STUDIES OF THE MORPHOLOGY AND PROTEOLYTIC ACTIVITY OF BUSH BABY SPERM

A Thesis

Presented to

The Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirement for the Degree

Master of Science

---

by

PRANEET CHANMANON

December 1976

TO MY PARENTS

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ABSTRACT

A standard electroejaculation procedure was developed to collect semen from bush babies. The method involved a gradual increase in voltage administered by a bipolar rectal electrode from 0 to 8 v, with a stimulus duration of 1.7 sec and interval between stimuli of 0.4 sec. Ejaculation was sequential with a clear viscous seminal fluid preceeding a less viscous fluid containing some sperm, then a sperm-rich fraction. The average volume of sperm-rich fractions was 12.9μ l $\pm$ 6.13 S.D. with an average concentration of 2.0×10^8 sperm/mℓ $\pm$ 1.4 S.D.

Bush baby sperm are 40μ long with heads about 3.6μ long and 2.7μ wide. Their general morphology conforms to a pattern common to primates with two striking exceptions, (1) the anterior portion of the acrosome is folded ventrally forming a hood, and (2) an intricate membranous complex in the neck region which contains a discrete phospholipid body demonstrable by staining with malachite green.

The proteolytic activity of guinea-pig and bush baby sperm was investigated by using the gelatin membrane technique. Activity was detected as a clear digested area around the head of the sperm on a red background. The activity was quantitated as the percentage of sperm exhibiting gelatin digestion. The time course of the expression of activity was pH dependent and species specific. At optimum pH (8), the activity of guinea-pig sperm was detected as early as 5 min and reached maximum in 1 hr. Activity was first detected in bush baby sperm at 30 min and reached maximum at 6 hr. No activity was detected at pH 2 or 4, and at pH 6 activity was retarded.

Observations of sperm morphology revealed that only sperm with disrupted acrosomes exhibited proteolytic activity. The presence of 1 mg/mℓ soybean trypsin inhibitor or 3.81×10^{-2} M benzamidine hydrochloride completely inhibited the expression of activity although acrosome disruption occurred. Heat pretreatment of sperm at 70 C completely inhibited expression of enzyme activity but also prevented disruption of the acrosome.

There was no difference in the time course of expression of enzyme activity before and after incubation of sperm in capacitating media (guinea-pig, MCM, 2-1/2 — 3 hr; bush baby, heat pretreated blood serum, 8 hr). However, the percentage of sperm exhibiting activity decreased during these incubations. The decrease in activity was apparently related to a decrease in the percentage of live sperm, and in the guinea-pig, also to the occurrence of acrosome reactions.

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INTRODUCTION

Use of primates as laboratory animals in biological research generally requires special laboratory and animal care facilities. In 1963, Austin called attention to the potential usefulness of small primates, such as the bush baby (Galago senegalensis), as laboratory animals, both from the practical viewpoint of more simplified handling and care and in consideration of their closer relationship to man than laboratory rodents of equivalent size. The suitability of the bush baby for in vitro sutdies of fertilization was investigated by Keating (1975), who demonstrated that the species can be reasonably maintained and studied without special physical facilities.

The present studies represent investigation of the morphology and proteolytic activity of bush baby sperm and consist of (1) the development of a standard electroejaculation procedure, using a circuit modification in the power supply to permit selection and automatic control of the duration and interval between stimuli, (2) electron microscopic examination of bush baby sperm, primarily intended to clarify the form and spatial relationship of the acrosome and nucleus but because of a special structural characteristic of the neck region of bush baby sperm, extended to test for the presence of a malachite gree stainable phospholipid which may relate to the fertility of sperm (Cummins and Teichman, 1974), and (3) the detection and investigation of acrosomal proteolytic activity of bush baby sperm, using a modification of the Benitez-Bribiesca and Velazquez-Meza (1972) gelatin membrane technique. Guinea-pig sperm were used in this series of experiments

for comparative purposes and as procedural controls, since proteolylic activity of guinea-pig sperm has been demonstrated (Gaddum and Blandau, 1970) and the ability of guinea-pig sperm to undergo capacitation and acrosome reaction in defined medium has been clearly established (Barros, 1974).

REVIEW OF PERTINENT LITERATURE

Electroejaculation

The first successful electroejaculation procedure was developed by Gunn (1936) to obtain semen from rams. The procedure involved intermittent stimulation (10-40 v a.c.) between a metallic rectal electrode and a lumbar needle electrode. Consequently, muscular contractions were severe and the animals had to be restrained on their sides.

A bipolar rectal electrode consisting of 30 metallic-ring contacts, each separated by an ebony disc and alternately charged was used by Thibault and his colleagues (1948, cited by Dziuk et al., 1954a) to electroejaculate bulls. Stimuli were administered at 3-5 sec intervals and were manually regulated from 0-30 v, a.c. and back to zero.

Subsequent design modifications in the bipolar rectal electrode were of two main types, (1) a plastic probe with four longitudinal brass-strip electrodes, alternately charged (Marden, 1954), and (2) a plastic probe with six metallic-band contacts, alternately charged (Dziuk et al., 1954a). The latter was adapted for use with boars, goats, and sheep as well as bulls (Dziuk et al., 1954b), and a similar but smaller electrode was later developed to electro-ejaculate guinea-pigs, mice and rats (Scott and Dzuik, 1959).

Attempts to collect semen from laboratory primates were largely unsuccessful until Mastroianni and Manson (1963) obtained ejaculates from macaques (rhesus monkeys) by electrical stimulation (20-40 v, a.c., 10-20 cps) of restrained animals with electrodes placed at

the base and glans of the penis. This method is still preferred by several investigators since it does not require sedation of the subject. In 1965, Weisbroth and Young constructed a bipolar rectal electrode for use with macaques. The probe was constructed as a ½ in dia. plastic rod in which were mounted four copper-band contacts. The contacts were grouped within a span of 2 in at the insertion end, with contacts 1 and 3 wired as one pole and contacts 2 and 4 the other. The electrical stimuli were varied manually from 0 to 9.1 v.

Fussell et al. (1967) constructed four similar bipolar rectal electrode of different sizes ranging in diameter from $^{1}/_{8}$ to 1 in. The two smaller electrodes contained only two contact bands instead of four. The four sizes were found adequate to electroejaculate 14 different species of primates, ranging from a small primate (tree shrew) to a full-grown chimpanzee (Roussel and Austin, 1968). The power source consisted of a variable transformer in circuit with a 10:1 stepdown transformer. This permitted selection of any electrode voltage between zero and 11 v (60 cps, a.c.). A duplicate of this power source was used by Keating (1975) to stimulate ejaculation of bush babies. However, the smallest bipolar electrode ($^{1}/_{8}$ in dia.) was altered to decrease the distance between the two copper contacts from 1.0 to 0.5 cm. Ejaculation of bush babies was obtained by intermittent application of stimuli, initially 1-2 v then increasing to 5-7 v in a rhythmic manner. In all, 86 ejaculates were obtained from 15 males.

Morphology of Mammalian Sperm

The morphology of mammalian sperm has been studied extensively at the electron microscope level (see review by Fawcett, 1975), but with the exception of humans (e.g., see Anberg, 1957; Chrzanowski, 1966; Pedersen, 1969, 1972; Zamboni et al., 1971) few detailed or comprehensive fine structure studies of primate sperm have been published. Zamboni et al. (1971) compared several morphological details of human and macaque sperm, and Bedford (1974) described the general structural organization and differences among sperm in representative species of eight nonhuman primate families. As shown by Bedford, the main structural components of primate sperm conform to the pattern which is common to all eutherian mammals. These features can be seen in figures 5-7 and 10-12 of bush baby sperm, and are: (1) the head which is usually ovoid and flattened dorsoventrally, and which consists of an anterior cap-shaped acrosome and the highly condensed nucleus, (2) the neck, which contains the proximal centriole of the flagellum and segmented connecting piece. These lie in an implantation fossa at the base of the nucleus and join the base of the nucleus to the flagellum, (3) the midpiece which represents the anterior portion of the tail or flagellum, and is delineated by a sheath of mitochondria arranged in gyres, and (4) the principal piece of the tail. This region is distinguished by a complex fibrous sheath. A 9+2 microtubular axial filament extends through the mid and principal pieces, terminating as a relatively short end piece. In mammals, the axial filament is surrounded by nine course fibers which extend from the neck into the principal piece. The entire sperm is enveloped by a plasma membrane.

Among the primate species examined by Bedford (1974) sperm of various cercopithecids (Old World monkeys) were quite similar in form, but different from those of hominoids (Gibbon chimpanzee and man) in having a more flattened nucleus, a longer head and midpiece (more mitochondrial gyres). Sperm of the gibbon and chimpanzee were intermediate between the cercopithecids and man in these characteristics. Sperm of platyrrhine species (New World monkeys) were very similar in form to those of cercopithecids except for marked species differences in the number of mitochondrial gyres (three species, 29, 38 and 53 gyres; five cercopithecids, 38-43 gyres).

The greatest departure in sperm form was found in prosimians and was most pronounced in the galagos, including the bush baby (Galago senegalensis). Unlike sperm of other primate groups, the sperm head in galagos is asymmetric and the acrosome was initially described as hook-shaped. A second distinguishing feature of galago sperm is the concentration of membrane and tubular elements in the neck region (Bedford, 1967, 1974).

During condensation of the mammalian spermatid nucleus, a portion of the nuclear envelope ("redundant nuclear envelope", Fawcett, 1965) becomes extended or folded back from the base of the nucleus into the neck region (Franklin, 1968). In several species, the excess nuclear envelope forms one or two elaborate membranous scrolls or concentric circles of membrane (Fawcett, 1970). The most extreme development of this membranous complex found to date is in the bush baby (Bedford, 1967). In view of the amount of membrane and the presence of tubular structures in the system, Bedford (1974)

has suggested that it derives from the manchette (a spermatid structure rich in microtubules) and the membranous contents of the cytoplasmic droplet rather than from the redundant nuclear membrane.

Teichman et al. (1972) demonstrated the presence of a material in sperm of several mammals, which is stained by malachite green (also by pyronine B and pyronine Y) and is not preserved during fixation unless fixed with glutaraldehyde containing the stain(s). The stainable material was localized in the postacrosomal region of the sperm head, between the nuclear envelope and postacrosomal dense lamina in rabbits, hamsters, rats and mice, but in sperm of the mongoose and guinea-pig, the material was localized below the base of the nucleus and in the mongoose was associated with the membranous complex in the neck region (Teichman et al., 1972; Cummins and Teichman, 1974). The malachite green stained material in rabbit sperm was characterized as a phospholipid, predominantly a choline plasmalogen (Teichman et al., 1974), and was shown to accumulate in rabbit sperm during maturation in the epididymis. The stainable material was retained in live sperm incubated up to 24 hr in vitro in modified Hank's solution, but was no longer present in live sperm incubated 12 hr in an estrous uterus, i.e., was utilized or lost during capacitation (Cummins and Teichman, 1974). Whether or not the sperm phospholipids demonstrable with malachite green may function as an energy source (see Mann, 1967) or are functionally linked to capacitation is not known.

Acrosomal Proteolytic Activity

In mammals, sperm must undergo some physiological change while in the female reproductive tract to become capable of fertilizing an egg ("capacitation", Austin, 1952).

The study of acrosomal enzymes has in recent years become an active and interesting field in reproductive biology. A variety of hydrolytic enzymes have been identified as acrosomal by histochemical methods and through studies of acrosomal extracts. During capacitation and as fertilization begins, acrosomal enzymes of the sperm must become activated, exposed and/or released as needed to assist sperm passage through each egg investment (see review by McRorie and William, 1974).

The acrosomal enzyme, hyaluronidase, can disperse the outermost investment, the cumulus oophorus (see Austin, 1948). Zaneveld *et al.* (1968, 1969a) and Zaneveld and Williams (1970) reported a corona penetrating enzyme in extracts of rabbit sperm, which disperses the next investing layer, the corona radiata. The zona pellucida of mouse eggs is dissolved *in vitro* by mercaptans (Inoue and Wolf, 1974), and sulfated polysaccharides have been detected in the zona pellucida and between cells of the corona radiata (Wislocki *et al.*, 1947; Konecny, 1959; Harper, 1970; cited by McRorie and Williams, 1974). This suggests a possible role for the acrosomal enzyme, aryl sulfatase, in sperm passage through the corona radiata and zona pellucida (see discussion by McRorie and Williams, 1974). The identification of neuraminidase in acrosomal extracts (Srivastava *et al.*, 1970) is of interest in view of the prevelance of neuramic

acid residues in cell membrane glycoproteins, but there is not yet any substantial indication of neuraminidase function in fertilization.

The most extensively studied acrosomal enzyme is a trypsin-like proteolytic enzyme (Stambaugh and Buckley, 1968, 1969; Zaneveld et al., 1969b) or acrosin (Zaneveld et al., 1971). Properties of the enzyme are similar to those of pancreatic trypsin (Stambaugh and Buckley, 1968, 1969, 1972; Zaneveld et al., 1972a; Polakoski et al., 1973). The activity of enzyme is inhibited by soybean and ovomucoid trypsin inhibitors (Stambaugh et al., 1969) and by the synthetic trypsin inhibitor α-N-tosyl-L-lysine chloromethylketone (TLCK) but not by the chymotrypsin analog α-N-tosyl-L-phenylalanine chloromethylketone (TPCK) (Polakoski et al., 1972). Stambaugh and Smith (1974) reported that the amino acid content of rabbit acrosin is quite similar to human pancreatic trypsin, and that immunological cross reactions of rabbit anti-bovine pancreatic trypsin antiserum with acrosin from rabbits, bulls, rhesus monkeys and humans also occur. Although acrosin is trypsin-like, it differs from pancreatic trypsin in sensitivity to ovomucoid trypsin inhibitor (less sensitive), undergoes rapid autoproteolysis when in highly purified form and hydrolyzes benzoyl arginine ethyl ester (BAEE) twice as rapidly as tosyl arginine methyl ester (TAME), where as pancreatic trypsin hydrolyses TAME at a 20 fold greater rate than BAEE (Williams, 1972).

Acrosin has been extracted with the cation detergent Hyamine, and Triton-X 100 from the sperm of rabbit (Stambaugh and Buckley, 1969; Zaneveld et al., 1969b; Zaneveld and Polakoski, 1971; Zaneveld et al., 1972a), boar (Zaneveld and Polakoski, 1971;

Polakoski and McRorie, 1973; Schleuning and Fritz, 1973; Schleuning et al., 1973), ram (Srivastava et al., 1965; Zaneveld and Polakoski, 1971; Brown et al., 1975), Stallion, rat (Zaneveld and Polakoski, 1971), bull (Garner et al., 1971; Multamaki and Niemi, 1972; Garner, 1973), hamster (Zaneveld et al., 1973a; Meizel and Mukerji, 1976), mouse (Zaneveld and Polakoski, 1971; Brown and Hartree, 1976), and human (Stambaugh and Buckley, 1970a, b; Zaneveld et al., 1972b). Acrosin has also been detected in fowl (Ho and Meizel, 1970; Palmer and Howarth, 1973) and in an amphibian (Penn and Gledhill, 1972b). It is well established that the role of acrosin in fertilization involves hydrolytic action on the zona pellucida. Stambaugh and Buckley (1968) observed complete dissolution of the zona pellucida of rabbit ova incubated in Tyrode's solution containing rabbit acrosin, and Meizel and Mukerji (1976) reported complete removal of the zona pellucida of hamster eggs incubated with hamster acrosin. The percentage of rabbit eggs fertilized *in vitro* by capacitated sperm decreased in the presence of soybean or ovomucoid trypsin inhibitors (Stambaugh et al., 1969) and Zaneveld et al. (1970a) obtained a 50% decrease in fertilization of rabbit eggs *in vivo* when capacitated sperm were incubated with bovine pancreatic trypsin inhibitor before insemination.

Acrosin was extracted from rabbit epididymal sperm by Stambaugh and Buckley (1969). Zaneveld et al. (1969b) at first failed to find acrosin activity in extracts of ejaculated rabbit sperm, but upon further purification of the extract (DEAE column chromatography) found activity. In the same study, acrosin activity was

found in extracts of capacitated rabbit sperm. This suggested that a seminal plasma inhibitor is added to acrosin during ejaculation (Zaneveld et al., 1970b) and is removed during capacitation (Zaneveld et al., 1969b; Polakoski et al., 1971). An acrosin inhibitor has been reported in human seminal plasma (Suomin and Niemi, 1972; Zaneveld et al., 1972b). Zaneveld et al. (1973a) suggested that the inhibitor is present both in free form and complexed with acrosin. Polakoski et al. (1971) had earlier demonstrated that an acrosin-inhibitor complex is present in boar ejaculates and that it dissociates under acidic conditions. The same property was demonstrated for the human acrosin-inhibitor complex (Zaneveld et al., 1973b).

