THE DEVELOPMENT AND EFFICACY OF A NEW STAIN FOR ASSESSING ACROSOMAL CHARACTERISTICS OF SPERMATOZOA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Impaired fertility in the male and female of farm animals has several causes, and spermatozoal abnormalities are known to contribute to lowered fertility.

There have been several studies dealing with acrosomal structure and functions. Anomalies of the acrosome cap are classified among the primary abnormalities of the spermatozoa (105). Abnormal acrosome caps in the bull and other farm animals, as well as in laboratory animals, have been defined by several workers to be a cause of complete sterility. In addition, some of the acrosomal malformations are known to be hereditary (46, 58, 60, 112). Therefore, because of its relationship to male fertility, the study of the acrosome cap is important. The acrosome cap apparently is the carrier of the enzyme hyaluronidase, which plays an important role in fertilization. In regard to this function of the acrosome cap, Jacquet et al (71) found a close relationship between the concentration of the enzyme in the semen and fertility. A possible similar correlation was also suggested by Johnston and Mixner (73).

Although there have been many investigations about the acrosome cap, more studies are needed to identify

clearly the nature of this tiny component of the spermatozoon, and to secure specific information about its relation to the male fertility. As an introduction to this study, a brief discussion of the sperm cell will follow.

The Sperm Cell

Mayer (83) has defined the sperm cell as follows:

> The mature mammalian spermatozoon is an atypical cell, unique both morphologically and physiologically. It is essentially a nucleus with a haploid chromosome number functioning without its full quota of cytoplasm. This unique cell is capable of independent motility, but is incapable of reproducing itself. It performs its chief function, union with an ovum, separated from and ungoverned by the parent organism.

Johan Ham, of Holland, was the first to see the human sperm cell and his observations were reported by the famous Leeuwenhoek in 1677 after the invention of the light microscope. The detailed structure and function of the spermatozoon could not be well defined until the later development of the ultraviolet, phase-contrast, and electron microscopes, and improved histochemical techniques.

The bull spermatozoon is about 70μ in length (105). The head, as described by Blom and Brich-Andersen (30), is 8.5-9.0μ in length, 4.5-5.0μ in width, 0.5μ thick at the base, 0.3μ thick at the middle, with its shape resembling that of a tennis racket, broad and flat.

The hereditary material of the sperm is coded in the deoxyribonucleic acid (DNA), which, according to Leuchtenberger et al (78) constitutes about 43 per cent of

the nuclear chromatin. The remaining 57 per cent is protein. The nucleus occupies most of the sperm head, forming a highly electron-dense material, and is covered by three membranes: (1) the nuclear membrane, (2) the acrosome cap anteriorly with the perforatorium at the apex of the nucleus beneath the inner membrane of the acrosome cap, and the post nuclear cap posteriorly, and (3) the cell membrane.

The tail is about 60μ in length (105) and is composed of three parts, the mid-piece, main piece, and end piece. The mid-piece connects to the head at the implantation region, which contains the proximal centriole (30). The mid-piece is made up of the axial filaments which are arranged in two concentric rings of nine fibrils each and surrounding two central filaments (9- 9- 2). It is also covered by the helix or spiral of mitochondria (The mitochondrial sheath) which, according to Blom and Brich-Andersen (28), contains the sperm enzymes that are important for the oxidative metabolism of the cell, and, thus, the mid-piece is regarded as the power house of the cell (57). The mid-piece ends in the ring centriole (105). The main piece is the longest part of the tail (about 30μ) and is responsible for sperm motility. The axial filaments in this portion are not covered by a mitochondrial sheath. The end piece (about 3μ) is composed of the axial filament fibrils that are not covered by a sheath.

Definitions

The Acrosome Cap

The acrosome cap is a relatively unstable and fragile structure that arises from the Golgi apparatus during spermiogenesis, and covers about two-thirds of the anterior part of the sperm cell head. In ejaculated living spermatozoa, it is closely adherent to the sperm surface, but it undergoes rapid changes that lead to its detachment upon the death of the cell, maltreatment, or incapacitated spermatozoa in the female reproductive tract. The acrosome cap is thought to be the site of an enzyme, or enzymes, that have an important role in fertilization.

The Perforatorium

The perforatorium is a modified thickened region of the spermatozoon nuclear membrane that lies on the apex of the nucleus beneath the inner membrane of the acrosome cap. The perforatorium is thought to be present, in varying forms, in all animals and probably plays a role in sperm penetration through the zona pellucida of the egg cell.

CHAPTER II

LITERATURE REVIEW

The Morphology of the Acrosome Cap

The acrosome cap has been investigated and its existence confirmed in several species of vertebrates and invertebrates. Rao and Berry (98) and Austin and Bishop (8) suggested that it is a universal structure of flagellate spermatozoa, and Nath (87) believes that the acrosome cap is present in all animal sperm from the sponges upward in the animal kingdom.

Bishop and Walton (24) stated that the term 'acrosome' seems to have been used by Lenhossek in 1898. Later, more than ten terms were used to refer to this structure. In this study, the term "acrosome cap" will be used in the sense adopted by Blom and Brich-Andersen (30).

The acrosome cap appears to originate from the Golgi apparatus of the spermatids during the process of spermiogenesis. Gattenby, as quoted by Schrader and Leuchtenberger (107), Bowen (31), and Gumbo (54) have stated that the acrosomal material might be secreted from the Golgi apparatus. Gresson (52) and Nath (87) suggested that the acrosome is formed from the proacrosome and the vacuoles

surrounding it. This was confirmed by the use of the periodic acid-Schiff technique. Leblond and Clermont (76) ascertained the presence of a PAS-reactive material in the acrosome and in the Golgi material of the spermatid. A similar finding was also stated by Schrader and Leuchtenberger (107). Several other workers have also described the Golgi apparatus as the origin of the acrosome cap in different species studied. After formation of the proacrosome, which gives rise to the acrosome cap, the Golgi remnant migrates to the posterior end of the maturing spermatid and eventually is shed from the cell with the residual cytoplasm (24).

Blom (26) demonstrated the presence of a sperm cap in the bull and stallion and identified it as the 'galea capitis'. This term was also used by Bretschneider (32, 33) who stated that the galea capitis of the mature spermatozoa of the bull is about 250-370 mμ in thickness, and is composed of inner and outer parts, the latter being slightly denser than the former which contained granules of the order of 50 to 80 mμ. Reed and Reed (99, 100) demonstrated the existence of several granules of the order of 0.25μ in the galea capitis of the bull spermatozoon.

The recent use of the electron, phase contrast and ultraviolet microscopes, the improved histochemical techniques and the more recent use of the thin-sectioning technique with electron microscopy have yielded valuable details of the formation, structure, and function of this

important component of the sperm cell. Considerable disagreement, however, still exists and more studies are needed.

The acrosome cap has been described by some workers as a single structure, others described it as composed of two components. The presence of one or more vacuoles in the acrosome was reported by some workers, thus Schnall (106) found one to three vacuoles in the human acrosome, and suggested that these vacuoles might be gaseous metabolic end-products on their way to escape from the sperm head. He also suggested that this rarely observed phenomenon may be a defect in the sperm head. In earlier studies, the acrosome itself was demonstrated to be absent in the human spermatozoon (67, 100), but later investigations confirmed its existence as a closely adherent cap on the human sperm head (50, 106).

Baylor et al (16) stated that the sperm head is always covered by a protoplasmic cap which becomes damaged or disappears if sperm are stained or fixed. Randall and Friedlaender (97) using the electron microscope, showed that the galea capitis of the ram becomes closely attached to the sperm head sides and detached from the tip upon drying of the spermatozoa. They also stated that the ram galea capitis has three components: (1) a transparent membrane, about 100 mμ in thickness, (2) a fibrous component, and (3) a globular component. Clermont and Leblond (38), studying spermiogenesis in man, monkey and ram, and

Burgos and Fawcett (34), working with the cat, found that the acrosomic granule flattens over the nuclear surface, giving rise to the acrosome. Gresson (52) described the galea capitis as a thin sheath-like structure present external to the acrosome of the ram spermatozoon. Clermont (37) described the rodents 'acrosomic system' to consist of a head cap and an acrosome. The cock acrosome, according to Grigg (53) consists of the apical spine, covered by the apical cap. Wu and Mckenzie (113) believe that the acrosome and galea capitis are two different structures and, thus, they disagreed with the conclusion of Hancock (59) that the terms acrosome and galea capitis have been used to describe the same structure in the bull sperm. These workers using the electron microscope, stated that the acrosome seems to consist of a tubular sheath and a central core, and is covered by the fragile galea capitis.

Hancock and Trevan (63) considered the bull acrosome to consist of the 'cap' and the 'bead'. Clermont and Leblond (38) described the acrosomic system of man, monkey, and ram spermatids to be composed of acrosome, head cap, and precursors originating from the Golgi zone of the spermatids. They suggested the existence of this system in all mammalian spermatozoa studied, and found it to consist of the head cap and acrosome in the mature spermatozoon of most species, but in the primates, they suggested the existence of only the head cap.

Hancock (59, 61) described the acrosome as a

cytoplasmic cap covering about two-thirds of the anterior of the spermatozoon head, and consisting of two components, the outer acrosome which is larger and having a straight posterior border coinciding with the anterior border of the post-nuclear cap, and the inner acrosome, which resembles the galea capitis of Blom (26), having a concave border directed caudally with its convex border following the outline of the front of the spermatozoon head. Hancock (61, 62) stated that the area between the posterior straight border of the outer acrosome and the posterior concave border of the inner acrosome is called the equatorial segment. These findings were confirmed by Lovell and Getty (79), but, as will be described later, differ from the recent observations of the structure of the acrosome cap and the equatorial segment. In a relatively similar description, Bishop and Austin (21) concluded that the acrosome consists of inner and outer caps, and that the equatorial segment is that part of the sperm head where the outer acrosome overlaps the inner acrosome. Karras (74) found the same structures, distinct outer and inner caps of the bull's acrosome. Fawcett and Burgos (48) presented evidence that the inner cap of the acrosome lies within the outer cap and not beneath it, and, thus, the acrosome is expected to behave as a single structure. Later, Fawcett (47) concluded that the 'acrosome' and 'head cap' in mammalian sperm cells are components of a single structure which he preferred to call the acrosomal

cap. Rahlman (96) suggested that the acrosomic cap of the bull overlaps the post nuclear cap.

Austin and Bishop (8), studying the acrosome in ten mammalian species with the phase-contrast and ultraviolet microscopes, found that there are marked differences in the size of the acrosome between species, the smaller acrosome was that of the bull and the larger was of the guinea-pig. From their observations on the breakdown of the acrosome, these authors confirmed the conclusion of Burgos and Fawcett (34) and Fawcett and Burgos (48) that the loss of the acrosome seems to involve the loss of the entire structure and not one part of it.

Bedford (17) found a significant reduction in the size of the rabbit acrosome during the passage of the sperm cells from the head of the epididymis to its tail, and suggested that the acrosome of the rabbit matures in the epididymis and not in the testis, as he found a significant correlation between the width of the acrosome and the position of the cytoplasmic droplet on the mid-piece. This reduction in acrosome size was suggested by the author to be a prerequisite for the acrosome to acquire its fertilizing ability. Dickey et al (44) found gradual changes in the acrosome cap of bull sperm, which took place in the caput, corpus and cauda epididymis and resulted in changing this cap from a wavy and loose fitting structure in testis sperm to a smooth structure closely adherent to the nucleus in ampullary and ejaculated sperm.

