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Title: CRYOGENIC PRESERVATION OF RAM SPERMATOZOA

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Investigations with ram semen were conducted to develop procedures of cyrogenic preservation that would provide long-term retention of fertilizing capacity in ram spermatozoa.

An isotonic extender consisting of egg yolk, sodium citrate, lactose, glycerol, and a broad spectrum antibiotic was selected from among several extenders tested in pilot studies. Using this extender, ram semen collected by artificial vagina was frozen in glass ampules at controlled rates in a Biological Freezing unit, in glass capillary tubes on dry ice, in plastic straws on dry ice and in pellet form on dry ice.

Factorial experiments were conducted to compare: (1) freezing containers, (2) freezing in ampules in a biological freezing unit at controlled rates with the freezing of pellets on dry ice, (3) the effect of enzymes added to the extender, (4) the effect of holding sperm in seminal plasma prior to extension, (5) the influence of

glycerol on acrosomal mophology of frozen semen, and (6) ram effects and interactions.

The viability of spermatozoa held in liquid nitrogen storage 18 to 21.8 months was examined. Fertilizing capacity of frozen semen was tested with ewes in natural estrus and with ewes in estrus induced by hormonal treatment.

Freezing in glass ampules at an optimum controlled rate and freezing in plastic straws on dry ice gave significantly higher post-freeze motility and percent survival than other methods used. Semen frozen in ampules at the controlled rate and thawed in ice water showed no apparent loss of viability compared to that thawed at 40°C. Semen frozen in plastic straws on dry ice and thawed at 75°C for 12 seconds gave viability equal to that frozen in glass ampules at the optimum controlled rate of freezing.

Alpha amylase, beta amylase, and beta glucuronidase had no significant effect upon postfreeze viability when added to the extender at the rate of 10 microgram per ml.

Holding ram spermatozoa in seminal plasma for four hours prior to extension caused no significant change in postfreeze motility or percent survival.

The addition of glycerol to the freezing medium caused a significant increase in acrosomal damage. The addition of glycerol was also found necessary to obtain postfreeze motility.

The insemination of a limited number of ewes with semen frozen and stored at -196°C for 30 days to 120 days failed to produce observed pregnancies. The probable causes of this are discussed.

The techniques developed in this study allowed ram spermatozoa to be frozen and to retain motility and percent survival in storage at -196°C for periods up to 21.8 months equal to that observed at one week of frozen storage.

Cryogenic Preservation of Ram Spermatozoa

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INTRODUCTION

The artificial insemination of farm animals has been practiced for some time, however, the successful use of frozen sperm is a comparatively new development made possible in 1949 when Polge, Smith and Parkes of England discovered that fowl spermatozoa could be treated with glycerol to provide resistance to freezing damage. Their technique was successful with bull semen and gave satisfactory conception rates. This work not only enhanced the use of artificial insemination in dairy cattle, but also provided the opportunity for storage of other living tissue. The use of glycerol as a life protector has been extended to the storage of blood cells, endocrine tissue, human cornea, bone marrow, and other tissues for surgical use. The long distance shipping of frozen bull semen, within and between countries, has become common practice and frozen semen is used for nearly all cattle artificially inseminated throughout the world.

The sperm of other domestic animals does not retain fertilizing capacity, after freezing and thawing, as well as does that of the bull. Limited success has been obtained to date with the semen of the ram. The reasons for this are not completely understood and work must therefore continue due to the need for acceleration of animal production occasioned by the impending world food crisis. Animal protein

is needed to help solve the problems of human malnutrition. Tremendous gains in animal protein values are obtainable by selective breeding of livestock. Throughout the world, there are vast acreages of arid lands producing forage which is of value to man only through the conversion of vegetable fiber to animal protein by ruminants.

Sheep are particularly adept at utilizing the forage on marginal lands. They convert browse and grass into meat and wool. The sheep population of the U. S. is currently at its lowest point since before the Civil War. Wentworth (1948) shows the 1850 sheep population of the U. S. at slightly under 22 million. The U. S. D. A. Economic Research Service (1972) shows the inventory of sheep and lambs on farms and ranches, January 1, 1972, at 18. 5 million. During this span of time the human population of the U. S. has increased from slightly over 23 million to 206. 5 million (World Almanac and Book of Facts, 1972). This changing ratio indicates a ready market for sheep products; however, it also indicates that the existence of the U. S. sheep industry is threatened. While U. S. numbers have declined, world sheep numbers have been increasing for a number of years. Improved management of U. S. flocks is needed for increased profits and a resurgence of the industry. Artificial insemination could play an important role in this improvement, but before artificial insemination can be practicable with sheep the problem of preserving the fertilizing capacity of frozen ram semen must be solved.

The ewe is seasonally polyestrus and therefore customarily lambs but once per year. Shortening the time interval between lambings holds promise as a method of increasing production and allowing more profits. This may be accomplished by genetic selection towards the ability to breed the year-around or by hormone treatment to induce ovulation during the non-breeding season. In either of these methods, artificial insemination could make worthwhile contributions.