Recently, Meizel and his co-workers have demonstrated that most of the acrosin in rabbit testis and cauda epidiymal sperm is present as an inactive zymogen (proacrosin, Huang-Yang and Meizel, 1975). The rate of autoactiviation of proacrosin was dependent upon pH, the concentrations of the active and inactive forms, and was enhanced by Ca^{2+} and inhibited by Zn^{2+} (Meizel, 1972; Meizel and Huang-Yang, 1973; Huang-Yang and Meizel, 1975; Mukerji and Meizel, 1975a,b; Meizel and Mukerji, 1975). Polakoski (1974) presented preliminary evidence of the presence of proacrosin in ejaculated boar sperm, and Meizel and Mukerji (1976) have obtained proacrosin from cauda epididymal sperm of the hamster

Polakoski et al. (1972) reported the molecular weight of highly purified rabbit sperm acrosin to be 55,000 as determined by Sephadex Chromatography and 27,300 by SDS-polyacrylamide gel electrophoresis indicating the possible existence of monomeric and

dimeric forms. Stambaugh and Buckley (1968) and Stambaugh and Smith (1974) obtained similar values, but values obtained by Meizel and Mukerji (1975) were considerably higher, i.e., 73,000 and 38,000. The latter investigators obtained similar values (70,000 and 41,000) for hamster sperm acrosin, and have suggested that the discrepancies stem from differences in methods of enzyme purification and molecular weight determinations. Other reported molecular weight values are human, 76,000 (Gilboa et al., 1973), 30,000 (Zaneveld et al., 1972b), boar, 30,000 (Polakoski et al., 1973), 34,000, 37,000 and 38,000 (Schlening and Fritz, 1974).

Localizations of proteolytic activity in sperm have involved different approaches. Yanagimachi and Teichman (1972) used the silver proteinate method described by Takamatsu et al. (1963) to identify sites of proteolytic activity in sperm of the golden hamster, mouse and deer mouse, rat , dog, guinea-pig, rabbit, bull, human and cock. In each case the final reaction product (silver bromide) was associated with the acrosome cap with no indication of activity in the equatorial segment or other regions of the sperm.

A more specific approach to acrosin localization was used by Stambaugh and Buckley (1970c) who employed fluorescein labelled trypsin inhibitors (soybean and lima bean). After treatment, green fluorescence was reported in the acrosome of rabbit, rhesus monkey and sea urchin sperm. No differences were detected among epididymal, ejaculated and capacitated (uterine) rabbit sperm. Garner et al. (1975) used the indirect immunofluorescent technique to localize bovine acrosin with rabbit anti-bovine acrosin immunoglobulin.

According to the authors, the entire acrosomal region, including the equatorial segment, was labelled. Similarly, Schill et al. (1975) used the indirect immunofluorescent technique to localize acrosin in sperm of the boar, ram, goat, bull and human with rabbit anti-boar acrosin immunoglobulin. As in the report by Garner et al., the entire acrosomal region appeared to be labelled. Interestingly, there was no cross reaction of the boar acrosin antibodies with hamster or rabbit sperm.

Gaddum and Blandau (1970) introduced a gelatin membrane method for demonstrating proteolytic activity of individual sperm. Sperm were smeared over a thin layer of gelatin impregnated with India ink. Proteolytic activity was observed directly as a lightening of the gelatin around the sperm head caused by dispersion of India ink particles as hydrolysis occurred. Release of proteolytic enzyme(s) was demonstrated for ejaculated sperm of the rabbit and human, and epididymal sperm of the guinea-pig, rat, mouse and hamster. Although the method is relatively simple and direct, results were somewhat inconsistent and cytological details of the sperm were obscure. Penn et al. (1972) further simplified the gelatin method by substituting commercial photographic film (Kodak AR-10 autoradiographic film) as the gelatin substrate. The film was exposed and processed. Then suspensions of rabbit, rat, mouse and guinea-pig sperm were applied to the gelatin membranes, which were mounted on clean slides with a camel's hair brush. Proteolytic activity was indicated by dissolution of the emulsion around the sperm heads. Allen et al. (1974) similarly substituted photographic plates

(Kodak P300, exposed, processed and fixed with glutaraldehyde) as the the substrate and expanded the species examined to include boars and rams.

In a simple method introduced by Benitez-Bribiesca and Velazquez-Meza (1972), sperm suspensions (human and rat) were spread on clean slides, air dried and immersed once in a 5% gelatin solution prepared in veronal acetate buffer. After incubation, the slides were stained and at the same time the sperm were fixed in Ponceau staining solution. This procedure gave good preservation of cytological detail and revealed regions of proteolytic activity as clear halos in a red background.

The gelatin substrate method has shown that the mode and time course of release of proteolytic activity from sperm is pH and species dependent. The activity of testicular sperm was found to be appreciably less than that of epididymal and ejaculated sperm (Penn et al., 1972; Allen et al., 1974). The expected difference in activity of epididymal and ejaculated rabbit sperm owing to the presence of an acrosin inhibitor in seminal plasma was not detected by this method (Penn, 1975), nor was any marked difference found between the activity of ejaculated and uterus incubated (capacitated) sperm of boars and rabbits (Landa, 1975).

MATERIALS AND METHODS

Electroejaculation of the Bush Baby

Ejaculates were obtained from adult males by the rectal electrode method of electrical stimulation described by Fussell et al. (1967). The electrode used was described by Keating (1975) and consisted of a $^1/_8$ in dia. plastic rod with two copper-band contacts separated by a distance of 0.5 cm. The power supply, described in detail by Fussell et al. (1967) was modified to permit selection and automatic control of the duration and interval between stimuli (Figure 4). The calibration of the power supply was performed with a Tektronix Oscilloscope Model 561D, Type 3A72, dual trace amplifier Type 354. The character of the stimulus was observed and photographed with a Tektronix Oscilloscope Camera Model C12 with polaroid land pack film type 107 (Figure 2).

The animals received no water during 24 hr preceding electroejaculation to minimize risks of urine contamination of the semen sample. The animals were anesthetized before proceeding by intraperitoneal injection of 0.5 mℓ Nembutal (Abbot Laboratory) previously diluted with normal saline to a concentration of 10 mg/mℓ. If necessary, the anesthetized state was maintained by inhalation of Metofane (Pittman-Moore). The rectal electrode was sterilized with 95% ethanol, dried, then coated with H-R water soluble jelly (Holland Rantos Company) as a lubricant, and inserted to a depth of about 0.5 cm beyond the copper contacts. The duration and interval between stimuli was selected and set for automatic control.

While the electrode was maintained in proper position with one hand, the stimulus voltage was slowly increased from zero to the desired trial voltage. If more than one trial was conducted, the animal was allowed to rest a minimum of 15 min between each 20 min trial period.

Ejaculates were collected from the glans with an Eppendorff pipette (5 µℓ or 10 µℓ tips to estimate volume), placed under mineral oil in a Falcon #3030 culture dish, and diluted with a known volume of normal saline. A small sample was withdrawn for assessment of sperm concentration and motility with a phase contrast microscope.

Preparation of Bush Baby Sperm for Morphological Studies

Samples of bush baby sperm obtained by electroejaculation were suspended in 10 times their volume of 0.9% normal saline, and were washed gently by centrifugation (MSE minor centrifuge, GT-2, 5 min, approx. 600 rpm). Sperm damage was assessed with a phase contrast microscope, and undamaged washed sperm samples were fixed 2 hr in Karnovsky's (1965) fixative modified to consist of 2.0% glutaraldehyde, 2.0% paraformaldehyde and 0.05% calcium chloride in 0.1 M sodium cacocylate buffer (pH 7.4). Fixed samples were washed in two changes of 0.2 M buffer and divided into two portions, for transmission and scanning electron microscopy.

For transmission microscopy, samples were post-fixed 1 hr in 1.0% osmium tetroxide in 0.1 M buffer, rinsed once in 0.2 M buffer and dehydrated in a graded series of acetone solutions (30% to

100%). Dehydrated specimens were infiltrated overnight with an equal volume mixture of acetone and Spurr's (1969) epoxy resin embedding medium. The samples were then transferred to pure embedding medium and polymerized (70C, 8 hr). Thin sections were cut with an LKB Ultrotome, recovered on 300 mesh copper grids and stained sequentially with aqueous uranyl acetate (Frasca and Parks,1965) and lead citrate (Reynolds, 1963). Specimens were examined and photographed with a Hitachi HS-8F-2 electron microscope.

For scanning microscopy, fixed samples were dehydrated in a graded series of acetone solutions and stored in 100% acetone. A small drop of sperm suspension in acetone was deposited on the surface of an aluminium specimen stub and allowed to dry. The specimens were then coated with gold in a vacuum evaporator and examined and photographed in a Cambridge S4-10 scanning electron microscope.

To test for the presence of malachite green stainable material in bush baby sperm, electroejaculated sperm were washed as previously described and fixed 3 hr in modified Karnovsky fixative buffered at pH 6.8 and containing 0.1% malachite green (Matheson Coleman & Bell). Control samples were fixed in an identical manner but without malachite green. After fixation specimens were washed in several changes of 0.2 M buffer (pH 6.8) and were post-fixed in 1.0% osmium tetroxide in 0.1 M buffer (pH 6.8), then processed as described for transmission electron microscopy.

Gelatin Membrane Procedure for Detection of Proteolytic Activity

Guinea-pig sperm were obtained from lacerations of the cauda epididymis and vas deferens. The sperm were allowed to disperse in Ca^{2+} free minimum capacitation medium (CF-MCM, Barros, 1974). Bush baby sperm were collected by electroejaculation as previously described and were suspended in Ham's F-10 cell culture medium (GIBCO). Sperm suspensions were washed twice by centrifugation (MSE minor centrifuge, GT-2, 5 min, approx. 600 rpm) and were resuspended in original media to a final concentration of 5×10^7 sperm/mℓ. The percentage of live-dead sperm was determined with a phase contrast microscope (50 counted/sample).

The gelatin membrane technique was modified from the procedure described by Benitez-Bribiesca and Velazquez-Meza (1972). Gelatin solutions (5.0%) were prepared at 40 C in veronal acetate buffers of different pH values ranging from 2.0 to 10.5. Gelatin solution (10 µℓ) was placed on a clean microscope slide, then 5 µℓ of a sperm suspension was added and dispersed in the gelatin by brief, vigorous agitation of the slide. The mixture was smeared in the same manner as a blood cell sample, and incubated in a humid chamber at room temperature for a specified time. After incubation, the slides were fixed and stained 2 min in a Ponceau S staining solution (10 mℓ of 1.0% Ponceau S, 1 mℓ glacial acetic acid, 89 mℓ deionized water). The stained slides were air dried and examined with a phase contrast microscope. Proteolytic activity was detected as a clear digested area on a red background. Percentage counts of proteolytic activity

were made for sperm populations at different time intervals and pH's, and the optimum pH was determined. The morphological state of the acrosome was carefully examined and correlated to enzyme activity.

To explore the effect of trypsin inhibitors on the expression of sperm proteolytic activity, soybean trypsin inhibitor (SBTI, SIGMA Chem. Co.), and benzamidine hydrochloride (Eastman Kodak Co.) were added to gelatin solutions buffered at optimum pH (8.0) in concentrations of 1.0 and 0.5 mg/mℓ (SBTI), and 1.27×10^{-2}M and 3.81×10^{-2}M (benzamidine HCℓ). Control slides lacking inhibitor were also prepared at pH 8.0. In these experiments, slide incubation time was 24 hr.

To test whether sperm motility is necessary for expression of enzyme activity, sperm were preheated in a water bath for 2 min at 50, 60 or 70 C. Heat inactivation of motility did not cause rupture or detachment of acrosomes. In these experiments, slides were prepared at pH 8.0 and incubated 24 hr.

The proteolytic activity of sperm incubated under conditions which support capacitation was compared with untreated samples. Samples (50 µℓ) of guinea pig sperm, twice washed and resuspended to a concentration of about 10^8 sperm/mℓ in CF-MCM (which does not support capacitation or acrosome reaction) were introduced into 450 µℓ of minimum capacitation medium (MCM) in Falcon #2095 culture tubes which were then sealed and incubated 2-1/2 — 3 hr at 37 C to effect capacitation and acrosome reaction. After incubation, the sperm were centrifuged and resuspended to a concentration of

5.0×10^7 mℓ and the percentages of live-dead and live acrosome reacted sperm were determined (50 counted/sample). Sperm smears were prepared in gelatin (pH 8.0) as previously described. Un-incubated controls were prepared by dilution of the original 10^8 sperm/mℓ samples to a concentration of 5.0×10^7 sperm/mℓ.

Ejaculated bush baby sperm were washed once by centrifugation in Ham's F-10 and innoculated into 50 µℓ of heat pretreated (60 C, 1 hr) human blood serum under mineral oil in Falcon #3001 culture dishes. Final sperm concentrations were adjusted to about 2.0×10^7/mℓ and the suspensions were incubated 8 hr at 37 C to support capacitation (Keating, 1975). After incubation, the sperm were washed by centrifugation and resuspended in Ham's F-10 to a concentration of about 5.0×10^7/mℓ. The percentage of live-dead sperm were determined, but no acrosome reactions (detachment, see Discussion) were detected. Smears were prepared in gelatin (pH 8.0) as previously described. Control slides were prepared before incubation from the original washed sperm suspension. The slides were incubated 24 hr.

RESULTS

Electroejaculation of the Bush Baby

Proper positioning of the bipolar rectal electrode was recognized as an important requirement in earlier development of ejaculation procedures for the bush baby (Keating and Darney, unpublished) and proved to be of paramount importance in development of the procedure used in this study. If positioning resulted in stimulation of the sciatic nerves to extend the legs, ejaculation did not occur. Proper positioning was indicated by flexure of the legs toward the abdomen together with contraction of the scrotum. Full erection did not usually precede ejaculation but instead was found to indicate too deep positioning of the electrode, and generally resulted in urination. Ejaculations were obtained after moderate erection in company with the muscular contractions mentioned previously.

The optimal electrode potential, duration of stimulus and interval between stimuli were determined empirically over a period of several months. Ejaculation attempts with any particular animal were not permitted at intervals less than two weeks because of the attendant anesthesia and non-expendable nature of the animal. Stimuli of 1.7 sec duration and an interval of 0.4 sec between stimuli were found to produce satisfactory results. The electrical stimulation was gradually increased from 0 to 8 v in the most successful trials (30 ejaculates in 38 trials). If the potential were maintained in the lower range (2-5 v), ejaculation did not occur (one exceptional instance, male No. 10, 2.0 v, ejaculated at 30 min). Electrode potentials greater than 14 v caused

TABLE I

CHARACTERISTICS OF SPERM-RICH FRACTIONS OBTAINED WITH ELECTRODE POTENTIAL 8.0 v, STIMULATIONS 1,7 SEC AT 0,4 SEC INTERVALS

Animal	Trial No.	Semen Volume (μℓ)	Sperm Concentration/mℓ	Estimated Percent Motile
3	1	10	4.1×10^7	85
	2	15	3.4×10^7	70
9	3	5	2.4×10^8	85
	4	15	2.6×10^8	85
	5	15	2.0×10^8	80
10	6	25	1.6×10^8	80
11	7	10	1.9×10^8	85
	8	8	4.8×10^8	80
Means	12.9 ± 6.13 S.D.		$2.0 \times 10^8 \pm 1.40$ S.D.	81.3 ± 5.18 S.D.

severe muscular contractions and in some instances, urination, but no ejaculations.

Ejaculation was generally observed as a three stage sequential discharge beginning with a clear, viscous seminal fluid, then with continuing stimulation, additional seminal fluid containing a few sperm and finally a milky white droplet of highly concentrated sperm. During the studies 38 electroejaculates were obtained from 11 different males in 52 attempts. Properties of some typical electroejaculates are presented in Table I. Ejaculates which contained fewer than 3.0×10^7 sperm/mℓ or with less than 70% progressively motile sperm were not used in the studies.

The viscosity of the seminal fluid varied, with the portion first ejaculated being most viscous. When sperm were occasionally present in the viscous portion they seemed immotile but became motile 10-15 min after addition of normal saline. When present in the less viscous portion of the ejaculate, as was the usual case, sperm exhibited a vigorous progressive motility. The firm coagulum which usually forms in ejaculates of primate species (Roussel and Austin, 1968) did not form in bush baby ejaculates. Irregularly shaped secretory granules (Fig. 3) were often present in the ejaculates.

Morphology of Bush Baby Sperm

Bush baby sperm are relatively small. They are about 40μ long with sperm heads about 3.6μ long by 2.7μ wide. In lateral views, the acrosome of the sperm head appears hook-shaped (Fig. 5), but when viewed dorsoventrally (Fig. 6) the head appears more ovoid.