According to Fawcett (47), the inconclusive results obtained by the electron microscope before 1954 were due to the inability of the electron beam to penetrate the dense head of the intact spermatozoon. The improvement of thin-sectioning techniques with the use of electron microscopy made it possible to obtain better information about the sperm cell. Thus, Blom and Brich-Andersen (29) described an "apical body" in the galea capitis of the bull and rabbit spermatozoa, based on their study of thin sections of the sperm heads. They stated that both structures, in addition to the post-nuclear cap, are covered by the cell membrane of the sperm which envelopes the entire sperm from the head to the tip of the tail. Later, Blom (27) suggested that the biconvex structure that is often observed in the middle of the sperm head is the middle part of the nucleus covered only by the cell membrane, and suggested that the galea capitis seems (1) to carry the necessary enzymes and (2) to protect the sharp front edge of the sperm nucleus until it reaches the egg cell.

The most recent and extensive investigations of the bovine sperm head are those of Blom (28), Blom and Brich-Andersen (30), and Saacke and Almquist (104). These authors have used the electron microscope and thin-sectioning techniques on sperm cells that allowed them to obtain new and rare sections clearly identifying the structure of the sperm head. Blom and Brich-Andersen used the term 'acrosome cap' to identify the acrosome, as a whole,

while Saacke and Almquist used the term 'head cap', and their observations are, generally, in close agreement.

The nucleus was found by the above workers to be covered by three membranes; the nuclear membrane, the acrosome (head) cap, and the cell membrane. The acrosome cap was found by Blom and Brich-Andersen (30) to cover about sixty per cent of the anterior part of the nucleus. This cap was described as an electron dense, about 0.1μ thick, double-walled structure. The apical body was a characteristic thickening along the front edge of the sperm head, and was composed of a more or less close fold, the apical fold, and a thickening containing a smaller body of smaller density, called the apical vacuole. By using the permanganate as a fixation material, better information concerning the acrosome cap membranes were obtained. For the study of these membranes, the authors divided the acrosome cap into a front part and a rear part. The front part was found to be composed of a double-walled sac containing a substance between them of low electron density after permanganate treatment. The outer membrane of this part shows a well-defined swelling which is the apical body, while the inner membrane is closely adherent to the nucleus. The perforatorium is found at the tip of the nucleus beneath the inner membrane of the front part of the acrosome cap. The rear portion of the acrosome cap appears to be identical with the equatorial segment often seen with the light microscope. In this

aspect, Blom and Brich-Andersen's electron micrographs of the sagittal sections of the sperm head clearly showed this segment as a part of the acrosome cap, in which the outer and inner membranes are paralleled and close to each other, containing a layer of high electron density. This indicated clearly that the equatorial segment is a part of the acrosome cap. They suggested that the outer membrane of the front part of the acrosome cap is identical with the galea capitis of Blom (26) and that only this membrane is shed off spontaneously, artifically in vitro, or during the fertilization process. When this occurs, the inner membrane remains on the perforatorium and the front part of the nucleus, and the equatorial segment also remains attached to the middle of the head of the spermatozoon. The detachment of the galea capitis releases the acrosomal enzymes.

The former description of the three layers of the acrosome cap is in agreement with the findings of Saacke and Almquist (104). These workers confirmed the presence of the apical body at the tip of the sperm head, and referred to this structure as the 'hook', but they could not confirm the presence of the apical vacuole within the apical body, and suggested that they likely were not able to get identical sagittal sections as those of Blom and Brich-Andersen or that the spermatozoa might show individual variation with respect to the structure of the apical body. The equatorial segment was also demonstrated by

Saacke and Almquist to be a part of the head (acrosome) cap, and they agreed with Blom and Brich-Andersen that the detachment of the acrosome cap includes only the outer membrane and not the entire structure.

Effect of Abstinence and Aging on the Acrosome Cap

In addition to its functions as a storage site of the sperm cells and a site for their maturation, the epididymis acts as a place for spermatozoa resorption. Nicander (88) observed sperm fragements found intracellularly in the ductuli efferentes of a bull and in the epididymis of a rabbit.

Spontaneous detachment of the acrosome is observed in a small proportion of ejaculated sperm cells in several species (65). In 1945, Blom (26) observed up to 18% spontaneous detachment of the galea capitis in fresh semen of normal Danish bulls collected after three or more weeks of abstinence. The percentage was reduced to zero upon regular collection of the semen. The same phenomenon was also reported by this worker in stallions, and, thus, he concluded that the acrosomal detachment was due to prolonged stay of the spermatozoa in the epididymis and it was among the first stages of spermatozoal disintegration. This interpretation seems logical if one considers the very fragile nature of the sperm cap.

Hancock (59, 61) stated that the detachment of the

acrosome is a sign of the death of the sperm, and this was confirmed by Austin and Bishop (8), who also added that this phenomenon resembles that of acrosomal loss within the female genital tract (which will be discussed later).

The detachment of the acrosome cap can be produced artificially in vitro. Iype et al (70) found that the acrosome was completely lost from bull and buffalo sperm cells suspended in water and subjected to cold treatment, and then rewarmed to room temperature. Clermont et al (39) and Hathaway and Hartree (65) caused detachment of the acrosome by treating the spermatozoa with weak solutions of sodium hydroxide. Austin (4) found that digitonin was effective in inducing the detachment of the acrosome.

Aging of the sperm cells has a remarkable effect on the acrosome. Lovell and Getty (79), working with the boar, stated that as the spermatozoa become older, the outer acrosome becomes enlarged and swollen and, in later stages, becomes wrinkled. More aging resulted in a great percentage of acrosomal detachment. These authors noted a great number of motile bacteria in the seminal fluid after 24 hours of storage and suggested that the detachment of the outer acrosome may be due to bacterial or enzymatic action. Onuma (91), studying acrosomal abnormalities in bulls and boars, found that the percentage of the acrosomal abnormalities increased upon storage. He also concluded that the susceptibility of the acrosome to temperature shock was greater in boars than in bulls.

Chemical Composition of the Acrosome Cap

There have been several attempts to define the chemical composition of the acrosome cap. A brief review of the most important and recent works might be necessary to complete the picture of this structure.

The application of the periodic acid-Schiff technique of McManus (85) and Hotchkiss (69) in the investigations of the acrosome cap led to a better knowledge of this structure. Chemical compounds in solutions or in tissues were found to give different reactions after treatment with the PAS. Hotchkiss found that polysaccharides containing 1:2 glycol grouping react with the periodic acid to give polyaldehyde as follows:

$$R - \overset{OH}{C} - \overset{OH}{C} - R \xrightarrow{HIO_4} 2R - CHO$$

The aldehydes which are formed after the PA oxidiation from 1:2 glycols form a colored complex with the Schiff reagent, Fuchsin sulfite. Using this technique, Schrader and Leuchtenberger (107) found a polysaccharide with 1:2 glycol groups in both the spermatozoal acrosome and Golgi material of the spermatids. They confirmed their results by blocking the 1:2 glycol groups with acetic anhydride, giving a negative PAS reaction. They also described the presence of a small amount of protein in the acrosome, and thus suggested that the acrosome material probably contains a polysaccharide-protein complex which is similar to mucopolysaccharides. Wislocki (111) stated that no

glycogen or other carbohydrates could be detected in human sperm cells by the PAS technique.

The presence of protein in the acrosome, which was suggested by Friedlaender and Fraser (51), have been confirmed by several other workers (39, 70). Melampy et al (86) found that the bull acrosome gave a slight positive reaction with the PAS procedure. Mucoproteins were demonstrated by Lovell and Getty (79), using the PAS technique, to be present in the outer acrosome of the boar.

According to Bishop and Smiles (23), Bishop and Austin (21), and Cole and Cupps (41), when spermatozoa are subjected to ultraviolet radiation after treatment with acridine orange, the acrosome fluoresces red to bright red. This reaction may suggest the presence of ribonucleic acid (RNA) in the acrosome, but chemical analysis have indicated the absence of RNA in the sperm cells, and the acrosome itself does not react with pyronine (43).

The acrosome can be successfully separated from the spermatozoa and, thus, its chemical nature can be effectively studied. Clermont et al (39) separated the large acrosome of the guinea pig with sodium hydroxide and found it to contain galactose, mannose, fucose, and hexosamine. They suggested that some of these units are responsible for the periodic acid-Schiff staining reaction. The presence of sugars in the acrosome was confirmed by Hartree and Srivastava (64) who found that the ram acrosome contains mannose, galactose, fucose, glucosamine,

galactosamine and sialic acid, in addition to amino acids of which glutamic acid predominates. These workers stated that all the sialic acid seems to be contained in the acrosome. Hathaway and Hartree (65) quoted work in which washed bull spermatozoa were extracted with sodium hydroxide and showed the existence of protein, phospholipid, cholesterol, glucosamine, and glucuronic acid in the extract.

Iype et al (70) concluded that mammalian spermatozoa can incorporate labelled amino acids into the proteins of the acrosome. The sperm extract was found to contain some antigens, one of these, according to Katsh and Katsh (75), is probably a polysaccharide and, thus, it might be an acrosomal material.

Mann (80) stated that the acrosomal material is not starch, glycogen, or hyaluronic acid, but suggested it might be the enzyme hyaluronidase which is a liable PAS-reactive enzyme known to be present in mammalian spermatozoa. The existence of this enzyme in the acrosome has been reported by several workers, and a brief review of their findings will be presented later in this study.

The Perforatorium

Several workers have used the term perforatorium synonymously with the acrosome (24), but recent investigations have identified the perforatorium as a distinctive structure in the spermatozoon head.

Blandau (25), working with the rat, found the perforatorium to be a pronged structure fitting over the anterior portion of the nucleus. Austin and Sapsford (11) described a clear region in the rat sperm head as a vesicle, but Clermont et al (40) denied that this clear region is a vesicle and demonstrated that it was a part of the perforatorium, which is in turn distinct from the acrosome. They considered the rat perforatorium to be a thickening of the nuclear membrane that fits tightly over the apex of the nucleus, and stated that it does not stain with the periodic acid-Schiff technique (while the acrosome does) and it is resistant to alkaline hydrolysis (while the acrosome is not). Bishop and Austin (21) found this structure, which they called the rod, in the cotton rat spermatozoa.

Austin and Bishop (7, 8, 9) described the perforatorium to be a specialized structure that enables the sperm to penetrate through the zona pellucida after the loss of the acrosome. They demonstrated the presence of the perforatorium in golden and Chinese hamsters, rats, cotton rats and mice. After considering the findings of Burgos and Fawcett (34) and Fawcett and Burgos (48), which showed distinct changes in the region of the nuclear membrane of the human and cat spermatids where it is covered by the acrosome, Austin and Bishop (8) suggested that the perforatorium is less developed in all mammalian spermatozoa. This view was supported by Nicander and Bane (89) and Bane (14) who studied the

perforatorium with the electron microscope in the bull, boar, ram, dog, rabbit, and guinea pig, and found small amounts of electron-dense material between the acrosome and the nuclear membrane of all these species, mainly along the anterior part of the sperm head. The findings of Nicander and Bane were also confirmed by Blom (28) and by Blom and Brich-Andersen (30) in their studies of the ultrastructure of the bull sperm head. Austin and Bishop (9) stated that the perforatorium may carry a lysin which enable the spermatozoon to penetrate the zona pellucida of the egg cell.

