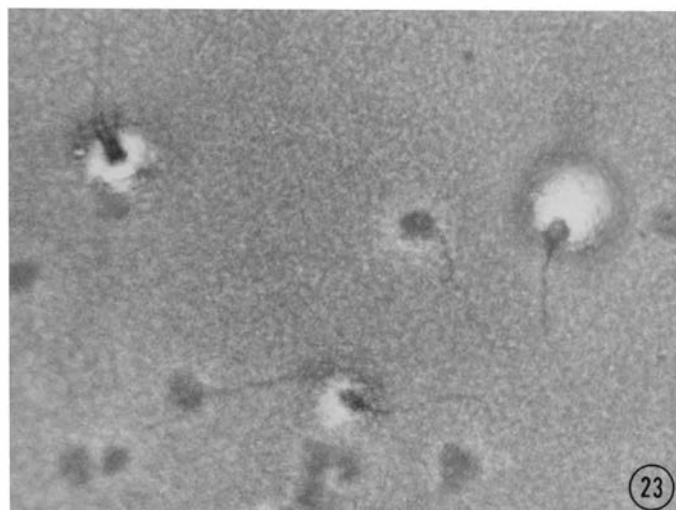


FIG. 25. Both reactive and non-reactive guinea pig spermatozoa were often observed in the same microscopic field of the gelatin membrane. These particular sperm were removed from the vagina and tested at a neutral pH. (pH 7.0) 400X.

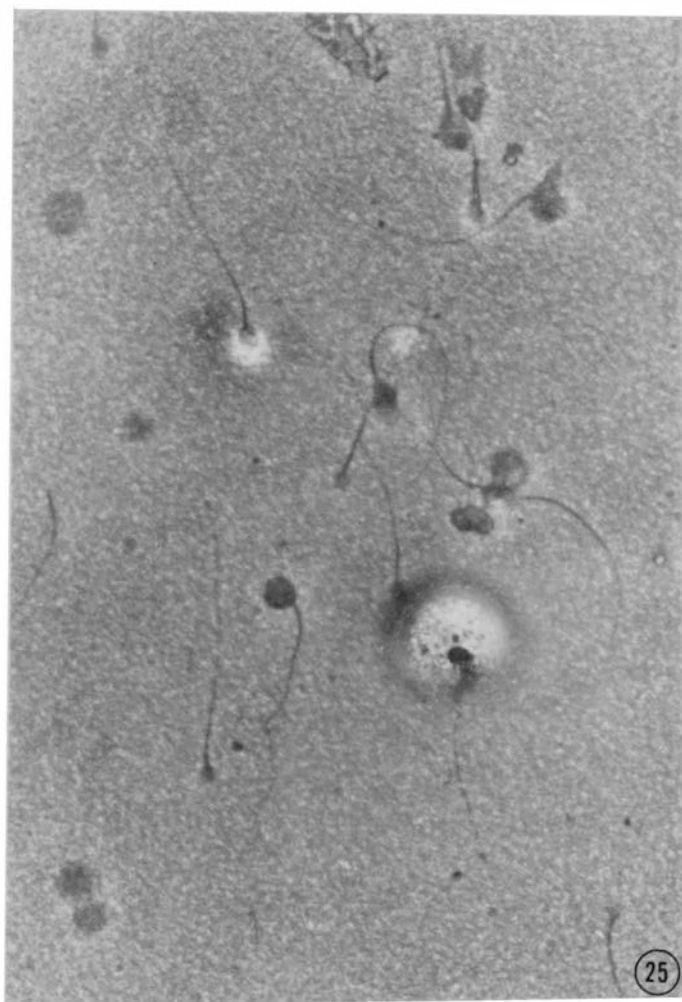


FIG. 26. The zone of lysis associated with this spermatozoon is representative of the greatest reactivity observed at a neutral pH for specimens removed from the vagina. Note the unidentified cellular debris in the field. (pH 7.0) 400X.

FIG. 27. Spermatozoa recovered from the vagina and tested at a basic pH showed variable degrees of reactivity. This guinea pig spermatozoon is representative of the maximal reactivity which was observed. (pH 8.4) 400X.

