# FACTORS CAUSING ANTERIOR ACROSOMAL SWELLING ON MOTILE BOVINE SPERM

by

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# PREFACE

My original goal when I began my graduate program for a Ph. D. degree was to discern what morphological or structural changes occur to bovine spermatozoa as a result of their exposure to the female reproductive tract. This would be an initial step in characterizing requirements for the fertilization process in the species most heavily subject to gamete manipulation on a commercial basis. To evaluate the morphological changes of bovine sperm exposed to the uterus, a supply of sperm were prepared for use over a few days by slowly cooling neat-semen to 4°C. After 1 day of storage, semen was extended in an isotonic Tris-fructose medium and incubated at 37°C. Sperm morphology was evaluated using differential interference contrast optics at 1250X. A distinct alteration of the acrosome was already apparent. The apical ridge and anterior acrosome of motile sperm appeared swollen. The apparent swelling could be very subtle or very extensive, involving up to 1/2 of the anterior acrosome. Sperm with such alterations retained their motility comparable to sperm without evidence of acrosomal modification. These types of changes in the acrosomal region had not been described previously for either viable or nonviable bovine sperm. Thus the physiological reasons for these acrosomal

modifications were unclear. These initial observations, while unrelated to the female at this time, were of sufficient importance to serve as a basis for this dissertation.

The objectives and related experiments undertaken for this dissertation are organized into two groups designated Series I and Series II Experiments. In Series I Experiments, the morphology of anterior acrosomal swelling (AS) was characterized with differential interference contrast and transmission electron microscopy. In addition, the importance of components of the original environmental conditions under which AS was first observed were evaluated. In Series II Experiments, major factors involved in AS formation were examined in detail. A mechanism for the induction of AS was also evaluated. In addition, an alternative method of quantitating acrosomal changes on viable sperm was developed.

The format of this dissertation differs from the norm in that it is organized and written to make more efficient utilization of the time associated with the review and publication of this work. I appreciate the indulgence of my committee in this endeavor and welcome their criticism. It is my intent to publish two papers from this dissertation. One paper is represented by the Series I Experiments and the other by the Series II Experiments. A general review of directly related literature is included. Each series of

experiments has introduction, materials and methods, results and discussion sections as they will appear in the journal manuscript. Additional information on experimental protocol and supportive statistics are relegated to the appendix. Discussion too speculative for publication and suggestions for future research are covered in an addendum to the discussion for each series of experiments. These addenda will also contain much of the relevant research normally presented in a review of literature.

# LITERATURE REVIEW

Changes in acrosomal morphology of sperm are classically associated with either the false acrosome reaction, a process of sperm deterioration, or the true acrosome reaction, which is understood to occur on viable sperm facilitating penetration of oocyte investments during fertilization (Bedford, 1970; Austin, 1975). For the bovine, Saacke and Marshall (1968) characterized the acrosomal modifications associated with sperm deterioration. Sperm were examined with bright field (fixed , stained preparations), differential interference contrast microscopy (unfixed, live preparations) and transmission electron microscopy. The differential interference contrast microscope permitted high resolution evaluation of acrosomal morphology on unfixed viable sperm without the potential for artifacts due to staining and/or fixation. From their collective observations they described the acrosome of a normal motile sperm as being intact with a smooth acrosomal surface and distinct crescent shaped apical ridge. The onset of deterioration occurred after loss of motility. There was a general swelling of all but the equatorial segment of the acrosome causing abrupt disappearance of the apical ridge. The integrity of the plasma membrane was lost and further swelling of the acro-

some occurred with breakdown of the outer acrosomal membrane. Eventually the acrosomal ground substance dispersed with only the equatorial segment and inner acrosomal membrane remaining. Such are the acrosomal changes known to occur in vitro on bovine sperm that can be associated with loss of sperm viability.

In the artificially inseminated bovine, the proportion of sperm with intact acrosomes in a dose of semen has been shown to have a positive relationship with fertility (Saacke and White, 1972; Saacke et al., 1980). Objective measurement of acrosomal integrity has become one of the bases upon which improvements have been made in commercial semen processing and handling procedures. It has also been used as criterion for selecting ejaculates to be used in artificial insemination.

In contrast to bovine sperm, localized swelling in the anterior acrosomal region of motile boar sperm has been observed and was postulated to be part of the deterioration process (Pursel et al., 1972a). This hypothesis is supported by the observation that affected boar sperm were infertile after normal intracervical insemination (Pursel et al., 1972b). While cold shock did not induce such morphology (Pursel et al., 1972a), storing sperm in various isotonic media at 15 or 25°C was effective. Media most conducive to acrosomal modification and to maintenance of sperm

motility were Tris based extenders containing either lactose (Pursel et al., 1974) or fructose (Pursel et al., 1972b).

Acrosomal changes occurring on viable sperm that are associated with the fertilization process have been conventionally referred to as the true acrosome reaction. The true reaction, as originally described for mammals by Barros et al. (1967) is a fusion and mutual vesiculation of the plasma and outer acrosomal membranes. Vesiculation involves all but the equatorial segment portion of the acrosome. Such acrosomal modifications allow for exposure and release of acrosomal enzymes that facilitate sperm penetration of ovum investments (McRorie and Williams, 1974). In contrast to the false acrosome reaction, sperm undergoing the true reaction have the potential to remain viable since the fusion of the plasma and outer acrosomal membranes at the equatorial segment results in maintenance of a continuous cell membrane over the entire cell.

In order for sperm to develop a true acrosome reaction they must first be capacitated. The concept of capacitation as reviewed by Johnson (1975) and Chang and Hunter (1975) encompasses the biochemical alterations that occur in the female reproductive tract to sperm in order that they may be able to fertilize the oocyte. By definition, capacitation is considered complete with the occurrence of the true acro-

some reaction and/or fertilization. Capacitation is normally characterized by the duration of exposure to the female reproductive tract that is required for the sperm to initiate the true reaction or fertilization.

Scientists are beginning to understand the process of capacitation and the true acrosome reaction in certain laboratory species. However, despite the attention given to artificial insemination in cattle, little is known about the modifications that bovine sperm undergo prior to and during fertilization. There is some indication that bovine sperm are able to fertilize oocytes after only a few h of exposure to the female reproductive tract (Iritani and Niwa, 1977; Trimberger, 1948). Wooding (1975) exposed bovine sperm to hyamine and/or incubated them in uterine fluid. Using the electron microscope, he observed a membrane fusion and vesiculation that was similar to the true reaction. Hyamine is a detergent that bypasses the need for capacitation by directly inducing membrane vesiculation (Hartree and Srivastava, 1965). Using stained smears and a light microscope, Breuer and Wells (1977) described an apparent vesiculation in the acrosomal region following incubation of bovine sperm with follicular fluid. However, in neither of these reports was an attempt made to relate the described acrosomal alterations to sperm viability nor the actual fertilization process.

The previous reports describing the membrane vesiculation attending the true acrosome reaction have done so based on transmission electron microscopy. This is required in that these acrosomal alterations are not resolvable with the light microscope. However, in some species there are acrosomal modifications, preparative to the true acrosome reaction, that can be described with the light microscope. Talbot et al. (1976) incubated guinea pig sperm in defined media and measured the percent motile sperm that had undergone the true acrosome reaction, i.e., lost their anterior acrosomal cap. They described the acrosome as being crenulated prior to completion of the true reaction. This crenulation may be a folding or wrinkling of the acrosome. Hamster sperm were recovered from the female reproductive tract by Yanagimachi and Noda (1970) and examined with the light microscope. Active sperm among the cummulus cells always had swollen, wrinkled or missing acrosomal caps. With electron microscopy they observed vesiculation characteristic of the true reaction indicating that the motile sperm with modified acrosomes were probably undergoing the true reaction. Following in vitro incubation in oviducal fluids, Franklin et al. (1970) described a general acrosomal swelling on motile hamster sperm prior to the true reaction. A more detailed description of acrosomal modifications prior to the

apparent true reaction on motile hamster sperm incubated in heat-treated blood sera was given by Talbot and Franklin (1976). The initial response was swelling along the anterior edge of the acrosome. The acrosomal cap then lifted and was detached from the motile sperm. They also indicated that the acrosome may appear crenulated on motile sperm before the true reaction. While they did not examine such sperm ultrastructurally, it appears from their light micrographs that crenulation is a localized expansion and folding of the anterior acrosome. It is also of interest that both Franklin et al. (1970) and Talbot and Franklin (1976) have indicated that the acrosomal modifications that they describe as preliminary to the true reaction can also occur on nonmotile sperm. Based on the lack of motility, both sets of authors think that these acrosomal modifications may also be a part of the false acrosome reaction.

Since the swollen apical ridges described for boar sperm (Pursel et al., 1972a) can occur on a large proportion of sperm in a highly motile population (Pursel et al., 1972b), it would seem that such could be a prelude to the true reaction. The lack of fertility of affected boar sperm populations after normal intracervical insemination (Pursel et al., 1972b) may be due to premature occurrence of the true reaction. The fertile life span of these reacted sperm

could be reduced in comparison to normal sperm populations. This concept is supported by the observations of Soupart and Orgebin-Crist (1966) who indicated that capacitated rabbit sperm may have a reduced life span. In addition, guinea pig sperm that undergo the apparent true reaction were reported to have a shorter motile life span when compared with unreacted sperm (Talbot et al., 1976).

Based on the apparent physiological importances of acrosomal swelling on motile sperm of other species it is plausible that the AS of motile bovine sperm could be either a unique form of sperm deterioration or a morphological prelude to the true acrosome reaction.

## SERIES I EXPERIMENTS: MORPHOLOGY OF AND FACTORS CONDUCIVE TO ANTERIOR ACROSOMAL ALTERATION OF MOTILE BOVINE SPERM

### INTRODUCTION

Acrosomal changes on bovine spermatozoa previously observed with brightfield, differential interference contrast and electron microscopy are associated with cellular degeneration (Saacke and Marshall, 1968). Sequentially, these changes involve disappearance of the apical ridge, a general swelling of the anterior acrosome, loss of the plasma membrane, breakdown of the outer acrosomal membrane and eventual loss of the acrosomal matrix. Such changes occur on nonmotile sperm (Saacke and Marshall, 1968) and have been referred to as the false acrosome reaction (Bedford, 1970). The true acrosome reaction, as reported in other species, involves viable sperm and is a fusion and vesiculation of the plasma and outer acrosomal membranes that occurs in association with the sperm penetration phase of fertilization (Bedford, 1970; Austin, 1975).

Under certain conditions, we have observed localized swelling of the anterior acrosome on motile bovine sperm. This anterior acrosomal swelling (AS), because it occurs on motile sperm, does not appear related to the false acrosome reaction. While it may represent a unique form of cell injury or deterioration, it could be a prelude to the true

acrosome reaction since the sperm are motile. Acrosomal modifications have been described prior to the true acrosome reaction in other species. For example, a general swelling of the anterior acrosome has been observed prior to what has been interpreted to be the true acrosome reaction on guinea pig (Yanagimachi and Usui, 1976) and hamster sperm (Yanagimachi and Chang, 1964; Yanagimachi and Noda, 1970; Franklin et al., 1970). The acrosome of guinea pig (Talbot et al., 1976) and hamster sperm (Talbot et al., 1974; Talbot, 1979) have also been described to be crenulated before the true acrosome reaction occurs. The described crenulation appears to be a wrinkling or folding of the anterior acrosome.

The conditions that sperm had been exposed to when AS was first observed were chosen by chance alone. Neat-semen from bulls had been slowly cooled to 4°C and stored for 1 day. After storage, semen was diluted in an isotonic Tris (hydroxymethylaminomethane)-fructose medium and incubated at 37°C. A majority of motile sperm in this suspension exhibited AS.

The present study was undertaken to morphologically characterize AS and elucidate the major environmental requirements for bovine sperm to exhibit this property.

## MATERIALS AND METHODS

### Experiment 1.

In the first experiment the relative importance of certain environmental conditions to AS were examined. These conditions included; the presence of seminal plasma (SP) during 4°C storage, the length of storage at 4°C in days, the influence of subsequent incubation (37°C) in other than a Tris-fructose based media and the effects of length of incubation at 37°C. Media and experimental protocol are described subsequently.

Semen storage and incubation media:

Egg yolk-citrate (EYC) was composed of 20% (v/v) egg yolk (Gallus domesticus) and 80% (v/v) 98.6 mM Na citrate. Egg yolk-Tris-fructose (EYT+F) contained 20% (v/v) egg yolk and 80% (v/v) of a buffer composed of 235 mM Tris, 54 mM fructose and 72 mM citric acid. These media also contained 1000 IU penicillin and 1000 ug dihydrostreptomycin sulfate/ml. Both EYC and EYT+F were adjusted to pH 6.8 with citric acid and centrifuged at 15,000 X g for 5 min to remove particulate matter. Osmolarities based on freezing point depression were 276±4 (range) mOsm for the media.

Experimental protocol:

The experiment was replicated 3 times. For each replicate three first ejaculates were collected with an artificial vagina from 3 of 5 bulls and pooled. No single bull contributed semen to more than 2 replicates. All treatments were derived from a single semen pool. Semen was allowed to gradually cool from 37°C to room temperature during formation of treatments, which required 1 3/4 h. SP was recovered after centrifugation of an aliquot of pooled semen at 3900 X g for 5 min. Sperm concentration of the remaining semen pool was determined turbidimetrically and adjusted to 1500 X $10^6$ sperm/ml by addition of SP. The adjusted semen pool was divided into two equal portions. One portion was washed to remove SP. This was accomplished by dilution (1:4) with EYC followed by centrifugation at 1000 X g for 5 min. A volume of EYC equal to that originally combined with the semen was removed. This process was repeated 2 more times leaving the semen at the original volume and concentration. This washing procedure replaced approximately 99% of the SP with EYC. Thus two storage media treatments were created, i.e. sperm in SP or EYC at 1500 X $10^6$ sperm/ml. Both received 500 IU or ug/ml of penicillin G or dihydrostreptomycin sulfate. The additional presence of antibiotics in the egg yolk based media insured a minimum of 500 and not

exceeding 1500 IU penicillin or ug dihydrostreptomycin/ml to prevent bacterial growth. The storage media treatments were cooled uniformly to 4°C in a 2 l 25°C water jacket and stored in a walk-in cold room. Semen reached 5°C in 5 1/2 h. After 1, 3 and 5 days of storage, aliquots of each storage media treatment were diluted to 25 X $10^6$ sperm/ml in either EYC or EYT+F and incubated at 37°C. Following incubation for 5 min, 2 and 4 h, percent motility and proportion of motile sperm with AS were estimated using phase contrast (100X) and differential interference contrast optics (1250X), respectively. AS was rated on a scale of 0-3. The estimated value of the scale units based on the percent motile sperm exhibiting AS were: 0<5%, 1=5-20%, 2=25-55% and 3≥60%.

Data were transformed for statistical analysis with the following formulas; 2X arcsin square root for percent motility values and the square root of ( X + 0.5) for AS values. Analysis of variance was conducted according to Barr et al. (1979).

Experiment 2

In this experiment the effect of storage temperature and use of SP as a storage medium upon AS formation was evaluated. In addition, the storage interval required for a max-

imum proportion of motile sperm to exhibit AS was determined. To address these objectives the following protocol was used. Bull semen was collected, pooled and prepared as previously described, i.e., two storage media treatments were produced with sperm in SP or EYC (SP removed by washing) at 1500 X $10^6$ sperm/ml. Sperm in the SP treatment received 1000 IU of penicillin and 1000 ug dihydrostreptomycin/ml. Antibiotics were present in all other culture media at these levels to prevent bacterial growth. Aliquots of sperm in SP or EYC media at 1500 X $10^6$ sperm/ml were stored in water baths at 37, 21 or 4°C. The latter treatment was cooled uniformly to 4°C using a 2 l 25°C water jacket placed in a 4°C walk-in cold room. Aliquots of each storage media treatment at each temperature were removed at 0, 2, 4, 6, 8, 10, 12 and 14 h, diluted in a Tris-fructose medium (TRIS+F) to 25 X $10^6$ sperm/ml and incubated at 37°C. TRIS+F was composed of 235 mM Tris, 54 mM fructose and 59 mM citric acid. It was adjusted to pH 7.8 at room temperature with citric acid and had a freezing point depression osmolarity of 291±3 (range) mOsm. Aliquots were removed from the 4°C treatments at the onset of cooling and during cooling and storage at 4°C. These treatments reached 5°C in 7 h. Percent motile sperm and proportion of motile sperm were estimated as described previously after 3 h of incubation. The experi-

ment was replicated three times. Data were transformed as previously described. Analysis of variance (Barr et al., 1979) was performed.

Experiment 3

Results of previous experiments demonstrated that storage of sperm in SP at 4°C was necessary for AS. However, use of the Tris-fructose based medium during 37°C incubation alleviated the need for a SP storage medium. Components of the Tris-fructose based medium that are common with SP are amines (Tris is a primary amine) and fructose (Mann, 1964). To discern which component(s) may be inducing AS, the effect of storing sperm at 4°C at neat-semen sperm concentrations in media containing Tris and/cr fructose was evaluated. The potential effect of glucose, a sugar not in bovine SP, was also tested. In addition, the influence of subsequent incubation at 37°C in a medium containing Tris was examined.

In pilot studies, it was established that sperm from the cauda epididymis were a valid model to further evaluate factors important to AS. In this experiment, cauda sperm were more suitable than ejaculated sperm due to their lack of exposure to seminal vesicle secretions that contain fructose, a factor of potential importance to AS.

Sperm culture media:

The EYC and EYT+F media described previously were prepared for this experiment. Additional media were also based on 20% (v/v) egg yolk and 80% (v/v) buffer. These included an egg yolk-Tris medium (EYT) with a buffer composed of 295 mM Tris and 95 mM citric acid and an egg yolk-Na citrate medium with a buffer that contained 78.2 mM Na citrate and 54 mM fructose (EYC+F) or 54 mM glucose (EYC+G). All media contained 1000 IU penicillin and 1000 ug dihydrostreptomycin/ml. Citric acid was used to adjust pH at room temperature to 6.8. Osmolarities of all media were 282±5 (range) mOsm. Particulate matter was removed by centrifugation of media at 15,000 X g for 10 min followed by filtration through a 0.45 um Millipore filter. SP was also used as a culture medium and served as a positive control. SP was recovered immediately after collection of an ejaculate as previously described and held on ice until needed.