Hafez (57) stated that the loss of the acrosome exposes the perforatorium which might have some unidentified enzyme that facilitates sperm passage through the zona pellucida. Austin (4) considered that the release of hyaluronidase from the acrosome during capacitation allows the sperm entry through the cumulus cells and then the removal of the acrosome which must precede sperm penetration through the zona pellucida.

The Acrosome Cap as a Carrier of Hyaluronidase

In order to fertilize the egg cell, the spermatoza have to penetrate through three barriers surrounding the ovulated ovum: (1) the cumulus oophorus, composing a mass of cells that are present in large amount in rodents, rabbits, and carnivores for several hours after ovulation.

In other species, including human and ungulates, most of these cells break down shortly after ovulation, (2) the zone pellucida, and (3) the vitelline membrane.

The old view of the role of the acrosome was suggested by Waldeyer in 1901 (cited by Bowen [31]), who stated that the position of the acrosome at the anterior part of the sperm head indicated that it has a mechanical function; that is, to act as a boring or cutting apparatus for sperm entry into the egg. This mechanical function was rejected in 1924 by Bowen (31) who suggested that some enzymes from the acrosome seems to activate the egg. Later investigations confirmed Bowen's conclusion.

Yamame (114), in in vitro studies, found that suspensions of rabbit sperm caused the dispersion of the cumulus cells from the ova. Pincus and Enzmann (95) found the rate of dispersal of the cumulus cells to be proportional to the sperm concentration in the suspensions used, and concluded that a heat-labile substance responsible for the dispersal of the follicle cells was present in the rabbit and rat spermatozoa. Hoffman and Duran-Reynals (68) stated that there is a 'spreading' factor in the testes and spermatozoa. Hyaluronidase was found by several workers to be present in considerable amount in mammalian spermatozoa. Mann (80) stated that the content of the enzyme per sperm differs among species. It is highest in rabbit and bull, less in human and boar, very little in dog and practically none in fowls and reptiles. Andersen (2) stated that this

enzyme is present in the sperm cells of all species (except perhaps the stallion and dog).

A close correlation between hyaluronidase content and sperm concentration in human, bull, boar and rabbit semen was found by Swyer (108). However, he did not find a similar correlation in fowl or dog semen. Bergenstal and Scott (20) found that the enzyme level is roughly proportional to the sperm concentration, and stated that the absence of spermatozoa was associated with the absence of the hyaluronidase, suggesting that the enzyme seems to be derived from spermatozoa or closely associated with them. McClean and Rowlands (84), working with rats, found a close correlation between the dispersion of the cumulus oophorus and the concentration of hyaluronidase, and suggested that the gel surrounding the ovum contains hyaluronic acid which is liquified by the enzyme so that the effective sperm can penetrate the ovum. They also stated that certain types of sterility may be the result of a deficiency of the enzyme. In this sense, Johnston and Mixner (73) confirmed that the level of hyaluronidase might be a factor in bull fertility. Feket and Duran-Reynals (49) confirmed the existence of large amounts of the enzyme in mammalian testes and spermatozoa, and its function in the dispersal of the follicular cells.

Masaki and Hartree (82) concluded that the enzyme is entirely present in spermatozoa at the time of ejaculation. Hechter and Hadidian (66) found that hyaluronidase

activity remained the same in washed spermatozoa, indicating that the enzyme is associated with the sperm cells. They also found that it is released from the sperm in the surrounding medium. Similar findings were obtained by Johnston and Mixner (73), Bergenstal and Scott (20), Perlman et al (92).

The association between the acrosome and the enzyme was suggested by several researchers. Rao and Berry (98) stated that the cytoplasmic cap is probably involved in the secretion of hyaluronidase. Leuchtenberger and Schrader (77), studying the chemical nature of the acrosome of the hemipteran insect "Arevelius albpunctatus", concluded that the acrosome material was not glycogen, starch, or hyaluronic acid, but it may be hyaluronidase. These workers compared bull sperm, which is a rich source of hyaluronidase, with snake sperm which is known to have no hyaluronidase. Using the periodic acid Schiff procedure, the snake sperm showed a very slight amount of PAS-positive material at the extreme tip of the acrosome, while the bull acrosome was completely stained by the PAS reaction, and, thus, they concluded that hyaluronidase may be constituting the acrosomal material. In a later paper, Schrader and Leuchtenberger (107) suggested that the enzyme is carried in the acrosome and it enables the sperm cells to penetrate through the hyaluronic acid matrix of the cumulus oophorus.

Bishop and Austin (21) suggested the existence of

hyaluronidase in the acrosome. Austin (4) treated rabbit semen with digitonin, a substance capable of removing the acrosome from the sperm heads, and treated another sample with formalin, a substance known to preserve the acrosome on the sperm heads. The supernatant from the first sample, which contained the acrosomes, had 22 units of hyaluronidase per ml., compared with 2.4 units per ml. in both untreated semen and the supernatant of semen treated with formalin. Austin (4,6) stated that hyaluronidase is released from the acrosome during sperm capacitation through changes in the properties of the acrosome, and this was confirmed by Blom (27).

Iype et al (70) found that storing aqueous suspensions of bull or buffalo spermatozoa at 0° C. and then rewarming them to room temperature resulted in the loss of acrosome. This treatment was shown by Swyer (109) to result in the release of hyaluronidase from the rabbit spermatozoa. The findings of these authors could be used to relate the enzyme location with the acrosome cap. More evidence of this may be gained from the findings of Hathaway and Hartree (65) and Piko' and Tyler (94). The former workers found that the disruption of the acrosome was accompanied by the release of hyaluronidase and, thus, suggested that at least a part of the enzyme is present in the acrosome. The latter workers found that the sperm cells which have just entered the zona pellucida had an opened acrosome with its contents missing and proposed

that the acrosome was opened by a break-down process with subsequent release of the acrosomal enzymes.

Acrosome Changes in the Female Reproductive Tract

Ovulation in farm animals occurs during or shortly after the estrus period. The relationship between the time of insemination and ovulation is very important to insure a high proportion of fertilization. Usually, insemination precedes ovulation for several hours. In the cow, for example, ovulation occurs about 12-14 hours after the end of the heat period. The best time for insemination is several hours before, preferably in mid or late estrus. In the rabbit, ovulation occurs about 9.5-10.0 hours after coitus. In the rat, coitus can precede ovulation by about 10 hours (12). The spermatozoa reach the fertilization site in the Fallopian tube within a few minutes and then wait for several hours for the ovum to be released. This period suggested that it may be necessary for the spermatozoa to acquire their final fertilization capacity within the female reproductive system. Evidence of this was found in the low conception rate, in several species of farm and laboratory animals, upon insemination shortly before or after ovulation. Austin (3) applied the term "capacitation" to refer to some unidentified physiological preparation of the spermatozoa within the female genital tract. Austin and Braden (10) found that the

minimum capacitation period, was four hours in the rabbit and two-four hours in the rat. Chang (36) found the minimum capacitation time to be six hours in the rabbit, two hours in the ferret, 20-30 hours in pigs, sheep and cattle, and up to several months in the bat.

There have been several reports about the changes of the spermatozoa during the capacitation process in laboratory animals. The literature indicated evidence of the morphological alteration in the acrosome cap and other parts of the spermatozoon head. Meves, as quoted by Dan (42), had reported as early as 1915 that the substance of the acrosome of Mytilus edulis dissolved very quickly and was absent in sperm cells that had just entered the egg cytoplasm. Popa, also quoted by Dan (42), was among the earlier workers to observe in 1927 that the acrosome of the sea urchin sperm showed some changes in the presence of substance from the egg cell.

Austin and Bishop (7) and Austin (4, 5) concluded that spermatozoa undergo capacitation before being able of penetrating the cumulus oophorus. Bishop and Austin (21) stated that capacitation might include the activation of the enzyme within the acrosome. It was suggested that changes in the acrosomal properties during capacitation result in the release of hyaluronidase (4, 6). The acrosome of the sperm cells which were recovered from the Fallopian tubes of the guinea pigs, golden hamsters, Chinese hamsters, and Libyian jirds had structural changes

and were removed from the sperm cells before entry into the egg cells, and, thus, it was suggested that as the sperm cells pass through the female reproductive tract the acrosome undergoes some changes and then is lost before sperm penetration through the zona pellucida (9). These acrosomal changes were also observed by Ohnuki (90) who stated that mouse sperm cells in the zona pellucida or in the internal area of the ova lack their acrosome.

No morphological changes were found in the acrosomes recovered from the Fallopian tubes of the rabbit (1, 6), and those recovered from the uterine sperm (19). Bedford (18) stated that the acrosomal changes detected were due to the effect of aging, rather than capacitation, but he confirmed that the acrosome cap is shed from the spermatozoa when they reach the perivitelline space of the ova. Austin (6), using phase-contrast microscopy, could not confirm the changes. However, with electron microscopy, he observed clear changes in the rabbit acrosome in the zona pellucida or the vitelline space, and concluded that the rabbit sperm loses the outer membrane of the acrosome upon entryinto the zona pellucida. These observations of Austin were confirmed by Hadek (55, 56) who also reported that the apical body of the rabbit acrosome is elongated after the loss of the head cap. Dickman (45) found that acrosomal detachment and sperm penetration into the zona pellucida in the rabbit may not occur if the full length of the sperm cell was attached to the zona pellucida.

However, detachment and penetration did occur when spermatozoa were attached to the zona by the acrosome.

Saacke and Almquist (104), working with the bovine spermatozoa observed that the shedding of the galea capitis might be involved as an early state in capacitation. However, they added that the changes in the head cap (acrosome cap) should not be considered solely as capacitation because some other structural and/or chemical changes are likely involved in this process. These findings were confirmed by Blom and Brich-Anderson (30) who suggested that the changes in the bull acrosome cap in the cow's genital tract includes: (1) the rupture of the outer membrane of the acrosome cap (which they called the galea capitis), which is shed off and, thus, the acrosome cap enzymes are released, (2) the detached galea capitis usually breaks down into two halves, and (3) the perforatorium remains on the tip of the sperm head covered by the inner membrane of the acrosome cap to play, probably, a role in sperm penetration into the egg.

Piko' and Tyler (93, 94), working with the rat, suggested that the sperm penetration into the ovum consists of opening-up of the acrosome, the release of the acrosomal enzyme(s) which enables the sperm to penetrate through the cumulus oophorus and zona pellucida.

Acrosome Abnormality in Relation to Male Infertility

A review of the different research results studying the abnormalities of the acrosome cap should clarify the relationship between these defects and infertility in farm animals.

Teunissen (110) reported that 82-96 per cent of the spermatozoa of a sterile bull in Holland had an abnormal formation at the anterior tip. This defect was also found in the spermatozoa of 60 other bulls which were either of low fertility or were sterile.

The evidence that acrosome abnormality is inherited was first presented by Hancock (58) who found that about 88 per cent of the spermatozoa of seven related Holstein-Friesian bulls had acrosomal deformities. The seven bulls were used in six different herds on 108 fertile females with no conceptions resulting. Two bulls participated in the pedigree of all these sterile animals. These findings were confirmed by Rollinson (102) and Rollinson and Makinson (103) who also found that this abnormality is frequently associated with infertility. They found that a certain bull with 5 per cent abnormal acrosomes was sterile, and suggested that 1 per cent or more abnormalities in the galea capitis in any bull would make him infertile. However, Bane and Nicander (15) stated that a small percentage of abnormal acrosomes, 1-2 per cent, might be found in the seminiferous tubules and in ejaculates

from normal fertility animals, including rabbits, stallions, rams, dogs, cats, and mice.