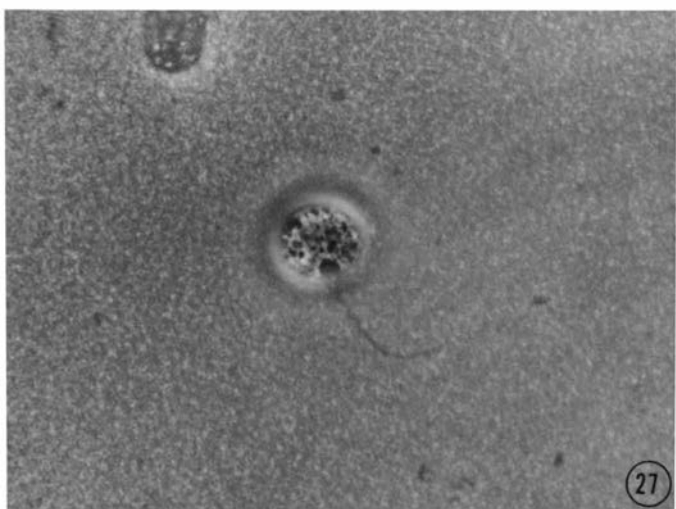
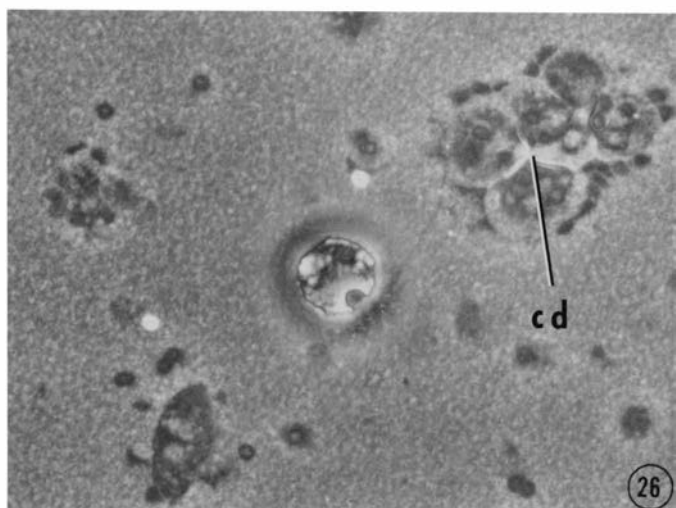


FIG. 28. Guinea pig spermatozoa recovered from the uterus 0 to 20 hours post coitus. (pH 4.5) 400X.

FIG. 29. The median reactivity observed in the majority of the experiments was the result of measuring zones of lysis similar to those shown here. These sperm were found in the uterus. (pH 4.5) 400X.

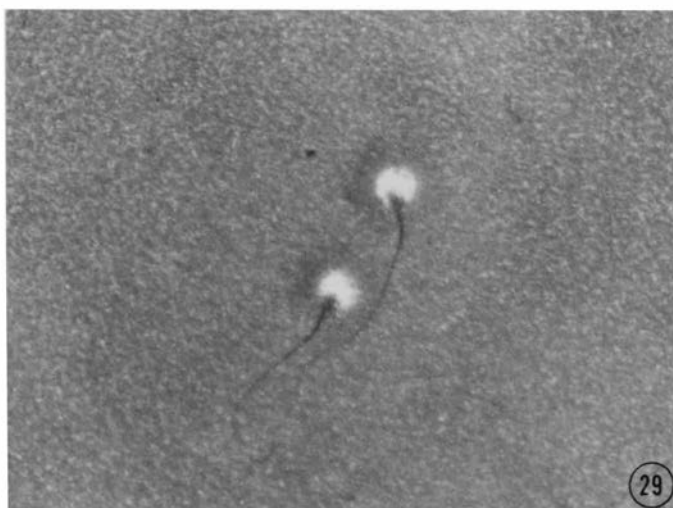
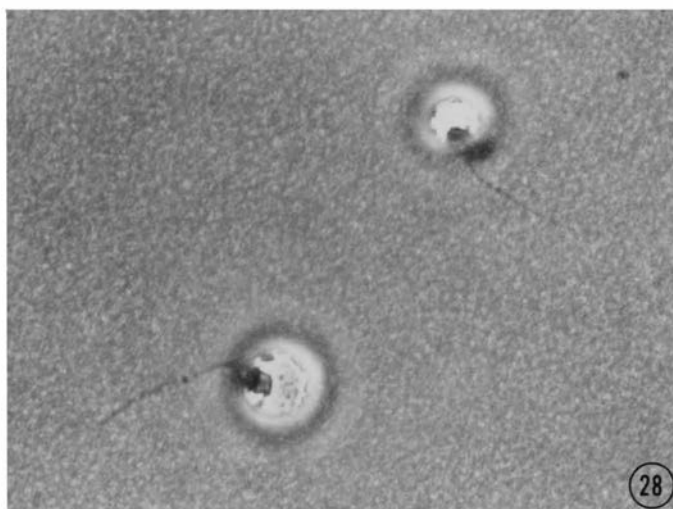