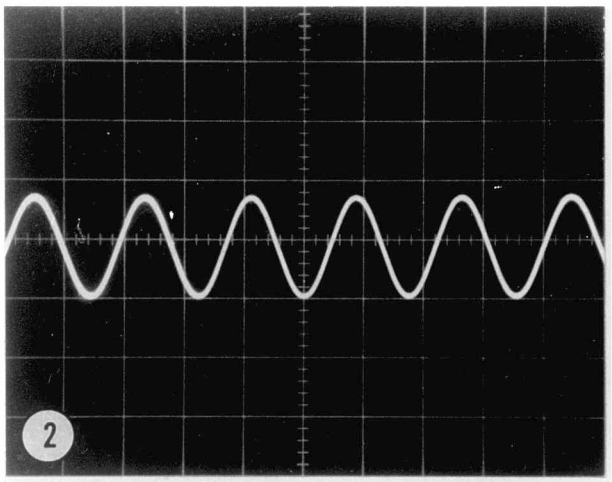
Figure 1. Bush Baby (Galago senegalensis), adult female and juvenile offspring.


1

Figure 2. The sine wave pattern of the electrical stimulus produced by the electroejaculator power supply. 60 cps, 8v.

Figure 3. Phase contrast micrograph of globular bodies usually found in the ejaculates. The source and nature of these bodies are not known. x280.

Figure 4. The electroejaculator used in this study. The stimulus duration and interval between stimuli are automatically controlled. Various voltages can be selected from 0 to 60 v. The rectal electrode is shown to the right of the power supply.



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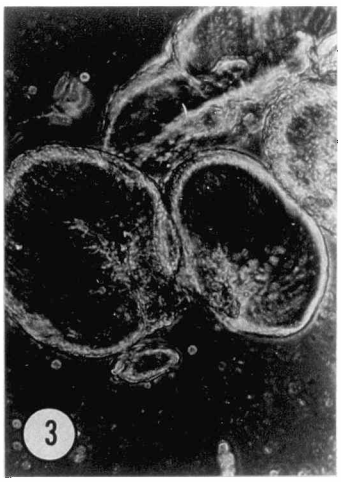

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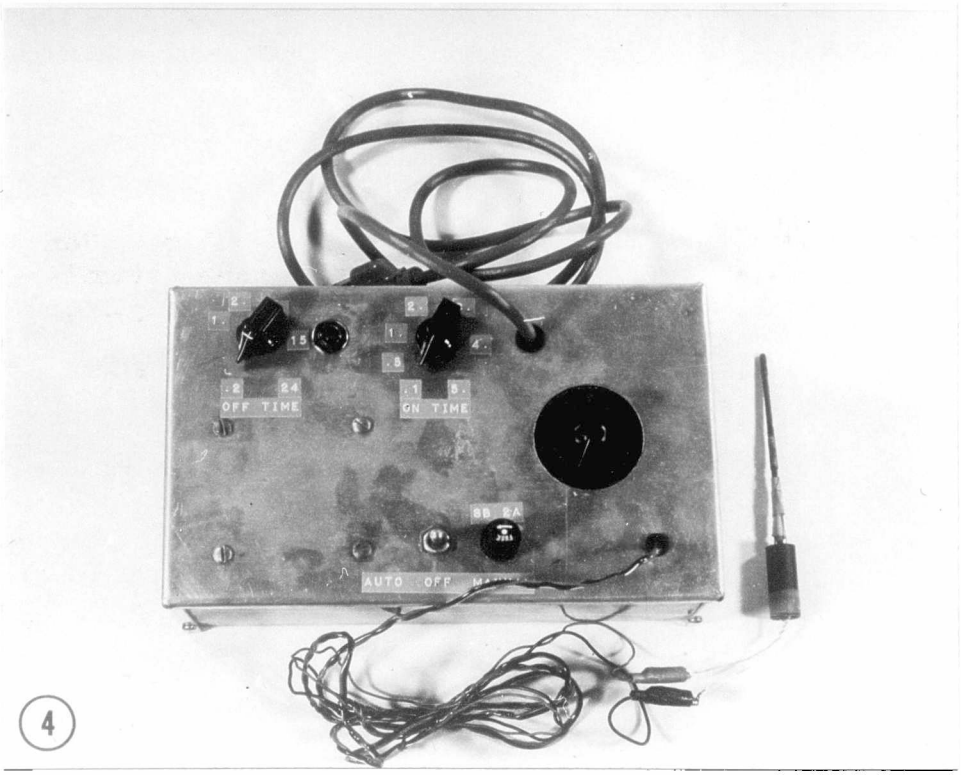

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A high concentration of membranous material gives the neck region a swollen appearance in ejaculated sperm viewed with the phase contrast microscope (Figs. 5 and 6). A second region of concentrated cytoplasm , the residual cytoplasmic droplet (Figs, 5 and 6), is generally present at the lower portion of the midpiece.

The hooked shape of the acrosome which is indicated in phase contrast micrographs was also seen in sagittal sections examined with the transmission electron microscope (Figs. 10 and 14), but observations with the scanning electron microscope revealed that the head is quite typically primate in form (Fig. 7) with the striking exception that the anterior region of the acrosome is folded ventrally as a hood (Figs. 8 and 9).

As shown in Figures 8 and 9, the acrosome comprises the anterior two-thirds of the head. The anterior segment of the acrosome represents the hood-like fold, the principal segment covers the anterior half of the nucleus (not visible in scanning micrographs), and the terminal or equatorial segment encircles the nucleus as a narrow, constricted band. The head tapers anteriorly from the relatively thick postacrosomal region (Fig. 8).

The residual cytoplasmic droplet was not retained by sperm prepared for scanning microscopy, and the membrane complex (MC) of the neck region, though retained by several sperm, was displaced back along the mid-piece (Fig. 7). The helical arrangement of sperm mitochondria delineate the mid-piece (Fig. 7) which constitutes the anterior fifth of the flagellum and contains 42-46 mitochondrial gyres.

The hood-like fold of the acrosome is quite apparent in sagittal sections of the sperm head (Figs. 10 and 14), and the acrosomal contents appear homogeneous and lack structural elements. The plasma membrane is closely applied to the sperm surface (Figs. 10—17) except in the acrosomal region where it was often observed separated from the ventral surface of the folded area, as in Figure 10 (P). Fragmentation of the plasma membrane was observed in the acrosomal region of several sperm (Fig. 14) but is generally regarded as a preparative artifact.

As in all mammalian sperm so far examined, the nuclear material is very condensed and amorphous in appearance (Figs. 10—14), and is ensheathed by a thin "postacrosomal dense lamina" (Fawcett, 1965) posterior to the acrosome (Fig. 12). The implantation fossa, a concavity in the base of the nucleus (Figs. 10—13) contains a dense basal plate closely applied to the base of nucleus (Figs. 11 and 12), the proximal centriole which lies at an oblique angle to the central axis of the head (Fig. 11), segmented connecting pieces (Figs. 12 and 13), and at least one longitudinally oriented mitochondrion (Fig. 12).

A simple fold of redundant nuclear envelope extends into the neck region from the base of the nucleus (Fig. 11), but the most conspicuous feature of the neck region is the complex array of concentric membranes which extend several microns from the neck region into the mid-piece (Figs. 10—13). At some points the membranes appear to coverge as tubular bundles (Figs. 13 and 15). Transverse sections of the membrane complex are shown in Figures 16 and 17.

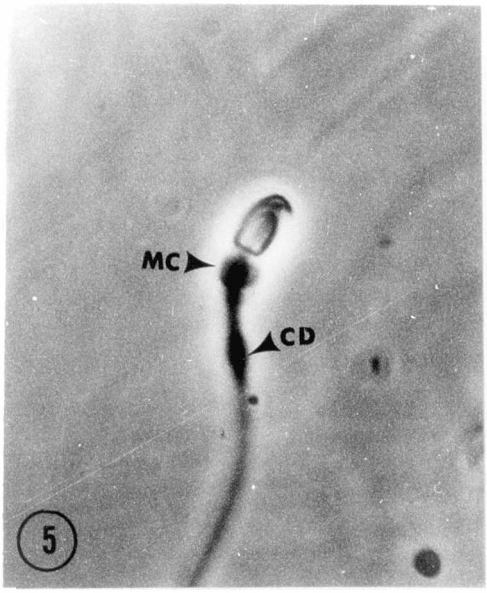
The mid-piece is relatively short (8-9μ) as is typical of primate sperm. Part of the residual cytoplasmic droplet is shown near the

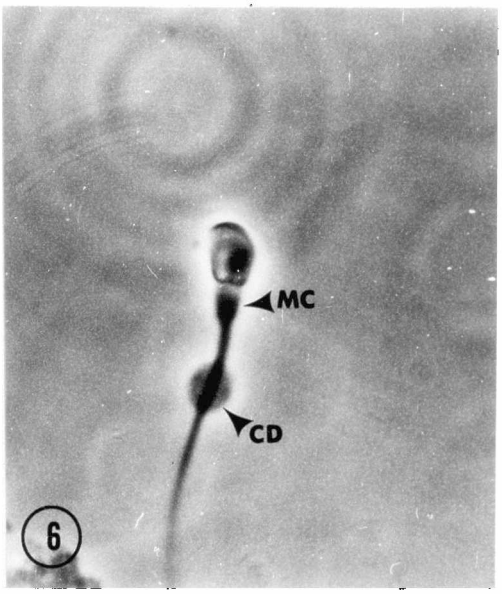
Figures 5 and 6 Phase contrast micrographs of bush baby sperm.

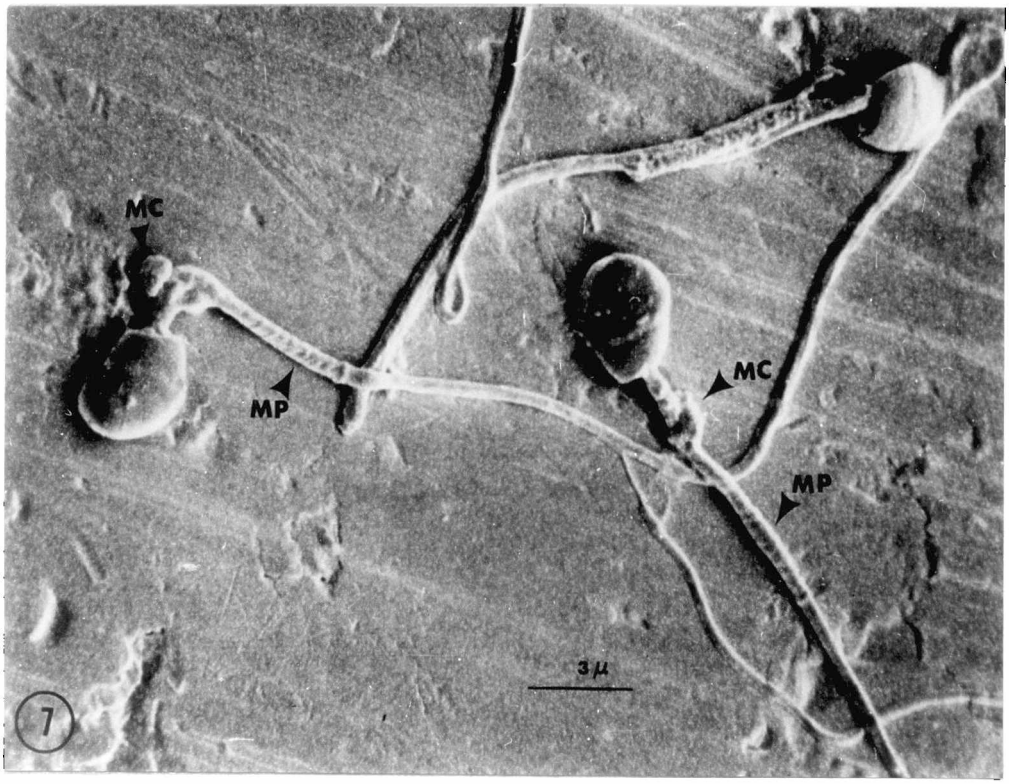
Fig. 5 Lateral view of bush baby sperm showing the hook-like acrosome; membraneous complex (MC) at the neck region and the cytoplasmic droplet (CD) at the lower midpiece. x2,750

Fig. 6 Bush baby sperm viewed ventrally, the true form of the acrosome is not clearly visible. x2,750

Figure 7 Low magnification scanning electron micrograph of bush baby sperm. Membraneous complex (MC), midpiece (MP). x4,670


MC
CD
5

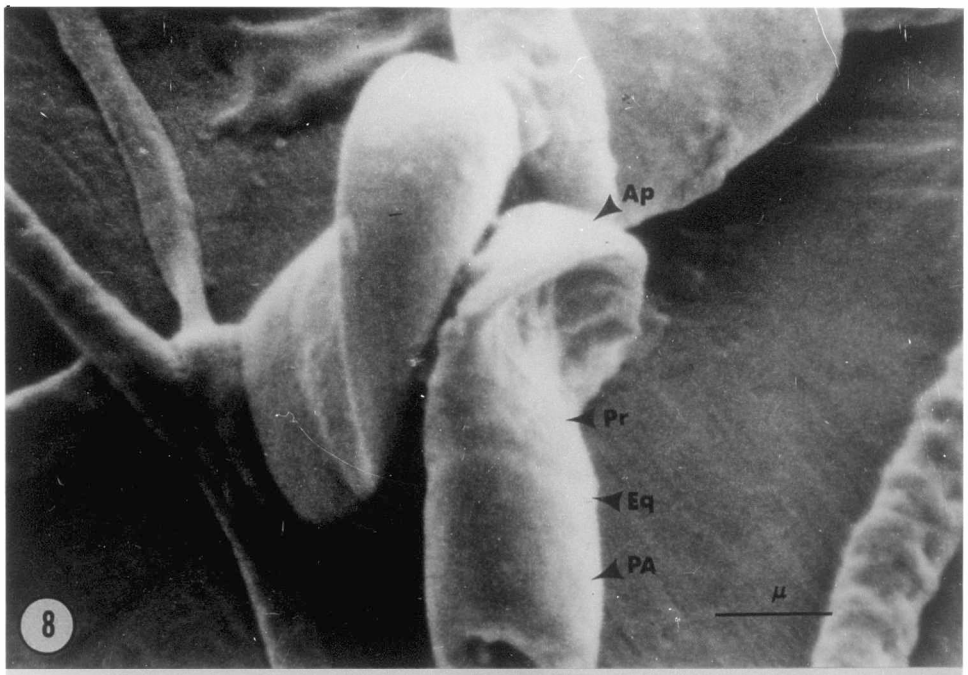

MC
CD
6


MC
MP
MC
MP
3 μ
7

Figures 8 and 9 Scanning electron micrographs of the head of the bush baby sperm. The anterior segment of the acrosome (Ap) is folded ventrally in the form of a hood. The principal segment of the acrosome (Pr) covers the anterior half of the nucleus and the equatorial segment (Eq) is apparent as a narrow band. The post acrosomal region is indicated (PA) and the posterior ring (PR) delineates the posterior limit of the head.

Fig. 8 lateral view. x15,930.

Fig. 9 ventral view. x16,370.