Hancock (60) and Donald and Hancock (46) demonstrated the "knobbed acrosome" as an abnormality in the spermatozoa of 17 Holstein bulls and suggested that it was due to an autosomal sex-linked recessive gene (kn), since 16 of those bulls were related to three common male ancestors. Knobbed acrosomes were also found by Jaskowski and Romaniuk (72) in 9 per cent of the spermatozoa of a sterile Black Lowland bull. A similar case in a Swedish Yorkshire boar was reported by Bane (13) where 92-95 per cent of the acrosomes had a backward-pointing, tongue-shaped segment at their anterior parts. Another type of abnormality was found in spermatozoa of that boar where 5-6 per cent of the sperm heads were malformed and had an abnormal distribution of the acrosomal substance on the heads of the sperm cells. Rob (101) found acrosomal defects in boars to be inherited, and Wohlfarth (112) presented evidence of the hereditary nature in this defect in six full-sib German Yorkshire boars, showing the defect (80-100 per cent abnormal acrosomes) to be due to a recessive gene. Buttle and Hancock (35) described the knobbed acrosome as a cause of sterility in boars and found that boars having this characteristic had a slightly higher percentage of dead spermatozoa than normal boars. These workers found few sperm cells on ova from sows mated to affected boars and they suggested that the failure of

fertilization was due to the failure of defective spermatozoa to attach to the ova. A similar interpretation of the abnormal acrosomes as a cause of sterility in boars was given by Bane and Nicander (15) who observed a high percentage of defective acrosomes in sterile boars.

In man, abnormal acrosome caps was also reported as a cause of sterility. Schnall (106) found that the acrosome was either smaller than normal or totally absent in a very high percentage of sterile men.

The discovery of the apical body in the acrosome cap of the bull spermatozoa (27, 28, 29, 30, 104) gave a simple and convincing interpretation of the knobbed acrosome previously described. The apical body of sperm cells of sterile bulls is changed into a cyst-like structure with inclusion bodies, six to eight times the normal size of the apical body. The perforatorium in such cells is bent. Blom and Brich-Andersen (30) concluded that the result of this malformation is an obstructing of the sloughing of the galea capitis, which, thus, prevent the penetration of the sperm into the ovum. They also suggested that the bent perforatorium may not allow the sperm head to break through the egg membrane.

Another type of acrosome malformation was described by Marx (81) as a "persistent acrosome". A German Improved Landrace boar had given good results with the first 72 sows he served. Later, on another farm, only 4 out of 15 sows he served became pregnant. The boar was castrated

and found to have 65% and 54% of the spermatozoa in his left and right vas deferens respectively to have a defect in the acrosome described as a "persistent acrosome". This defect differs from others mentioned before in that it did not appear until several mating seasons were passed. This case apparently was not hereditary since a son of the defective boar was used with good results.

Summary

In summarizing the evidence available, it appears that the acrosome cap is a double-walled structure that arises from the Golgi apparatus during spermiogenesis, and covers about 60 per cent of the sperm head. The acrosome cap undergoes quick changes upon the death of the cell, after a period of abstinence, maltreatment, or in capacitated spermatozoa. Recent studies have shown this cap as a single structure of two membranes, the outer membrane or galea capitis, and the inner membrane, with the equatorial segment being the rear portion of that cap, and the perforatorium at the tip of the nucleus between the inner membrane of the acrosome cap and the nuclear membrane (Figure 1).

The acrosome cap undergoes morphological changes during the process of capacitation, with the release of the enzyme hyaluronidase which plays an important role in fertilization. However, it seems that little is known with certainty about the changes that occurs within the surface

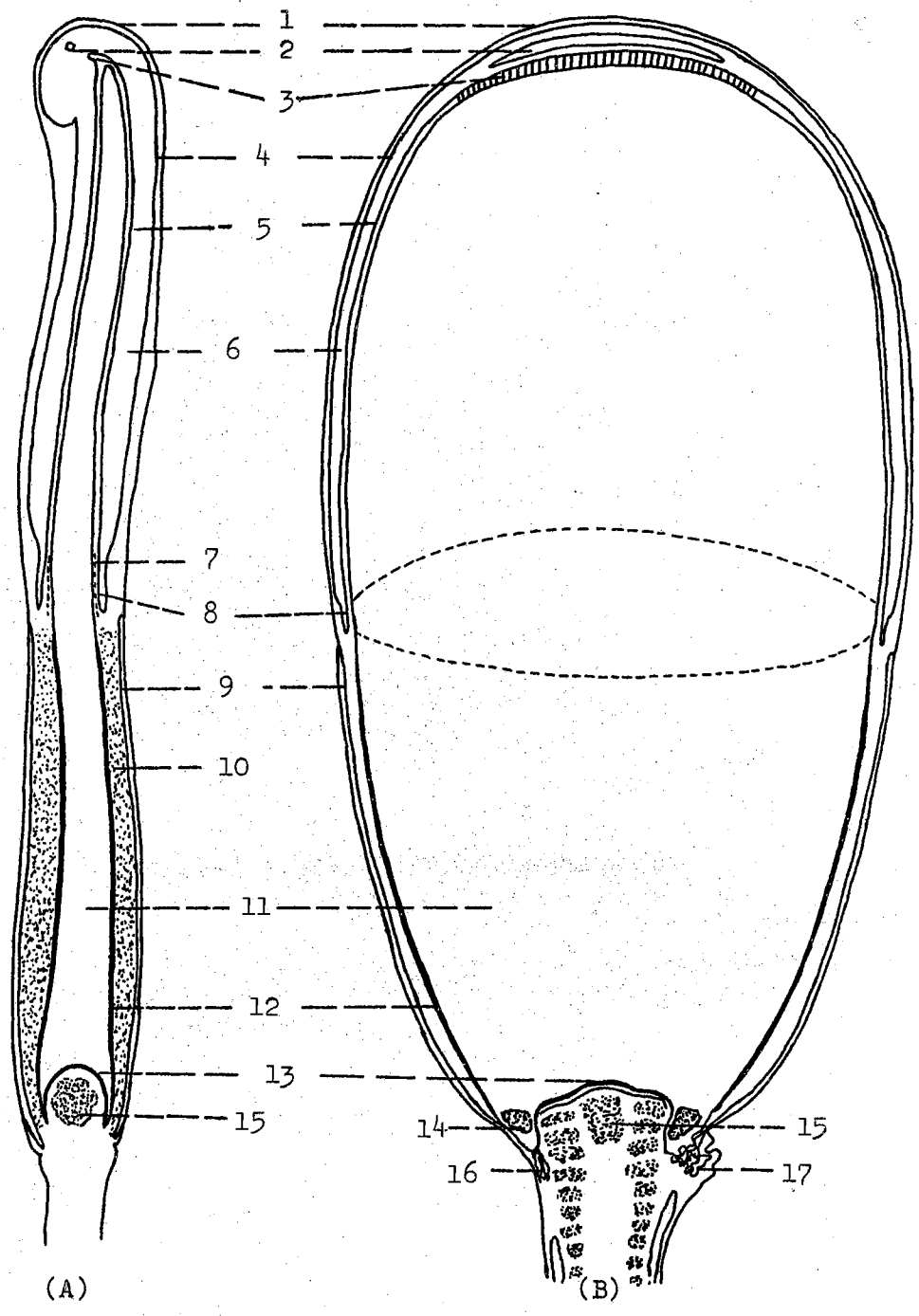

Figure 1. Schematic Drawing of the Normal Bull Sperm Head (30)
(A) Median-Sagittal Section
(B) True Coronal Section

1. Cell Membrane; 2. Apical Vacuole; 3. Perforatorium; 4. Outer Membrane of the Acrosome Cap; 5. Inner Membrane of the Acrosome Cap; 6. Acrosome Cap (contents); 7. Intermediate Layer; 8. Equatorial Segment; 9. Post-Nuclear Cap; 10. Intermediate Layer; 11. Nucleus; 12. Nuclear Membrane; 13. Basal Plate; 14. Basal Knob; 15. Centriole; 16. Evaginated Nuclear Membrane; 17. Evaginated Nuclear Membrane (Convulated Type)

of the sperm head during capacitation.

Abnormality of the acrosome cap, such as the knobbed acrosome may result in complete sterility. Some of the acrosomal abnormalities have been found to be hereditary. This indicates the possible role of the acrosome cap as a cause of impaired fertility. Further studies are needed to clearly elucidate the relationship of the state of the acrosome to the fertility of the animal.

CHAPTER III

EXPERIMENTAL PROCEDURE

The importance of the relationship of the acrosome cap to the fertility of the animal has been known for several years. However, few commercial artificial insemination studs evaluate the ejaculate for acrosomal characteristics. The basic reason is that the acrosome cap is not visible with the routine light microscope and the techniques typically used to evaluate semen characteristics. As was indicated in several studies in the literature review, the acrosome cap was studied with techniques involving ultraviolet, phase-contrast or electron microscopy. Such tools are not normally found in laboratories of bull studs. The studies reported herein center around a differential acrosome stain by which the acrosome cap can be viewed with the ordinary light microscope.

The stain used in these studies was developed by Dr. Milton Wells and the author in a series of studies on different stains of spermatozoa. The primary research interest was to develop a stain that would easily and effectively stain the sperm cells, particularly the acrosome cap. Several stains were used, with eosin B and fast green FCF being the eventual stains of choice. After

considerable research, the two stains were combined as follows:

Solution (A): 1% solution of water-soluble eosin B (total dye content 88%) in glass distilled water.

Solution (B): 1% solution of water-soluble fast green FCF (total dye content 90%) in glass distilled water.

1 volume of solution (A) was mixed with 2 volumes of solution (B) and 1.7 volumes of ethyl alcohol, giving a final pH of 6.8 to 7.0.

The smears were made, stained, and examined according to the following procedure:

1. One-tenth ml of the semen was diluted in 0.9 ml of 2.9% sodium citrate dihydrate at the same temperature of the semen. This rate of dilution was narrowed if the concentration of spermatozoa in the initial ejaculate was low.
2. A small drop of this diluted semen was placed on a clean slide and mixed with one equal drop of the stain, and then smeared in a thin layer.
3. After the smear was air-dried, it was mounted in Diaphane.
4. Two or more smears were made, and not less

than 150 sperm cells per slide were counted, unless sperm concentration in the smear was too low.

5. The acrosome cap studies were made with the light microscope under oil, at a magnification of 970X. A blue filter was used.
6. Photographs were taken with a Pony Kodak 35 mm camera, model 635, mounted on a trinocular Fluorestar microscope, model L16TG-FW. Kodacolor II film was used with color prints then made from the 35 mm slides.

The semen was obtained from bulls of the Oklahoma State University dairy and beef herds by means of artificial vagina or electro-ejaculator. Ejaculates were held at 30° C and maximum care was taken to avoid temperature shock. When sperm were to be held in refrigerator, this was done by putting the semen tube in a beaker containing water at 30° C and transferring it to the refrigerator. Cooling of this semen was gradual. A few ampules of bovine frozen semen were also utilized in these studies.

The efficacy of the developed stain (hereafter called the Wells-Awa stain) for assessing acrosomal characteristics was tested by treating spermatozoa in a variety of ways. These tests, though not exhaustive, were sufficiently varied so as to reveal the effectiveness of the stain. Limited studies were conducted to determine the efficacy of the stain in staining the acrosome cap of the

sperm cells of the ram and the boar. A description of the various studies follows.