FIG. 30. Reactive spermatozoon from the uterus of the female guinea pig. Note the apparent dislocation of the tail from the nucleus and the segmentation (arrow) of the tail in the region of the principal piece. (pH 7.0) 400X.

FIG. 31. This rather large zone of lysis was produced by a spermatozoon recovered from the uterus. The tail, although faint, may be seen to project from the nucleus towards the upper left hand corner of the picture. (pH 8.4) 400X.

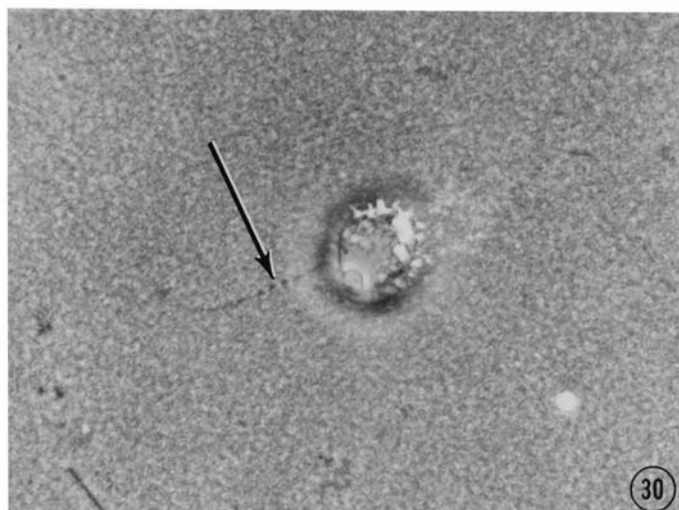


FIG. 32. Guinea pig spermatozoon removed from the oviduct.  
(pH 4.5) 400X.

FIG. 33. These spermatozoa were recovered from the oviduct  
of the female guinea pig. Note the relatively weak  
activity of the spermatozoon to the right when  
compared with that to the left (pH 7.0) 400X.

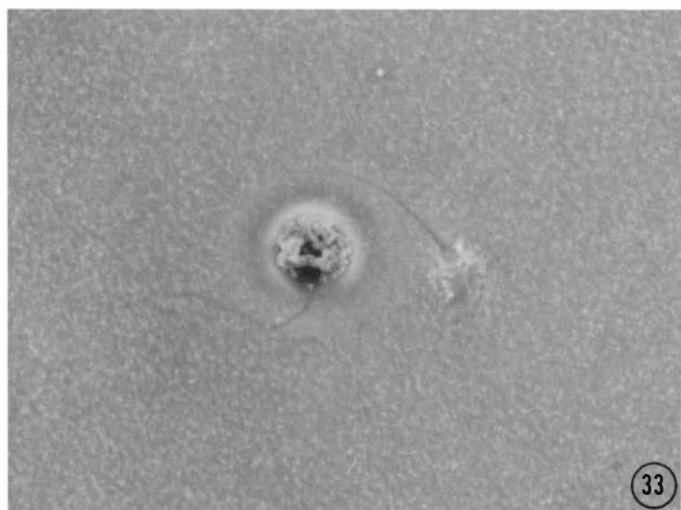
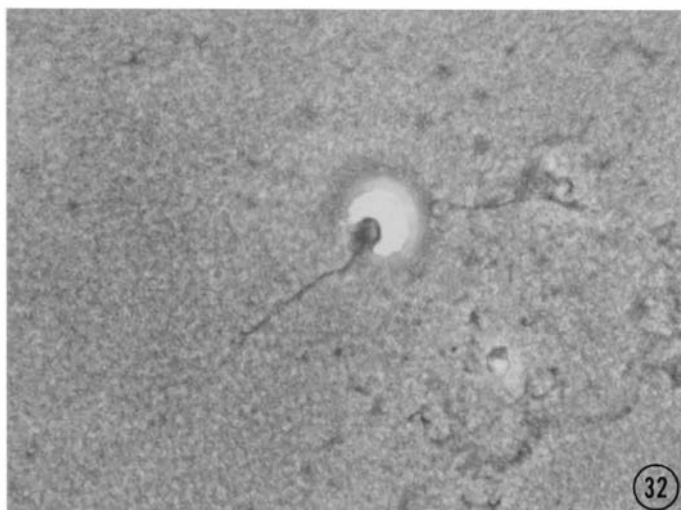