Experimental Protocol:

To obtain epididymal sperm, each bull was slaughtered within 45 min of semen collection. The vas deferens and cauda epididymis were removed. The surface of the epididymis was trimmed and cleaned with flush media. Flush media was isotonic EYC without antibiotics (based on 101 mM Na

citrate). A blunt 25 ga. needle was attached to the vas deferens with a hemostat. Sperm were flushed with media in a retrograde manner from a small cut in the distal cauda epididymis. Care was taken to avoid contamination with blood. Cauda sperm were maintained at 34°C from slaughter until the onset of the experiment. Sperm concentration was determined using a Coulter Counter. Cauda sperm were adjusted initially to 3500 X $10^6$ sperm/ml with EYC flush medium. Penicillin and dihydrostreptomycin were added at 1000 IU and ug/ml, respectively, to this solution of cauda sperm. Treatments were then created by further diluting sperm to 1000 X $10^6$/ml with the test medium, i.e., SP, EYC, EYC+F, EYC+G, EYT or EYT+F. These treatments were slowly cooled to 4°C. Cooling was conducted in a step-wise fashion. Treatments were initially placed in a 300 ml water jacket which cooled from 37 to 25°C over 2 h while being held at room temperature. They were then placed in a 2 l 25°C water jacket which cooled to 5°C in 7 1/2 h while being held at 4°C in a walk-in cold room. After 24 h of storage at 4°C, aliquots of each storage treatment were extended to 50 X $10^6$ sperm/ml in either EYC or EYT and incubated in a 37°C water bath. Following 5 min, 2 and 4 h of 37°C incubation, treatments were evaluated for percent motility and proportion of motile sperm with AS. The entire experiment

was replicated 5 times, each replicate represented by a different bull. Analysis of variance was performed as described for the previous experiments utilizing transformed data. Treatment means were compared with Duncan's new multiple range test (Duncan, 1955).

Morphological evaluation

A population of motile sperm with AS was generated by storing neat-semen at 4°C for 1 day followed by incubation at 50 X $10^6$ sperm/ml in TRIS+F at 37°C for 2 h. For comparative purposes a control population of sperm without AS was created by storing sperm adjusted to 375 X $10^6$ sperm/ml with EYC for 24 h at 4°C followed by 2 h incubation at 50 X $10^6$ sperm/ml at 37°C in EYC. In addition to centrifugation (15,000 X g for 10 min), sperm culture media used to prepare sperm for electron microscopy were passed through a 0.45 um Millipore filter to remove particulate matter. After incubation each treatment was centrifuged at 1000 X g for 5 min and sufficient supernatant volume removed to provide a sperm concentration of 1.5 X $10^9$/ml. Two drops of semen were fixed as a suspension in Karnovsky's fixative (Karnovsky, 1965) Sperm were pelleted at 1700 X g, rinsed in Na phosphate buffer and post fixated with osmium tetroxide. Sperm pellets were subsequently treated with tannic acid (Simion-

escu and Simionescu, 1976). Sperm were dehydrated and embedded in Epon 812 and Araldite. Thin sections were cut and stained with uranyl acetate and with lead citrate (Venable and Coggeshall, 1965). Preparation of sperm for electron microscopy is described in further detail in Appendix Tables 9 and 10.

Differential interference contrast photomicrographs of sperm with AS and normal intact acrosomes were taken after motility was arrested by fixation with addition of 10 ul of 5% (w/v) paraformaldehyde in 0.01 M Na phosphate and 137 mM NaCl (pH 6.9) to 1 ml of semen. This procedure did not visibly affect sperm morphology.

## RESULTS

### Morphological Observations

From the image produced using the differential interference contrast microscope, the normal viable bovine sperm head (control) was characterized by a smooth acrosomal surface and a distinct crescent shaped apical ridge (Fig. 1A). In contrast, motile sperm exhibiting AS demonstrated an apparent expansion of the apical ridge (Fig. 1B-E). The degree of apparent acrosomal involvement varied. Subtle to extensive amounts of acrosomal alteration are shown successively in Fig. 1B-E. Additional acrosomal modifications

associated with AS were fine order irregularities (Fig. 1C and D) and expansion of the lateral edge of the acrosome (Fig. 1D and E).

Electron microscopy confirmed that the morphology observed with the light microscope was a result of localized swelling of the anterior acrosome. Ultrastructural comparison to a normal sperm (Fig. 2A) indicated that those with AS (Fig. 2B-C) possessed an anterior acrosomal matrix that was expanded into folds and projections. A moderate form of AS is shown in Fig. 2B. Alteration of the acrosomal matrix can become quite complex (Fig. 2C) and even involve the lateral edges of the acrosome (Fig. 2D). The integrity of the plasma membrane and outer acrosomal membrane appeared to be maintained regardless of the complexity of AS (Fig. 2B-C).

Experiment 1

The effects of storage media, duration of storage, incubation media and duration of incubation on AS are presented in Table 1. Analysis of variance is shown in Appendix Table 11. There was an interaction between storage media, incubation media and length of incubation ($P \leq 0.05$). Storing sperm in SP always resulted in a maximum AS response regardless of length of storage, incubation media used or length of incubation. In contrast, if SP was replaced with EYC as

FIG 1. Differential interference contrast micrographs of the bovine sperm head x 2000.

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A. Acrosomal morphology of a normal (control) sperm. Note the distinct smooth crescent shape of the apical ridge (►) and the smooth acrosomal surface (]).

B. Sperm heads showing early and moderate forms of AS. Note the posterior expansion in the apical ridge region.

C. Sperm head showing moderate AS and fine order irregularities on the acrosomal surface.

D. Sperm head showing more extensive AS. Note the involvement of the lateral edges of the acrosome. Fine order irregularities are also present on the acrosomal surface.

E. Sperm head showing the maximum degree of AS observed in these studies.

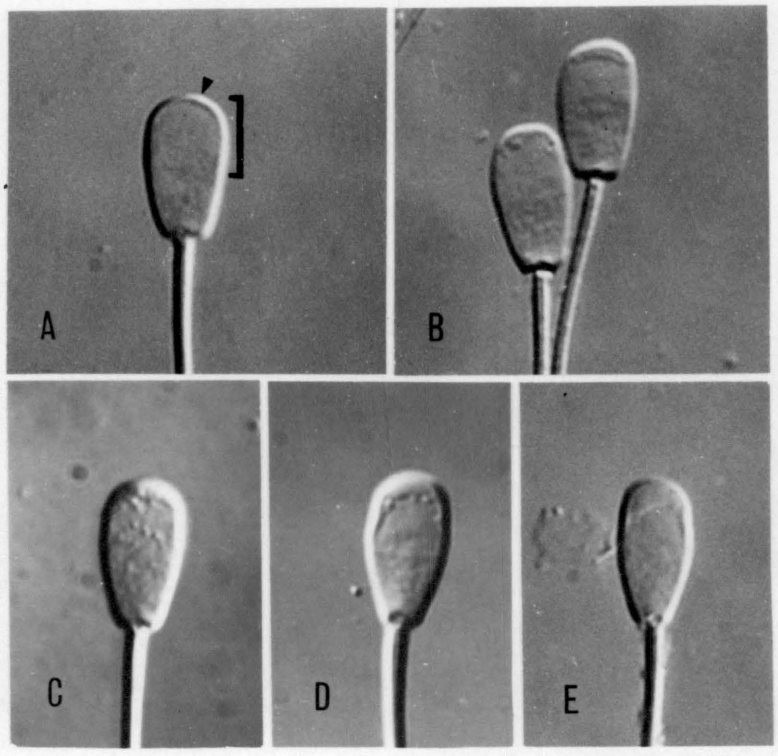
A
B
C
D
E

FIG. 2. Transmission electron micrographs of the bovine sperm head x 26,000.

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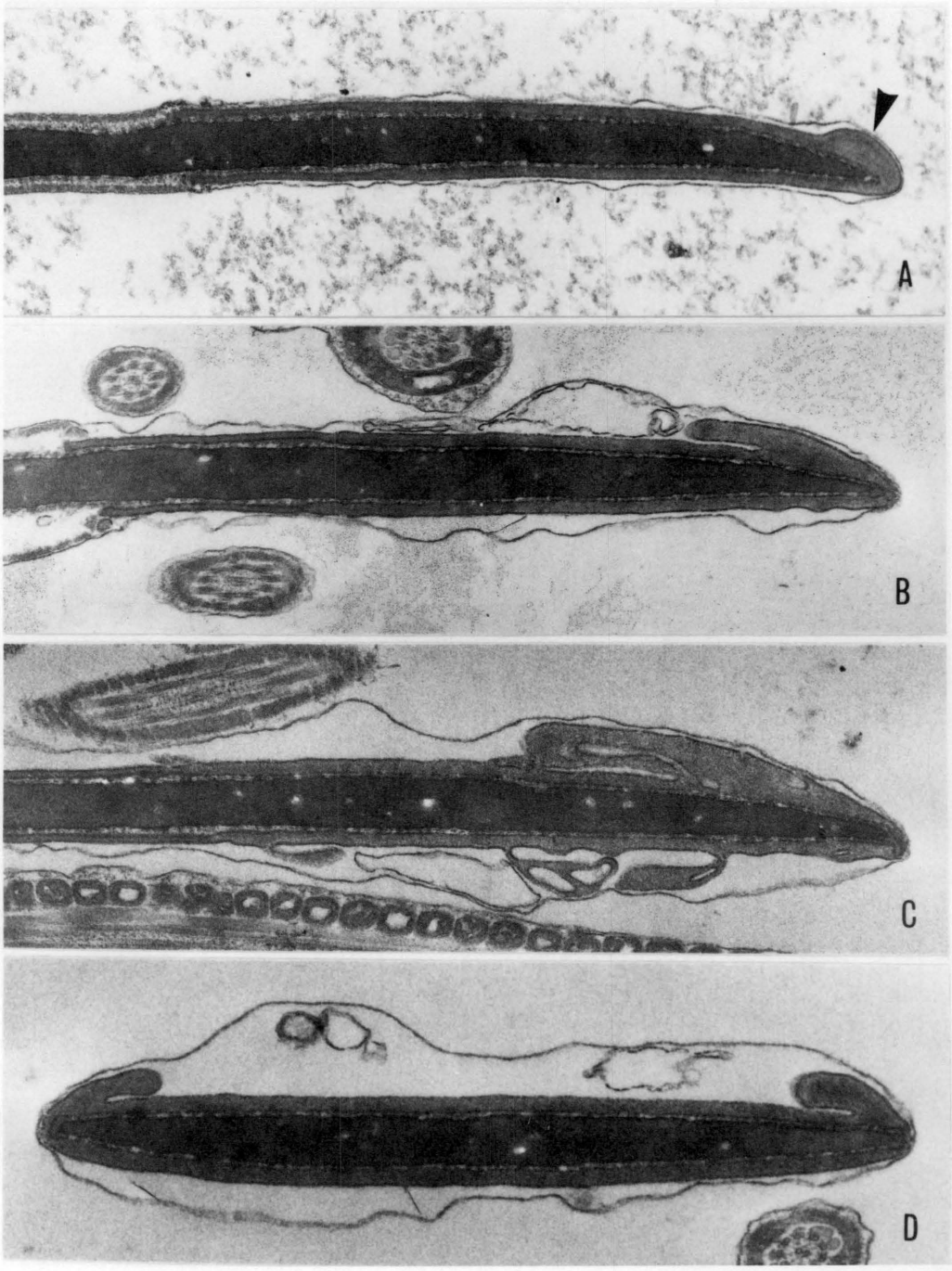
A. Morphology of the acrosome from a sagittal section of a normal sperm head (control). Note the small but distinct apical ridge (➤).

B. A sagittal section of moderate anterior acrosomal swelling. Note the posterior expansion of the apical ridge as it folds back over the acrosomal surface. Both outer acrosomal and plasma membranes are intact.

C. A sagittal section showing extensive anterior acrosomal swelling. Note the expansion and complex folding of the apical ridge. It was also evident that AS can develop on both sides of the acrosome, in this case in the form of projections. The outer acrosomal membrane was intact as it covered the complex acrosomal surface. Plasma membrane integrity was also apparent.

D. A cross section in the anterior acrosomal region showing AS on the lateral edges of the acrosome.


A
B
C
D

a storage medium, the AS response was altered. Increasing storage in EYC or incubation in EYC caused little AS. However, sperm stored in EYC and subsequently incubated in EYT+F revealed marked increases in AS in relation to storage and incubation. There was a high proportion of motile sperm with AS after 3 days of storage in EYC. Maximum AS was observed after 5 days storage and 2 h of incubation.

The effects of storage media, storage interval, incubation media and incubation interval on percent motility are presented in Table 2. Analysis of variance is shown in Appendix Table 12. An interpretable interaction ($P \leq 0.01$) existed among the effects of storage medium, length of storage and incubation medium. Storage in SP followed by incubation in EYC resulted in the lowest motility. Motility under these conditions decreased rapidly with storage and/or incubation. Very few sperm were motile after 3 days of storage. While incubation in EYT+F improved percent motility after storage in SP, the precipitous decline in motility over storage was still evident. In contrast, if SP was replaced with EYC and sperm were incubated in EYC, the percent motile sperm was substantially improved and there was little change with incubation. In comparison to other combinations of storage and incubation conditions, percent motility was supported best by storage of sperm in EYC with

TABLE 1. Effect of storage media, length of storage, incubation media, and length of incubation on AS.[a,b]

| Incubation Media | Incubation Interval | Storage Media (4°C) | | | | | |
|---|---|---|---|---|---|---|---|
| | | SP | | | EYC | | |
| | | Days of Storage (4°C) | | | Days of Storage (4°C) | | |
| | | 1 | 3 | 5 | 1 | 3 | 5 |
| EYC | 5 min | 3.0 ± 0.0 | 3.0 ± 0.0 | 3.0 ± 0.0 | 0.0 ± 0.0 | 0.3 ± 0.3 | 0.3 ± 0.3 |
| | 2 h | 3.0 ± 0.0 | 3.0 ± 0.0[c] | - - - | 0.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.6 |
| | 4 h | 3.0 ± 0.0 | - - - | - - - | 0.0 ± 0.0 | 0.7 ± 0.3 | 0.7 ± 0.7 |
| EYT + F | 5 min | 3.0 ± 0.0 | 3.0 ± 0.0 | 3.0 ± 0.0 | 0.0 ± 0.0 | 0.3 ± 0.3 | 0.7 ± 0.3 |
| | 2 h | 3.0 ± 0.0 | 3.0 ± 0.0 | 3.0 ± 0.0 | 0.7 ± 0.3 | 2.3 ± 0.3 | 3.0 ± 0.0 |
| | 4 h | 3.0 ± 0.0 | 3.0 ± 0.0 | - - - | 1.7 ± 0.3 | 2.7 ± 0.3 | 3.0 ± 0.0 |

[a]Proportion of motile sperm with AS based on a scale of 0-3 (0<5%, 1=5-20%, 2=25-55%, and 3≥60% motile sperm with AS).

[b]Mean ± SEM (n=3)

[c]n=2, No motile sperm in one replicate.

- - - No motile sperm to evaluate.

subsequent incubation in EYT+F. Under these conditions motility varied little over incubation or storage.

Experiment 2

The effects of storage temperature (37, 21 or 4°C) on AS of sperm stored in SP or EYC at 1500 X $10^6$ sperm/ml is shown in Figs. 3 and 4 respectively. Analysis of variance is presented in Appendix Table 13. There was an interaction between storage media, storage temperature and storage interval ($P \leq 0.01$). Most of the interaction can be attributed to the absence of AS when sperm were stored in SP at 37°C. This was due to the fact that motility was not sustained beyond 2 h of storage. In contrast, after storage in EYC at 37°C, proportionately more sperm exhibited AS as storage interval increased. A maximum response was apparent by 10 h. After 12 and 14 h of storage there was a slight reduction in AS. Very little AS was apparent after 21°C storage in SP. Storing sperm at 21°C in EYC resulted in a moderate proportion of motile sperm with AS after 10 or more h of storage. AS was first evident following 4 h of 4°C storage in SP and increased thereafter reaching the maximum level by 12 h. A moderate level of AS was observed after 8 h of storage in EYC at 4°C. This did not change with subsequent storage.

TABLE 2. Effect of storage media, length of storage, incubation media and length of incubation on percent motility.[a]

| Incubation Media | Incubation Interval | Storage media (4°C) | | | | | |
|---|---|---|---|---|---|---|---|
| | | SP | | | EYC | | |
| | | Days of Storage (4°C) | | | Days of Storage (4°C) | | |
| | | 1 | 3 | 5 | 1 | 3 | 5 |
| EYC | 5 min | 38 ± 4 | 15 ± 3 | 2 ± 1 | 53 ± 2 | 38 ± 9 | 35 ± 3 |
| | 2 h | 40 ± 5 | 5 ± 3 | 0 ± 0 | 55 ± 0 | 42 ± 6 | 27 ± 7 |
| | 4 h | 23 ± 3 | 0 ± 0 | 0 ± 0 | 55 ± 0 | 47 ± 2 | 22 ± 6 |
| EYT + F | 5 min | 42 ± 4 | 18 ± 2 | 5 ± 0 | 62 ± 1 | 50 ± 0 | 43 ± 3 |
| | 2 h | 45 ± 8 | 37 ± 7 | 5 ± 0 | 60 ± 3 | 53 ± 2 | 53 ± 2 |
| | 4 h | 40 ±10 | 20 ± 6 | 0 ± 0 | 58 ± 3 | 53 ± 2 | 48 ± 4 |

[a]Mean ± SEM (n=3)

FIG. 3. AS of sperm stored in SP at 37, 21 and 4°C up to 14 h. Means ± SEM (n=3) are indicated. No motile sperm at 37°C after 2h. Proportion of motile sperm with AS is based on a scale of 0-3 (0< 5%, 1 = 5-20%, 2 = 25-55% and 3 $\geq$ 60% motile sperm with AS).