The Knobbed Acrosome Cap

During these investigations, the "knobbed acrosome" condition was found in the spermatozoa of a Jersey bull. Slides were made using the Wells-Awa stain and the nigrosin-eosin stain, a differentiation live-dead stain. Smears were made with this stain as follows: One drop of the fresh undiluted semen was diluted in eight drops of the live-dead stain for five minutes. Then, a drop of the suspension was smeared on a clean slide on a warm stage, mounted in Diaphane and examined with the light microscope under oil (970X), using a blue filter. The nigrosin-eosin stain was made by dissolving 5g of water-soluble eosin B (total tye content 88%), in 300 ml of a 10% solution of nigrosin in distilled water (59)

The Effect of Aging on the Acrosome Cap

This investigation was conducted on semen collected from a Hereford bull having a low level of fertility and known to produce low concentration of spermatozoa. Two smears of the electro-ejaculator collected semen were made immediately after semen collection to determine the proportions of elevated acrosome cap and capless sperm cells in the initial ejaculate. The percentage of dead sperm was calculated with the nigrosin-eosin stain.

The ejaculate was divided into two parts, one of them was stored at 5° C for six days. Smears were made daily from this semen and stained with the Wells-Awa stain. Counts of sperm cells were made under oil (970 X), and the average percentage of capless cells were computed. The second portion of the ejaculate was incubated in a water bath at 37° C for 11 hours. Smears were made and stained with the same staining mixture every two hours to determine the effect of sustained high temperature on acrosomal characteristics. The average percentage of capless sperm cells in these smears was also determined under oil (970 X).

The Effect of Abstinence on the Acrosome Cap

The effect of abstinence was studied in the semen of a Jersey bull which was not used for semen collection for about six months.

Semen was collected by artificial vagina, and smears were made and stained with the Wells-Awa stain and examined under oil (970 X) where counts of the capless sperm cells and assessment of acrosomal characteristics were made.

The Effect of Freezing of Semen on the Acrosome Cap

This part of the studies was made using commercially frozen bovine semen. The semen was thawed in ice water,

then centrifuged for 5-10 minutes at 5000 RPM. The supernatant was discarded and the sperm cells were resuspended in 2.9% sodium citrate to 1 ml volume. Smears of the suspensions were prepared in the described method, and the per cent of detached acrosomes was determined.

CHAPTER IV

RESULTS AND DISCUSSION

The Wells-Awa Stain

An effective stain and a simple technique for preparing smears of spermatozoa for examination with the light microscope, and particularily the study of the acrosome cap, was developed by Dr. M. Wells, the author's adviser, and the author, and was called the Wells-Awa Stain.

Three levels of eosin B (0.5, 0.75, 1.0 per cent) were tested in combination with three levels of fast green FCF (1.0, 1.25, 1.5 per cent). In each combination, one volume of eosin B was mixed with two volumes of fast green FCF and 1.7 volumes of ethyl alcohol. Bull semen of different breeds was used to test these combinations. The smears were made and stained as explained earlier (Chapter III). Best results were obtained with 1 per cent eosin B (total dye content 88%) and 1 per cent fast green FCF (total dye content 90%) with ethyl alcohol. Substituting distilled water for ethyl alcohol did not give satisfactory staining. The stain, therefore, is composed of (a) 1 volume eosin B, (b) 2 volumes fast green FCF, and (c) 1.7 volumes ethyl alcohol. Mixing the dyes every few days

gave better results than using aged stain.

Due to the small size of the acrosome cap and its strong attachment to the sperm head in the fresh and normal ejaculate of the bull, the acrosome cap appears as a thin greenish sheath around the anterior two-thirds of the sperm head, with a very slight thickening on its tip. Aging or maltreatment of the spermatozoa lead to an easier identification of the acrosome cap (Figure 2).

The developed stain has no harmful effects on the sperm cells, no detached heads or other abnormalities that could be attributed to the stain or the staining technique were observed. A few sperm cells, however, were observed to take the stain very slightly and, thus, to be stained with a very pale red color. It is assumed that such cells had been dead for a long time. In such spermatozoa, it was difficult to identify the acrosome cap.

The stain results in the spermatozoon head and tail being stained red while the acrosome cap is stained green. Detached acrosome caps, which is an abnormality in the fresh ejaculate, can be easily seen in the smear, having a green color and a bathing-cap shape, as described by Blom and Brich-Anderson (30). Elevated acrosome caps are also easily observed (Figure 3). The capless sperm cells show the nuclei as elongated bright red structures protruding above the post-nuclear cap, showing clearly that the acrosome cap has been detached.

Among the other features of the stain are the uniform

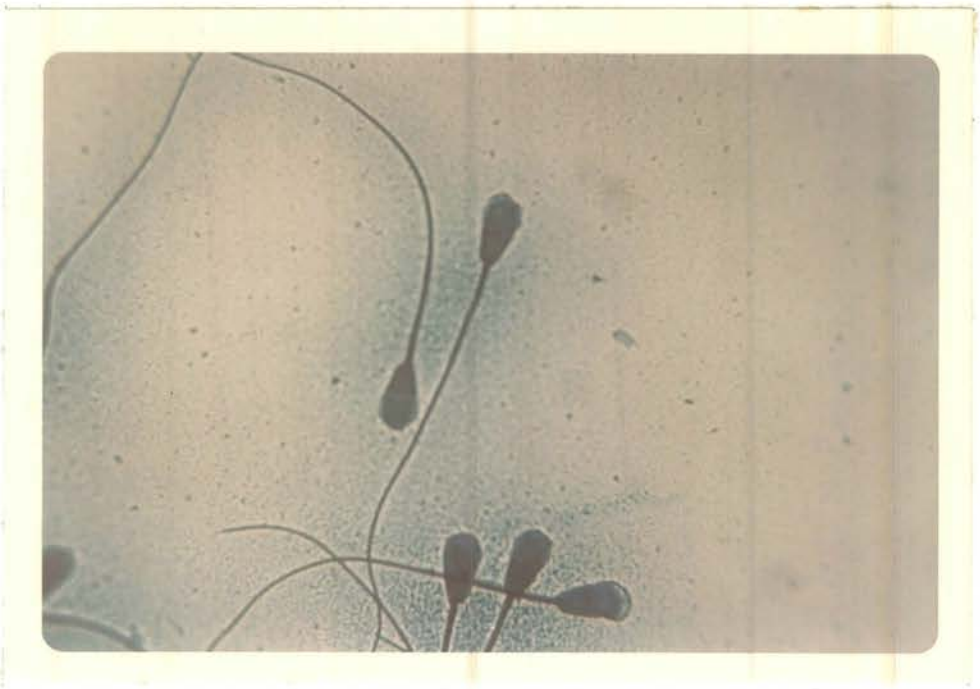

Figure 2. Elevation of the Acrosome Cap in Bovine Semen Stored at 5° C for Five Months

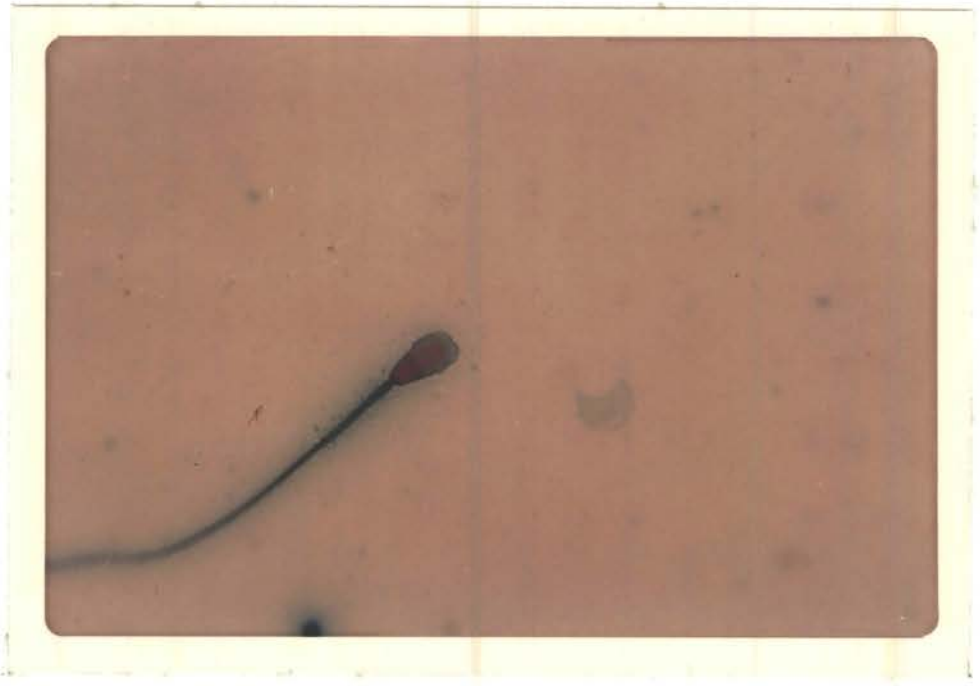

Figure 3. Elevated and Detached Acrosome Caps in an Ejaculate of a Bull

and relatively clearly stained slides, and the short time needed for preparing and staining the smears. The slides could be prepared for microscopic examination within a few minutes. The main suggested use of this stain is the detection of acrosomal abnormalities in fresh ejaculates, as will be discussed later.

The majority of the investigations were carried out on bovine semen; however, the effectiveness of the stain was tested on the ram and boar semen, using the same technique described for bull semen. The stain was found to be effective in staining ram semen, where it yielded clearly stained sperm cells. The acrosome cap of the ram spermatozoon was not, however, quite clear in the fresh ejaculate, and was observed to be closely adherent to the sperm head. Capless sperm cells of the ram had the same general appearance as that of the bull, and detached acrosomes could be easily seen in the smear (Figure 4).

Since these investigations did not include the effects of aging on the acrosome cap of the ram, and in order to be sure that the technique would show the acrosome cap, a rapid test was done by subjecting a few drops of the ram semen to cold shock (0° C for 15 minutes). The acrosome caps became relatively more visible (Figure 5). This cold-shock treatment suggested that the technique may be useful in staining the acrosome cap of the ram sperm. However, further studies are needed.