FIG. 34. This large zone of lysis was observed for a spermatozoon recovered from the oviduct and tested at a basic pH. Note the segmentation (arrow) of the tail. (pH 8.4) 400X.

FIG. 35. Guinea pig spermatozoon removed from the oviduct. (pH 4.5) 400X.

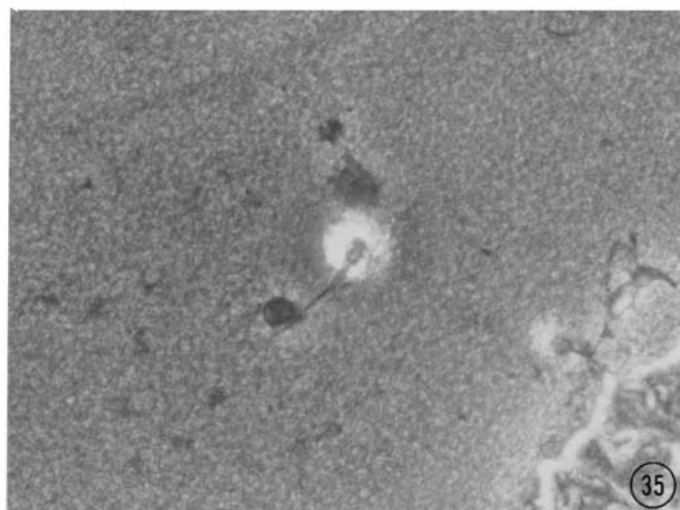
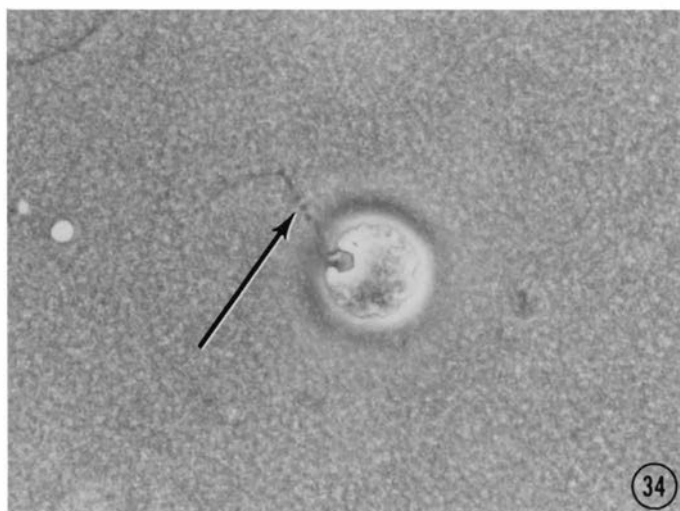


FIG. 36. Guinea pig spermatozoon recovered from the oviduct. Note the large zone of lysis and the segmentation (arrow) of the tail. (pH 7.0) 400X.

FIG. 37. Guinea pig spermatozoa recovered from the oviduct. Note the large difference in area between the zones of lysis. (pH 8.4) 400X.