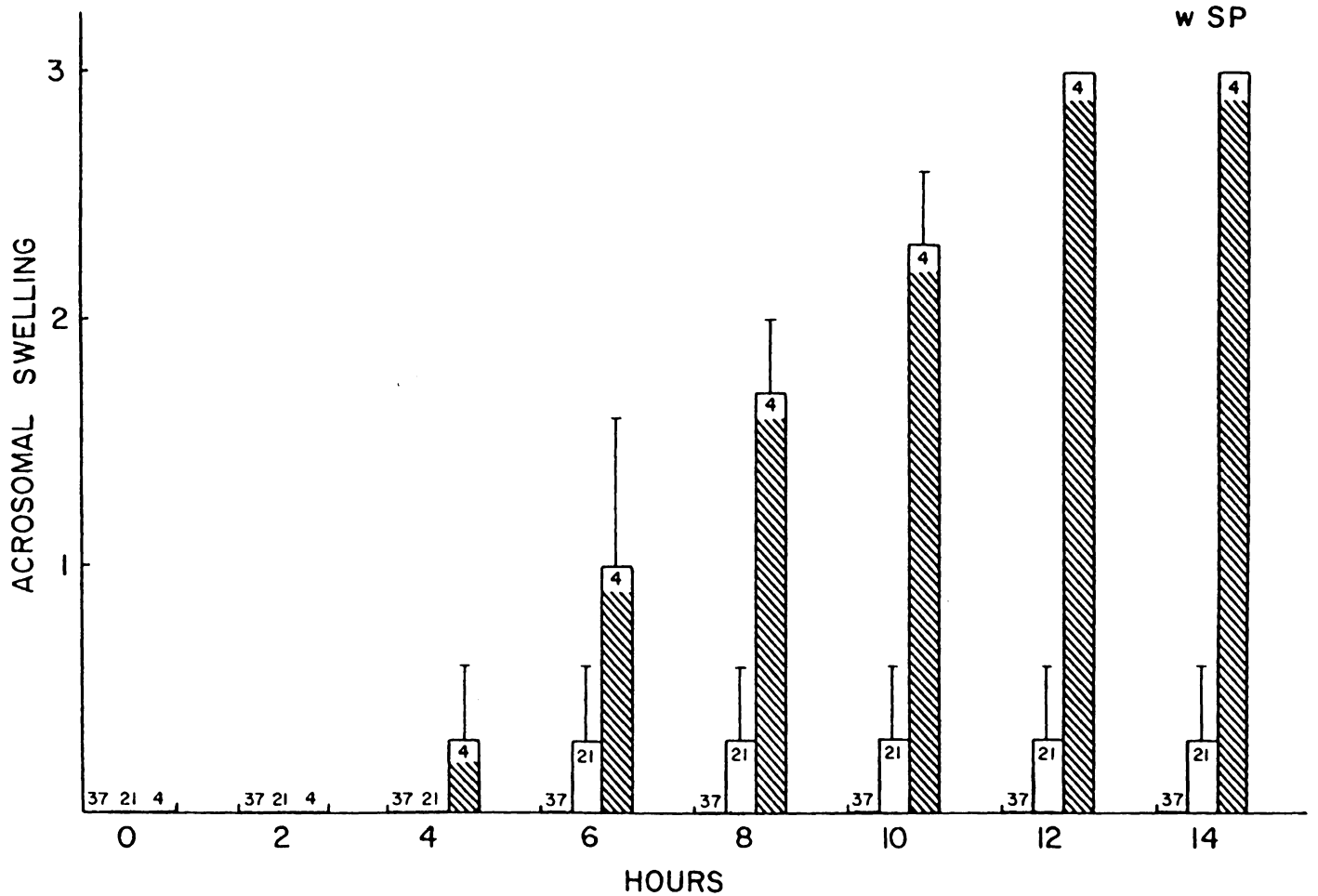
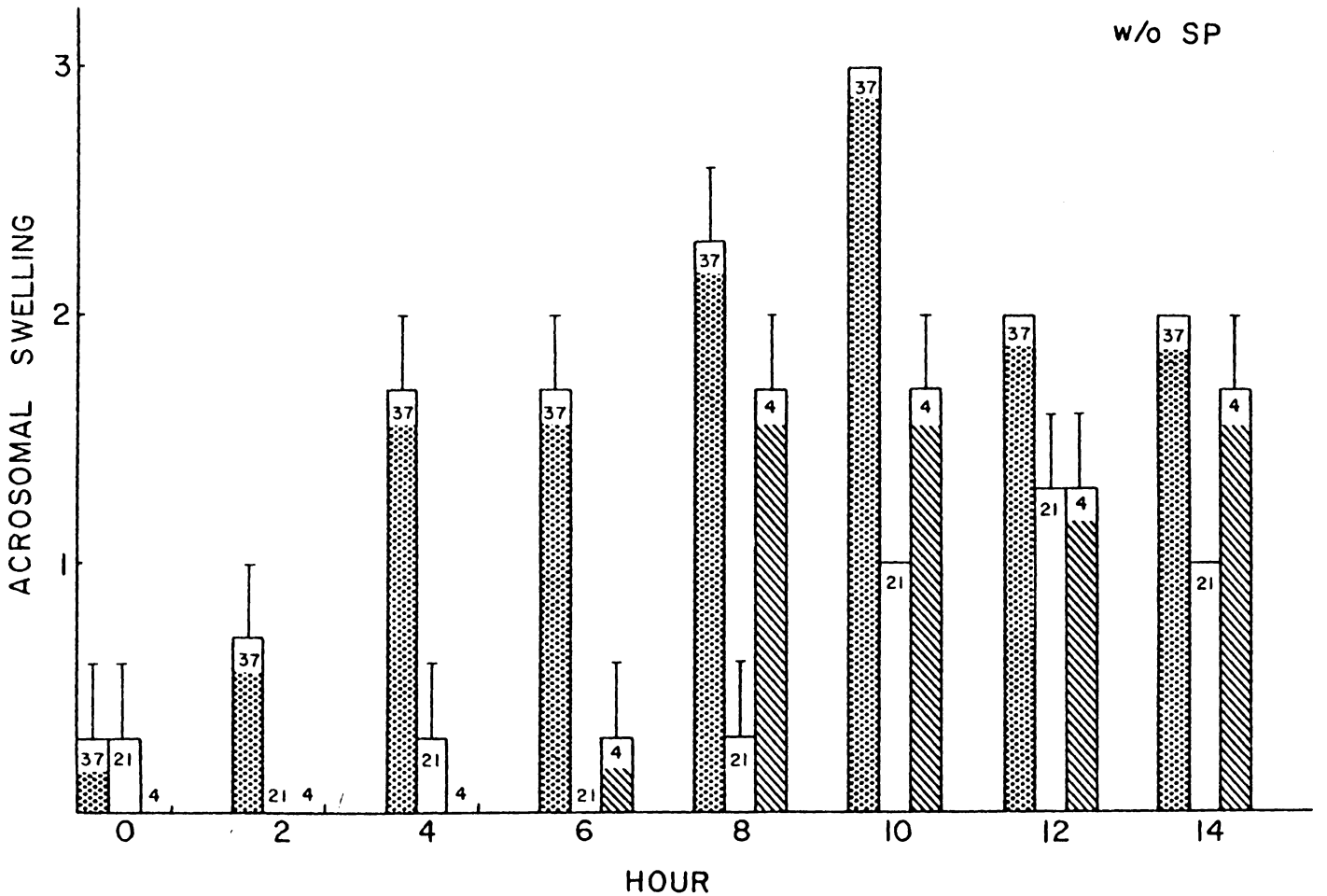

w SP
ACROSOMAL SWELLING
3
2
1
37 21 4
37 21 4
37 21
4
37
21
4
4
37
21
4
4
37
21
4
4
37
21
4
4
37
21
4
HOURS
0
2
4
6
8
10
12
14

FIG. 4. AS of sperm stored in EYC at 37, 21, and 4°C up to 14 h. Means ± SEM (n=3) are indicated. Means at 10 and 12 h are of 2 replicates and at 14 h of 1 replicate due to lack of motility. Proportion of motile sperm with AS is based on a scale of 0-3 (0 < 5%, 1= 5-20%, 2 = 25-55% and 3 $\geq$ 60% motile sperm with AS).


w/o SP
ACROSOMAL SWELLING
3
2
1
37
21
4
0
2
4
6
8
10
12
14
HOUR

The percent motility response of sperm stored at 37, 21 or 4°C in SP or EYC is presented in Figs. 5 and 6, respectively. Analysis of variance is shown in Appendix Table 14. A storage media X storage temperature X storage interval interaction existed ($P \leq 0.01$). Percent motility decreased very rapidly with 37°C storage in SP. Use of EYC as the storage medium at 37°C slowed the loss of motility. Motility after storage in EYC cooled to 4°C was sustained better than after storage in SP at 4°C. These are the major differences accounting for the interaction. There was little difference in percent motility following storage in SP at 21°C and EYC at 21 or 4°C. The sperm motility in these latter treatments was the highest of all storage conditions and remained fairly stable as storage interval increased.

Experiment 3

The AS response of cauda sperm stored in SP, EYC, EYC+F, EYC+G, EYT and EYT+F followed by dilute incubation in EYC and EYT for 5 min, 2 or 4 h is presented in Table 3. Analysis of variance is presented in Appendix Table 15. Storage media affected AS ($P \leq 0.01$). Storing sperm in SP, EYC+F, EYC+G and EYT+F induced a strong AS response ($P \leq 0.01$) that was greater than that following storage in either EYC or EYT. While use of EYT as a storage medium had little influ-

FIG. 5. Percent motility of sperm stored in SP at 37, 21 and 4°C up to 14 h. Means ± SEM (n=3) are indicated.

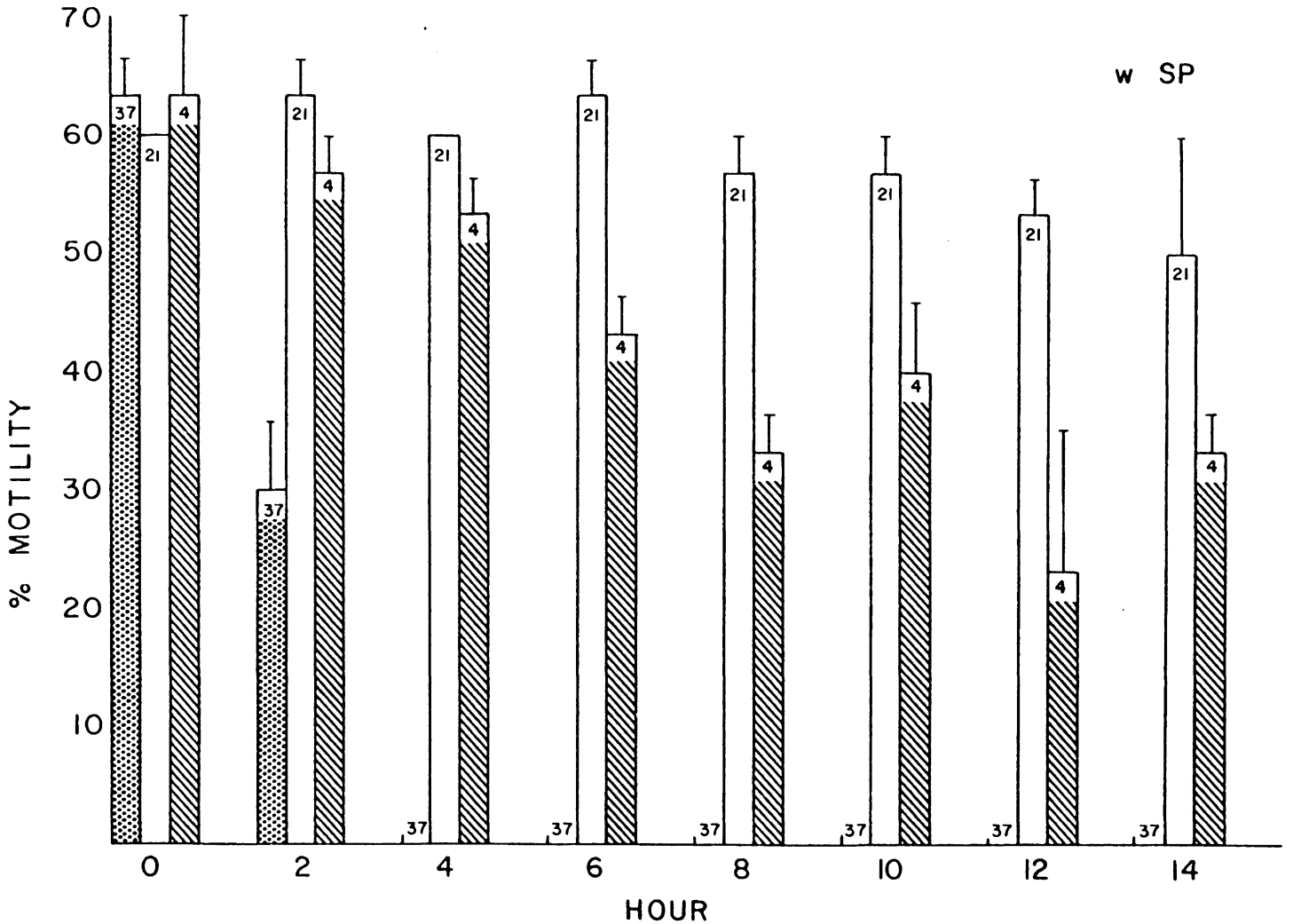
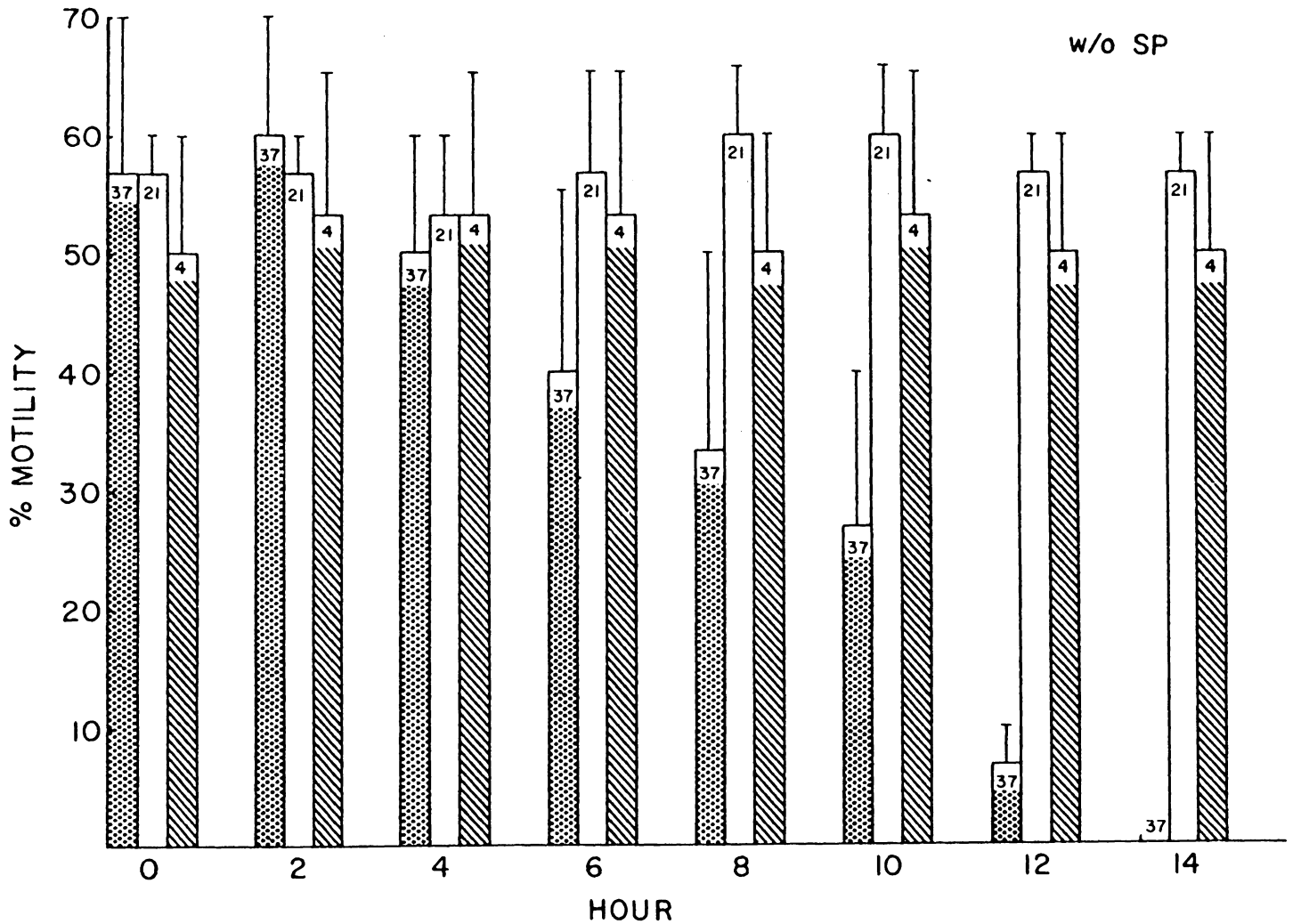

w SP
% MOTILITY
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
HOUR
37
21
4

FIG. 6. Percent motility of sperm stored in EYC at 37, 21 and 4°C up to 14 h. Means ± SEM (n=3) are indicated.


w/o SP
% MOTILITY
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
HOUR
37
21
4

ence on AS, it did facilitate a slight increase in AS in comparison to EYC ($P \leq 0.01$) when used as an incubation medium. With respect to the entire experiment, there was a small increase in AS with incubation at 37°C ($P \leq 0.01$).

The effects on percent motility of storing cauda sperm in SP, EYC, EYC+F, EYC+G, EYT and EYT+F followed by dilute incubation in EYC and EYT for 5 min, 2 or 4 h is presented in Table 4. Analysis of variance is shown in Appendix Table 16. There was a storage media X length of incubation interaction ($P \leq 0.01$). Percent motility of sperm stored in SP, EYC+F and EYT+F decreased more with incubation than when sperm were stored in EYC, EYT and EYC+G. Most of the decrease, though small, came between 2 and 4 h. This was more evident with use of EYC as the incubation medium. In general, incubating sperm in EYT resulted in slightly higher motility ($P \leq 0.01$).

## DISCUSSION

Ultrastructural investigation confirmed that AS observed on motile sperm using differential interference contrast microscopy was due to a localized expansion of the acrosomal matrix. Based on electron microscopy it was evident that despite the dramatic change in acrosomal morphology, outer acrosomal and plasma membrane integrity was unaffected. The

Table 3. Effect of tris, fructose and glucose in the storage media on AS during subsequent incubation in EYC or EYT for 5 min, 2 and 4 h.[a,b]

| Incubation Interval | Incubation Media | Storage media (4°C) | | | | | |
|---|---|---|---|---|---|---|---|
| | | SP | EYC | EYC + F | EYC + G | EYT | EYT + F |
| 5 min | EYC | 2.0 ± 0.3 | 0.0 ± 0.0 | 1.0 ± 0.5 | 0.8 ± 0.6 | 0.0 ± 0.0 | 1.0 ± 0.6 |
| | EYT | 2.6 ± 0.4 | 0.0 ± 0.0 | 1.6 ± 0.7 | 1.6 ± 0.5 | 0.2 ± 0.2 | 1.2 ± 0.5 |
| 2 h | EYC | 2.6 ± 0.4 | 0.0 ± 0.0 | 2.0 ± 0.3 | 1.6 ± 0.7 | 0.0 ± 0.0 | 1.4 ± 0.4 |
| | EYT | 2.8 ± 0.2 | 0.6 ± 0.4 | 2.2 ± 0.4 | 2.0 ± 0.4 | 0.6 ± 0.2 | 2.4 ± 0.4 |
| 4 h | EYC | 2.4 ± 0.2 | 0.2 ± 0.2 | 2.0 ± 0.3 | 1.8 ± 0.4 | 0.0 ± 0.0 | 2.4 ± 0.4 |
| | EYT | 3.0 ± 0.0 | 0.6 ± 0.2 | 2.4 ± 0.4 | 2.6 ± 0.4 | 0.8 ± 0.4 | 2.6 ± 0.2 |
| | Grand Mean ± SEM (n = 30) | 2.1 ± 0.1[c] | 0.2 ± 0.1[d] | 1.9 ± 0.2[c] | 1.7 ± 0.2[c] | 0.3 ± 0.1[d] | 1.8 ± 0.2[c] |

[a]Proportion of motile sperm with AS based on a scale of 0-3 (0 < 5%, 1 = 5-20%, 2 = 25-55%, and 3 ≥ 60% motile sperm with AS).