One ram ejaculate was held at room temperature (20° C)

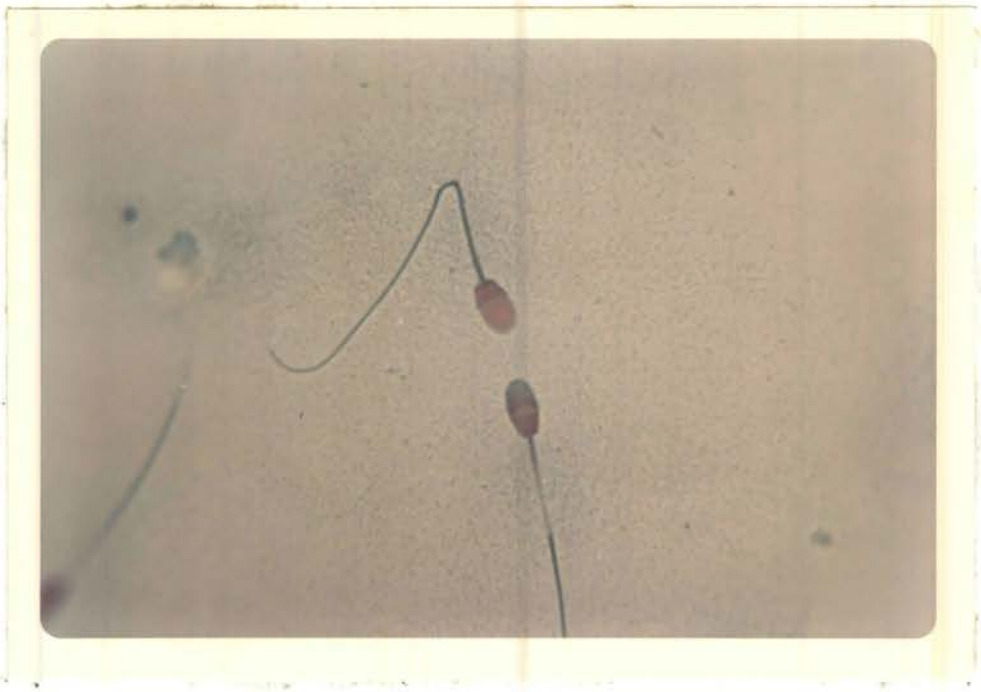

Figure 4. Capless Spermatozoa in the First Ejaculate of a Ram

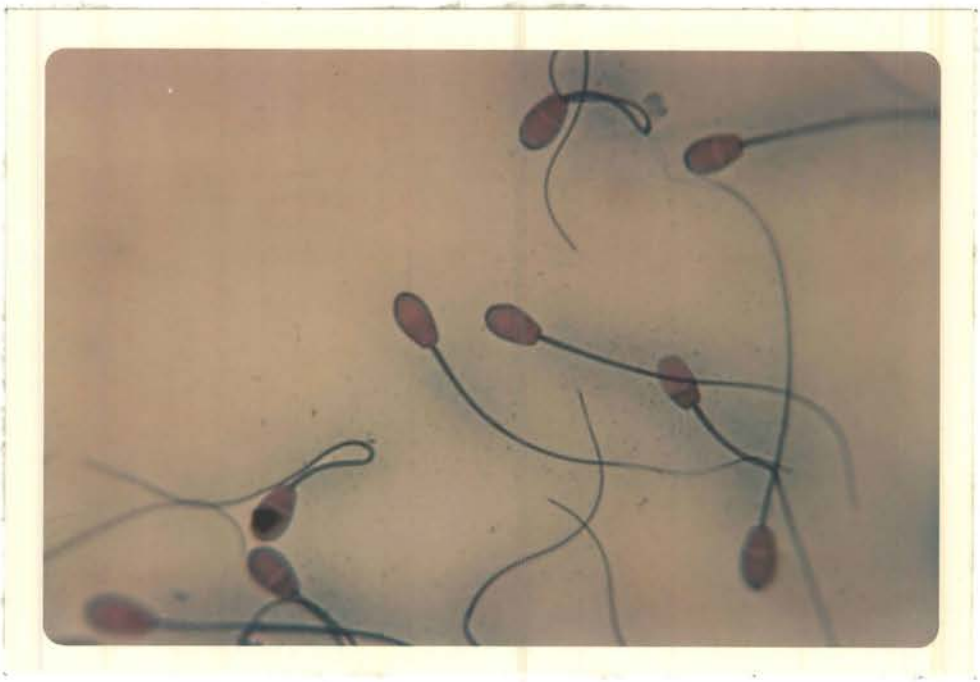

Figure 5. Effect of Cold Shock on the Acrosome Cap of the Ram

for 12 hours and the sperm cells showed a very evident elevation of the acrosome caps (Figure 6). The outline of these elevated caps, however, was smoother than that of aged sperm cells of the bull.

With boar semen, the stain was not successful for staining either the sperm cells or the acrosome cap. Many of the sperm cells were unstained and some cells were heavily and irregularly stained. The acrosome caps were not visible. This indicates that further studies and modifications are needed to use the stain on this specie of farm animal.

The Knobbed Acrosome Cap

In the course of these investigations, the knobbed acrosome cap was observed in the semen of a 3-year Jersey bull. This defect was clearly identified under oil with the light microscope in smears stained with the Wells-Awa stain (Figure 7). It was also noticed that the defect occurs mostly on sperm cells with tapered heads, a primary abnormality. An average of 43 per cent of these cells in the ejaculate exhibited the knobbed acrosome characteristic (Table II). It was rarely observed on the normal spermatozoa, again illustrating the usefulness of the stain in assessing acrosomal characteristics of spermatozoa.

Another observation on this defect was that in aged sperm cells, where the acrosome caps become gradually

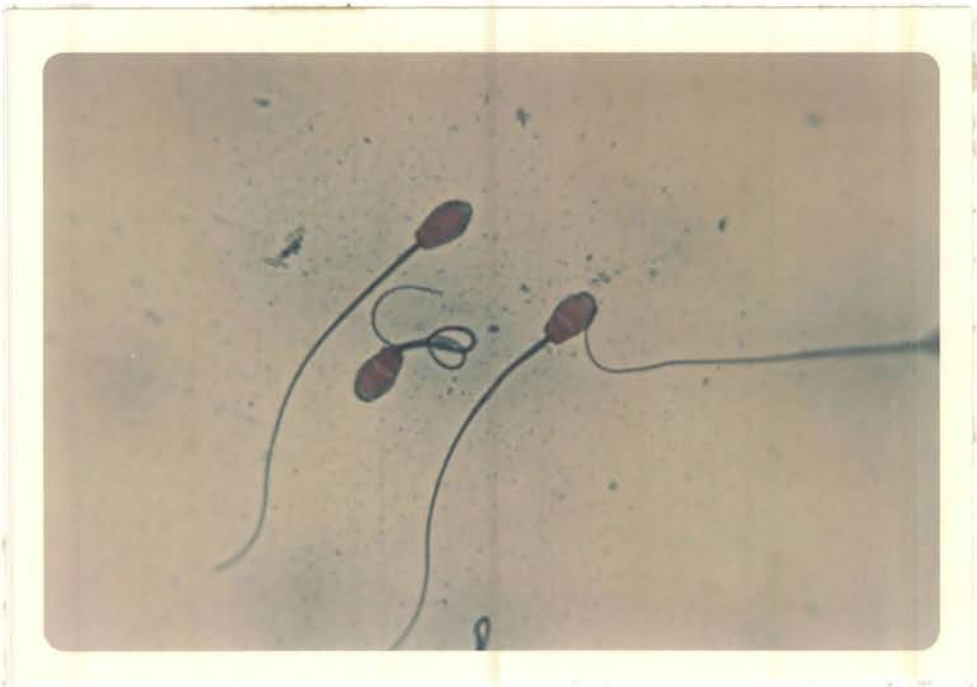

Figure 6. Effect of Storage of the Ram Semen at Room Temperature for 12 Hours on the Acrosome Cap

Figure 7. Fresh Spermatozoa of a Jersey Bull With the Knobbed Acrosome Cap (The Wells-Awa Stain)

Figure 8. Fresh Spermatozoa of a Jersey Bull With the Knobbed Acrosome Cap (The Nigrosin-Eosin Stain)

elevated, the elevation of the knobbed acrosome caps seemed to be impaired. This resulted in a reduced percentage of detached acrosome caps in such spermatozoa.

This defect was also noticeable with the light microscope (under oil) by using the nigrosin-eosin stain of Hancock (59), where the knobbed acrosome appeared as a bright small dot on the tip of the sperm heads (Figure 8). A similar comparison made with this stain revealed an average of 26.3 per cent (Table III) of the tapered sperm exhibited the knobbed acrosome. From these data, it appears that the Wells-Awa stain is more precise in revealing the knobbed acrosome defect.

Aging of the Acrosome Cap

Several workers have reported the changes that occur in the acrosome cap in the female reproductive system and this is known as the capacitation process. The activation of the enzyme, hyaluronidase, that exists in the acrosome cap is likely a part of this process. Capacitation is thought to be essentially a change in the properties and structure of the acrosome cap that leads to its detachment from the sperm head and the exposure of the perforatorium. Similar changes also occur upon the death of the sperm cells. Other authors, however, stated that the acrosome cap alterations should not be considered alone as capacitation (104).

It is of interest to study the changes of this cap in

vitro, since there are some similarities with those in vivo. In the normal fresh ejaculate of the bull, the acrosome cap is closely adherent to the anterior part of the sperm head. The outline of such caps is seen, with the Wells-Awa Stain, to be smooth with some slight thickening in the anterior portion of the acrosome cap. Aging of this cap, either in vivo or in vitro, results in some changes of its structure and functions. In these in vitro studies, sperm cells were incubated at 37° C for 12 hours or stored, undiluted, at 5° C for several days. A gradual elevation of the acrosome cap could be easily detected with the developed stain (Figures 9, 10, 11, 12). The majority of the acrosome caps lost their smooth outline and became, instead, wrinkled and irregular as well as elevated. This is in agreement with the findings of Lovell and Getty (79). Incubated sperm cells exhibited these changes much faster than refrigerated spermatozoa. The equatorial segment of the sperm head became more visible upon aging of the sperm cells. Spermatozoa stored beyond 10 days at 5° C did not stain well, with sperm outline and acrosomal features being quite indistinct.

Detachment of the acrosome cap was not observed to be the major effect of aging. The percentage of detached caps in spermatozoa of two bulls studied was less than 20 per cent when stored at 5° C or incubated at 37° C. On the other hand, the proportion of elevated acrosomes increased, reaching a percentage of about 70 per cent in

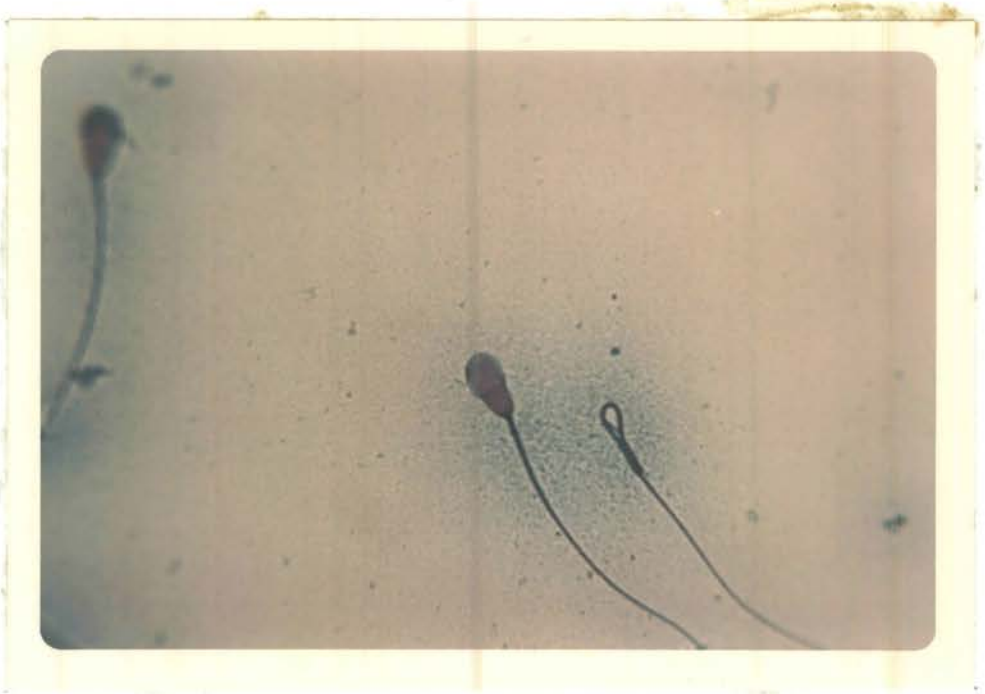

Figure 9. Effect of Semen Incubation at 37° C for Six Hours on the Acrosome Cap of the Bull