Ap
Pr
Eq
PA
μ
8

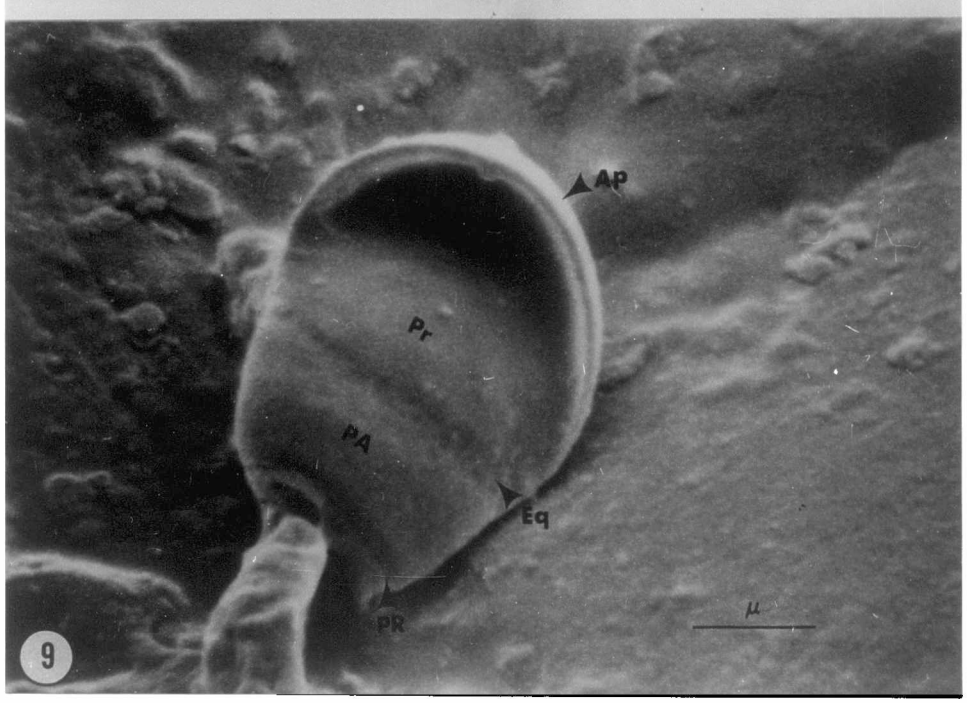

Ap
Pr
PA
Eq
PR
μ
9

Figure 10 Sagittal section of the bush baby sperm. The acrosomal(A) is folded ventrally and appears hook-shaped. The plasma membrane (P) is closely applied to the sperm except along the ventral surface of the folded region of the acrosome. The membraneous complex (MC) is seen at both sides of the neck region. Nucleus (N). x20,160

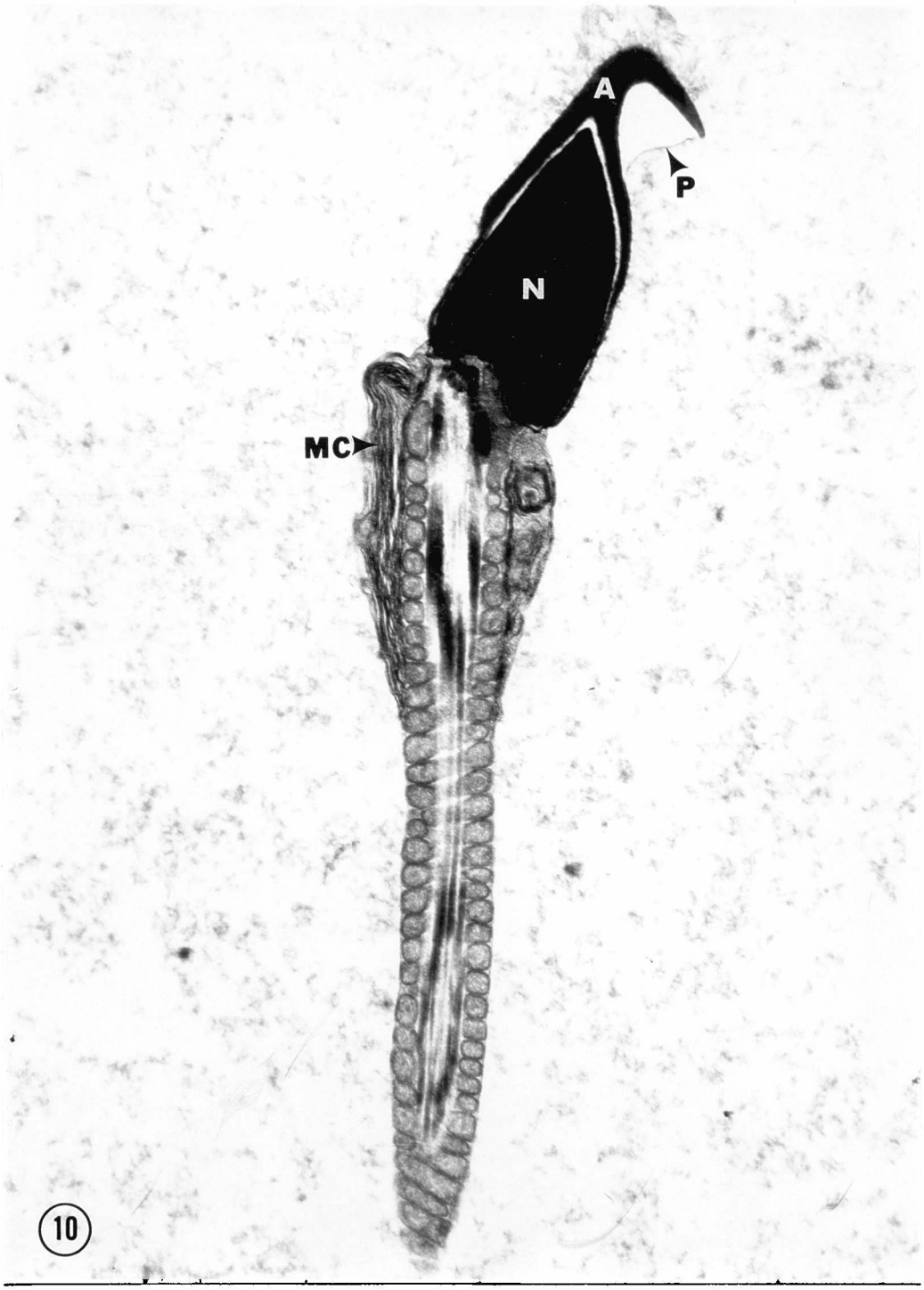
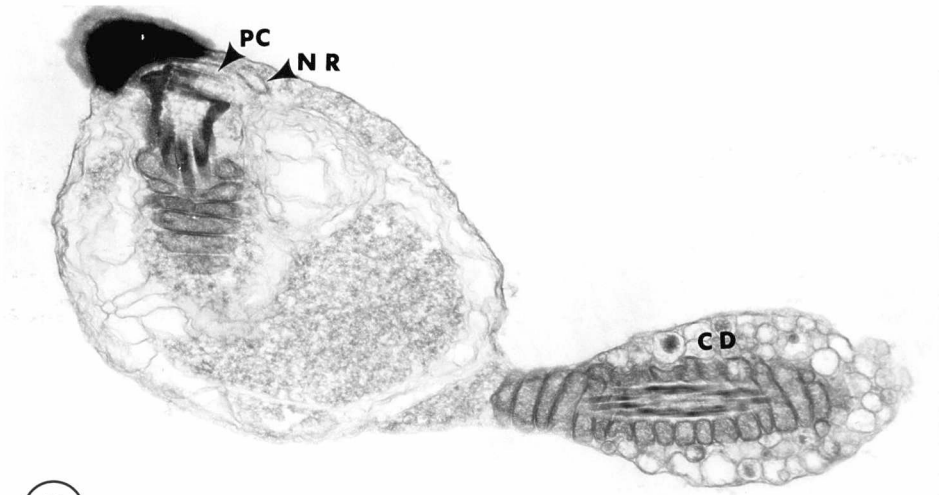

A
P
N
MC
10

Figure 11 Horizontal section of a bush baby sperm. The redundant nuclear envelope (NR) extends into the neck region. The obliquely oriented proximal centriole (PC) is shown at the base of nucleus. The cytoplasmic droplet (CD) is shown at the lower portion of the midpiece. x18,707.

Figure 12 Sagittal section of the neck region of a bush baby sperm. Part of the basal plate (BP) is shown at the base of the Nucleus (N) which is surrounded by the nuclear envelope (NE). Segmented connecting piece (SC). Membranous complex of the neck region (MC). x45,000.



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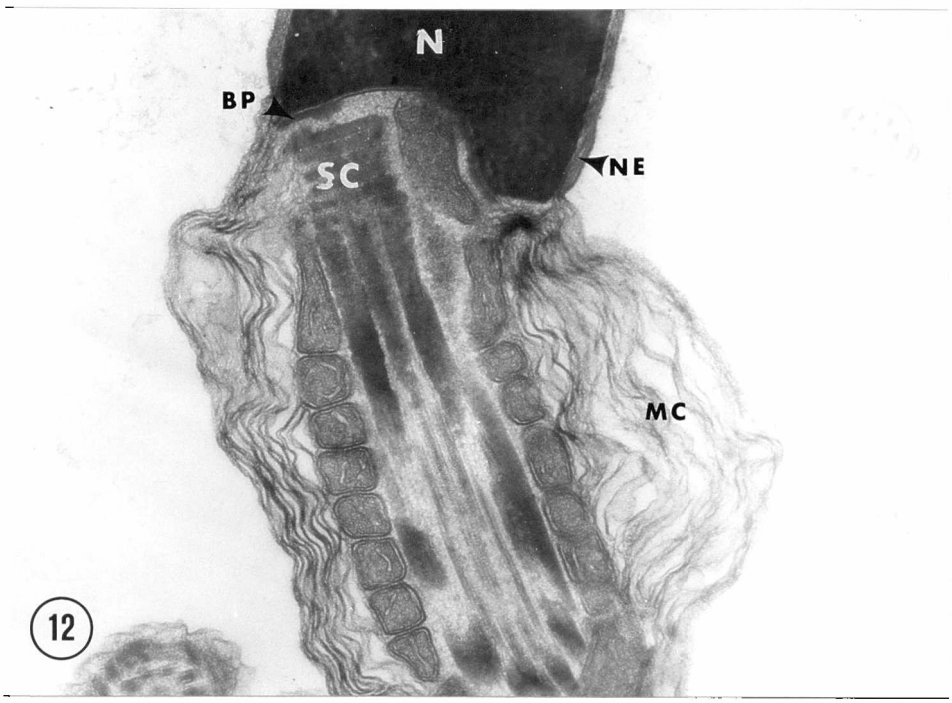

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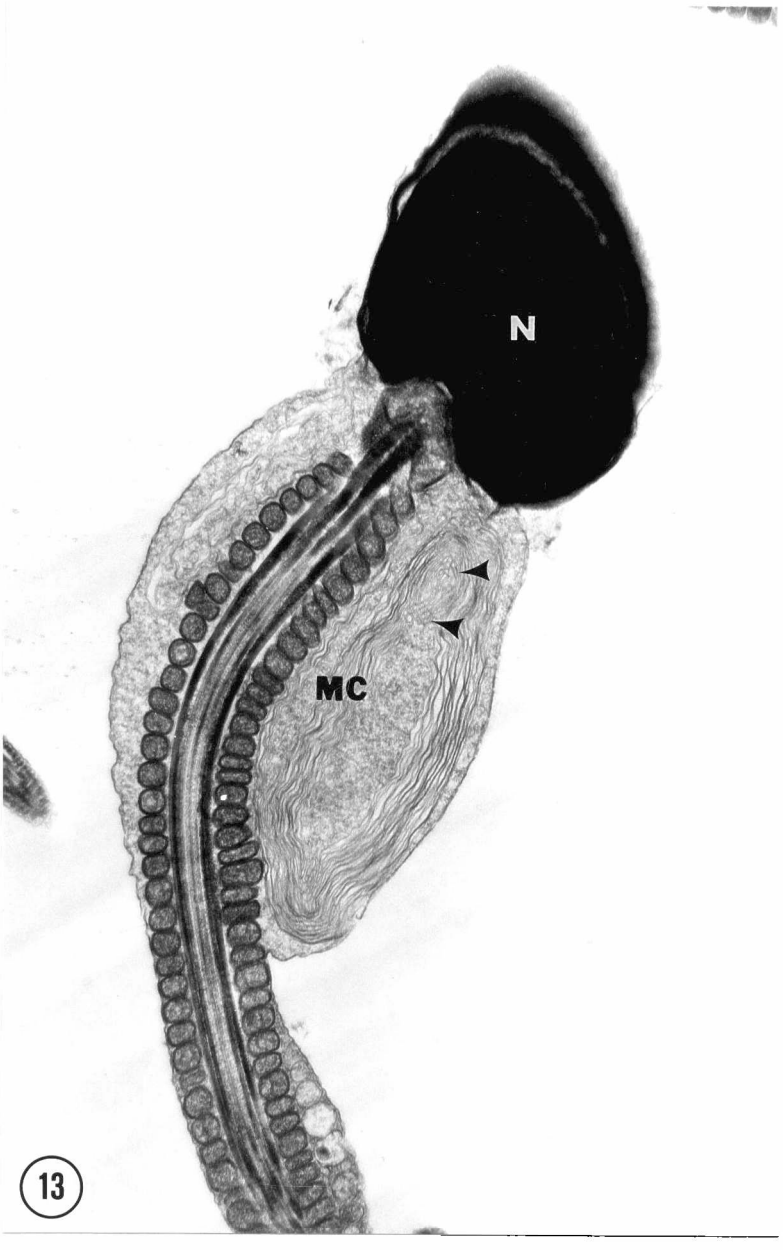
Figure 13 Bush baby sperm prepared as a control for the malachite green staining technique. No electron dense malachite green stainable material was detected in the membraneous complex of the neck region of control sperm, but the structural details of the sperm are otherwise comparable to those stained with malachite green (Figs. 14-16). The arrows show regions of tubular profiles of the membranous complex (MC). Nucleus (N). x26,300.



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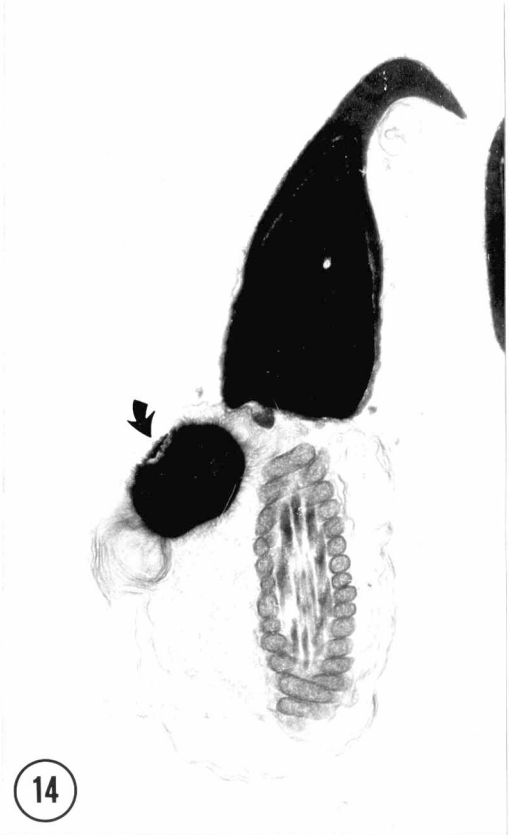
Figures 14-16 Bush baby sperm showing the electron dense malachite green stainable material (arrows) in the membranous complex of the neck region. Note the convergence of membranes into the stained material.

Fig. 14 Sagittal section of the sperm. x17,780.

Fig. 15 Section showing the membrane complex at the upper midpiece. x27,830.

Fig. 16 Cross section through the midpiece. x26,250.

Figure 17 Cross section through the midpiece of a bush baby sperm at a level which does not pass through the stainable material. x33,000.



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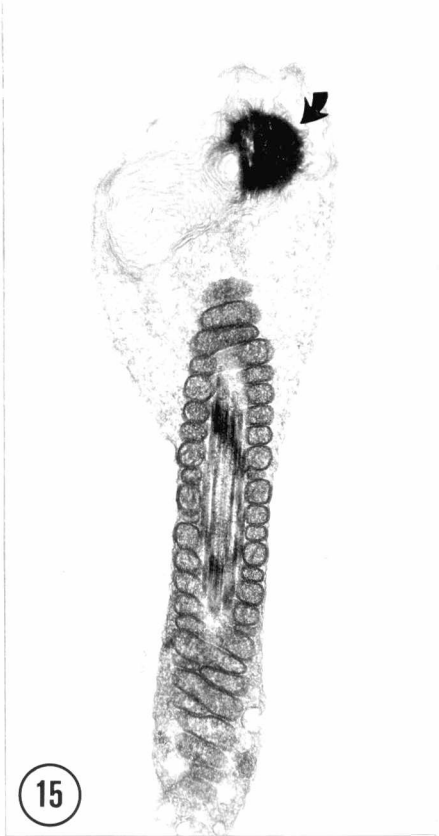

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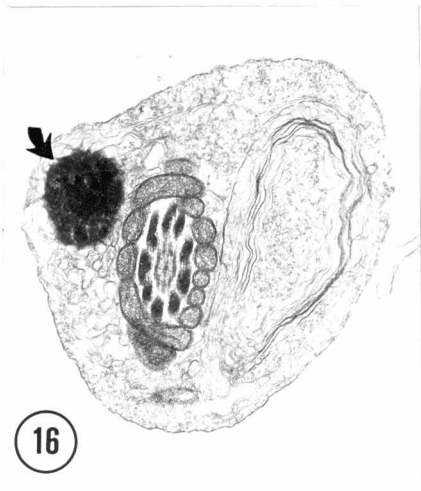

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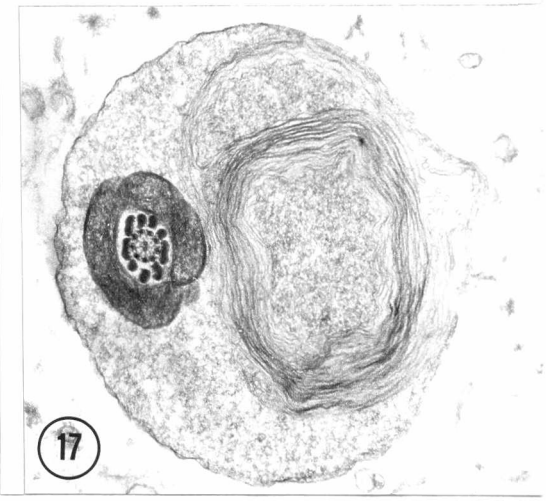


17

posterior limit of the midpiece in Figure 11. The axial filament, which contains the 9+2 arrangement of microtubules common to cilia and flagella, can be seen in cross-section in Figures 16 and 17. Figure 17 also demonstrates the nine dense fibers which are peripheral to the axial filament and extend from the segmented connecting piece, through the midpiece (Fig. 13), and terminate at some point in the principal piece of the flagellum. The principal piece and end piece of the flagellum are not pictured but have the same structural organization found in all eutherian sperm (see Fawcett, 1970, 1974).