[b]Mean ± SEM (n=5)

[c,d]Means without common superscripts are different ($P \leq 0.01$).

TABLE 4. Effect of tris, fructose and glucose in the storage media on percent motility during subsequent incubation in EYC or EYT for 5 min, 2 and 4 h.

| Incubation Interval | Incubation Media | Storage Media (4°C) | | | | | |
|---|---|---|---|---|---|---|---|
| | | SP | EYC | EYC + F | EYC + G | EYT | EYT + F |
| 5 min | EYC | 53 ± 2[a] | 48 ± 3 | 48 ± 2 | 53 ± 2 | 55 ± 2 | 58 ± 3 |
| | EYT | 58 ± 1 | 57 ± 1 | 53 ± 2 | 53 ± 1 | 59 ± 1 | 51 ± 3 |
| | Overall Mean ± SEM (n = 10) | 56 ± 1 | 53 ± 2 | 51 ± 2 | 53 ± 1 | 57 ± 1 | 50 ± 2 |
| 2 h | EYC | 49 ± 2 | 53 ± 1 | 50 ± 3 | 50 ± 2 | 53 ± 1 | 50 ± 3 |
| | EYT | 55 ± 2 | 60 ± 0 | 53 ± 3 | 54 ± 2 | 57 ± 1 | 54 ± 2 |
| | Overall Mean ± SEM (n = 10) | 52 ± 2 | 57 ± 1 | 52 ± 2 | 52 ± 2 | 55 ± 1 | 52 ± 2 |
| 4 h | EYC | 34 ± 5 | 52 ± 1 | 39 ± 4 | 51 ± 3 | 49 ± 1 | 40 ± 4 |
| | EYT | 53 ± 1 | 56 ± 2 | 51 ± 3 | 55 ± 2 | 56 ± 1 | 51 ± 2 |
| | Overall Mean ± SEM (n = 10) | 48 ± 3 | 54 ± 1 | 45 ± 3 | 53 ± 2 | 53 ± 1 | 46 ± 3 |

[a]Mean ± SEM (n = 5).

retention of membrane integrity supports the observation that that AS need not be associated with a loss of sperm viability. A most interesting aspect of AS was the apparent expansion of the outer acrosomal membrane necessary to cover what would seem to be a marked increase in acrosomal surface area. Such an apparent increase in the outer acrosomal membrane is without physiological explanation at this time.

A form of acrosomal swelling similar to but more subtle than AS has been ultrastructurally characterized for bull sperm by Jones and Stewart (1979). The swelling was restricted to the apical ridge. While it did not invclve the extensive acrosomal projections described in this study, vesicles or vacuoles within the matrix were reported and outer acrosomal membrane integrity was maintained in most cases. Such morphology was apparent on sperm following dilution and cooling to 5°C in a skimmilk-egg yolk-glycerol-fructose medium. Morphological changes in sperm were still evident after cryopreservation (-196°C). Since Healey (1969) reported normal ultrastructural morphology of the acrosome following processing and freezing of bull sperm in an egg yolk-Na citrate-glycerol medium, it would appear that the morphological alterations observed by Jones and Stewart (1979) were related to their semen processing system. The identical sperm populations examined and found to have swol-

len apical ridges by Jones and Stewart (1979) were previously used by Linford et al. (1976) for artificial insemination and appeared to have normal fertility. This prompted Jones and Stewart to speculate that this subtle swelling of the apical ridge may be a change preparative to the true acrosome reaction and not a phase of sperm deterioration.

In other species, acrosomal modifications have been observed prior to the true acrosome reaction. Based on ultrastructural observations, Yanagimachi and Usui (1974) and Green (1978a) indicated that there was a general swelling of the anterior guinea pig acrosome prior to the apparent true acrosome reaction. The former authors induced the general swelling after several h of incubation in defined media by adding calcium to the culture system. Green (1978a) used the ionophore A23187 and calcium to cause this response. Talbot et al. (1976) described the guinea pig acrosome as being crenulated just prior to exhibiting the true acrosome reaction. They evaluated acrosomal loss on motile sperm as indication of the true reaction during incubation in defined media. A general swelling of the anterior acrosome has been observed on fixed and motile hamster sperm prior to the true reaction (Franklin et al., 1970). Sperm had been incubated in vitro in oviducal fluid. Talbot and Franklin (1976) examined the acrosomal changes of motile

hamster sperm with respect to the true acrosome reaction during incubation with heat-treated blood sera. They noted that swollen hamster acrosomes can appear scalloped, rippled or crenulated before the true reaction.

Aside from the possible physiological role of AS in the events of fertilization, this acrosomal alteration could be a unique form of bovine sperm deterioration not to be confused with the false acrosome reaction which occurs on non-motile cells. For example, motile boar sperm have been reported to develop swollen apical ridges (Pursel et al., 1972a). The gross morphology appears similar to AS. A high proportion of sperm exhibited an AS like property following storage at 15 or 25°C in Tris-lactose or Tris-fructose media (Pursel et al., 1974; Pursel et al., 1972b). They suggested that these acrosomal modifications were a phase of boar sperm deterioration. This concept is supported by the fact that affected boar sperm were not fertile following normal intracervical insemination (Pursel et al., 1972b).

While AS could be a unique form of sperm deterioration, viability, as interpreted by level of percent motility, was inconsistently affected by the occurrence of AS. High levels of AS were associated both with substantially reduced motility as well as some of the highest levels of motility. The occasional decrease in sperm motility may be related to

the storage environments used to induce AS rather than AS itself.

From Experiment 1, the condition most conducive to AS was storing sperm at 4°C as neat-semen, i.e., in SP at 1,500 X $10^6$ sperm/ml. This might be considered contradictory to the possibility that AS is an event preliminary to the true acrosome reaction which is presaged by capacitation. Capacitation is considered to be the biochemical changes that a sperm must undergo in order for the true reaction and fertilization to occur (Chang and Hunter, 1975). The SP of most species is known to contain a factor(s) that prevents capacitation and has been thought to reverse the process once it has occurred (Chang and Hunter, 1975; Williams et al., 1970). Dukelow et al. (1967) and Chang (1957) demonstrated the presence of a decapacitation factor in bovine SP using rabbit sperm as a model. However, biochemical differences do appear to exist between the active factor in bovine SP and rabbit SP (Williams et al., 1967). The bovine component was water soluble and of much lower molecular weight than that found in rabbit SP. Since capacitation has not been well characterized for bovine sperm, the effect of decapacitation factor(s) is also unclear.

With respect to a possible AS relationship with fertilization, it is of interest that holding neat bovine semen at

37°C for a few h facilitates the in vitro fertilization process in a system described by Brackett et al. (1980, 1981). Furthermore, the effects of factors in hamster cauda epididymal plasma that inhibit the true acrosome reaction are diminished during in vitro incubation with sperm (Bavister et al., 1978). Perhaps storage conditions pertinent to AS override or neutralize the effect of decapacitation factors.

It was evident in experiment 1 that dilute incubation in EYT+F but not EYC would partially replace the necessity of storing sperm at 1500 X $10^6$ sperm/ml in SP with respect to induction of high levels of AS. A comparison of EYT+F and EYC in relation to SP indicates that the common components were a primary amine in the Tris molecule and the sugar fructose. Experiment 3 with cauda epididymal sperm demonstrated that having fructose present in the storage media was of major importance to the induction of AS. The presence of Tris in the storage media was of no consequence.

The level of sugar in which the sperm were stored in EYC or EYT to induce AS was 30.8 mM. This is equal to 555 mg% which is an average fructose concentration for bovine semen (Mann, 1964). Thus fructose is probably the component in SP inducing AS. Although fructose is uniquely the predominate sugar in bovine SP, the formation of AS was not restricted

to the inclusion of fructose in defined media. Glucose also induced a strong response and glucose is actually metabolized preferentially by bull sperm (VanTienhoven et al., 1952). Perhaps other glycolytic substrates would have comparable effects.

In addition to culture media composition, storage temperature did influence the AS response. While AS was very evident after 37 and 4°C storage it is unclear why storage at 21°C was relatively ineffective. Storage media was a factor with 37 and 4°C storage. While SP was required during 4°C storage for a strong AS response, sperm in SP at 37°C did not survive long enough to develop AS. The strong response associated with 37°C storage in EYC may have been associated with the fructose component of the 37°C incubation media.

With regard to a strong AS response in the first two experiments it was apparent that time was an important component of the storage process. With storage at 37°C in EYC, 10 h was required for a maximum response to develop. Reducing storage temperature to 4°C necessitated 12 h in SP. In contrast, sperm stored in EYC at 4°C had a strong AS response after 3 days if subsequent incubation at 37°C was in EYI+F.

Temperature has been reported to influence acrosomal changes in other species. Decreasing temperature from 37 to

20°C increased the time required for the true acrosome reaction to occur during in vitro incubation of hamster sperm (Mahi and Yanagimachi, 1973). A further reduction to 16°C prevented the true acrosome reaction. Increasing temperature from 15 to 25°C reduced the time required for a high proportion of boar sperm to develop swollen apical ridges (Pursel et al., 1974).

In summary, it was evident that localized swelling can occur to the acrosome of motile bovine sperm without an influence on membrane integrity or viability as indicated by motility. Storage in SP at 1500 X $10^6$ sperm/ml at 4°C was most conducive to this acrosomal modification. If fructose or glucose was present during storage in defined media, AS also occurred. This indicated that fructose was probably the active component in SP.

Whether the AS on motile bovine sperm is a unique form of deterioration or a spermatozoal change related in a positive way to the fertilization process remains to be established. Current findings in the literature implicate the latter possibility. Future research will be devoted to more definative characterization of the conditions that results in AS.

ADDENDUM TO DISCUSSION

Swelling of the apical ridge and anterior acrosome have been demonstrated in a few instances without a direct or implied relationship to sperm viability. A subtle swelling of the apical ridge, that can involve vesicles or vacuoles within the matrix, was observed with transmission electron microscopy of ram sperm following storage at 5°C in a medium composed of glucose, NaCl, Na and K phosphate and egg yolk (Jones and Martin, 1973). The response increased over 3 days of storage. Comparable modifications to the apical ridge were observed on boar sperm after storage at 5°C in an egg yolk-glycine-Iris-glycerol medium or after in utero incubation (Jones, 1973). Processing and cryopreservation (-196°C) of boar sperm (Larsson et al., 1976) caused similar swelling of the apical ridge. This was observed with both phase contrast and transmission electron microscopy. Using scanning electron microscopy Yasuda and Tanimura (1974) reported that cryopreservation of boar sperm induced projections of the acrosomal matrix. A localized expansion of the acrosomal matrix of human spermatozoa was evident after exposure to blood serum or bovine follicular fluid upon examination with the transmission electron microscope (Roomans and Afzelius, 1975). The physiological importance of these forms of acrosomal swelling was unclear due to the absence of demonstrated relationships with sperm viability.

The lack of a distinct relationship between AS and sperm deterioration indicates that AS could be a controlled cellular change of perhaps active rather than passive nature. Systems of microfilaments and microtubules have been related to somatic cell function and shape (Threadgold, 1976). Clarke and Spudich (1977) reviewed the role of actin and myosin in somatic cell shape. Johnson (1975) has suggested that if actin like material existed in the acrosomal region it may facilitate the membrane vesiculation of the true acrosome reaction. Microfilaments and microtubules have been demonstrated in the acrosomal region of boar sperm (Peterson et al., 1978) in relation to comparable membrane vesiculation. Rabbit and monkey sperm (Stambaugh and Smith, 1978) also have microtubules and/or microfilaments in their acrosomal regions. The polymerization of actin has been associated with formation of an acrosomal process during the acrosome reaction of echinoderm sperm (Tilney et al., 1973). While Tamblyn (1980) could not demonstrate actin in the acrosomal region of boar and bull sperm, other structural elements may be present or develop in relation to capacitation and the true acrosome reaction. Perhaps a system of microtubules and filaments could be responsible for controlled extension of the acrosomal matrix associated with AS. Either formation or breakdown of these structural proteins could be related to the development of AS.

Bedford (1974) drew attention to some unique properties of the apical and lateral margins of the mammalian sperm acrosome in lieu of the possibility that the true acrosome reaction may be initiated in these regions. One such property is the close association between the outer acrosomal and plasma membranes in these areas. Such is commonly observed when the rest of the plasma membrane swells away from the acrosomal surface on sperm prepared for and examined with the electron microscope. This is evident in the cross and sagittal sections in Fig. 2B-D. Gordon et al. (1974) demonstrated that the cephalic tip of the sperm head was modified by capacitating rabbit sperm with 15 h of in utero incubation after mating. Concanavalin A binding on the plasma membrane in this region was reduced. Friend (1977) indicated that calcium was concentrated in the apical acrosome of guinea pig sperm. The requirements of calcium for the true acrosome reaction (Yanagimachi and Usui, 1974) denotes the potential importance of Friend's observation. These qualities and others not yet defined may not only be related to the true reaction, but could account for the localized expansion of the acrosomal matrix on the apical and lateral edges of the acrosome as described for AS.

Green (1978a) and Yanagimachi and Usui (1974) describe a general swelling of the guinea pig anterior acrosome prior

to the true acrosome reaction. The disruption and dispersion of the matrix within the acrosome were characterized as cavitation of the acrosome. It has been shown that uncapacitated guinea pig sperm exposed to hypotonic media will develop a similar acrosomal response (Green, 1978b). This indicates that the outer acrosomal and plasma membranes are permeable to water. Based on these observations Green (1978c) offered an explanation for the acrosomal swelling associated with the true acrosome reaction. He felt that the matrix of the acrosome was normally hypotonic and thus reduced to its smallest volume which was limited by the compressability of the matrix. With the onset of the true acrosome reaction, the constituents of the matrix would be activated. Proacrosin for example, an inactive zymogen, is converted upon activation into several smaller molecular weight forms (Parrish and Polakoski, 1979). An increase in molecular entities would increase acrosomal tonicity and cause an influx of water and swelling. Increased acrosin like activity has been related to the occurrence of the true acrosome reaction (Green, 1978d; Meizel and Lui, 1976; Lui and Meizel, 1979) and penetration of the zona pellucida (McRorie and Williams, 1974). While the density and integrity of the matrix has not been lost with the occurrence of AS, an influx of water could at least partially explain the localized swelling.

Regardless of the fluid media or temperature components of the environment in which sperm were stored or incubated to induce AS, several h or a few days of storage and/or dilute incubation for a few h was necessary for a majority of motile sperm to respond. Mann and Lutwak-Mann (1975) proposed that aging of sperm was part of the maturational process. From this point of view they have suggested that capacitation and aging may be interrelated. Capacitation is often defined as the in vivo or in vitro incubation time period required for sperm to undergo the true acrosome reaction and/or fertilize the oocyte (Chang and Hunter, 1975). From this perspective the subsequent observations are of interest. Holding bovine semen extended at 5°C for 1 day improves the fertility when used as liquid semen for artificial insemination (Almquist et al., 1954; Shannon, 1964; Salisbury and Flerchinger, 1967). Incubating the neat-semen of bulls with lower fertility at 26°C for 20 min prior to processing and freezing (-196°C) was sufficient to improve their fertility (Rajamannan et al., 1971). To facilitate in vitro fertilization, Brackett et al. (1980) held bovine sperm as neat-semen at 37°C for 1 to 3 h. Thus it seems feasible that the conditions which induce AS may be facilitating sperm capacitation through a controlled aging process and AS would then be the subsequent prelude to the true acrosome reaction.

The presence of either fructose or glucose in defined storage medium was required for a high proportion of motile sperm to have AS. In other species the relationship between glucose and fructose and in vitro fertilization or associated spermatozoal changes has been investigated and the findings vary. Anderson et al. (1980) demonstrated that human acrosin was inhibited by both fructose and glucose. Fructose was twice as effective as glucose. Fructose concentrations comparable to that used in this research reduced acrosin activity by less than 50%. Both glucose and fructose impair while pyruvate and lactate encourage the development of the true acrosome reaction in the guinea pig (Rogers and Yanagimachi, 1975). Rogers et al. (1979) indicated that such an effect may be due to a glucose or fructose induced decrease in respiration. In contrast, the proportion of hamster sperm with the true acrosome reaction was increased with addition of glucose to media containing lactate and pyruvate (Bavister and Yanagimachi, 1977). With regard to in vitro fertilization in rats, glucose proved to be a necessary component of the medium whereas fructose could not support the same response (Niwa and Iritani, 1978). A similar observation was made by Hoppe (1976) for capacitation of mouse sperm. Fraser (1981) also found glucose facilitative of mouse sperm capacitation. Mahi and

Yanagimachi (1978) indicated that the presence of glucose in the medium was beneficial to the occurrence of the true acrosome reaction in dog sperm. It is also of interest that glucose is present in the media developed by Brackett and Oliphant (1975) for in vitro fertilization with rabbit gametes. This same media has been used for in vitro capacitation and fertilization of bovine gametes (Brackett et al., 1980). Reducing sugars have also been found in the uterine and oviducal secretions of the cow (Guay and Lamothe, 1970; Heap, 1962; Schultz et al., 1971; Olds and VanDemark, 1957). Consequently, the fact that both fructose or glucose have a positive effect on AS does not necessarily preclude and may well favor the relationship of AS with the events in bovine fertilization.

In experiment 2, the low proportion of motile sperm with AS after storage at 21°C is perplexing when compared to the strong response after 37 or 4°C storage. A review of the procedures indicates some possible explanations. Sperm had been slowly cooled to room temperature during processing prior to the onset of storage at a given temperature. Sperm to be stored at 37°C were placed in such a water bath and thus warmed rapidly from room temperature. Sperm to be stored at 4°C were cooled slowly (7 1/2 h to 5°C). However, sperm stored at 21°C essentially underwent no thermal

change. The rate and/or the magnitude of the temperature change encountered in the 37 or 4°C treatments may have been an important factor. Evaluation of such effects in future experiments may explain why so few sperm responded after storage at 21°C.

In experiment 3 it was evident that Tris as a component of the storage media had no effect on AS. However, it did augment the response slightly during dilute incubation at 37°C. Incubation in a Tris buffered medium also resulted in higher percent motility. In addition, the motility style was altered by Tris buffer and appeared much like the activated motility described in association with capacitation in other mammalian species (Yanagimachi, 1970; Katz et al., 1978; Fraser, 1977). Catecholamines (Cornett and Meizel, 1978; Meizel and Working, 1980; Bavister et al., 1979) have been shown to cause activated motility and the acrosome reaction of hamster sperm. Cornett et al. (1979) further demonstrated that epinephrine facilitates in vitro fertilization in the hamster indicating that capacitation and the true acrosome reaction have been induced. Perhaps during dilute incubation at 37°C the amine nature of Tris is partially capacitating bovine sperm and in this way facilitating AS development. This hypothesis is tempered by the observation of Barros and Berrios (1977) that activated

motility can be induced on hamster sperm without a subsequent acrosome reaction or fertilization of oocytes.