Figure 10. Effect of Semen Incubation at 37° C for Ten Hours on the Acrosome Cap of the Bull

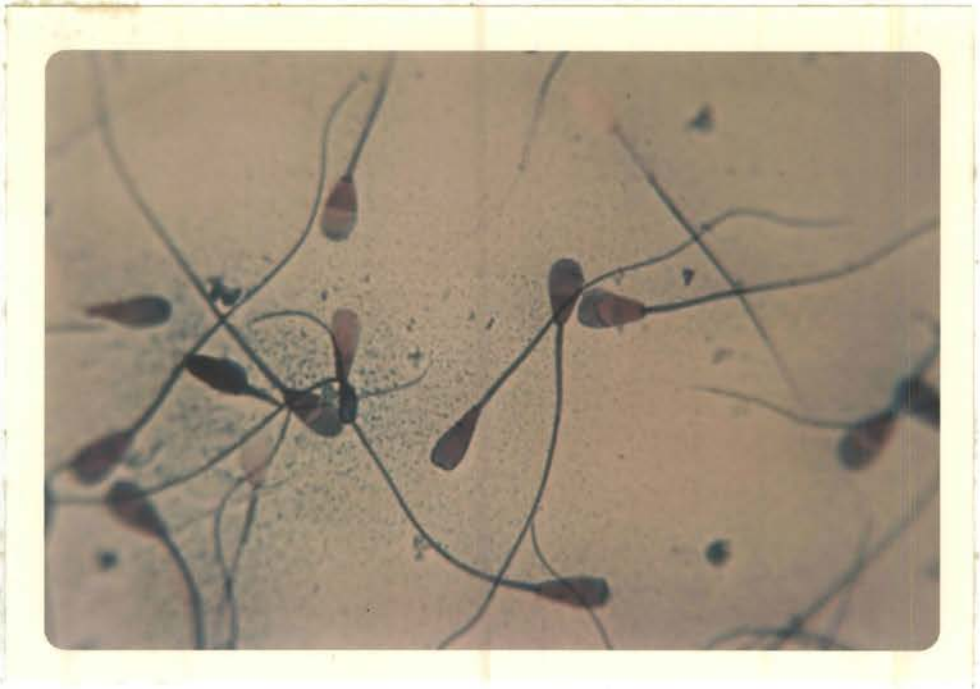

Figure 11. Effect of Semen Storage at 5° C for Three Days on the Acrosome Cap of the Bull

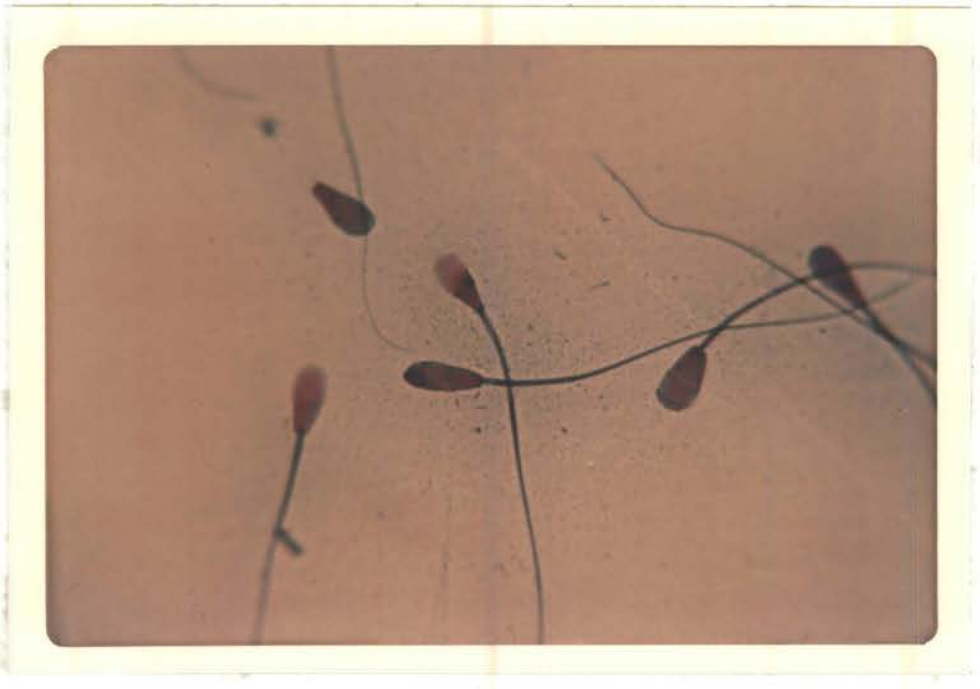

Figure 12. Effect of Semen Storage at 5° C for Six Days on the Acrosome Cap of the Bull

stored semen after about 6 days, and the same percentage after about 8 hours in incubated semen. This elevation was accompanied by disintegration in a small proportion of the acrosome caps.

It was noted that this pattern of morphological changes of the acrosome cap was not the same for all bulls studied, and it was possible to correlate the initially "changed" acrosome of a Hereford bull to his fertility. This bull was two years old and was known to have a low level of semen production and fertility. An ejaculate of this bull was studied with the developed stain which showed clearly some abnormal features of the acrosome cap.

The initial ejaculate, which had a low concentration of spermatozoa, contained a relatively high proportion of capless sperm cells (8.06 per cent) and particularly a very high percentage of spermatozoa with partially or completely elevated acrosomes (46.4 per cent) (Figure 13). Normal ejaculates, by contrast, contain a low percentage of capless cells or elevated acrosome caps. The effect of aging on the detachment of the acrosome caps of this bull was studied using the split-ejaculate technique. One fraction was stored at 5° C and sampled daily while the other was incubated at 37° C and sampled every two hours. The results are shown in Tables IV and V and in Figure 14. At the end of six days storage at 5° C, 34% of the sperm cells had detached caps. The cells incubated at 37° C showed rapid detachment of acrosomes, with 74.52%

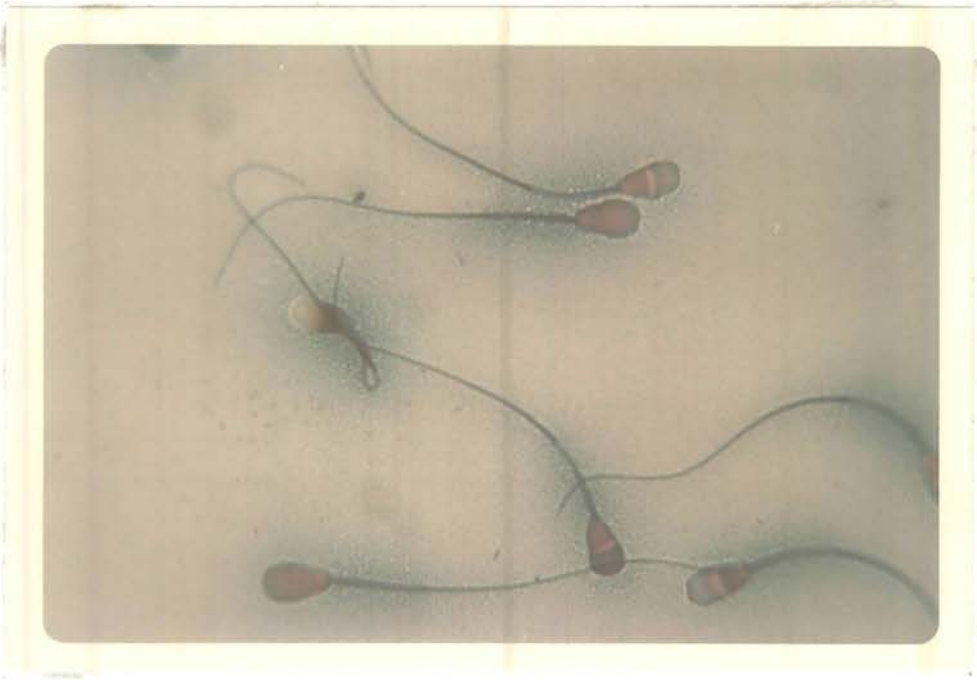

Figure 13. Initial Elevation and Detachment of the Acrosome Cap in the Semen of a Hereford Bull

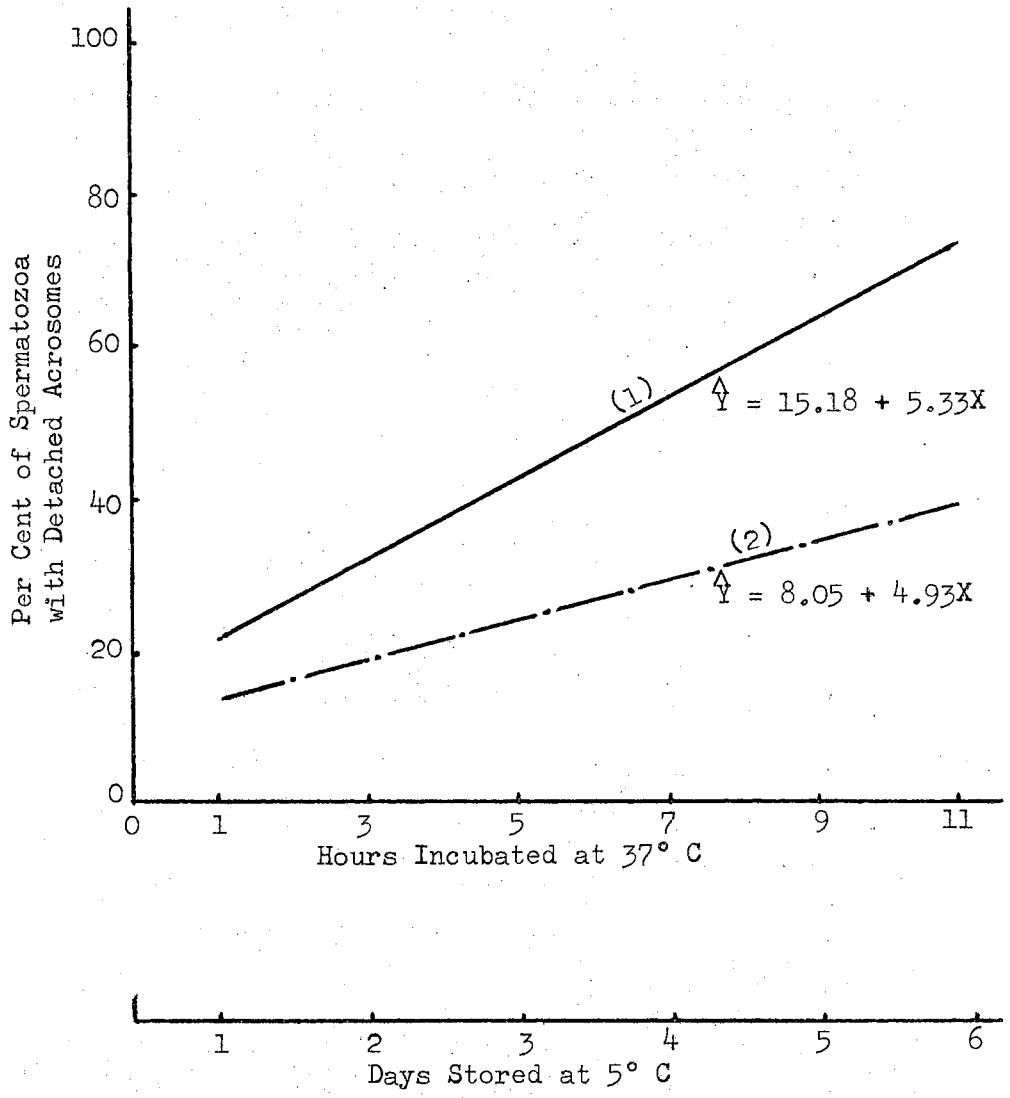

Figure 14. Effect of Semen Incubation at 37° C (Line 1) and Storage at 5° C (Line 2) on the Detachment of the Acrosome Cap of a Hereford Bull

showing detachment after 11 hours of incubation. These data clearly show that the stain is effective in visualizing changes in the acrosomal characteristics in ejaculated and stored sperm cells.