Malachite green stainable material was found as a spherical electron dense body within the membrane complex of the neck region (Figs. 14—16). The body was discernable in thick sections at the light microscope level as well as in thin sections viewed with the electron microscope. The material was invariably situated at the dorsal side of the neck region (Fig. 14). Surrounding membranes apparently converge into the material (Figs. 14 and 15).

No comparable body or region of membrane convergence was found in control sperm. In other respects control and malachite green treated sperm were indistinguishable.

Photeolytic Activity of Bush Baby and Guinea-Pig Sperm

In the gelatin membrane digestion experiments, proteolytic activity of sperm were evidenced by a clear digested area on a red background. Enzyme activity under different conditions was quantitated as the percentage of sperm which exhibited activity. Proteolytic activity of

guinea-pig sperm was detected as early as 5 min after incubation at pH 7.0, 8.0, 9.0 and 10.5, with highest activity (13.6% ± 7.40 S.D.) occurring at pH 8.0 (Fig. 18a). Proteolytic activity of bush baby sperm was first detected after 30 min of incubation in gelatin at pH 7.0, 8.0, 9.0 and 10.5, with highest activity (16.0% ± 7.07 S.D.) expressed at pH 8.0 (Fig. 18b).

Activity was first detected as a small digested area around the acrosome (Figs. 20 and 32). The digested area became larger in time (Figs. 23, 33 and 34) and the percentage of sperm showing digestive activity also increased (Figs.18a,b).

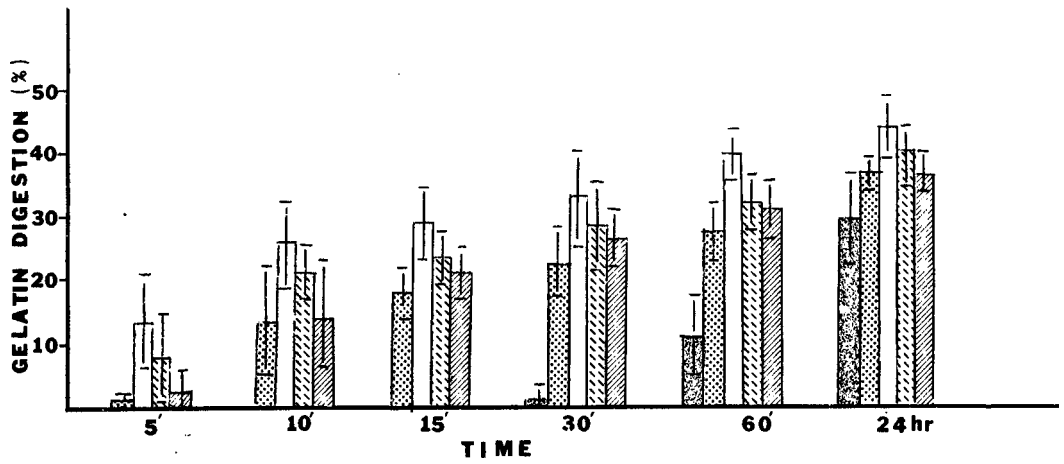
The effect of pH was clearly noticeable. No enzyme activity was detected at pH 2.0 or 4.0. At pH 6.0, activity was retarded, i.e., was first detected at 30 min in guinea-pig sperm and 60 min in bush baby sperm, and the size of the digested area was smaller in comparison to those incubated at higher pH values for the same period of time (Figs. 24 and 25). The percentage of sperm showing digestive activity at pH 6.0 did not reach the maximum obtained much earlier at optimum pH (pH 8.0), even after 24 hr of incubation. Sperm incubated in gelatin at higher pH values than 6.0 approached the maximum percentage of expression of proteolytic activity shown at pH 8.0 after 24 hr incubation (Fig. 18a,b).

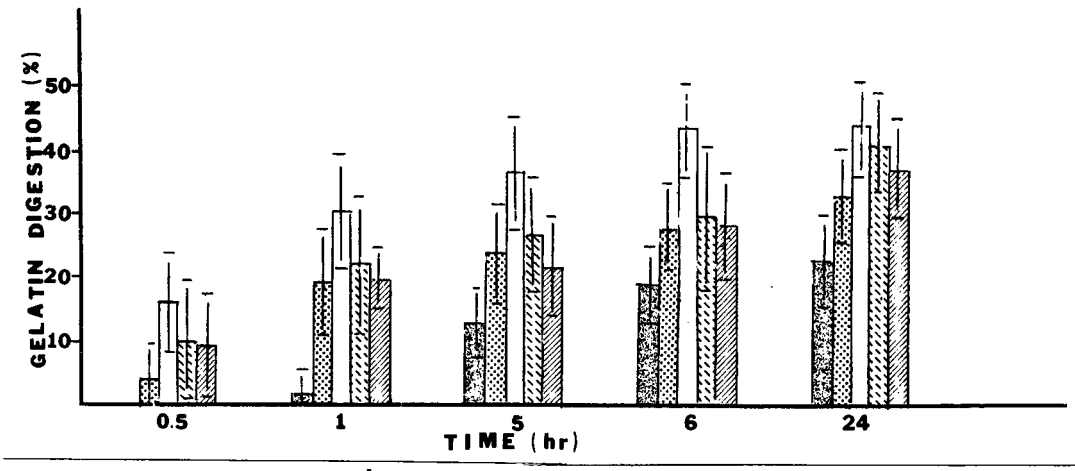
The data from incubation periods of 1 hr and less for guinea-pig sperm (Fig. 18a) and 6 hr and less for bush baby sperm (Fig. 18b) more clearly show that optimum activity was expressed at pH 8.0. At this pH, guinea-pig sperm exhibited activity within 5 min (13.6% ± 7.40 S.D.) and reached the maximum level within 1 hr (44.4% ± 5.37 S.D.); bush baby

Figures 18a and b — The percentage of sperm showing gelatin digestion at different time intervals and at different pH values. At pH values of 2 and 4, no digestion was observed even after 24 hr of incubation (not shown in the figure). The bar graph represents pH values of 6, ▒ ; 7, ▒ ; 8 □ ; 9, ▒ ; and 10.5, ▨ .

Figure 18a — Guinea-pig sperm. Each point represents the mean ± standard deviation of 5 different experiments.

Figure 18b — Bush baby sperm. Each point represents the mean ± standard deviation of 5 different experiments.

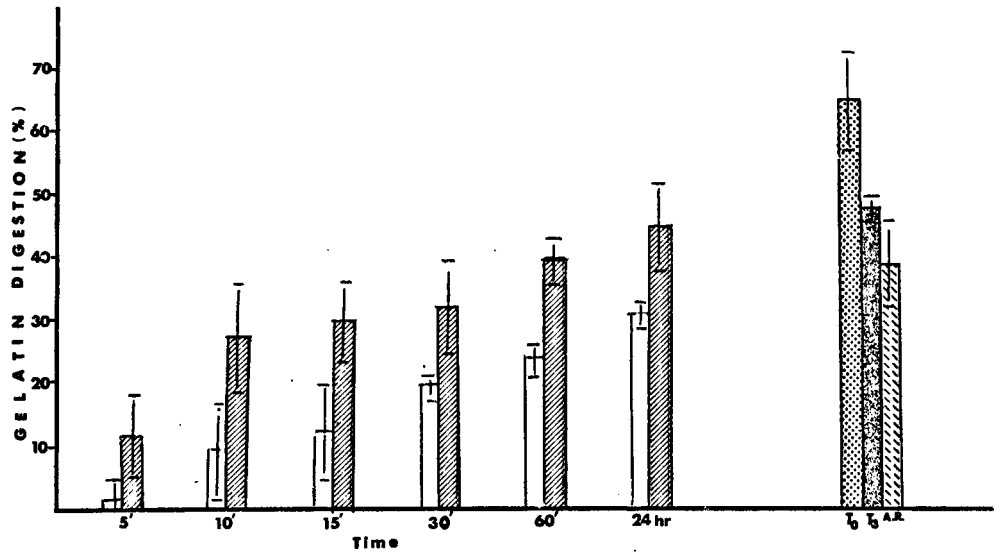

GELATIN DIGESTION (%)
10
20
30
40
50
5′
10′
15′
30′
60′
24hr
TIME

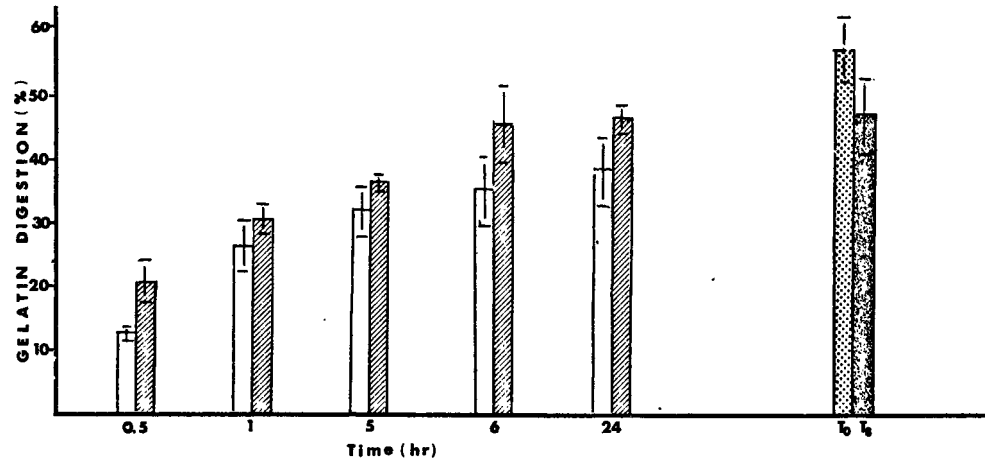

GELATIN DIGESTION (%)
10
20
30
40
50
0.5
1
5
6
24
TIME (hr)

Figures 19a and b — The percentage of sperm showing gelatin digestion (40 C, pH 8.0 for 24 hr) before, ▨ ; and after, □ ; incubation in capacitating media (2-1/2 — 3 hr in MCM for guinea-pig sperm and 8 hr in blood serum for bush baby sperm).

Figure 19a — Guinea-pig sperm. The percentage of live sperm before (T_0) and after (T_3) incubation in MCM, and the percentage of acrosome reacted sperm (A.R.) are shown at the far right. Each point represents the mean $\pm$ standard deviation of 4 different experiments.

Figure 19b — Bush baby sperm. The percentage of live sperm before (T_0) and after (T_8) incubation in blood serum is shown at the far right. Each point represents the mean $\pm$ standard deviation of 4 different experiments.


GELATIN DIGESTION (%)
70
60
50
40
30
20
10
5'
10'
15'
30'
60'
24 hr
T0
T8
A.R.
Time


GELATIN DIGESTION (%)
60
50
40
30
20
10
0.5
1
5
6
24
T0
T8
Time (hr)

sperm showed activity after 30 min (16.0% ± 7.07 S.D.) and reached a maximum level by 6 hr (42.4% ± 7.67 S.D.).

The morphology of guinea-pig sperm in the gelatin films buffered at different pH's was carefully examined. The state of the sperm head was assigned to one of three categories, (1) sperm showing various degrees of acrosome disruption (Figs. 20, 21, 22 and 23), (2) sperm showing a completely intact acrosome (Fig. 26), and (3) sperm showing complete loss of the acrosome (Fig. 27). Digestion of the gelatin was caused only by sperm showing different degrees of acrosome disruption (Table II and Figs. 20, 21,22 and 23). Sperm which showed no proteolytic activity were found to have either an intact acrosome or had completely lost the acrosome (Figs. 26 and 27) although a few inactive sperm with disrupted acrosomes were observed (Table II).

The small size of bush baby sperm was previously mentioned. Since bush baby sperm heads are only one-fourth the width of guinea-pig sperm heads, morphological details of acrosome disruption in bush baby sperm were obscure to a greater degree by the gelatin film and were more difficult to discern. However, in sperm showing proteolytic activity the heads were more elongate and noticeably larger (Figs. 32, 33 and 34), whereas heads of inactive sperm were smaller and round in appearance (Fig. 35).

The effects of trypsin inhibitors on expression of proteolytic activity are shown in Table III. All incubations were at pH 8.0 for 24 hr. At concentrations of 0.5 mg/mℓ soybean trypsin inhibitor (SBTI) in the gelatin, both the diameter of the digested regions and the percentage of sperm showing proteolytic activity slightly decreased. At SBTI concentrations of 1 mg/mℓ, gelatin digestion was completely

TABLE II

RELATION OF GELATIN DIGESTION TO THE MORPHOLOGY OF THE ACROSOME OF GUINEA-PIG SPERM INCUBATED IN GELATIN (40 C, pH 8.0, FOR 24 HR)

Morphology of the Acrosome	Gelatin Digestion (%)	Non Digested (%)
Acrosome disrupted	44.4 ± 5.37	2.0 ± 2.0
Completely intact acrosome	0	8.0 ± 1.41
Complete loss of acrosome	0	45.6 ± 7.92
Total	44.4 ± 5.37	55.6 ± 5.37

Each calculation represents the mean of 4 experiments ± S.D.

TABLE III

THE EFFECT OF TRYPSIN INHIBITORS AND TEMPERATURE ON THE PROTEOLYTIC ACTIVITY OF GUINEA-PIG AND BUSH BABY SPERM INCUBATED IN GELATIN (40 C, pH 8.0, FOR 24 HR)

Treatment	Gelatin Digestion (%)	
	Guinea-pig sperm	Bush baby sperm
Inhibitors		
0.5 mg/mℓ soybean trypsin inhibitor	35.0 ± 2.58	31.5 ± 5.26
1.0 mg/mℓ soybean trypsin inhibitor	0	0
1.27×10^{-2}M Benzamidine. HCℓ	36.5 ± 3.41	33.0 ± 1.41
3.81×10^{-2}M Benzamidine, HCℓ	0	0
Heat (2 min)		
50 C	44.0 ± 1.63	40.5 ± 7.19
60 C	34.0 ± 2.83	28.5 ± 4.44
70 C	0	0
Control	46.5 ± 3.00	43.0 ± 8.72

Each calculation represents the mean of 4 experiments ± S.D.

inhibited. The effects of benzamidine.HCℓ were similar. At concentrations of 1.27×10^{-2}M, the digested areas were smaller and fewer sperm showed activity . Complete inhibition of activity occurred at concentrations of 3.81×10^{-2}M. Examination of the acrosomes of guinea-pig sperm in these experiments revealed that disruption of acrosomes occurred (Fig. 29) in all preparations, including those where expression of proteolytic activity was completely inhibited.

The effects of heat immotilization of sperm on expression of proteolytic activity are also shown in Table III. Sperm of both species were completely immotilized by preheating at 50 C, 2 min in a water bath, but expression of activity was not significantly decreased ($P > 0.1$). In sperm preheated at 60 C, the digested areas were slightly smaller and the percentage of sperm showing activity was significantly ($P<0.01$) decreased. Complete inhibition of enzyme activity was obtained when sperm were preheated at 70 C. Unlike the sperm completely inhibited by SBTI and benzamidine.HCℓ, no disruption of acrosomes occurred in preparations completely inactivated by heat (Fig. 31).

Preincubation of guinea-pig and bush baby sperm in capacitating media (2-1/2 — 3 hr in MCM, and 8 hr in heat pretreated blood serum, respectively) did not alter the pattern and time course of expression of proteolytic activity (Fig. 19a,b), i.e., in guinea-pig sperm, activity was still detected at 5 min incubation in gelatin and reached the maximum level by 1 hr, and in bush baby sperm, activity was detected by 30 min and reached the maximum level at 6 hr. However, the percentage of sperm showing proteolytic activity was decreased, 14.0% $\pm$ 7.10 S.D.

in the guinea-pig and 8.0% ± 0.05 S.D. in the bush baby. Acrosome reactions occurred in 38.3% ± 7.20 S.D. of the live MCM incubated guinea-pig sperm, but no reactions were detected in bush baby sperm incubated in blood serum.