## SERIES II EXPERIMENTS: FRUCTOSE CONCENTRATION AND PH EFFECTS ON ANTERIOR ACROSOMAL ALTERATION OF MOTILE BOVINE SPERM

### INTRODUCTION

Previous research (Series I Experiments) has shown that the acrosome of motile bovine sperm can undergo localized swelling. This expansion of the acrosomal matrix was in the form of folds and projections in the anterior region of the acrosome with integrity of the plasma and outer acrosomal membranes maintained. By virtue of membrane integrity, this anterior acrosomal swelling (AS) can be considered different from the process described for bovine sperm deterioration (Saacke and Marshall, 1968) where both plasma and acrosomal membrane integrity is lost in relation to acrosomal change.

Since AS occurs on viable sperm and appears unrelated to sperm deterioration, it may be associated with the true acrosome reaction. The true reaction is important to penetration of oocyte investments during fertilization (Bedford, 1970; Austin, 1975). In gaining the ability to fertilize the ovum sperm must first be capacitated, a process apparently involving biochemical changes that allow the formation of a true acrosome reaction (Chang and Hunter, 1975; Meizel, 1978). The true reaction, as initially described for mammalian sperm, is a fusion and vesiculation of the plasma and outer acrosomal membranes over the anterior acrosome (Barros

et al., 1967). In addition to these acrosomal changes, there are reports of general swelling of the anterior acrosome of guinea pig (Yanagimachi and Usui, 1974; Green, 1978a) and hamster sperm (Yanagimachi and Noda, 1970; Yanagimachi and Chang, 1964; Franklin et al., 1970) just prior to what was interpreted to be the true reaction. Thus understanding the cause and nature of AS in bovine sperm may provide further insight into the fertilization process in the bovine and other species.

Research reported in Series I experiments indicated that after 1 day of 4°C storage at ejaculate sperm concentrations in seminal plasma (SP) or with 30.8 mM fructose or glucose, a majority of motile sperm developed AS during subsequent incubation at 37°C. Fructose or glucose were the active components in the storage environment responsible for AS. In this series of experiments the relative importance of these sugars to AS formation was further examined with respect to the effects of fructose concentration in the storage medium. The role of pH as a mechanism through which these sugars may influence AS was also studied. Sperm may produce sufficient lactic acid from the metabolism of fructose to reduce the pH of the storage medium. In addition, a vital staining procedure used in conjunction with the differential interference contrast microscope, was developed as a means of more objec-

tively quantifying the proportion of viable sperm exhibiting AS. Previous experiments (Series I) were based on a subjective estimate of the proportion of motile sperm exhibiting acrosomal alteration.

## MATERIALS AND METHODS

### Experiment 1.

Cauda epididymal sperm were stored with increasing concentrations of fructose to more clearly establish the relationship of fructose to the AS development of motile bovine sperm. The pH of the storage medium at the termination of storage was measured to evaluate the possibility that fructose may influence the acrosome through alteration of pH.

Sperm culture media:

SP was obtained from an ejaculate collected by artificial vagina. Semen was immediately centrifuged at 3900 X g for 5 min and SP was recovered and held on ice until used. Egg yolk-Tris(EYT) medium was prepared by combining 20% (v/v) egg yolk (Gallus domesticus) and 80% (v/v) buffer. Egg yolk contains 0.7% (w/v) free glucose (Romanoff and Romanoff, 1949) and thus contributes about 8 mM glucose to all media. Buffer was composed of 172 mM Tris (hydroxymethylaminomethane), 55 mM citric acid and either 0, 6.8, 13.5, 27.0, 54.0

or 108.0 mM fructose. NaCl was added to the buffer solutions to adjust osmolarity to 289±5 (range) mOsm and thus accommodate varying fructose concentrations. Additional citric acid was used to adjust pH to 6.8 at 23°C. Egg yolk based media were centrifuged at 15,000 X g for 10 min and the supernatant passed through a Millipore filter (0.45 um pores) to remove particulate matter and bacteria. Antibiotics were added to SP and included in EYT media at 1000 IU penicillin and 1000 ug dihydrostreptomycin/ml.

Experimental protocol:

Cauda epididymal sperm were used in this experiment due to their lack of prior exposure to seminal plasma and fructose. Bulls were slaughtered about 45 min after semen collection (where SP was obtained). The surface of the epididymis was cleaned with flush media (EYT medium without fructose or antibiotics). Flush medium was introduced into the vas deferens with a 3 ml syringe and 25 ga. blunt needle. A small cut was made in the distal cauda and sperm were expressed in a retrograde manner with the flush medium. Sperm concentration was determined with a Coulter Counter and semen was diluted to 3.5 X $10^9$ sperm/ml with the flush medium. Antibiotics were included in this dilution at 1000 IU/ml and 1000 ug/ml of penicillin and dihydrostreptomycin,

respectively. Cauda sperm were further extended to 1 X $10^9$ sperm/ml in the various EYT storage media and the final stored volumes were 0.5 ml. Final fructose concentrations after dilution were 0, 3.9, 7.7, 15.4, 30.8 or 61.7 mM fructose. Cauda sperm were also extended in SP to serve as a positive control treatment. The fructose concentrations represented in the EYT media more than span the naturally occurring range reported for bovine semen (Mann, 1964). Sperm in these 7 storage treatments were cooled step-wise from 37 to 25°C over 2 h (at room temperature) and from 25 to 5°C over an additional 8 h (at 4°C in a walk-in refrigerator). After 24 h of storage at 4°C, two aliquots of each treatment were diluted in EYT to 50 X $10^6$ sperm/ml and incubated at 37°C for 5 min. This allowed duplicate evaluation of each treatment. After incubation, percent motility was estimated from wet smears with phase contrast microscopy (100X) and the proportion of motile sperm with AS was scored subjectively on a scale of 0-3 using differential interference contrast microscopy (1250X). The estimated value of the scale units based on percent of motile sperm exhibiting AS were; 0<5%, 1=5-20%, 2=25-55% and 3≥60%. In addition, an objective method of evaluating AS on viable sperm (described below) was compared with the subjective method described above. All treatments were coded to prevent observer bias.

At the end of storage, the pH of each storage treatment was measured at 4°C with a Corning Research pH meter and a Sigma calomel probe designed for Tris buffered solutions. The entire experiment was replicated 5X using sperm from a different bull for each replicate. Data were analysed by analysis of variance (Barr et al., 1979). The data were transformed for analysis using the following calculations; the square root of the sum of an AS value plus 0.5 and 2X the arcsin square root of a percent motility value.

Quantitation of AS from vitally stained semen smears

Objective quantification of the proportion of motile sperm with AS was not feasible in the Series I experiments due to sperm motility. However, quantitative data would be more reliable and sensitive for evaluating treatment effects. On this basis, vital staining of sperm was examined as a means to allow quantitative determination of the proportion of viable sperm with AS. Preliminary experiments indicated that it was possible with differential interference contrast microscopy to simultaneously observe the outcome of vital staining and the acrosomal integrity of sperm.

Vital stains were originally developed for use with neat-semen. However, in the present application, sperm were extended in defined media that differs considerably from SP

in composition. The staining of sperm has been shown to be adversely influenced by some extender components such as glycerol (Mixner and Saroff, 1954), glucose and various ions (Mayer et al., 1951). Consequently, the staining system was evaluated to ascertain its ability to differentially stain sperm based upon viability. The integrity of the acrosome, based on the presence of an apical ridge (Saacke and Marshall, 1968), was the criterion for sperm viability. The ability to quantify the proportion of viable sperm with AS was also evaluated by examining the staining properties of sperm with AS and comparing the percent unstained sperm with AS to estimates of the proportion of motile sperm with AS.

The vital staining system was evaluated in conjunction with Experiment 1. This afforded examination of the staining response among several different storage media and wide variations in the proportion of viable sperm and viable sperm with AS.

Staining procedure:

The stain used was developed by Dr. H. P. VanKrey at V.P.I. & S.U. for use with poultry semen. Fast Green FCF (0.5g) and Eosin B (0.2g) were dissolved in 25 ml of glutamate based buffer solution. This combination of Fast Green FCF and Eosin B was developed by Mayer et al. (1951). The

glutamate buffer solution used in this stain solution is diluent A as previously described by Lake (1960). Stain solution pH was adjusted to 7.3-7.4 with NaOH and HCl. The stain composition is presented in detail in Appendix Table 17. At the time of evaluation in experiment 1, two stained smears of each sample were prepared. Five ul of diluted semen were mixed with 10 to 15 ul of stain on one end of a slide. Another slide, placed at a 45° angle to the admixture, was used to draw the preparation into a smear. The smears were immediately dried in a 62°C jet of hot air (Oster model 202C Air Jet) for 30 sec. The slide was held at an oblique angle to the air flow 7 cm from the duct opening. Dried smears were protected with resin mounted cover slips. All smears were coded to prevent observer bias. Smear preparation is described in Appendix Table 18.

Each smear was examined twice using differential interference contrast microscopy (1250X). At each examination, 100 sperm were differentially characterized based on the criteria described subsequently. Sperm were catagorized as being stained, unstained or half-stained. The totals were expressed as the percent of sperm in each category. By definition, half-stained sperm were unstained in the acrosomal region and eosinophilic in the post acrosomal region. Half-staining of sperm is commonly thought to be an artifact

of the staining procedure as it affects the viable sperm population such that some become partially eosinophilic. However, it has not been clearly demonstrated that such sperm were viable at the time of staining. Within each stain category sperm were characterized as having an intact apical ridge (referred to as an intact acrosome), which is characteristic of a viable sperm, or acrosomal morphology associated with sperm undergoing deterioration. Since AS occurs on motile sperm with retention of membrane integrity, such sperm were considered to have an intact apical ridge for the purpose of this study. From these data, the percent of unstained, half-stained and stained sperm with an intact acrosome were calculated.

In a simultaneous differential characterization, the same sperm were categorized as to how they stained and whether or not they had acrosomal morphology characteristic of AS (Series I Experiments). From these data, the percent of unstained, half-stained and stained sperm with AS was calculated.

The percentage values of the data were transformed for statistical analysis by taking 2X the arcsin square root of a value. Overall means of smears and counts within smears for each duplicate evaluation of a treatment were submitted for analysis. Analysis of variance was performed according

to Barr et al. (1979) on each parameter. Means were compared using Duncan's new multiple range test (Duncan, 1955).

Experiment 2

Results from Experiment 1 indicated that sperm did respond with AS as fructose concentration in the storage media increased. However, there was a concommitant reduction in media pH during cooling and storage as fructose level increased. Consequently, it was unclear whether the reduction in media pH and/or the increase in fructose concentration was the cause of AS. This relationship was evaluated using a 5 X 2 factorial arrangement of treatments where cauda epididymal sperm were exposed to 5 different media at each of two different pH ranges during storage. The 5 media were SP, egg yolk-citrate and egg yolk-Tris with fructose (EYC+F, EYT+F) or without fructose (EYC, EYT). Based on the results of Experiment 1, the media pH during storage was maintained in either a normal range (6.6-6.8) associated with the absence of fructose and minor AS or a low pH range (5.7-6.0) related to the presence of fructose and a strong AS response. Sperm culture media and experimental protocol are described below.

Sperm culture media:

SP was obtained and EYT prepared as described previously. The EYT+F was prepared from the previously defined buffer that contained 54 mM fructose. EYC media were composed of 20% (v/v) egg yolk and 80% (v/v) of a buffer solution composed of 78.2 mM sodium citrate and either 54 mM fructose (EYC+F) or 32 mM NaCl (EYC). After dilution with cauda sperm, the final fructose concentration was 30.8 mM. All other media characteristics were as described for Experiment 1.

Experimental protocol:

Cauda epididymal sperm were obtained and prepared as previously described except that 150 mM NaCl was the flush medium instead of EYT. Sperm concentration was determined with a Coulter Counter and adjusted to 3,500 X $10^6$ sperm/ml with flush medium. Penicillin (1000 IU/ml) and dihydrostreptomycin (1000 ug/ml) were included in the dilution. Sperm were further diluted to 1 X $10^9$/ml in the five storage media, i.e., SP, EYC, EYC+F, EYT and EYT+F. Two preparations were made for each storage medium. Both preparations were subsequently cooled to 4°C and stored as previously described. One preparation of each storage medium was allowed to undergo its normal pH change. This pH change was

monitored throughout cooling and storage. Based on the previous experiment, media pH's with sperm stored in SP, EYC+F or EYT+F were expected to decrease and be in a range of 5.7 to 6.0 by the end of storage. In contrast, the environmental pH for sperm stored in EYT or EYC was expected to change very little and be in a range of 6.6-6.8. The other preparations for sperm stored in EYC+F, EYT+F and SP were maintained at a pH comparable to those of sperm stored in the absence of fructose, i.e., 6.6-6.8. This was accomplished with periodic addition of ul quantities of 1 M NaOH. The pH was reduced to 5.7-6.0 for the other aliquots of sperm stored in EYC and EYT in a manner comparable to that which would have occurred if fructose was present. This was accomplished with periodic addition of ul quantities of 1 M HCl. This created 10 storage media treatment conditions, i.e., 5 different storage media at both normal and reduced pH. Following dilution, pH measurements and appropriate pH adjustments were made after 12, 28, 42, 70 and 120 min (during the period of initial cooling from 37 to 25°C). This was continued at 3, 6, 12 and 24 h after dilution (during the next cooling step from 25 to 4°C and subsequent storage at 4°C). Semen reached 5°C 7.5 h after the second cooling step was initiated. pH was also measured at the beginning and end of the experiment (34 h after the onset of cooling).

The storage treatment receiving the largest volume of NaOH and or HCl served as a basis for which the volume of all other treatments were adjusted with ul quantities of 0.5 M NaCl to equalize changes in osmolarity and dilution among all treatments. Total adjustments in volume due to addition of NaOH and HCl ranged from 8-12 ul per 0.5 ml of diluted semen. Actual pH changes for each storage medium over the time course of the experiment are presented in Fig. 7. At the end of storage two aliquots of each of the 10 storage treatments were diluted at random in EYT (50 X $10^6$ sperm/ml) and incubated in a 37°C water bath. This allowed duplicate evaluations of each storage treatment for AS and percent motility after 5 min and 4 h of incubation. During evaluation of each aliquot of a treatment, two smears of vitally stained sperm were prepared to determine the percent live sperm and the percent live sperm with AS. Results of the previous experiment indicated that half-stained sperm should be considered viable and thus half-stained sperm were combined with unstained sperm in this experiment. This combination will be referred to as percent live sperm. One hundred sperm from each of the two smears were characterized for each parameter. If either the percent live sperm or the percent live sperm with AS differed by ≥9% between the two smears of an aliquot, then both of the smears were

recounted. In such instances, both sets of counts for a smear were averaged to arrive at a parameter value for that smear. All evaluations were done on coded samples and slides to prevent observer bias. The entire experiment was replicated 5 times using semen and SP from one of five different bulls for each replicate. Data were transformed for analysis as described previously. Overall means of smears for each duplicate evaluation of a treatment were submitted for analysis. Analysis of variance was performed (Barr et al., 1979).

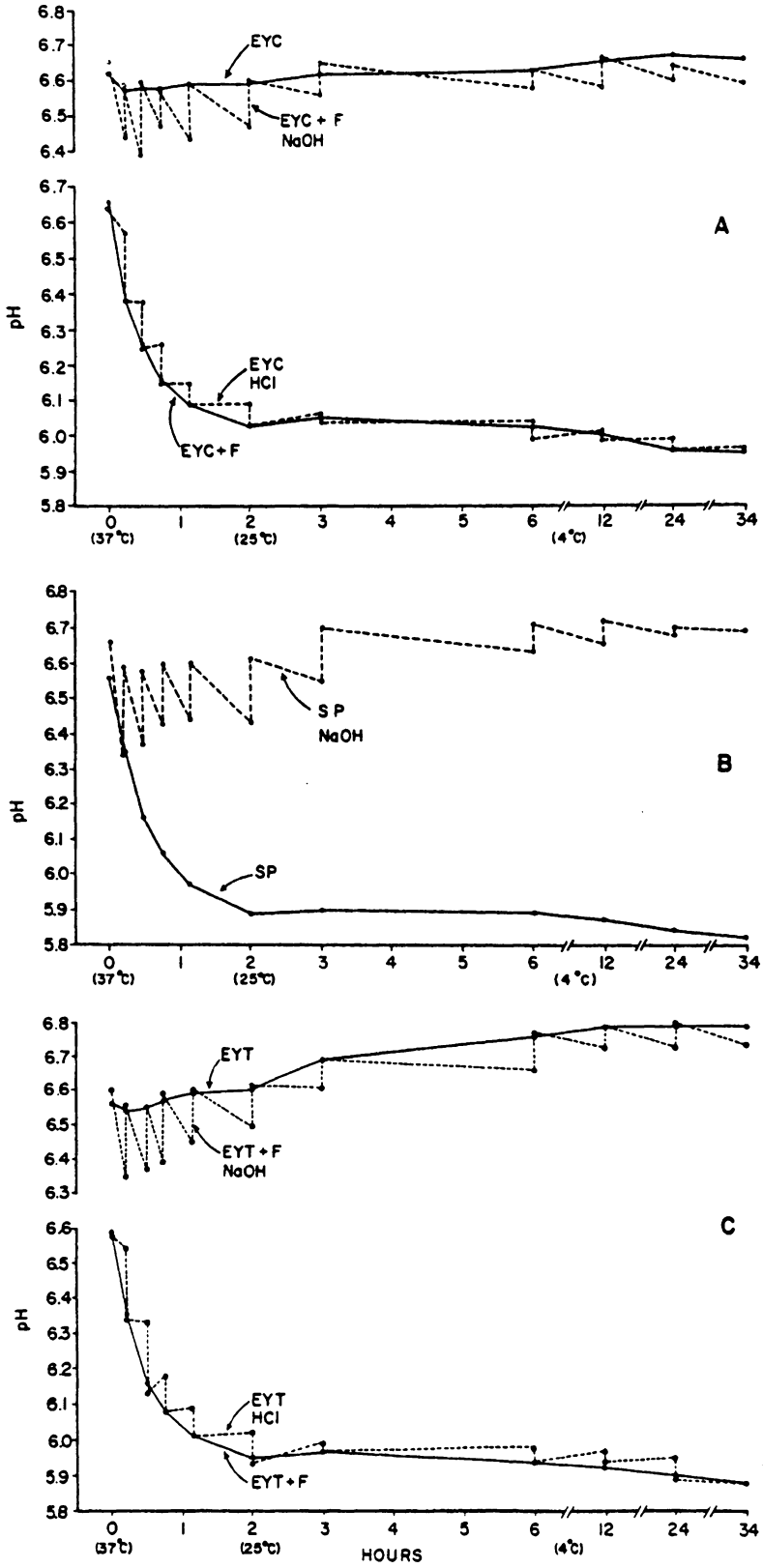
## RESULTS

### Experiment 1.

The proportion of motile sperm with AS after storage in EYT with increasing concentrations of fructose or in SP is presented in Fig. 8 along with the pH achieved by the end of the storage period (Fig. 8). Analysis of variance is presented in Appendix Tables 19 and 20 for AS and pH respectively. Both AS and pH were influenced by fructose concentration in the storage medium and SP ($P \leq 0.01$). Sperm storage in EYT without fructose had minimal AS and pH was reduced slightly during storage (6.8 to 6.7). The proportion of motile sperm with AS increased sharply and pH decreased rapidly with increases in media fructose concen-

FIG. 7. Storage media treatment pH changes during cooling and storage. Means of 5 replicates are shown. Standard deviations averaged 0.05 pH units and ranged from 0.01 to 0.19 units. Media temperatures in relation to cooling and storage are indicated.