The Effect of Abstinence on Acrosomal Detachment

According to Salisbury and VanDemark (105), periodically resting of bulls routinely collected in artificial insemination centers will restore sexual interest of bulls fertility, however, this opinion lacks strong support. Too, there is some evidence that semen collected from the bull after a long period of sexual rest may not have good fertility. In the present studies, the semen of a Jersey bull, collected after several months of sexual rest, had many sperm cells that had no acrosome. No semen was collected from that bull for another six months. The first ejaculate from this bull after the long period of abstinence was characterized by a large proportion of capless spermatozoa (19.49%), as shown in smears of this ejaculate stained with the Wells-Awa stain. The nigrosin-eosin stain showed the ejaculate to have 16.84% dead sperm. This high level of detached acrosome caps should be considered as an abnormal character of the sperm cells that may have some detrimental effects on its fertility, particularly when compared to an average of 2.32% capless sperm in fresh ejaculates of bulls that contained 27.2% dead cells

(59), and to an average of 4.67% capless sperm in samples of commercially frozen semen studied in these investigations. This high level of detached acrosomes seems to be the result of aging of spermatozoa in the epididymis that leads to acrosomal detachment as the first step in sperm degeneration in the epididymis. This is in agreement with the findings of Blom (26) who stated that up to 18 per cent of the spermatozoa had detached acrosome caps in a bull ejaculate collected after a long period of abstinence; however, regular use of the bull reduced this percentage to zero. It is of interest to notice a similar high proportion of detached acrosomes occurs in semen collected for the first time. For example, 14.6 per cent of the sperm cells of a 15-month ram collected for the first time by the electro-ejaculator had no acrosome caps. This suggests that acrosomal detachment may be an intermediate stage of sperm cell degeneration in the epididymis upon prolonged storage. A reduction of this defect should be expected upon regular collection (at least weekly) of ejaculates from the male.

The high percentage of detached acrosome caps found in the semen of the bull mentioned above does not mean that a shorter period of sexual rest may have such a large effect. Therefore, the opinion of Salisbury and VanDemark (105) appears to be reasonable, provided that too long an abstinence period is avoided. Too, the effect of abstinence may be different in beef bulls than dairy bulls, and

the proper length of such sexual rest should be studied more carefully.

Capless Spermatozoa in Frozen Semen

With frozen semen, the stain did not give good results and it was very difficult to identify the acrosome caps. This was due to the masking effect of the diluent components of the frozen semen, and, therefore, it was necessary to remove the diluter. This was done by thawing the frozen semen and centrifuging it for 5-10 minutes at 5,000 RPM. The spermatozoa were resuspended to a volume of 1 ml with a 2.9% sodium citrate solution. Smears were made from these suspensions by mixing one drop of the suspension with one drop of the stain. This treatment resulted in a clear microscopic field and differential staining of the sperm cells and acrosome caps.

In the course of this part of the studies, it was evident that freezing semen had no harmful effects on the acrosome caps. An average of 4.67 per cent of capless cells was found in the commercially frozen semen of five bulls (Table I). This would be considered a low level of acrosomal loss. The acrosome caps were observed, in general, to be slightly elevated. However, this may be due either to the effect of centrifugation or could well be due to the effect of freezing the spermatozoa, since cold shock of fresh semen results usually in slight elevation of the acrosome cap.

TABLE I

CAPLESS SPERMATOZOA IN FROZEN SEMEN

Bull	Replicate	No. Sperm Counted	Capless Sperm No.	Capless Sperm %
1	1	128	5	3.90
	2	71	3	4.22
	3	109	4	3.66
	4	103	4	3.93
2	1	205	8	3.90
	2	244	13	5.32
	3	140	7	5.00
	4	173	9	5.20
3	1	125	5	4.00
	2	91	6	6.59
	3	121	7	5.78
	4	128	6	4.68
4	1	215	15	6.97
	2	157	6	3.82
	3	170	10	5.88
	4	164	10	6.09
5	1	345	10	2.89
	2	422	18	4.26
Mean				4.67

The Importance of Acrosomal Evaluation

The literature contains detailed information concerning the results of acrosomal investigations during the last twenty years, and the existence of the acrosome cap has been confirmed in all species of farm animals. Today, much is known about the morphology, chemical composition, and some functions of this cap. This knowledge has been aided by the use of the electron, phase-contrast, and ultraviolet microscopes, modern histochemical techniques, and the recent methods of thin-sectioning of sperm cells. However, not all the functional aspects of this tiny, fragile, and important component of the spermatozoa are known with certainty and much disagreement still exists.

With the increased use of artificial insemination throughout the world, the importance of the use of high-quality semen of bulls having greater producing potential is very important in the field of cattle improvement. Thus, there is found several methods of evaluating the bull semen that is intended for use in artificial insemination. However, although there exists several criteria that were developed for such evaluation, most bull studs use only those criteria that are adaptable to their laboratory time schedules and competence. The ejaculate volume, concentration of the sperm cells, rate of sperm motility, and some consideration of abnormal spermatozoa are the principal criteria employed (22). The acrosome cap gets no attention in any evaluation of the semen

quality, although there have been numerous reports based on careful investigations that stressed the real importance of this structure. Some of the acrosomal defects, for example, the knobbed acrosome, was found to be hereditary (46, 58, 60, 112), or sometimes acquired (81), leading to impaired fertility or even to complete sterility. This indicates clearly the necessity of including the acrosomal evaluation in the present used criteria of semen evaluation.

Some of the possible reasons for the exclusion of evaluation of the acrosome cap is due to its extremely small size and the impossibility of observing this cap in routine semen preparations. Some compounds can be used for the staining and identification of the acrosome cap, but they require complicated procedures and relatively a long time, before yielding the final semen preparations. For routine commercial use, the evaluation of semen characteristics needs to be a rapid and easy procedure. The need for such a stain, as developed by Dr. M. Wells and the author, that can stain the acrosome cap effectively and rapidly is, in their point of viewpoint, a necessity. The staining technique should be rapid and give a clear field of observation that leads to minimal error in any evaluation of the nature of the initial acrosome cap in fresh ejaculates. These were the main objectives of this new specific bovine stain for the sperm cells and the acrosome caps.

The preliminary studies of this stain indicate the possibility of its use as a new means for acrosomal evaluation. The in vitro studies of the acrosomal defects such as the knobbed acrosome cap and initially elevated caps, and the acrosomal structural changes in stored semen, in incubated semen or that subjected to cold shock, and in frozen semen indicate the possibilities of the effectiveness of this stain and staining techniques as a quick procedure for including acrosomal evaluation in the present methods of semen evaluation.

The main work in these investigations was done on bull semen. The stain worked reasonably well with the ram acrosome, but did not yield satisfactory results with the porcine acrosome cap. Species differences obviously exist and future work may result in some modifications that make the suggested staining technique of greater adaptability to all farm animal species.

CHAPTER V

SUMMARY AND CONCLUSIONS

This study was conducted to determine the effectiveness of a new stain for bovine sperm cells, specifically, the acrosome cap. Certain characteristics of the acrosome caps were examined in this study.

The new stain, which was developed after testing three levels of eosin blue and three levels of fast green FCF, mixed with alcohol, led to a staining mixture consisting of (a) 1 volume of 1% eosin B, (b) 2 volumes of 1% fast green FCF, and (c) 1.7 volumes of ethyl alcohol. A simple and rapid staining technique was developed which consists of mixing one drop of the stain with one drop of diluted semen on a microscopic slide. The semen is initially diluted with a 2.9 per cent solution of sodium citrate at the rate of one volume semen: nine volumes of sodium citrate.

The stain gave good results with bull spermatozoa, was slightly less effective on ram spermatozoa, but was not effective in staining boar acrosomal caps.

The stain was effective in identifying one of the defects of the acrosome caps, the knobbed acrosome, which was stated by several authors to affect male fertility.

A count of this defect showed that almost all of the knobbed acrosomes were found on cells showing abnormal head structure.

The effect of aging on the acrosome cap was studied in a Hereford bull that had a low level of fertility. The acrosome cap of this bull was loosely attached to the sperm head and, as a result, the proportion of capless cells increased markedly upon incubating the semen at 37° C or storing it at 5° C. This abnormal feature of the acrosome cap could be a contributing factor to the bull's low level of fertility.

The effect of abstinence, studied in the first ejaculate of a Jersey bull that had not been used for breeding purposes for six months, was reflected in a high percentage of capless ejaculated sperm cells that were easily detectable with the new stain.

A few samples of commercially frozen bull semen showed, after being washed, resuspended in sodium citrate, and stained, a low proportion of detached acrosomes, that was in relatively close agreement with proportion of capless sperm cells in fresh semen reported by several workers.

This study indicates the potential usefulness of this new stain in assessing the state of the acrosome cap in semen. Further studies will be conducted to fully investigate the usefulness of the Wells-Awa stain.

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APPENDIX

TABLE II

THE RELATIONSHIP BETWEEN THE KNOBBED ACROSOME CAP AND THE ABNORMAL SPERM CELLS IN THE SEMEN OF A JERSEY BULL[1]

No. of Abnormal Sperm Counted	Abnormal Sperm Having Knobbed Caps	
	No.	%
186	105	56.45
168	72	42.85
183	84	45.90
183	63	34.42
252	102	40.47
180	69	38.33
Mean ± S.D.		43.07 ± 7.63 %

[1]Using the Wells-Awa Stain.

TABLE III

THE RELATIONSHIP BETWEEN THE KNOBBED ACROSOME CAP AND THE ABNORMAL SPERM CELLS IN THE SEMEN OF A JERSEY BULL[1]

No. of Abnormal Sperm Counted	Abnormal Sperm Having Knobbed Caps	
	No.	%
210	66	31.42
115	35	30.43
200	35	17.50
110	31	28.18
100	24	24.00
Mean ± S.D.		26.30 ± 5.68%

[1]Using the nigrosin-eosin stain.

TABLE IV

THE EFFECT OF INCUBATION AT 37° C ON ACROSOME DETACHMENT IN BOVINE SPERMATOZOA

Hours incubated after semen collection	Average cells counted/slide	Capless sperm cells Avg.	%
0[a]	105.5	8.5	8.06
1	86.6	23.6	27.25
3	64.6	22.0	34.05
5	82.0	50.6	61.70
7	65.0	44.3	68.15
9	49.3	39.5	80.12
11	83.6	62.3	74.52

[a]Values of the initial ejaculate.

TABLE V

THE EFFECT OF STORAGE AT 5° C ON ACROSOME DETACHMENT IN BOVINE SPERMATOZOA

Days stored after semen collection	Average cells counted/slide	Capless sperm cells Avg.	Capless sperm cells %
0[a]	105.5	8.5	8.06
1	103.3	11.6	11.22
2	74.3	14.6	19.65
3	112.5	23.5	20.88
4	148.0	45.0	30.40
5	117.0	42.0	35.89
6	136.0	46.5	34.19

[a]Values of the initial ejaculate.

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