Phase contrast microscopic examination of the 2-1/2 — 3 hr MCM preincubated guinea-pig sperm in the gelatin film again revealed that only sperm with disrupted acrosomes exhibited enzyme activity. No sperm which lacked the acrosome exhibited activity.

Figure 20 Guinea-pig sperm incubated 30 min in gelatin (40 C, pH 8.0). A small digested area surrounds the disrupted acrosome. x1,960.

Figure 21 Guinea-pig sperm incubated 1 hr in gelatin (40 C, pH 8.0). The digested area has become larger. The acrosome is disrupted. x1,960.

Figure 22 Guinea-pig sperm incubated 1 hr in gelatin (40 C, pH 8.0) as in Fig. 21 but exhibiting different degree of fragmentation of the acrosome. x1,960.

Figure 23 Guinea-pig sperm incubated 24 hr in gelatin (40 C, pH 8.0). The area of digestion is quite large and the acrosome quite fragmented, but the remnants of the acrosome can still be seen. x1,960.

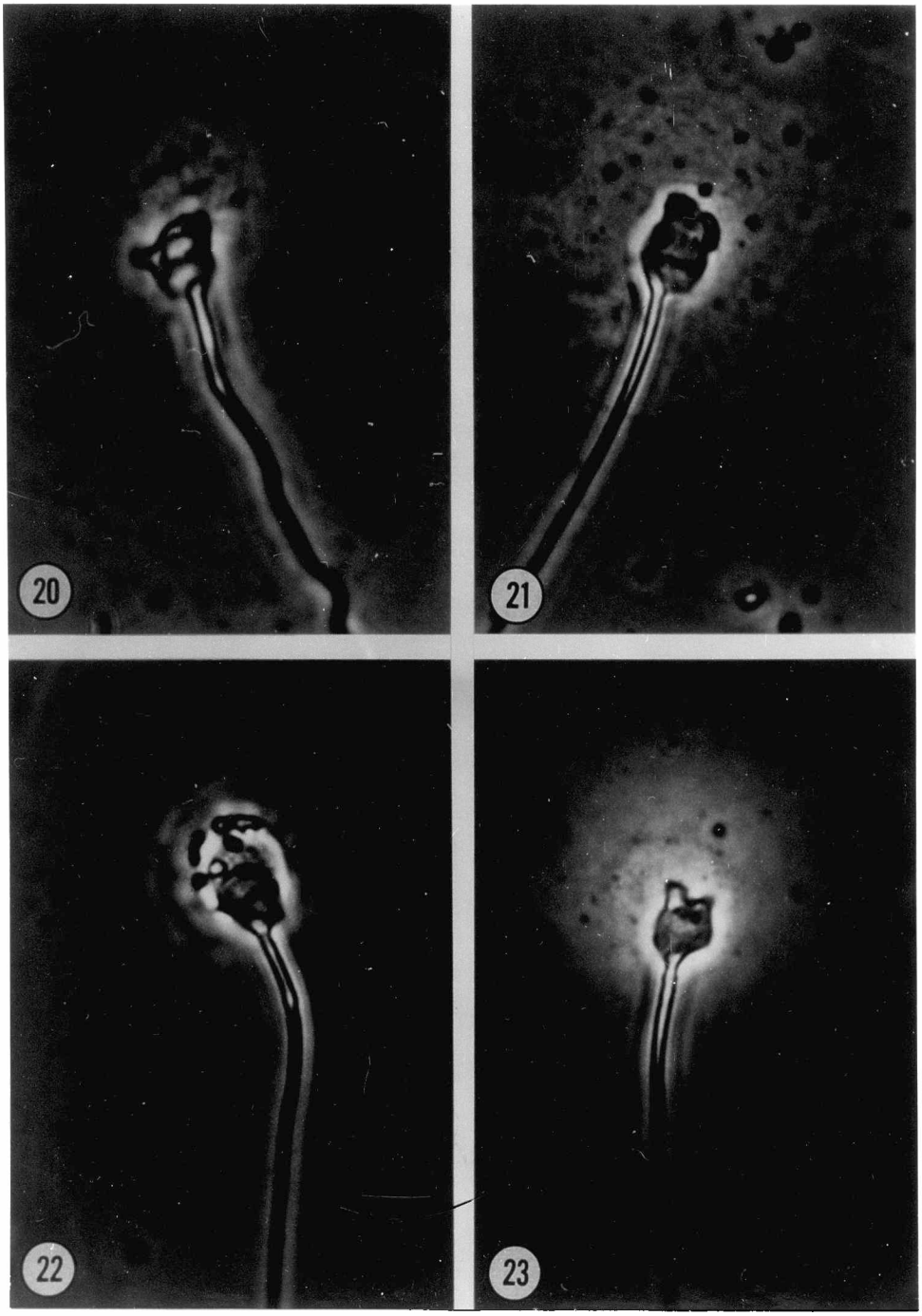

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Figure 24 Guinea-pig sperm incubated 24 hr in gelatin (40 C, pH 8.0). Most of the acrosome is still present. The digested area surrounds the entire head. x1,960.

Figure 25 Guinea-pig sperm incubated 24 hr in gelatin (40 C, pH 6.0). The digested area is small in comparison to sperm incubated at pH 8.0 for 24 hr (see Figs. 23 and 24). x1,960.

Figure 26 Guinea-pig sperm with a completely intact acrosome. No digestion has occurred. The lighter region around the sperm is an optical artifact. The sperm was incubated 24 hr in gelatin (40 C, pH 8.0). x1,960.

Figure 27 Guinea-pig sperm which lacks an acrosome. No digestion has occurred. The lighter area around the sperm is an optical artifact. The sperm was incubated 24 hr in gelatin (40 C, pH 8.0). x1,960.

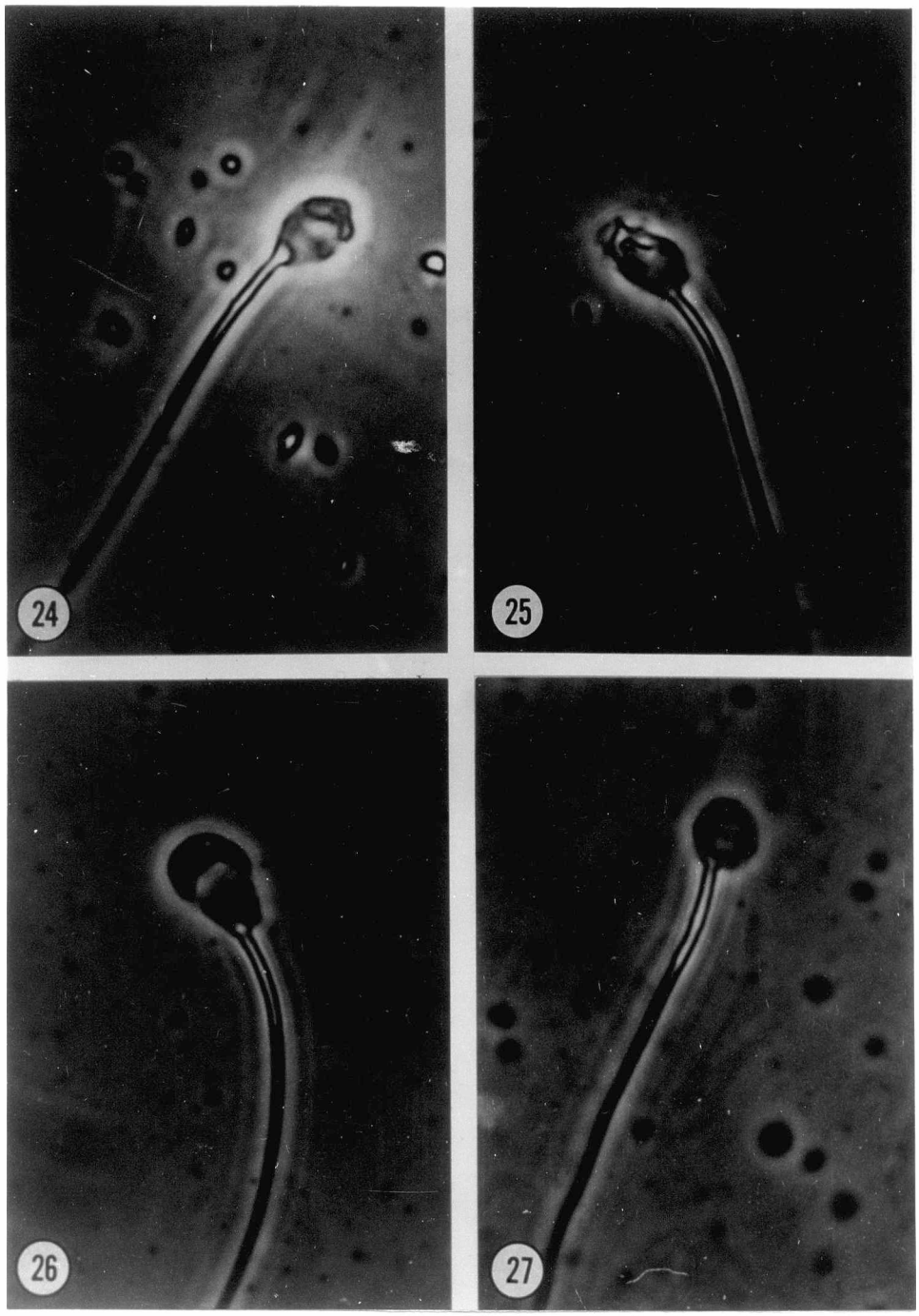
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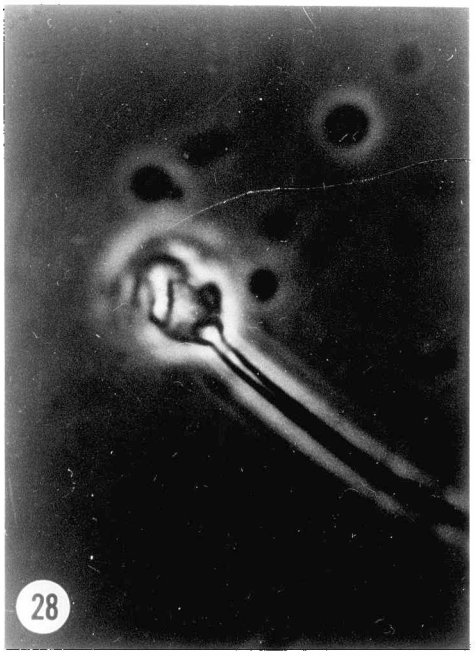
Figure 28 Guinea-pig sperm preincubated 3 hr in MCM before incubation 24 hr in gelatin (40 C, pH 8.0). The digested area is small and the acrosome is disrupted. x1,960.

Figure 29 Guinea-pig sperm incubated 24 hr in gelatin (40 C, pH 8.0) containing 1 mg/mℓ soybean trypsin inhibitor. No digestion has occurred, but the acrosome is disrupted. x1,960.

Figure 30 Guinea-pig sperm preheated at 60 C for 2 min before incubation 24 hr in gelatin (40 C, pH 8.0). Enzyme activity is slightly impaired as evidenced by the smaller halo than in controls. (c.f. Fig. 24). x1,960.

Figure 31 Guinea-pig sperm preheated at 70 C for 2 min before incubation 24 hr in gelatin (40 C, pH 8.0). Proteolytic activity is completely inhibited. The acrosomes are intact. x1,960.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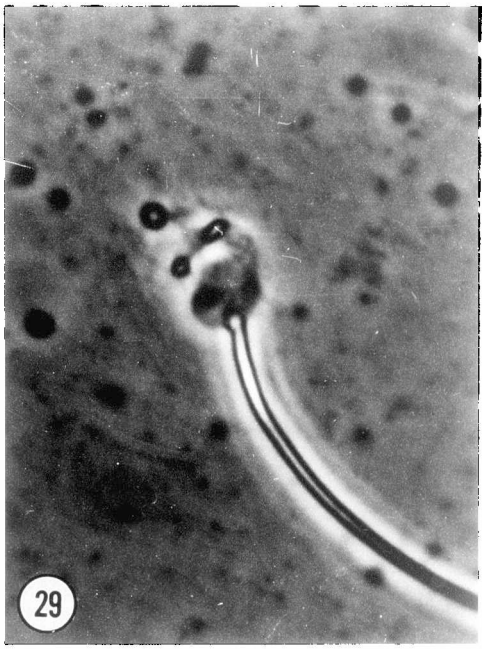
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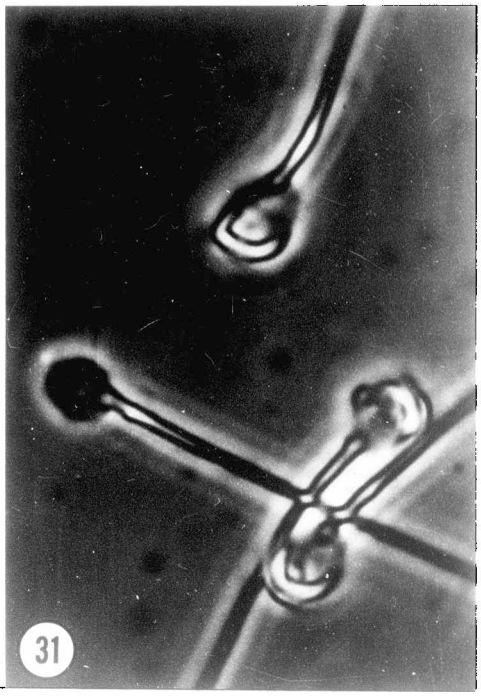
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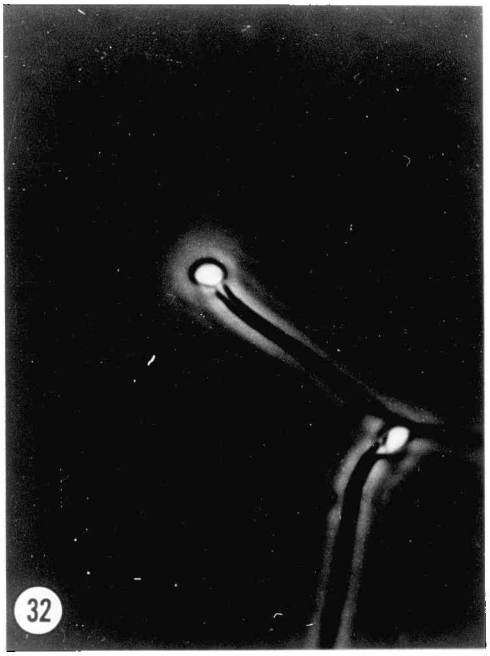
Figure 32 Bush baby sperm incubated 30 min in gelatin (40 C, pH 8.0). Only a small digested area is detected around the head region. x2,000.

Figure 33 Bush baby sperm incubated 6 hr in gelatin (40 C, pH 8.0). The digested area is larger. The acrosome is not clearly distinguishable, but a protrusion of the head portion indicates the presence of the acrosome. x2,000.

Figure 34 Bush baby sperm incubated 24 hr in gelatin (40 C, pH 8.0). The digested area is large compared to Figs. 32 and 33. x2,000.

Figure 35 Bush baby sperm incubated 24 hr in gelatin (40 C, pH 8.0). No digestion is detected. The head of the sperm is small compared to those which exhibit proteolytic activity and indicates that the acrosome is absent. x2,000.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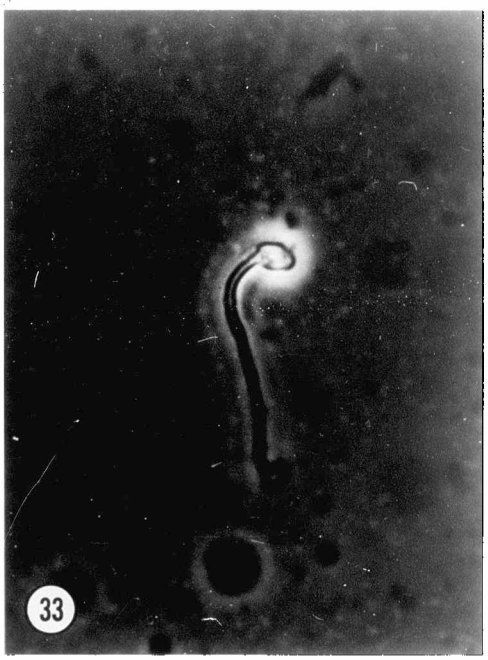
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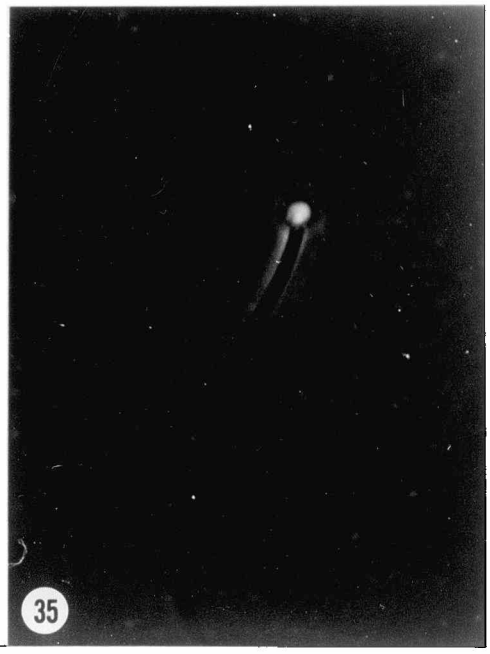
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DISCUSSION

Bush babies are small primates (about 300 gm) and can be electrically stimulated to ejaculate while completely anesthetized. These circumstances together with incorporation of automatic stimulation controls into the electroejaculator power supply permitted development of a simple, standard electroejaculation procedure. The standard values of electrode potential, durations of stimulus and interval between stimuli were selected through monitoring appropriate muscular responses of the subjects. The values 8 v, 1.7 sec and 0.4 sec for these three variables elicited ejaculations within 15-20 min. The significance of the procedure is that it is highly repeatable, involves a stress period of less than half an hour and does not require assistance in handling the animal.