A). pH changes for EYC based storage media treatments.
B). pH changes for SP based storage media treatments.
C). pH changes for EYT based storage media treatments.


EYC
EYC + F
NaOH
A
pH
EYC
HCl
EYC+F
(37°C)
(25°C)
(4°C)
B
S P
NaOH
SP
C
EYT
EYT + F
NaOH
EYT
HCl
EYT+F
HOURS

tration. AS was maximum at 15.4 mM fructose. Fructose concentrations of 7.7, 15.4, 30.8 and 61.7 mM induced strong and comparable AS responses similar to that achieved by storage in SP. Media pH's for these conditions ranged from 5.97 to 5.74 with the lowest levels associated with the SP storage medium (Fig. 8).

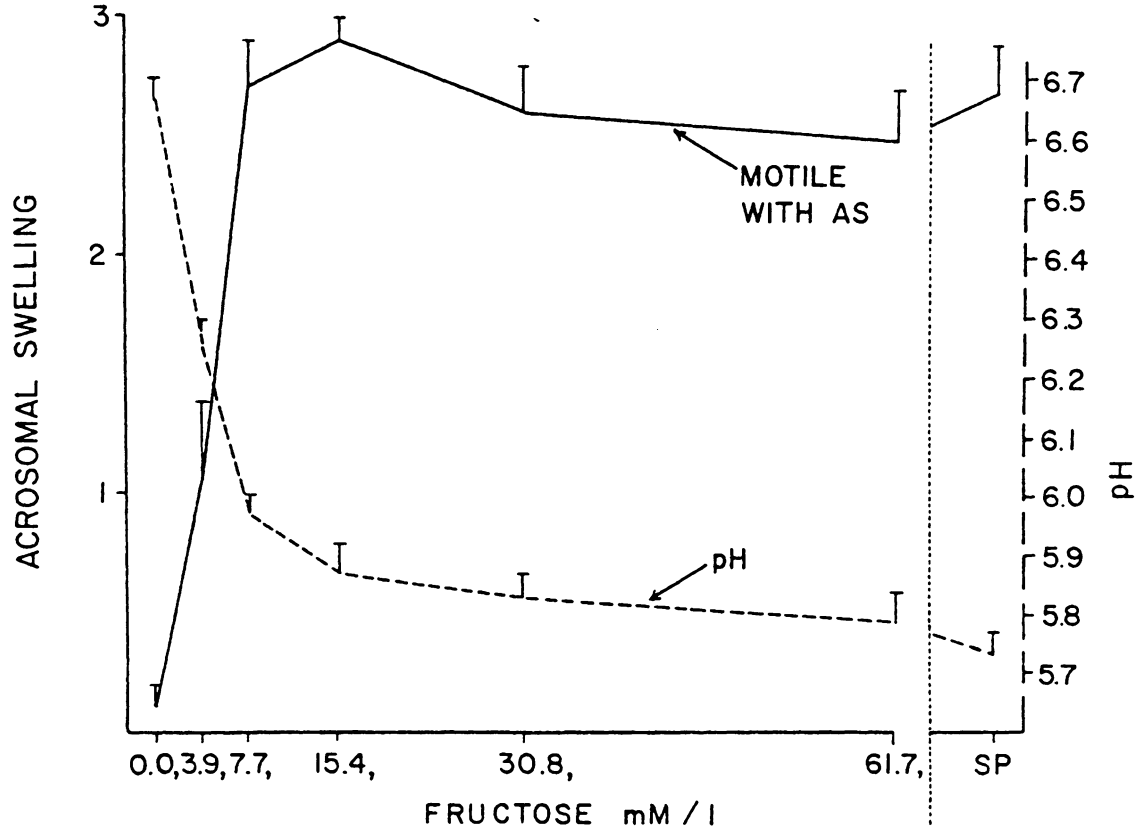
Percent motility was not affected by the fructose concentration in the storage medium or SP. Over the entire experiment motility averaged 60±5% S.D. for all fructose levels and SP. Analysis of variance is presented in Appendix Table 21.

Quantitation of AS from vitally stained semen smears

The effect of the presence of various fructose concentrations in the storage medium and SP on the percent unstained, half-stained and stained sperm is presented in Table 5. The majority of sperm were unstained while very few were stained or half-stained. Storage medium did affect ($P \leq 0.01$) the percent unstained and stained sperm. Based on the staining, storage in SP was associated with depressed sperm viability. Analysis of variance for these parameters is expressed in Appendix Tables 22-24.

The percent of unstained, half-stained and stained sperm with intact acrosomes is also presented in Table 5. Almost

FIG. 8. Effect of fructose concentration and SP on AS and storage media pH after 24 h at 4°C. Means ± SEM of 5 experimental replications and duplicate treatment evaluations are indicated. AS was rated on a scale of 0-3. Arbitrary scale values were estimated to be 0<5%, 1=5-20%, 2=25-55% and 3≥60% motile sperm with AS.


ACROSOMAL SWELLING
3
2
1
MOTILE
WITH AS
pH
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6.0
5.9
5.8
5.7
pH
0.0,3.9,7.7,
15.4,
30.8,
61.7,
SP
FRUCTOSE mM / l

all unstained sperm had an intact acrosome. While there was a low percent of half-stained sperm, a high percent of these also had intact acrosomes indicating that such sperm were most likely viable at the time of staining. Very few stained sperm had intact acrosomes. Percent unstained sperm with intact acrosomes was increased slightly by storing sperm in SP ($P \leq 0.01$). Fewer half-stained sperm had intact acrosomes after storage in EYT ($P \leq 0.01$). Analysis of variance is presented in Appendix Tables 25-27.

Since half-stained and unstained sperm can be considered viable they were combined for each of the storage media treatments and referred to as percent live sperm for comparison to the total percent of sperm with intact acrosomes (Table 5). Analysis of variance is shown in Appendix Tables 28 and 29, respectively. Storing sperm with fructose decreased the percent live sperm slightly ($P \leq 0.01$) SP had even a greater depressing effect on percent live sperm ($P \leq 0.01$). The total percent sperm with intact acrosomes was reduced by storage in SP ($P \leq 0.01$). Within each storage media treatment there was little difference between these parameters as to the percentage of sperm that were viable. The linear correlation between percent intact acrosomes and percent live sperm was high ($r = 0.93$, $P \leq 0.01$) and had a regression coefficient of 0.99 ($P \leq 0.01$).

TABLE 5. Effect of fructose concentration and SP on the vital staining characteristics and acrosomal integrity of sperm.[a]

| Storage Media mM Fructose | % US[b] | %HS | %ST | %US with intact acrosomes | %HS with intact acrosomes | %ST with intact acrosomes | Total % live (US + HS) | Total % intact Acrosomes |
|---|---|---|---|---|---|---|---|---|
| 0.0 | 94.4 ± 0.3 | 2.2 ± 0.3 | 3.4 ± 0.4 | 97.9 ± 0.3$^{d}$ | 76.5 ± 3.5$^{d}$ | 6.5 ± 2.7 | 96.7 ± 0.4$^{c}$ | 94.4 ± 0.3$^{c}$ |
| 3.9 | 92.2 ± 0.6 | 3.5 ± 0.6 | 4.3 ± 0.5 | 98.1 ± 0.3$^{d}$ | 86.6 ± 2.3$^{c}$ | 3.9 ± 2.9 | 95.7 ± 0.5$^{cd}$ | 93.7 ± 0.6$^{c}$ |
| 7.7 | 87.9 ± 0.6 | 5.3 ± 0.9 | 6.7 ± 0.8 | 97.4 ± 0.3$^{d}$ | 82.6 ± 4.1$^{cd}$ | 5.8 ± 1.6 | 93.3 ± 0.8$^{d}$ | 90.7 ± 0.6$^{c}$ |
| 15.4 | 87.1 ± 1.2 | 6.5 ± 1.3 | 6.5 ± 0.8 | 97.1 ± 0.4$^{d}$ | 89.9 ± 3.4$^{c}$ | 6.6 ± 3.0 | 93.6 ± 0.8$^{d}$ | 91.0 ± 0.8$^{c}$ |
| 30.8 | 86.8 ± 0.7 | 6.3 ± 1.1 | 7.0 ± 0.9 | 97.3 ± 0.4$^{d}$ | 89.0 ± 3.5$^{c}$ | 4.2 ± 1.3 | 93.0 ± 0.9$^{d}$ | 90.5 ± 0.7$^{c}$ |
| 61.7 | 88.9 ± 0.9 | 4.9 ± 0.9 | 6.3 ± 0.4 | 97.8 ± 0.3$^{d}$ | 89.5 ± 3.8$^{c}$ | 6.9 ± 2.2 | 93.8 ± 0.4$^{d}$ | 92.0 ± 0.7$^{c}$ |
| SP | 74.6 ± 2.0 | 4.7 ± 0.7 | 20.7 ± 2.0 | 98.9 ± 0.2$^{c}$ | 89.2 ± 2.5$^{c}$ | 2.0 ± 0.8 | 79.3 ± 2.0$^{e}$ | 78.5 ± 2.1$^{d}$ |

[a]Mean ± SEM of 5 replications and 2 evaluations per treatment.

[b]US = unstained, HS = half-stained, and ST = stained.

[c,d,e]Means within columns with different superscripts are different ($P < 0.01$).

The percent of unstained, half-stained and stained sperm with AS as influenced by storage at 4°C in EYT with various fructose concentrations or with SP is presented in Table 6. Analysis of variance can be examined in Appendix Tables 30-32. Regardless of the storage medium, very few stained sperm had AS. Increasing the fructose concentration or storing sperm in SP caused a large increase in the percent unstained sperm with AS ($P \leq 0.01$). While only a few sperm were half-stained (Table 5), increasing fructose affected the percent half-stained sperm with AS ($P \leq 0.01$) in a manner comparable to that of unstained sperm.

Since both the percent of half-stained and unstained sperm with AS reflected the effects of the storage media in a similar manner and that half-stained sperm can be considered to be viable based on acrosomal integrity (Table 5), both parameters were combined to indicate the percent live sperm with AS (Fig. 9). Analysis of variance is presented in Appendix Table 33. Fructose concentration and SP affected the AS response ($P \leq 0.01$). Increasing fructose concentration caused a rapid increase in the percent live sperm with AS. The AS response was high and comparable after storage with 7.7, 15.4, 30.8 and 61.7 mM fructose and SP.

TABLE 6. Effect of fructose and SP on the percent unstained, half-stained and stained sperm with AS.[a]

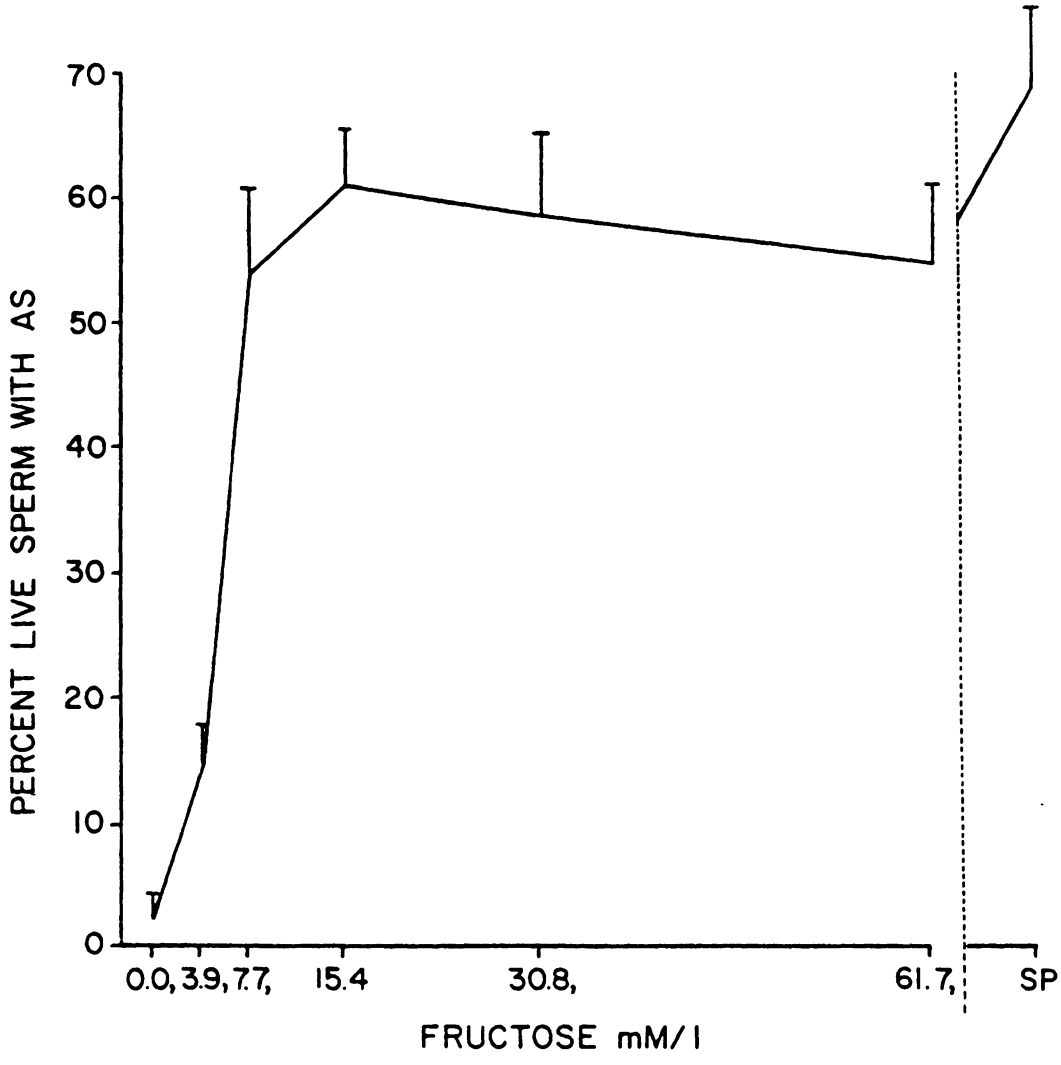
| Staining Characteristic | Fructose Concentration (mM) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.0 | 3.9 | 7.7 | 15.4 | 30.8 | 61.7 | SP |
| %US[b] | 2.7 ± 3.3[f] | 14.7 ± 9.7[e] | 55.0 ± 14.1[d] | 62.0 ± 8.5[cd] | 59.6 ± 13.4[cd] | 55.0 ± 13.7[d] | 70.4 ± 15.2[c] |
| %HS | 3.8 ± 1.9[e] | 19.5 ± 3.3[d] | 29.8 ± 5.3[cd] | 43.7 ± 4.6[c] | 43.3 ± 7.7[c] | 44.9 ± 6.3[c] | 46.2 ± 5.2[c] |
| %ST | 3.8 ± 1.9 | 0.0 ± 0.0 | 2.4 ± 1.3 | 2.2 ± 1.5 | 3.3 ± 1.2 | 2.7 ± 1.1 | 1.2 ± 0.7 |

[a]Mean ± SEM of 5 replicates and 2 evaluations per treatment.

[b]US = unstained, HS = half-stained, and ST = stained.

[c,d,e,f]Means within rows with different superscripts are different ($P < 0.01$).

FIG. 9. Effect of fructose level and SP on the percent live (half-stained plus unstained) sperm with AS. Means ± SEM include 5 replications and duplicate evaluations of each treatment.


70
60
50
40
30
20
10
0
PERCENT LIVE SPERM WITH AS
0.0, 3.9, 7.7, 15.4 30.8, 61.7, SP
FRUCTOSE mM/l

A comparison of the percent live sperm with AS (Fig. 9) to the proportion of motile sperm with AS (Fig. 8) shows that both parameters define the effects of fructose concentrations and SP in a similar direction and magnitude. There was a correlation coefficient between these parameters of 0.88 ($P \leq 0.01$) and a regression coefficient expressed as a change of 22% live sperm with AS for each unit of motile sperm with AS ($P \leq 0.01$).

Experiment 2

pH of storage media throughout the cooling and storage period is presented in Fig. 7. The inclusion of 30.8 mM fructose (EYC+F, EYT+F) in the storage medium or the use of SP as the storage medium resulted in a rapid decrease in pH. The majority of the pH decrease occurred during the 2 h of cooling from 37 to 25°C. For EYC and EYT media in the normal pH range there was a slight increase in pH during cooling and storage. Although occurring in a step-wise fashion, the addition of HCl to EYC or EYT storage media treatments over cooling and storage produced reductions in pH comparable to those achieved with inclusion of 30.8 mM fructose in either storage media. By addition of NaOH throughout cooling and storage of sperm in SP, EYC+F and EYT+F, it was possible to arrest the pH decrease and maintain a pH level com-

parable to that of EYC and EYT treatments in the normal pH range (Fig. 7).

The effects of storage media pH and storage media on the proportion of motile sperm with AS and percent motility is presented in Table 7. Analysis of variance for AS is in Appendix Table 34. In general, it is evident from the data in Table 7 that reduced storage media pH (5.83- 5.96) and not the presence of fructose per se was the major factor responsible for AS on motile sperm. While incubation interval did not influence the AS response, there was an interaction ($P \leq 0.01$) between storage medium and storage medium pH. The interaction was due to the intermediate proportion of sperm with AS after storage in SP and EYC at normal pH (6.69-6.86). All other media held at normal pH during cooling and storage resulted in only slight AS.