Various factors affect electroejaculation, including the physical condition of the animal (Weisbroth and Young, 1965) and the type anesthetic used (Roussel and Austin, 1968). Electroejaculates from two of the eleven males used in this study contained no sperm. One of these (male No. 6) had previously had its left epididymis excised, and the other, though of adult size and age, has noticeably smaller testes than other adults of the colony.

Sperm of male No. 14 were immotile in 2 of 5 ejaculates, and ejaculates of male No. 2 were of poor quality (< 30% motile sperm). Although the reasons for these failures are not clear, male No. 14 died some time later from causes unknown and male No. 2, which was purchased in 1973, had been repeatedly subjected to electroejaculation

(21) prior to this study and was perhaps approaching old age. The fertile life span of bush babies is not known but has been estimated from a zoo specimen to be about 10 yr (see Butler, 1971).

According to Roussel and Austin (1968), the type anesthetic used affects the volume of semen obtained by electroejaculation. Best results in their experiments were obtained with Sernylan (Parke, Davis & Co.) but they did not test the anesthetics that were used in this study. Keating (1975) commented that the volume of ejaculates obtained from the bush baby by electroejaculation may not represent the normal volume but is within the range obtained by electrical stimulation of rats (Scott and Dziuk, 1959) and mice (Snyder, 1966).

Normal ejaculation of boars involves discharge of a sperm-free seminal fluid then a sperm-rich fraction (Nalbandov, 1964). The same sequential pattern of ejaculation has been observed in farm animals when semen is colelcted with an artifical vagina (Weisbroth and Young, 1965). The same ejaculation sequence was observed during electroejaculation of bush babies, but does not occur during electrical stimulation of rhesus monkeys (Weisbroth and Young, 1965).

Secretory particles of various sizes and forms have been found in semen of several marsupials (Rodger and White, 1975) and in tree shrews (Barros, unpublished). The nature of the globular bodies found with sperm in ejaculates of the bush baby is not known, nor has their accessory gland origin been determined. The motility of sperm in samples which contain the globular bodies was found to be more vigorous, suggesting that these globules may represent an exogenous source of energy for the sperm.

Although bush baby sperm conform to the basic anatomical pattern common to primates and other eutherian mammals, they exhibit striking morphological departures in their small size (about two-thirds the size of human sperm), the hooded form of their acrosomes and the highly developed system of membranes in the neck region.

The sperm head in primate species is typically ovoid, flattened dorsoventrally and has bilateral symmetry (see Bedford, 1974). The head of bush baby sperm, however, has been described as asymmetric (Bedford, 1967, 1974; Keating, 1975), owing to the hook-like appearance of the acrosome in sagittal sections (e.g., Figs. 10 and 14) and in sperm suspensions viewed with the phase contrast microscope (e.g., Figs. 5 and 6). Since the bush baby is a prosimian and sperm of most rodent species have hook-shaped acrosomes, the form of the bush baby acrosome at first seemed to suggest retention of a rodent-like characteristic. However, the scanning electron microscope observations described in the present study revealed that the bush baby sperm head is actually ovoid, has bilateral symmetry, and that the acrosome has the form of a hood. This particular acrosomal configuration is unique to sperm of the Galagidae among the various primate families, and consequently the dorsal and ventral sperm surfaces can only be distinguished in members of that family (Bedford, 1967, 1974). Bedford (1967, 1974) demonstrated the presence of a large aggregate of flocculent material on the ventral surface of the folded acrosome in epididymal sperm. The material was not observed in ejaculated sperm which were used in the present study, but was found in sections of epididymal sperm cut from samples provided by Dr. Keating. Whether or not there are

differences in the molecular composition of the dorsal and ventral acrosomal surfaces, differences in surface absorbed components acquired during sperm passage through the epididymis or any functional differences in the two surfaces is only a matter of speculation at the present time.

After condensation of the nucleus, sperm of several mammals exhibit excess or redundant portions of nuclear envelope which extend back into the neck region (Fawcett, 1965). In some species the scroll or concentric circles of excess membrane are too extensive to be reasonably accounted for as redundant membrane resulting from nuclear condensation (Fawcett, 1970). In sperm of the bush baby, the array of neck region membranes is developed to an extent not seen in an other species (Bedford, 1967, 1974). According to Bedford (1974) microtubes are associated with the membrane complex, and he suggests that the complex may derive from microtubules of the manchette which is a transient cytoplasmic structure in spermatids, and from membranous elements of the cytoplasmic droplet rather than redundant nuclear envelope. No microtubles were observed within the membrane complex during the present study. However, in some sections, tubular profiles which represent closely associated or converging membranes were observed (Figs. 13 and 15).

The presence of malachite green stainable material in sperm of various species was demonstrated by Teichman et al. (1972). In sperm of the mongoose, which contain a scroll-like concentration of membrane in the neck region, the material was localized within the scroll. In the bush baby, it was similarly localized within the membranous complex of the neck (Figs. 14, 15 and 16). The material has recently

been characterized in rabbit sperm as phospholipid, predominantly a choline plasmalogen (Teichman et al., 1974). According to Mann (1964) sperm utilize endogenous phospholipids as an energy source, under aerobic conditions in the absence of exogenous carbohydrate, and semen phospholipids, which are rich in choline plasmalogen are primarily associated with the sperm fraction. Fawcett (1970) had suggested consequently that the concentrations of membrane in the neck region of several species of sperm may represent an endogenous lipid energy reserve. However, Cummins and Teichman (1974) found that live rabbit sperm retain malachite stainable material during 24 hr incubation in Hank's solution, whether or not glucose is present. In the same study, the stainable material was reported to disappear from rabbit sperm incubated 12 hr in the estrous uterus, a treatment known to capacitate rabbit sperm (see Bedford, 1970).

A possible relationship between malachite green staining material and the infertility of sperm was also suggested by Cummins and Teichman (1974) from observations that sperm of rabbits made artificially cryptorchid for 3 or 4 days appeared normal but lacked the stainable material. Such sperm are known to be incapable of fertilizing eggs (Cummins and Glover, 1970). Although Cummins and Teichman's observations are of interest, the presence, absence or disappearance of malachite green stainable material from rabbit sperm has not yet been shown to have a functional relationship to either infertility of sperm or the capacitation process.

The gelatin membrane technique used was modified from Benitez-Bribriesca and Valezquez-Meza (1972) and is a relatively simple, inexpensive method. It has an advantage over India ink impregnated gelatin (Gaddum and Blandau, 1970) and photographic emulsions (Penn et al., 1972; Allen et al., 1974) in that morphological details are clearly visible both in sperm which show proteolytic activity and those that do not. Gaddum-Rosse and Blandau (1972) subsequently used clear gelatin to facilitate observations of the morphology of sperm which exhibited enzyme activity.

In the cited methods, sperm were smeared or brushed onto dry gelatin membranes or were smeared on glass slides and air dried before application of the gelatin film. In the studies where sperm were smeared onto fixed gelatin membranes, observations were restricted to the area near the origin of the smear, because onset of digestion and the size of digested areas varied along the length of the smear (see Gaddum and Blandau, 1970; Allen et al., 1974). At the beginning of this study, direct smearing of guinea-pig sperm was observed to cause detachment of acrosomes. Consequently, a technique was developed to maintain the original, live state of the sperm through the period of slide preparation. Sperm were mixed with the warm (40 C) gelatin solution before the smear was made. This innovation provided more uniform preparations and protected the sperm during smearing.

Although proteolytic activity has been detected over a wide pH range, most species exhibit rapid and intense activity at alkaline pH values, i.e., rabbit, human, hamster from 7.5 to to 9.5; rat from 8.55 to 9.5 (Gaddum and Blandau, 1970), human at 8 and rat at 9

(Benitez-Bribiesca and Valezquez-Meza, 1972). No activity was detected in sperm of several species at pH values below 3.6 (Penn and Gledhill, 1972). In all previous studies, relative activity was determined from the size of the digested area around the sperm head. In the present study, proteolytic activity was quantitated as the percentage of sperm showing activity. The preparation had to be assessed with a phase contrast microscope since enzymatically inactive sperm were not clearly visible with standard bright field illumination. This suggests that inactive sperm may have been obscure and overlooked in studies where gelatin containing ink or photographic emulsions were used as substrates.

Bush baby and guinea-pig sperm incubated at pH values from 7.0 to 10.5 approached the same maximum activity after prolonged incubation (24 hr). Consequently, the optimum pH was determined on the basis of earliest detectable activity. Both bush baby and guinea-pig sperm were found to show optimum activity at pH 8.0, which is within the range reported previously for most species of sperm.

The time course of expression of activity was reported to be species specific (Gaddum and Blandau, 1970; Allen et al., 1974). Data presented (Fig. 18a,b) show that the time course of expression of activity is pH dependent as well, i.e., at optimum pH the activity of guinea-pig sperm was detected as early as 5 min and reached a maximum level within 1 hr, and in bush babies was first detected at 30 min and reached maximum within 6 hr. Though activity was also detected in the sperm of both species when incubated at pH 7.0, 9.0, and 10.5, it was highest at pH 8.0. On the other hand, no

activity was apparent at pH 2.0 or 4.0, and at pH 6.0 activity was retarded and did not reach the normal maximum level even after 24 hr of incubation.

In the case of the guinea-pig, the gelatin procedures used by Gaddum-Rosse and Blandau (1972) and Allen et al. (1974) lead to somewhat different results than the procedure used in this study and by Penn et al. (1972) and Penn (1975). The former investigators fixed gelatin films with 0.05% buffered glutaraldehyde before use, whereas substrate used in this study were unfixed, and Penn's (photographic film) were simply exposed and processed with Kodak D-19 developer and hypo. Gaddum-Rosse and Blandau reported good reactions (digestion) by 1 hr but with considerable variation. Allen et al. reported onset of digestion at 2—2-1/2 hr. Both reported initial digestion at the equatorial segment region (posterior segment of the acrosome) without acrosomal disruption, in most instances, until sometime later. Penn reported onset of digestion at 1—5 min and data from this study indicated onset by 5 min. Penn also stated that in most cases digestion was first detected at the anterior edge of the head (acrosome). In the present study activity at 5 min and all later stages was invariably restricted to sperm which had disrupted acrosomes and the digested area surrounded the whole acrosome. It is tempting to suggest that the discrepancies may relate to use of glutoraldehyde by Gaddum-Rosse and by Allen et al. Glutaralhyde is a non-coagulating histological fixative but gels proteins by forming cross linkages within and between the molecules. It might have stabilized the acrosomal membrane to some extent. It might also be expected to partially denature the released enzyme and render the

the gelatin less susceptable to enzymatic digestion.

Morphological observations with the phase contrast microscope revealed that activity was detected from sperm with disrupted acrosomes, suggesting that the release of enzyme(s) into the medium results from acrosomal disruption. No specific point of origin of proteolytic activity was observed. When detected even at 5 min, the digested area uniformly surrounded the acrosome. The sperm which lacked acrosomes did not show any activity. This seems to suggest that the enzyme(s) is located within the contents of the acrosome or is associated with the outer acrosomal membrane. Proteolytic activity has been localized with silver proteinate at the outer acrosomal membrane of rabbit sperm at the electron microsope level (Yanagimachi and Teichman, 1972) and within the acrosome at the phase contrast microscope level (Stambaugh et al., 1975).

The expression of proteolytic activity only by sperm which had disrupted acrosomes also held in the experiments which involved use of trypsin inhibitors and heat pretreatment. At the highest concentrations of both trypsin inhibitors, disruption of acrosomes still occurred, but no enzymatic activity was detected. This suggests that the enzyme(s) was released into the medium but that its activity was inhibited by the presence of the inhibitors in the substrate. To the contrary, heat pretreatment at 70 C stabilized the acrosome so that no disruption occurred. Whether the heat pretreatment inactivated the enzyme(s) is not known, but no activity was detected.

Gaddum-Rosse and Blandau (1972) earlier reported that the degree of proteolytic activity expressed (size of digested area around

the sperm head) by guinea-pig sperm was quite variable within a given preparation. This was also observed in the present study and in the bush baby sperm preparations, but to a lesser degree.

Landa (1975) reported that onset of gelatin digestion occurred 5 min earlier in rabbit and boar sperm which had been incubated in the uterus but no marked change in the sizes of digested regions was observed. No change in the time-course of expression of activity was found in guinea-pig or bush baby sperm following incubation in capacitating media (MCM, and heat pretreated blood serum, respectively). There was, however, a significant decrease in the percentage of sperm which exhibited activity. In the bush baby the decrease in activity may be explained by the decrease in the percentage of live sperm during incubation in serum (Fig. 19b), since no acrosome reactions were observed. In guinea-pigs, the decrease in percentage of enzymatically active sperm following incubation in MCM appears to represent the percentage of live sperm (47.5% $\pm$ 1.9 S.D.) minus the percentage of live acrosome reacted (acrosome lacking) sperm (38.25% $\pm$ 7.23). The resulting percentage of live sperm with acrosomes (29.3% $\pm$ 3.30 S.D.) was very close to the observed percentage of enzymatically active sperm (30.5% $\pm$ 1.91 S.D.). This suggests that virtually all the live non-acrosome reacted sperm may be capable of expressing enzyme activity, presumably as a result of incubation in MCM, since not all the unincubatd live sperm showed enzymatic activity. These interpretations are consistent with the observation that only sperm with acrosomes (disrupted) exhibited proteolytic activity.

SUMMARY

(1) A standard procedure for collecting semen samples from the bush baby by electroejaculation was developed.

(2) The average volume of the sperm rich portions of ejaculates was 12.9µℓ ± 6.13 S.D. and the average concentration was 2.0×10^8 sperm/mℓ ± 1.4 S.D.

(3) The general morphology of bush baby sperm was found to conform to the pattern common to primates with the striking exceptions of the hood-like shape of the acrosome and the highly developed membranous complex of the neck region.

(4) Malachite green stainable phospholipid which may be involved in capacitation was detected in the membraneous complex of the neck region.

(5) Proteolytic activity of bush baby and guinea-pig sperm was studied using a modification of gelatin membrane technique described by Benitez-Bribiesca and Valezquez-Meza (1972).

(6) Proteolytic activity was evidenced as a clear digested area surrounding the head of the sperm. Activity was expressed only by sperm which had disrupted acrosomes.

(7) Both bush baby and guinea-pig sperm exhibited optimum proteolytic activity at pH 8.

(8) The time course of the expression of the activity was pH dependent and species specific.

(9) Proteolytic activity of bush baby and guinea-pig sperm was completely inhibited by soybean trypsin inhibitor (1 mg/mℓ) and

benzamidine hydrochloride (3.81×10^{-2}M) and by heat pretreatment at 70 C.

(10) There was no difference in the time course of expression of proteolytic activity of sperm before and after incubation in capacitating media. However, the percentage of sperm exhibiting proteolytic activity decreased. The decrease in activity apparently related to a decrease in the percentage of live sperm, and in the guinea-pig also to the occurrence of the acrosome reaction.

BIBLIOGRAPHY

Allen, G. J., M.W.H. Bishop and T. E. Thompson. 1974. Lysis of photographic emulsions by mammalian and chicken spermatozoa. J. Reprod. Fertil.36:249-252.

Anberg, A. 1957. The ultrastructure of the human spermatozoa. Acta Obstet. Gynec. Scand. 36:Supple 2.

Austin, C. R. 1948. Function of hyaluronidase in fertilization. Nature 162:63-64.

Austin, C. R. 1952. The 'capacitation' of the mammalian sperm. Nature 170:326.

Austin, C. R. 1963. Introducing new animals to the laboratory. New Scientist 17:117-120.

Barros, C. 1974. Capacitation of mammalian spermatozoa. In: Physiology and Genetics of Reproduction Part B, E. Coutinho and F. Fuchs, eds., pp 3-24. Plenum Publishing Co., New York, New York.

Bedford, J. M. 1967. Observations on the fine structure of spermatozoa of the bush baby (Galago senegalensis), the African green monkey (Cercopithecus aethiops) and Man. Am. J. Anat 121:443-460.

Bedford , J. M. 1970. Sperm capacitation and fertilization in mammals. Biol. Reprod. Suppl. 2:128-158.

Bedford, J. M. 1974. Biology of primate spermatozoa. Contributions to Primatology. 3:97-139.

Benitez-Bribiesca, L. and S. Velazquez-Meza. 1972. Cytochemical demonstration of proteolytic activity of human and rat spermatozoa. J. Reprod. Fertil. 29:419-420.

Brown, C. R., Z. Andani and E. F. Hartree. 1975. Studies on Ram Acrosin: Isolation from spermatozoa, activation by cations and organic solvents, and influence of cations on its reaction with inhibitors. Biochem. J. 149:133-146.

Brown, C. R. and E. F. Hartree. 1976. Identification of acrosin in mouse spermatozoa. J. Reprod. Fertil.46:249-251.

Butler, H. 1971. Oogenesis and Folliculogenesis. In: Comparative Reproduction of Nonhuman Primates, Ed., E.S.E. Hafez. Chas. C. Thomas. Ill. Chap. 9, p. 243.

Chrzanowski, S. 1966. Ultrastructure of human spermatozoan. Pol. Med. J. vol. V:482-491.

Cummins, J. M. and T. D. Glover. 1970. Artificial cryptorchidism and fertility in the rabbit. J. Reprod. Fertil. 23:423-433.

Cummins, J. M. and R. J. Teichman. 1974. The accumulation of malachite green stainable phospholipid in rabbit spermatozoa during maturation in the epididymis, and its possible role in capacitation. Biol. Reprod. 10:555-564.

Dziuk, P. J., E. F. Graham and W. E. Petersen. 1954a. The technique of electroejaculation and its use in dairy bulls. J. Dairy Sci. 37:1035-1041.

Dziuk, P. J., E. P. Graham, J. D. Donker, G. B. Marion and W. E. Petersen. 1954b. Some observations in collection of semen from bulls, goats, boars and rams by electrical stimulation. Vet. Med. 69:455.

Fawcett, D. W. 1965. The anatomy of the mammalian spermatozoon with particular reference to the guinea-pig. Z. Zellforsh. 67:279-296.

Fawcett, D. W. 1970. A comparative view of sperm ultrastructure. Biol. Reprod. (suppl.) 2:90-127.

Fawcett, D. W. 1975. The mammalian spermatozoon. Dev. Biol. 44: 394-436.

Franklin, L. E. 1968. Formation of the redundant nuclear envelope in monkey spermatids. Anat. Rec. 161:149-162.

Frasca, J. M. and V. R. Parks. 1965. A routine technique for double staining ultra-thin sections using uranyl and lead salts. J. Cell. Biol. 25:157-161.

Fussell, E. N., J. D. Roussel and C. R. Austin. 1967. Use of the rectal proble method for electrical ejaculation of apes, monkeys and a prosimian. Lab. Anim. Care 17:528-530.

Gaddum, P. and R. J. Blandau. 1970. Proteolytic Reaction of mammalian spermatozoa on gelatin membranes. Science 170:749-751.

Gaddum-Rosse, P. and R. J. Blandau. 1972. Comparative studies on the proteolysis of fixed gelatin membranes by mammalian sperm. Am. J. Anat. 134:133-143.

Garner, D. L. 1973. Partial characterization of bovine acrosomal proteinase. Biol. Reprod. 9:71.

Garner, D. L., G. W. Salisbury and C. N. Graves. 1971. Electrophoretic separation of bovine acrosomal proteins and proteinase. Biol. Reprod. 4:93-100.

Garner, D. L., M. P. Easton, M. E. Munson and M. A. Doane. 1975. Immuofluorescent localization of bovine acrosin. J. Exp. Zool. 191:127-131.

Gilboa, E., Y. Elkana and M. Rigbi. 1973. Purification and Properties of human Acrosin. Eur. J. Biochem. 39:85-92.

Gunn, R.M.C. 1936. Artificial production of seminal ejaculation and the characters of the spermatozoa contained therein. Bull. Coun. Sci. Industr. Res. (Australia) 94:116.

Ho, J.J.L. and S. Meizel. 1970. Electrophoretic detection of multiple forms of trypsin-like activity in spermatozoa of the domestic fowl. J. Reprod. Fertil. 23:177-179.

Huang-Yang, Y. H. and S. Meizel. 1975. Purification of rabbit testis proacrosin and studies of its active form. Biol. Reprod. 12:232-238.

Inoue, M. and D. P. Wolf. 1974. Comparative solubility properties of zonae pellucidae of unfertilized and fertilized mouse ova. Biol. Reprod. 11:558-565.

Karnovsky, M. J. 1965. A formaldehyde glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol: 137A-138A.

Keating, R. J. 1975. Evaluation of the prosimian Galago Senegalensis as a laboratory primate for in vitro studies on early gamete interactions. Ph.D Thesis, University of Houston, Houston, Texas.

Landa, V. 1975. Activity and subcellular localization of proteolytic enzymes of boar and rabbit spermatozoa. Folia Biologica 21:192-195.

Mann, T. 1964. The biochemistry of semen and of the male reproductive tract. Methuen. London.

Mann, T. 1967. Sperm metabolism. In "Fertilization" (C.B. Metz and A. Monroy, ed.), vol. I, pp. 99-116, Acad. Press, New York.

Marden, W.G.R. 1954. New advances in electroejaculation of the bull. J. Dairy Sci. 37:556-561.

Mastroianni, L., Jr. and W. A. Manson, Jr. 1963. Collection of monkey semen by electroejaculation. Proc. Soc. Exp. Biol. Med. 112: 1025-1027.

McRorie, R. A. and W. L. Williams. 1974. Biochemistry of mammalian fertilization. Ann. Rev. Bioch. 43:777-803.

Meizel, S. 1972. Biochemical detection and activation of inactive form of a trypsin-like enzyme in rabbit testes. J. Reprod. Fertil. 31:459-462.

Meizel, S. and Y. H. Huang-Yang. 1973. An inactive form of a trypsin-like enzyme in rabbit testes and epididynaml sperm. Anat. Rec. 175:387-388.

Meizel, S. and S. K. Mukerji. 1975. Proacrosin from rabbit epididymal spermatozoa: Partial purification and initial biochemcial characterization. Biol. Reprod. 13:83-93.

Meizel, S. and S. K. Mukerji. 1976. Biochemical studies of proacrosin and acrosin from hamster cauda epididymal spermatozoa. Biol. Reprod. 14:444-456.

Mukerji, S. K. and S. Meizel. 1975a. The molecular transformation of rabbit testis proacrosin into acrosin. Arch. Biochem. Biophys. 168:720-721.

Mukerji, S. K. and S. Meizel. 1975b. Conversion of rabbit testis proacrosin to acrosin. FEBS. 54:269-273.

Multamaki, S. and M. Niemi. 1972. Trypsin-like proteolytic activity in an acrosomal extract of bull spermatozoa. Int. J. Fert. 17: 43-52.

Nalbandov, A. U. 1964. Reproductive Physiology: Comparative Reproductive Physiology of Domestic Animals and Laboratory Animals, and Man. 2nd ed. Freeman Co., San Francisco. 316p.

Palmer, M. B. and B. Howarth, Jr. 1973. The requirement of a trypsin-like acrosomal enzyme for fertilization in the domestic fowl. J. Reprod. Fertil. 35:7-11.

Pedersen, H. 1969. Ultrastructure of the ejaculated human sperm. Z. Zellforsch 94:542-554.

Pedersen, H. 1972. Further observations on the fine structure of human spermatozoon. Z. Zellforsch. 123:305-315.

Penn, A., B. L. Gledhill and Z. Darzynkiewiez. 1972 . Modification of gelatin substrate procedure for demonstration of acrosomal proteolytic activity. J. Histochem. Cytochem. 20:499-506.

Penn, A. 1975. Studies on trypsin-like enzymes in sperm and early embryos. Ph.D. Thesis, University of California, Livermore, California.

Penn, A. and B. L. Gledhill. 1972 . Acrosomal activity of amphibian sperm. Exp. Cell Res. 74:285-288.

Polakoski, K. L. 1974. Partial purification and characterization of proacrosin from boar sperm. Fed. Proc. 33:1308.

Polakoski, K. L., L.J.D. Zaneveld and W. L. Williams. 1971. An acrosin-acrosin inhibitor complex in ejaculated boar sperm. Biochm. Biophys. Res. Commun. 45:381-386.

Polakoski, K. L., L.J.D. Zaneveld and W. L. Williams. 1972. Purification of a proteolytic enzyme from rabbit acrosomes. Biol. Reprod. 6:23-29.

Polakoski, K. L., R. A. McRorie and W. L. Williams. 1973. Boar Acrosin. I Purification and preliminary characterization of a proteinase from boar sperm acrosomes. J. Biol. Chem. 248:8178-8182.

Polakoski, K. L. and R A. McRorie. 1973. Boar Acrosin II Classification inhibition and specificity studies of a proteinase from sperm acrosom. J. Biol. Chem. 248:8183-8188.

Reynolds, E. S. 1963. The use of lead citrate and high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208-212.

Rodger, J. C. and I. G. White. 1975. Electroejaculation of Australian marsupials and analyses of the sugars in the seminal plasma from three macropod species. J. Reprod. Fert. 43:233-239.

Roussel, J. D. and C. R. Austin. 1968. Improved electroejaculation of primates. J. Inst. Anim. Tech. 19:22-32.

Schill, W. B., W. D. Schleuning, H. Fritz. V. Wendt and N. Heimburger. 1975. Immunofluorescent and localization of acrosin in spermatozoa by boar acrosin antibodies. Naturwissenchaften. 11:540.

Schleuning, W. D., H. Schiessler and H. Fritz. 1973. Highly purified acrosomal proteinase (boar acrosin): Isolation by affinity chromatography using benzamidine-cellulose and stabilization. Hoppe-Seyler's Z. Physiol. Chem. 354:550-554.

Schleuning, W. D. and H. Fritz. 1974. Some characteristics of highly purified boar acrosin. Hoppe Seyler's Z. Physiol. Chem. 355:125-130.

Scott, J. V. and P. J. Dziuk. 1959. Evaluation of electroejaculation techniques and spermatozoa thus obtained from rat, mice and guinea-pigs. Anat. Rec. 133:655.

Snyder, R. L. 1966. Collection of mouse semen by electroejaculation. Anat. Rec. 155:11.

Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.

Srivastava, P. N., C. E. Adams and L. F. Hartree. 1965. Enzymatic action of acrosomal preparations on the rabbit ovum, in vitro. J. Reprod. Fertil. 10:61-67.

Srivastava, P. N., L.J.D. Zeneveld and W. L. Williams. 1970. Mammalian sperm acrosomal neuraminidases. Biochem. Biophys. Res. Comm. 39:575-582.

Stambaugh, R. and J. Buckley. 1968. Zona pellucida dissolution enzymes of the rabbit sperm head. Science 161:585-586.

Stambaugh, R. and J. Buckley. 1969. Identification and subcellular localization of the enzymes effecting penetration of zona pellucida by rabbit spermatozoa. J. Reprod. Fert. 19:423-432.

Stambaugh, R., B. G. Brackett and L. Mastroianni. 1969. Inhibition of in vitro fertilization of rabbit ova by trysin inhibitors. Biol. Reprod. 1:223-227.

Stambaugh, R. and J. Buckley. 1970a. Acrosomal enzymes of mammalian spermatozoa effecting fertilization. Fed. Proc. 30:1184.

Stambaugh, R. and J. Buckley. 1970b. Comparative studies of the acrosomal enzyme of rabbit, rhesus monkey and human spermatozoa. Biol. Reprod. 3:348-352.

Stambaugh, R. and J. Buckley. 1970c. Histochemical subcellular localization of the acrosomal proteinase effecting dissolution of the zona pellueida using fluorescent-labeled inhibitors. Fert. Steril. 23:348-352.

Stambaugh, R. and J. Buckley. 1972. Studies on Proteinase of rabbit spermatozoa. Biochem. Biophys. Acta 284:473-477.

Stambaugh, R. and M. Smith. 1974. Amino acid content of rabbit acrosomal proteinase and its similarity to human trypsin. Science 186:745-746.

Stambaugh, R., M. Smith and S. Faltas. 1975. An organized distribution of acrosomal proteinase in rabbit sperm acrosomes. J. Exp. Zool. 193:119-123.

Suomin, J. and M. Niemi. 1972. Human seminal trypsin inhibitor. J. Reprod. Fertil. 29:

Takamatsu, H., M. Itok, K. Mizoguchi and J. Iketani. 1963. New histochemical demonstration of proteinase: Silver protein method. Proc. Jap. Histochem. Assoc. 4:146-147.

Teichman, R. J., M. Fujimoto and R. Yanayimachi. 1972. A previously unrecognized material in mammalian spermatozoa as revealed by malachite green and pyroninc. Biol. Reprod. 7:73-81.

Teichman, R. J., J. M. Cummins and G. H. Takei. 1974. The characterization of a malachite green stainable, glutaraldehyde extractable phospholipid in rabbit spermatozoa. Biol. Reprod. 10:565-577.

Weisbroth, S. and F. A. Young. 1965. The collection of primate semen by electroejaculation. Fert. Steril. 16:229-235.

William, W. L. 1972. Biochemistry of capacitation of spermatozoa. In: Biology of Mammalian Fertilization and Implantation. Eds. K. S. Moghissi, E.S.E. Hafez. pp. 19-53. Springfield: Thomas

Yanagimachi, R. and R. J. Teichman. 1972. Cytochemical demonstration of acrosomal proteinase in mammalian and avian spermatozoa by a silver proteinate method. Biol. Reprod. 6:87-97.

Zaneveld, L.J.D., R. A. McRorie and W. L. Williams. 1968. A sperm enzyme that removes the corona radiata from ova and its inhibition by decapacitation factor. Fed. Proc. Fed. Amer. Soc. Exp. Biol. 27:567.

Zaneveld, L.J.D., R. A. McRorie, W. L. Williams. 1969a. Enzymatic removal of corona radiata of the ovum. Fed. Proc. 28:705.

Zaneveld, L.J.D., P. N. Srivastava and W. L. Williams. 1969b Relationship of a trypsin-like enzyme in rabbit spermatozoa to capacitation. J. Reprod. Fert. 20:337-339.

Zaneveld, L.J.D., R. T. Robertson, M. Kessler, P. N. Srivastava and W. L. Williams. 1970a. Inhibition of fertilization in vitro by mammalian trypsin inhibitors. Fed. Proc. 29:644.

Zaneveld, L.J.D., P. N. Srivastava and W. L. Williams. 1970b. Inhibition by seminal plasma of acrosomal enzymes in intact sperm. Proc. Soc. Exp. Biol. Med. 133:1172-1174.

Zaneveld, L.J.D. and W. L. Williams. 1970. A sperm enzyme that disperse the corona radiata and its inhibition by decapacitation factor. Biol. Reprod. 2:363-368.

Zaneveld, L.J.D., R. T. Robertson, M. Kessler and W. L. Williams. 1971. Inhibition of fertilization in vitro by pancreatic and seminal plasma trypsin inhibitors. J. Reprod. Fertil.25:387-392.

Zaneveld, L.J.D. and K. L. Polakoski. 1971. Characterization of sperm acrosomal hyaluronidase and acrosin. Biol. Reprod. 5:85.

Zaneveld, L.J.D., K. L. Polakoski and W. L. Williams. 1972a. Properties of a proteolytic enzyme from rabbit sperm acrosomes. Biol. Reprod. 6:30-39.

Zaneveld, L.J.D., B. M. Dragoje and G.F.B. Schumacher. 1972b. Acrosomal proteinase and proteinase inhibitor of human spermatozoa. Science 177:702-703.

Zaneveld, L.J.D., K. L. Polakoski and W. L. Williams. 1973a. A proteinase and proteinase inhibitor of mammalian sperm acrosome. Biol. Reprod. 9:219-225.

Zaneveld, L.J.D., G.F.B. Schumacher, H. Fritz, E. Fink and E. Jaumann. 1973b. Interaction of human sperm acrosomal proteinase with human seminal plasma proteinase inhibitors. J. Reprod. Fertil.32:525-529.

Zamboni, L., R. Zemjanis and M. Stefanini. 1971. The fine structure of monkey and human spermatozoa. Anat. Rec. 169:129-154.