Based on the motility data in Table 7, storing sperm at reduced pH (5.83-5.96) and the subsequent formation of AS appears to be inconsistently associated with loss of viability. Analysis of variance for percent motile sperm is presented in Appendix Table 35. There was an interaction ($P \leq 0.01$) between storage medium pH, storage medium and incubation interval with respect to motility. After 5 min of incubation, decreased motility was associated with previous storage in EYC and EYT at reduced pH and in SP. At 4 h of

TABLE 7. Effect of storage media pH and fructose on AS and the percent motile sperm after 5 min and 4 h of 37°C incubation.[a]

| Incubation Interval at 37°C | Storage media and media pH at end of storage as affected by addition of NaOH and HCL | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SP 5.83[b] | SP + NaOH 6.69 | EYC 6.86 | EYC + F + NaOH 6.59 | EYC + F 5.95 | EYC + HCl 5.96 | EYT 6.78 | EYT + F + NaOH 6.73 | EYT + F 5.88 | EYT + HCl 5.88 |
| | AS[c] | | | | | | | | | |
| 5 min | 1.7 ± 0.4 | 0.3 ± 0.3 | 1.2 ± 0.4 | 0.0 ± 0.0 | 2.2 ± 0.2 | 2.9 ± 0.9 | 0.4 ± 0.2 | 0.0 ± 0.0 | 2.6 ± 0.2 | 3.0 ± 0.0 |
| 4 h | 2.6 ± 0.2 | 1.9 ± 0.3 | 1.6 ± 0.0 | 0.3 ± 0.2 | 2.1 ± 0.2 | 2.9 ± 0.1 | 0.9 ± 0.3 | 0.1 ± 0.1 | 2.6 ± 0.2 | 2.8 ± 0.1 |
| | % Motility | | | | | | | | | |
| 5 min | 42 ± 3 | 49 ± 2 | 60 ± 1 | 62 ± 1 | 63 ± 1 | 54 ± 1 | 64 ± 2 | 62 ± 1 | 63 ± 1 | 59 ± 1 |
| 4 h | 34 ± 3 | 42 ± 3 | 64 ± 1 | 58 ± 1 | 60 ± 1 | 42 ± 2 | 63 ± 1 | 58 ± 1 | 53 ± 2 | 34 ± 2 |

[a] Means ± SEM of 5 replicates and duplicate evaluation of each treatment.

[b] Mean pH of 5 replicates.

[c] AS is rated on a scale from 0-3 with arbitrary scale values being, 0<5%, 1=5-20%, 2=25-55%, and 3≥60% motile sperm with AS.

incubation reduced motility was apparent after storage at reduced pH in EYC, EYT and EYT+F as well as in SP.

The effect of storage medium pH and storage medium on the percent of live (half-stained plus unstained) sperm with AS and percent live sperm is presented in Table 8. Analysis of variance for percent live sperm with AS can be evaluated in Appendix Table 36. There was an interaction ($P \leq 0.01$) between storage medium, storage medium pH and incubation interval. Reduced storage pH (5.83-5.96) was always associated with pronounced AS on live sperm regardless of presence or absence of 30.8 mM fructose in the storage medium. Conversely, maintaining pH in a normal range (6.59-6.86), even with fructose in the storage medium, resulted in only a few sperm with AS after 5 min of incubation. However, after 4 h of 37°C incubation there was an increase in AS for sperm that had been stored at normal pH's. Sperm stored at normal pH's in SP, EYC and EYT were affected to a greater degree than those stored in EYC+F and EYT+F.

AS measured subjectively on motile sperm or objectively on live sperm resulted in a comparable description of storage treatment effects. The measurements were highly correlated ($r=0.83$; $P \leq 0.01$). For each unit of motile sperm with AS there was a 19% increase in AS on live sperm ($P \leq 0.01$).

Based on vital staining, the percent live sperm data (Table 8) indicates that in general sperm viability was high and comparable after storage in any of the defined media but was suppressed by SP regardless of storage media pH or the occurrence of AS. Analysis of variance for percent live sperm is presented in Appendix Table 37. There was a storage medium, storage medium pH and incubation interval interaction ($P \leq 0.01$). Inclusion of fructose in the storage medium and control of medium pH with either NaOH or HCl appeared to depress the percent live sperm slightly.

## DISCUSSION

The ability of the Fast Green-Eosin B live-dead staining system to distinguish live sperm from dead was examined in conjunction with Experiment 1. The disposition of half-stained sperm, a known property in vital staining systems, was also examined. Salisbury et al. (1978) suggested in their review that any form of sperm eosinophilia should be considered nonviable. However, Brochart (1953) indicated that half-staining may be an artifact related to the manipulations associated with the staining process. Data from Experiment 1 demonstrated that while very few sperm were half-stained, the majority possessed an intact apical ridge at the time of staining. Thus half-stained sperm were most

TABLE 8. Effect of storage media pH and fructose on the percent live (half-stained plus unstained) sperm with AS and percent live sperm after 5 min and 4 h of incubation at 37°C.[a]

| Incubation Interval at 37°C | Storage media and media pH at end of storage as affected by addition of NaOH or HCl | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SP 5.83[b] | SP + NaOH 6.69 | EYC 6.86 | EYC + F + NaOH 6.59 | EYC + F 5.95 | EYC + HCl 5.96 | EYT 6.78 | EYT + F + NaOH 6.73 | EYT + F 5.88 | EYT + HCl 5.88 |
| | % Live Sperm with AS | | | | | | | | | |
| 5 min | 54 ± 6 | 5 ± 1 | 5 ± 1 | 1 ± 0 | 53 ± 7 | 69 ± 3 | 4 ± 1 | 1 ± 0 | 71 ± 3 | 78 ± 2 |
| 4 h | 58 ± 3 | 37 ± 5 | 53 ± 4 | 18 ± 3 | 61 ± 4 | 70 ± 1 | 44 ± 5 | 24 ± 4 | 66 ± 3 | 70 ± 2 |
| | % Live Sperm | | | | | | | | | |
| 5 min | 55 ± 3 | 60 ± 1 | 96 ± 0 | 89 ± 1 | 94 ± 1 | 89 ± 1 | 95 ± 1 | 90 ± 1 | 92 ± 1 | 88 ± 1 |
| 4 h | 49 ± 3 | 54 ± 2 | 90 ± 1 | 83 ± 1 | 86 ± 1 | 84 ± 1 | 90 ± 1 | 83 ± 1 | 85 ± 2 | 83 ± 1 |

[a]Means ± SEM of 5 replicates and duplicate evaluations of each treatment.

[b]Mean pH of 5 replicates.

likely viable when the smear was made. From this perspective, half-stained sperm were considered viable in these experiments.

Further examination of the vital staining system demonstrated that it was a suitable approach for quantifying the percent of viable sperm with AS. In experiment 1 it was evident that sperm with AS were not eosinophilic. In both experiments 1 and 2, which had wide variations in the number of sperm with AS, there was a strong positive relationship between the proportion of motile sperm with AS and the percent live (unstained plus half-stained) with AS. Both parameters reflected the storage treatment effects in the same direction and magnitude.

In addition to being a more objective method for determining the proportion of live sperm with AS, evaluating vitally stained sperm with differential interference contrast microscopy may allow closer scrutiny of acrosomal changes. Incubating sperm that had been stored at normal pH's (Experiment 2) for 4 h caused an increase in the percent live sperm with AS. It was appeared that 30 to 50% of this increase was due to sperm that had very subtle forms of AS. These forms were not apparent after 5 min of incubation and would be difficult to detect on motile sperm. This combination of a differential interference contrast image of

sperm morphology with vital stain identification of sperm viability maybe useful in other types of research involving acrosomal changes on viable sperm.

The positive influence of the presence of fructose in the storage medium on AS formation was demonstrated in a previous experiment (Series I, Experiment 2). This effect was also evident in these experiments. In addition, it was apparent that a wide range of concentrations from 7.7 to 61.7 mM fructose were comparable and as effective in the induction of AS as storing sperm in SP. Such concentrations of fructose could be considered physiological as they barely exceed the range in fructose concentrations reported for bovine semen (Mann, 1964). While little difference existed in the strong AS response associated with 7.7 to 61.7 mM fructose, decreasing fructose to 3.9 mM sharply reduced the proportion of sperm with AS. This indicated that a rather narrow range existed for the minimum fructose concentration required for a high proportion of sperm to develop AS under the culture conditions used.

In Experiment 1, it was evident that a decrease in storage medium pH occurred as fructose concentration increased. Storage with SP or fructose concentrations conducive to a strong AS response was associated with pH's of 5.7 and 6.0. Apparently there was enough metabolic production of lactic

acid by sperm to override media buffering capacity and reduce the pH. Storing 500 X $10^6$ sperm in 0.5 ml of media in a 1.5 ml covered tube would likely provide very anaerobic conditions conducive to lactic acid formation.

The implied interrelationship between fructose, reduced media pH and AS formation proved to be real based on results from Experiment 2. Preventing the pH decline during sperm storage in SP, EYC+F and EYT+F by addition of NaOH inhibited AS formation. Conversely if sperm were stored in EYC or EYT without fructose and the pH was reduced by addition of HCl to levels normally associated with the presence of fructose, a high proportion of viable sperm developed AS. Thus neither fructose nor the lactic acid per se were directly related to AS formation. Storing sperm at low pH's in the range of 5.7 to 6.0 would seem to be the major cause of AS. Such an observation explains why glucose, also a metabolic substrate for sperm, was found to be as effective as fructose for inducing AS in a previous experiment (Series I, Experiment 2).

In both experiments 1 and 2 and the Series I experiments it was evident that occurrence of a high proportion of sperm with AS was not necessarily associated with reduced sperm viability. In fact, sperm with AS possess intact outer acrosomal and plasma membranes (Series I experiments). The

apparent lack of an effect of AS on sperm viability was further supported by the absence of eosinophilia in sperm with AS. Reduced sperm viability may be associated more with the environments that induce AS rather than AS itself.

Although AS does not appear to affect sperm viability, its physiological importance is not known at this time. It is possible that AS is a unique and/or preliminary form of sperm deterioration. Pursel et al. (1972a) described an acrosomal change on motile boar sperm that appears similar to AS. They felt this was an initial step in boar sperm degeneration. In fact strongly affected populations of boar sperm were not fertile when used for intracervical insemination (Pursel et al., 1972b).

The fusion between and vesiculation of the outer acrosomal and plasma membranes during the true acrosome reaction are acrosomal changes understood to occur on viable sperm during the fertilization process (Bedford, 1970; Austin, 1975). Since AS involves viable sperm without loss of viability, perhaps it is a prelude to the true reaction. Swollen anterior acrosomes have been observed on motile guinea pig (Yanagimachi and Usui, 1974) and hamster sperm (Yanagimachi and Noda, 1970; Franklin et al., 1970; Talbot and Franklin, 1976; Talbot, 1979) prior to what was thought to be the true reaction. Others have indicated that before the

true reaction, the acrosome may appear crenulated on guinea pig (Talbot et al., 1976) and hamster sperm (Talbot and Franklin, 1976 ). Based on light microscopy, the described crenulation appears to be a wrinkling or folding of the apical acrosome with some similarities to AS.

Regardless of the comparisons that can be made between AS and the morphological as well as physiological changes that occur to sperm in preparation for fertilization, the property described herein as AS has vague physiological implications. It has the potential of being an aspect of the fertilization process in the bovine or a unique form of sperm deterioration. Additional research is needed to explain the importance of AS to the function of spermatozoa.

## ADDENDUM TO DISCUSSION

pH's of 5.7-6.0, while conducive to AS formation on viable sperm in the Series II experiments, would seem unphysiological since there is no indication that sperm would normally be exposed to such pH's during transport through the female reproductive system. The pH of the secretions from various regions of the cow reproductive tract has been measured in vitro by several researchers. The clear strands of gelatinous cervical mucus that fill the cervix and the anterior vagina during estrus has been reported to have pH's

ranging from 7.4 to 8.9 (Lardy et al., 1941; Akhtar and Singh, 1979). Estimates of the pH of uterine fluid ranged from 6.8-7.1 (Lardy et al., 1940; Sergin et al., 1940; Olds and VanDemark, 1957). However, there is the potential that these in vitro measurements may be influenced by methods of collection and exposure of the fluids allowing loss of carbon dioxide which would increase pH. Mather (1975) measured the in vivo pH of the bovine uterine lumen throughout the estrous cycle. Average pH values were slightly higher but comparable to in vitro measurements and ranged from 7.22-7.38 with the lower pH's associated with maximum follicular development just prior to ovulation.

While pH's in the range of 5.7 to 6.0 are lower than a sperm would normally encounter in the female reproductive system there is some evidence that these pH's may have a positive influence on the fertilizing ability of bovine sperm. Brackett et al. (1980,1981) commented that holding neat bovine semen for 1-3 h at 37°C facilitates in vitro fertilization in their system. Based on data reported herein the pH of SP would have dropped rapidly during incubation and potentially been in the range of 5.7-6.0 for 0.5-2.5 h of the incubation period. Rajamannan et al. (1971) found that incubation of neat-semen for 20 min prior to processing and freezing (-196°C) for artificial insemina-

tion improved the conception rates of low fertility bulls. Perhaps pH's of 5.7-6.0 cause a physiological response through an unphysiological mechanism.

It is possible that media pH's in the range of 5.7-6.0 may induce acrosomal changes by altering the pH of acrosomal constituents. Meizel and Deamer (1978) used indirect methods to suggest that acrosomal pH's <5 occur in the hamster. A preliminary report by Working and Meizel (1981) indicated that increasing acrosomal pH of capacitated hamster sperm encourages the true acrosome reaction. In vitro capacitation was required for a response to occur. While they also felt that neither the outer acrosomal nor plasma membranes of noncapacitated sperm were permeable to $H^+$, the effects of pH's between 5.7-6.0 on this selective membrane permeability is not known. If an increase or change in acrosomal pH does take place, increased enzyme activity and/or zymogen activation could occur and manifest itself morphologically as AS with a potential relationship to the true reaction. Several enzymes or classes of enzymes have been associated with the acrosome (Zaneveld, 1975; Stambaugh, 1978). The proteolytic activity of extracts from ram sperm acrosomes was evident at pH 5.6 (Allison and Hartree, 1970). Neuraminidase like factors from the acrosome of bull sperm have a pH optima close to 6.0 (Srivastava et al.,

1970). Acrosin from bull sperm can be active at pH's of 5.0-6.0 (Garner et al., 1971). Polakoski and Zaneveld (1976) indicated that an arginyl hydrolase, that was active at pH 5.3, existed in crude acrosomal extracts. The pH optima of this enzyme appears closer to 6.0 (Polakoski, personal communication). Acrosin exists in the normal acrosome in a proacrosin form (see review by Parrish and Polakoski, 1979). The arginyl hydralase that is active at pH's close to 6.0 will activate proacrosin. An increase in acrosin activity has been associated with the occurrence of the true acrosome reaction of hamster (Meizel and Lui, 1976; Lui and Meizel, 1979) and guinea pig sperm (Green, 1978d). Acrosin activity was implicated in hamster sperm as a causal agent of the true reaction. The guinea pig research indicates that it is related not to membrane vesiculation but to dispersal of the acrosomal matrix.

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# APPENDIX

TABLE 9. Sperm preparation protocol for electron microscopy.

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1. Remove large particulate matter from egg yolk media by centrifugation (15,000 x g for 10 min). Pass all culture media through a 0.45 um pore size Millipore filter to remove small particulates.
2. After culture, concentrate sperm to $1.5 \times 10^9$ sperm/ml with centrifugation (1000 x g for 5 min. at room temperature). A total of $250 \times 10^6$ sperm is sufficient.
3. Suspend 1-2 drops of semen in 1 ml of fixative (Appendix, Table 10) in a 1.5 ml plastic Eppendorf centrifuge tube with rapid mixing.
4. After 30-45 min. form a sperm pellet by centrifugation (1700 x g for 5 min.).
5. Remove pellet with sharpened applicator stick. With careful handling throughout the procedure, the sperm pellet will remain intact.
6. Rinse the pellet 3 times, 10 min each, in pH 7.2 0.1M phosphate buffer. Buffer is composed of 23 mM $NaH_2PO_4$, 77 mM $Na_2HPO_4$, 0.005% (w/v) $CaCl_2$ and sucrose. Sucrose is added to make buffer isotonic with Karnovsky's fixative ($\sim$520 mOsm).
7. Post-fix in 0.5% (w/v) osmium tetroxide (Appendix Table 10) for 30 min.

TABLE 9. (Cont'd.)

| |
|---|
| 8. Wash 2 times (10 min each) in the same buffer without osmium (Appendix Table 10) and once in 0.1 M Na cacodylate (pH 6.0). |
| 9. Treat with 1% (w/v) tannic acid (Mallinckrodt) in 0.1 M Na cacodylate (pH 6.0) for 30 min. Prepare solution just prior to use. |
| 10. Wash in 0.1 M Na cacodylate (pH 6.0) containing 1% (w/v) Na sulfate (clearing agent) for 5 min. |
| 11. Dehydrate the pellet in a % (v/v) graded ethanol series; 30% for 5 min, 50% for 7 min, 80% for 10 min, 95% for 10 min, and 100% (2 times) for 10 min. |
| 12. Wash in 100% (v/v) propylene oxide (3 times) for 10 min. |
| 13. Immerse the pellets in equal volumes of propylene oxide and a 50:50 mixture of Epon and Araldite. Evaporate proplyene oxide at 60°C for 1 h. |
| 14. Imbed in the Epon Araldite mixture at 60°C for 3 days. |
| 15. Cut thin sections with a diamond knife on a Porter-Blum MT2-B ultramicrotome and mount on 300 mesh copper grids. |
| 16. Stain thin sections first with 3.0% (w/v) uranyl acetate and then with 0.3% lead citrate (Appendix Table 10). |

TABLE 9. (Cont'd.)

| |
|---|
| 17. Examine the thin sections with a Jeol 100-C transmission electron microscope. |

TABLE 10. Preparation of fixatives and lead citrate stain for electron microscopy.

---

Karnovsky's Fixative[a]

1. Combine 0.53 g paraformaldehyde with 53 ml of 60-70°C water.
2. Clear the solution with 1-2 drops of 1N NaOH.
3. Cool to room temperature and add 2 ml of 70% (w/v) glutaraldehyde.
4. Adjust to pH 7.2 with 0.1 N NaOH or 0.1 N HCl.
5. Add equal volume of 0.2 M phosphate buffer (46 mM $NaH_2PO_4 \cdot H_2O$ and 154 mM $Na_2HPO_4$).

Osmium tetroxide

1. Dissolve 0.5 g of osmium tetroxide in 25 ml of water overnight at 4°C.
2. Increase volume to 50 ml with addition of water.
3. Mix 1:1 with 0.2 M of phosphate buffer (46 mM $NaH_2PO_4 \cdot H_2O$ and 154 mM $Na_2HPO_4$).

Lead citrate[b]

1. Combine 0.3 ml of 10 N NaOH with 30 ml of distilled water.
2. Add 0.09 g lead citrate and mix until dissolved. Do not shake—this forms lead carbonate crystals.

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[a]Karnovsky (1965)

[b]Venable and Coggeshall (1965)

Table 11. Analysis of variance for the proprotion of motile sperm with AS (Series I-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square[a] |
|---|---|---|
| Replicate | 2 | 0.04659 |
| Treatment[b] | 1 | 11.81536** |
| Day[c] | 2 | 0.60988** |
| Diluent[d] | 1 | 1.59856** |
| Hour[e] | 2 | 0.79963** |
| Treatment X Day | 2 | 0.23844** |
| Treatment X Diluent | 1 | 0.35938** |
| Treatment X Hour | 2 | 0.35713** |
| Day X Diluent | 2 | 0.04659 |
| Day X Hour | 4 | 0.08148 |
| Diluent X Hour | 2 | 0.45137** |
| Treatment X Day X Diluent | 2 | 0.00744 |
| Treatment X Day X Hour | 3 | 0.03708 |
| Treatment X Diluent X Hour | 2 | 0.10721* |
| Diluent X Day X Hour | 4 | 0.00586 |
| Treatment X Diluent X Day X Hour | 1 | 0.00166 |
| Residual | 61 | 0.03395 |

[a]Based on partial sums of squares

[b]Storage media treatment.

[c]Day of storage.

[d]Incubation media.

[e]Incubation interval.

**$P < 0.01$

*$P < 0.05$

TABLE 12. Analysis of variance for the percent of motile sperm (Series I-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Replicate | 2 | 0.082784* |
| Treatment[a] | 1 | 15.12984** |
| Day[b] | 2 | 4.90135** |
| Diluent[c] | 1 | 2.18833** |
| Hour[d] | 2 | 0.21814** |
| Treatment X Day | 2 | 1.02971** |
| Treatment X Diluent | 1 | 0.02589 |
| Treatment X Hour | 2 | 0.18151** |
| Day X Diluent | 2 | 0.15603** |
| Day X Hour | 4 | 0.00911 |
| Diluent X Hour | 2 | 0.12248* |
| Treatment X Day X Diluent | 2 | 0.24789** |
| Treatment X Day X Hour | 4 | 0.03045 |
| Treatment X Diluent X Hour | 2 | 0.03721 |
| Diluent X Day X Hour | 4 | 0.03975 |
| Treatment X Diluent X Day X Hour | 4 | 0.11339** |
| Residual | 70 | 0.02537 |

[a]Storage media treatment.

[b]Day of storage.

[c]Incubation media.

[d]Length of incubation interval.

**$P < 0.01$

*$P < 0.05$

TABLE 13. Analysis of variance for the proportion of motile sperm with AS (Series I-Experiment 2).

| Source of Variation | Degrees of Freedom | Mean Square[a] |
|---|---|---|
| Replicate | 2 | 0.36655** |
| Medium | 1 | 0.00438 |
| Temperature | 2 | 2.32197** |
| Hour[b] | 7 | 1.07632** |
| Medium X Temperature | 2 | 0.41012** |
| Medium X Hour | 7 | 0.02779 |
| Temperature X Hour | 14 | 0.16448** |
| Medium X Temperature X Hour | 8 | 0.09285** |
| Residual | 78 | 0.03316 |

[a]Based on partial sums of squares.

[b]Hour of storage at 4°C.

**P < 0.01

TABLE 14. Analysis of variance for the percent motile sperm (Series I-Experiment 2).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Replicate | 2 | 1.57140** |
| Medium | 1 | 2.87264** |
| Temperature | 2 | 11.48848** |
| Hour[a] | 7 | 1.09647** |
| Medium X Temperature | 2 | 1.54485** |
| Medium X Hour | 7 | 0.21399** |
| Temperature X Hour | 14 | 0.50061** |
| Medium X Temperature X Hour | 14 | 0.20656** |
| Residual | 94 | 0.06107 |

[a]Hour of storage at 4°C.

**$P < 0.01$

TABLE 15. Analysis of variance for the proportion of motile sperm with AS (Series I-Experiment 3).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 1.08851** |
| Treatment[a] | 5 | 4.27768** |
| Medium[b] | 1 | 1.61795** |
| Hour[c] | 2 | 1.43331** |
| Bull X Treatment | 20 | 0.16002** |
| Bull X Medium | 4 | 0.04558 |
| Bull X Hour | 8 | 0.15693** |
| Treatment X Medium | 5 | 0.02903 |
| Treatment X Hour | 10 | 0.07834 |
| Medium X Hour | 2 | 0.01233 |
| Bull X Treatment X Medium | 20 | 0.05020 |
| Bull X Treatment X Hour | 40 | 0.06786 |
| Bull X Medium X Hour | 8 | 0.06199 |
| Treatment X Medium X Hour | 10 | 0.03654 |
| Residual | 40 | 0.03452 |

[a]Storage medium used at 4°C.

[b]Incubation medium used at 37°C.

[c]Hour of incubation at 37°C.

**P <0.01

TABLE 16. Analysis of variance for the percent motile sperm (Series I-Experiment 3).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.09231** |
| Treatment[a] | 5 | 0.07837* |
| Medium[b] | 1 | 0.59086** |
| Hour[c] | 2 | 0.09714* |
| Bull X Treatment | 20 | 0.02656** |
| Bull X Medium | 4 | 0.00590 |
| Bull X Hour | 8 | 0.01349* |
| Treatment X Medium | 5 | 0.00835 |
| Treatment X Hour | 10 | 0.01911** |
| Medium X Hour | 2 | 0.02616 |
| Bull X Treatment X Medium | 20 | 0.00665 |
| Bull X Treatment X Hour | 40 | 0.00579 |
| Bull X Medium X Hour | 8 | 0.00913 |
| Treatment X Medium X Hour | 10 | 0.00799 |
| Residual | 40 | 0.00552 |

[a]Storage medium used at 4°C.

[b]Incubation medium used at 37°C.

[c]Hour of incubation at 37°C.

*$P < 0.05$

**$P < 0.01$

TABLE 17. Fast Green-Eosin B live-dead stain.[a]

| | | mM/l |
|---|---|---|
| **Buffer Composition**[b] | | |
| Sodium Glutamate | ($C_5H_8NNaO_4 \cdot H_2O$) | 102.6 |
| Sodium Acetate | ($CH_3COONa \cdot 3H_2O$) | 62.6 |
| Potassium Citrate | ($K_3C_6H_5O_7 \cdot H_2O$) | 3.9 |
| Fructose | ($OCH_2(CNOH)_3COHCH_2OH$) | 55.5 |
| Magnesium Chloride | ($MgCl_2 \cdot 6H_2O$) | 3.3 |
| **Stain Composition**[c] | | |
| Buffer Solution | | 100 ml |
| Fast Green FCF[d] | (93% total dye content) | 2.0 gr |
| Eosin B[d] | (99% total dye content) | 0.8 gr |

[a]Developed by Dr. H. P. VanKrey, Virginia Polytechnic Institute and State University.

[b]Diluent A, Lake (1960)

[c]Adjust the pH of the stain solution to 7.3 with 10 and 1N NaOH.

[d]Mayer et al. (1951)

TABLE 18. Fast Green-Eosin B live-dead staining procedure.

1. Store stain at 3 to 5°C.
2. Warm stain to room temperature before use.
3. Place 10-15 ul of stain at end of clean slide.[a]
4. Add and mix 1 ul neat semen or 5 ul of extended semen ($50 \times 10^6$ sperm/ml) with the drop of stain.[a]
5. Smear the slide. A blood smear technique works best. The edge of smearing slide is drawn up to the drop so it spreads across the edge of the slide. The smearing slide is at a 45° angle to the bottom slide with the drop spread out inside the acute angle. The drop of stain and sperm is drawn out, not pushed, across the bottom slide. Pushing the drop will damage the sperm.
6. The slide is dried either on a 60°C hot plate with air from a fan moving over the side or in hot air from an Air Jet[b] (56°C). The air jet is the faster and preferred method. Use the maximum air flow and heat settings for the Air Jet.
7. Mount cover slips on the slides to prevent scratching and deterioration.

[a]Eppendorf pipettes.

[b]Oster Model 202 Series C Air Jet.

TABLE 19. Analysis of variance for the proportion of motile sperm with AS (Series II-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.24853** |
| Treatment[a] | 6 | 1.67504** |
| Bull X Treatment | 24 | 0.03658 |
| Residual | 35 | 0.02149 |

[a]Storage media treatments.

**P < 0.01

TABLE 20. Analysis of variance for storage media pH (Series II, Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.00336* |
| Treatment[a] | 6 | 0.54455** |
| Residual | 24 | 0.00102 |

[a]Storage media treatments.

*P < 0.05

**P < 0.01

TABLE 21. Analysis of variance for the percent motile sperm (Series II-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.04952** |
| Treatment[a] | 6 | 0.02791 |
| Bull X Treatment | 24 | 0.01153** |
| Residual | 35 | 0.00346 |

[a]Storage media treatments.

**P $<$ 0.01

TABLE 22. Analysis of variance for the percent unstained sperm (Series II-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.03147** |
| Treatment[a] | 6 | 0.33259** |
| Bull X Treatment | 24 | 0.01410** |
| Residual | 35 | 0.00407 |

[a]Storage media treatments.

**P < 0.01

TABLE 23. Analysis of variance for the percent half-stained sperm (Series II-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.14312** |
| Treatment[a] | 6 | 0.04736 |
| Bull X Treatment | 24 | 0.02136** |
| Residual | 35 | 0.00721 |

[a]Storage media treatments.

**P < 0.01

TABLE 24. Analysis of variance for the percent stained sperm (Series II-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.05348** |
| Treatment[a] | 6 | 0.36371** |
| Bull X Treatment | 24 | 0.01317** |
| Residual | 35 | 0.00440 |

[a]Storage media treatments.

**P < 0.01

TABLE 25. Analysis of variance for the percent unstained sperm with an intact acrosome (Series II-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.06137** |
| Treatment[a] | 6 | 0.03079** |
| Bull X Treatment | 24 | 0.00543 |
| Residual | 35 | 0.00301 |

[a]Storage media treatments.

**P < 0.01

TABLE 26. Analysis of variance for the percent half-stained sperm with an intact acrosome (Series II-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.49096** |
| Treatment[a] | 6 | 0.23636** |
| Bull X Treatment | 24 | 0.05269 |
| Residual | 35 | 0.09118 |

[a]Storage media treatments.

**$P < 0.01$

TABLE 27. Analysis of variance for the percent stained sperm with an intact acrosome (Series II-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.07399 |
| Treatment[a] | 6 | 0.02785 |
| Bull X Treatment | 24 | 0.07933 |
| Residual | 35 | 0.05477 |

[a]Storage media treatments.

TABLE 28. Analysis of variance for the percent live (half-stained plus unstained) sperm (Series II-Experiment 1).[a]

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.05347** |
| Treatment[a] | 6 | 0.36422** |
| Bull X Treatment | 24 | 0.01318** |
| Residual | 35 | 0.00444 |

[a]Storage media treatments.

**$P < 0.01$

TABLE 29. Analysis of variance for the percent sperm with an intact acrosome (Series II-Experiment 1).[a]

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.03016** |
| Treatment[b] | 6 | 0.25672** |
| Bull X Treatment | 24 | 0.01341** |
| Residual | 35 | 0.00188 |

[a]Values used are the sum of all sperm with an intact acrosome.

[b]Storage media treatments.

**$P < 0.01$

TABLE 30. Analysis of variance for the percent unstained sperm with AS (Series II—Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.33208** |
| Treatment[a] | 6 | 0.67598** |
| Bull X Treatment | 24 | 0.01625** |
| Residual | 35 | 0.00221 |

[a]Storage media treatments.

**$P < 0.01$

TABLE 31. Analysis of variance for the percent half-stained sperm with AS (Series II-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 1.40032** |
| Treatment[a] | 6 | 2.44381** |
| Bull X Treatment | 24 | 0.15121 |
| Residual | 35 | 0.12919 |

[a]Storage media treatment.

**P < 0.01

TABLE 32. Analysis of variance for the percent stained sperm with AS (Series II, Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.04952 |
| Treatment[a] | 6 | 0.02449 |
| Bull X Treatment | 24 | 0.02436 |
| Residual | 35 | 0.02199 |

[a]Storage media treatments.

**$P < 0.01$

TABLE 33. Analysis of variance for the percent live (half-stained plus unstained) sperm with AS (Series II-Experiment 1).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 1.64032** |
| Treatment[a] | 6 | 4.10080** |
| Bull X Treatment | 24 | 0.06715** |
| Residual | 35 | 0.01196 |

[a]Storage media treatments.

**$P < 0.01$

TABLE 34. Analysis of variance for the proportion of motile sperm with AS (Series II—Experiment 2).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.51614** |
| Medium | 4 | 0.90052** |
| pH | 1 | 26.39127** |
| Hour[a] | 1 | 1.08342 |
| Bull X Medium | 16 | 0.07556 |
| Bull X pH | 4 | 0.14612* |
| Bull X Hour | 4 | 0.24362** |
| Medium X pH | 4 | 0.58207** |
| Medium X Hour | 4 | 0.41404* |
| pH X Hour | 1 | 0.49321 |
| Bull X Medium X pH | 16 | 0.09932* |
| Bull X Medium X Hour | 16 | 0.10013** |
| Bull X pH X Hour | 4 | 0.08805 |
| Medium X pH X Hour | 4 | 0.03223 |
| Bull X Medium X pH X Hour | 16 | 0.04337 |
| Duplicate[b] (Bull X Medium X pH) | 50 | 0.05012 |
| Residual | 50 | 0.03554 |

[a]Hour of incubation at 37°C.

[b]Duplicate evaluation of each storage media X pH combination.

*P $<$ 0.05

**P $<$ 0.01

TABLE 35. Analysis of variance for the percent motile sperm (Series II—Experiment 2).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.13384** |
| Medium | 4 | 0.91101** |
| pH | 1 | 1.22404** |
| Hour[a] | 1 | 1.01659** |
| Bull X Medium | 16 | 0.05159** |
| Bull X pH | 4 | 0.01645* |
| Bull X Hour | 4 | 0.01071 |
| Medium X pH | 4 | 0.24555** |
| Medium X Hour | 4 | 0.05968** |
| pH X Hour | 1 | 0.45468** |
| Bull X Medium X pH | 16 | 0.01291** |
| Bull X Medium X Hour | 16 | 0.00913 |
| Bull X pH X Hour | 4 | 0.00685 |
| Medium X pH X Hour | 4 | 0.10981** |
| Bull X Medium X pH X Hour | 16 | 0.00550 |
| Duplicate[b] (Bull X Medium X pH) | 50 | 0.00463 |
| Residual | 50 | 0.00606 |

[a]Hour of incubation at 37°C.

[b]Duplicate evaluation of each storage medium X pH combination.

*$P < 0.05$

**$P < 0.01$

TABLE 36. Analysis of variance for the percent live (half-stained plus unstained) sperm with AS (Series II-Experiment 2).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.97328** |
| Medium | 4 | 1.03448** |
| pH | 1 | 60.13921** |
| Hour[a] | 1 | 10.26109** |
| Bull X Medium | 16 | 0.04502** |
| Bull X pH | 4 | 0.17997** |
| Bull X Hour | 4 | 0.11819** |
| Medium X pH | 4 | 0.46066** |
| Medium X Hour | 4 | 0.09284 |
| pH X Hour | 1 | 10.55648** |
| Bull X Medium X pH | 16 | 0.08000** |
| Bull X Medium X Hour | 16 | 0.05338** |
| Bull X pH X Hour | 4 | 0.30172** |
| Medium X pH X Hour | 4 | 0.23342** |
| Bull X Medium X pH X Hour | 16 | 0.1446 |
| Duplicate[b] (Bull X Medium X pH) | 50 | 0.00885 |
| Residual | 50 | 0.00911 |

[a]Hour of incubation at 37°C.

[b]Duplicate evaluation of each storage medium X pH combination.

**$P < 0.01$

TABLE 37. Analysis of variance for the percent live (half-stained plus unstained) sperm (Series II-Experiment 2).

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Bull | 4 | 0.07768** |
| Medium | 4 | 5.18381** |
| pH | 1 | 0.25245* |
| Hour[a] | 1 | 1.75237** |
| Bull X Medium | 16 | 0.03140** |
| Bull X pH | 4 | 0.02654** |
| Bull X Hour | 4 | 0.01734* |
| Medium X pH | 4 | 0.28787** |
| Medium X Hour | 4 | 0.01823 |
| pH X Hour | 1 | 0.00006 |
| Bull X Medium X pH | 16 | 0.01049* |
| Bull X Medium X Hour | 16 | 0.01037 |
| Bull X pH X Hour | 4 | 0.00523 |
| Medium X pH X Hour | 4 | 0.01981** |
| Bull X Medium X pH X Hour | 16 | 0.00343 |
| Duplicate[b] (Bull X Medium X pH) | 50 | 0.00542 |
| Residual | 50 | 0.00649 |

[a]Hour of incubation at 37°C.

[b]Duplicate evaluation of each storage medium x pH combination.

*$P < 0.05$

**$P < 0.01$

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# FACTORS CAUSING ANTERIOR ACROSOMAL SWELLING ON MOTILE BOVINE SPERM

by

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(ABSTRACT)

Swelling of the apical ridge and anterior acrosome of motile bovine sperm was observed using differential interference contrast optics. Transmission electron microscopy indicated that the acrosomal matrix was extended into complex folds and/or projections. Outer acrosomal and plasma membrane integrity was retained. Anterior acrosomal swelling (AS) was first observed after neat-semen had been slowly cooled to 4°C and stored for 1 day. With subsequent dilution (25 X $10^6$ sperm/ml) and incubation of sperm in a Tris-fructose medium at 37°C, a majority of the motile sperm had AS. Of these unique conditions, storing sperm (1500 X $10^6$/ml) in seminal plasma (SP) at 4°C was very conducive to AS. Replacing SP with egg yolk-citrate (EYC) inhibited AS. Using a Tris buffer with 54 mM fructose as an incubation medium eliminated the necessity of storing sperm in SP provided storage in EYC at 4°C was ≥3 days. AS occurred after storage at 37 or 4°C but not at 21°C. Storing cauda epididymal sperm at 4°C with and without 30.8 mM fructose or glu-

cose in EYC or egg yolk-Tris (EYT) media demonstrated that AS formation required the presence of either sugar. Storing cauda sperm in EYT at 4°C with 0, 3.9, 7.7, 15.4, 30.8, and 61.7 mM fructose indicated that a minimum of 7.7 mM fructose was necessary for a strong AS response. Storage media pH was measured at the end of storage. Media pH was 6.7 with 0 mM fructose and had decreased during storage to 5.7-6.0 in media giving a strong AS response. Further experimentation with cauda sperm demonstrated that storage at reduced media pH would induce AS with or without the presence of fructose. Conversely, storage at normal pH's (6.6-6.8) inhibited AS even with fructose in the media.

The proportion of motile sperm with AS was estimated from wet smears initially. Evaluation of acrosomal morphology from dry smears of vitally stained sperm was developed as a means of quantifying the proportion of viable sperm with AS. This technique also allowed closer scrutiny of the acrosomal morphology of viable sperm.

The physiological importance of AS is unclear. It may be a unique form of sperm deterioration or a prelude to the true acrosome reaction since AS occurs without loss of sperm viability.