The gene pool for year-around breeding exists within the 450 plus breeds of sheep found throughout the world (Ryder and Stephenson, 1968). The Merino, Dorset Horn, Karakul, Tunis, Blackheaded Persian, Ille-de-France, Hangyang of China and Bikaner of India have a tendency to breed the year-around. The Finnish Landrace (Finnsheep) is extraordinarily prolific and also possesses the tendency to conceive independent of season. Due to quarantine restrictions, it is difficult to import foreign breeds of sheep in sizeable numbers. One worthwhile contribution that artificial insemination might make to the U.S. sheep industry would be the long distance shipping of frozen semen from foreign born rams. Terrill (1970) stated that the use of exotic breeds offers hopes of rapid and dramatic gains in lambs per ewe. Ideal control of reproductive capacity in sheep will occur when we obtain through selection or crossbreeding the genetic ability of ewes to cycle throughout the year. Artificial insemination could assist the industry in reaching this goal by facilitating the crossing of desired

breeds and by increasing the progress to be made by selection.

The treatment of ewes with hormones to synchronize estrus, during the natural breeding season, has been successfully accomplished so that ewes lamb at approximate 17 day intervals. In large flocks this results in large numbers of ewes coming into estrus at the same time with a corresponding heavy requirement for sudden natural matings. This requirement can only be met by using a very large number of rams or by artificial insemination. Ram fertility and semen quality have been found to decline quite rapidly when there is a greatly increased number of natural matings over a limited period of time (Hulet, 1968a). Due to these factors, it appears that controlled estrus and artificial insemination could go together to make a contribution. The need for dependable artificial insemination will increase when satisfactory hormonal procedures are developed to obtain fertile breeding from synchronized estrus out of season.

The most important advantage of artificial insemination could be the greatly extended use of superior rams - possibly years after their death. If dependable frozen ram semen was available, sheepmen would be motivated to keep more and better production records - to locate the truly outstanding rams. Progeny tests would then become more precise and progress toward desired breeding goals would be rapid.

Before artificial insemination of sheep is practicable the techniques for long term storage of semen in the frozen state with the retention of fertilizing capacity after thawing must be worked out. The investigations contained herein were initiated to study the methods used by others and to develop procedures that will allow ram semen to be frozen and thawed with satisfactory retention of fertilizing capacity.

LITERATURE REVIEW

This review includes appropriate literature concerning the factors that affect viability of ram spermatozoa from the moment of semen collection until the time of thawing for insemination or evaluation.

The Collection of Semen From Rams

Four methods have been used to collect semen from rams. These are: (1) allow the ram to serve a restrained ewe, then collect the semen from the vagina, (2) by hand-held artificial vagina using a restrained ewe or another ram as the mount animal, (3) by placing an artificial vagina in a dummy ewe, (4) by electrical stimulation of the reproductive system with a device known as an electro-ejaculator. This device makes use of a bipolar or multipolar electrode that is inserted into the animal's rectum.

The first named method is the simplest and requires the least equipment. It is also fast and usually less difficult than methods requiring the artificial vagina or electro-ejaculator. Terrill (1968) states that collection by this method should be done using a ewe out of heat so that the vagina will be free from mucus. The ewe must be firmly secured in a stanchion and fluid in the vagina should be removed before service. The semen is removed immediately after

each service with a pipette. Use of a plastic disposable pipette is advisable to eliminate the danger of breakage while in the ewes reproductive tract. Care should be taken to remove all semen and to prevent contamination with urine or foreign material. Although repeated collections can be made from a single ewe, it is advisable to use a different ewe for each ram in order to prevent the mixing of semen and uncertain determinations of volume, motility and parenthood. Rams can be induced to serve the anestrous ewe if they are first allowed to serve a ewe in heat in the same stanchion that is to be used later. The advantages of this method are its simplicity, speed, and low requirement for specified equipment. The disadvantages are: (1) the ejaculation always contains some amount of fluid from the ewes reproductive tract and it is impossible to withdraw all of the semen, (2) a different mount animal must be used for each ram to avoid the mixing of semen. Further information concerning this method and a description of some of the early work accomplished with it may be found in Terrill (1937) and Terrill and Gildow (1937).

The ovine artificial vagina consists of a heavy rubber outer casing and a thin rubber inner liner (Figure 3). A thin rubber funnel is attached to one end of the A. V. and the other end of this funnel holds the collection tube. This tube may be graduated to assist in volume measurement. An adjustable valve in the outer casing allows the space between the casing and liner to be filled with warm water.

It also allows adjustment of air pressure to facilitate ejaculation. The inner liner and the rubber funnel are held in place with heavy rubber bands. Such an artificial vagina is described by Terrill (1968). It may be hand held beside the ewe or placed inside a dummy.

Most rams can be easily trained for collection with the artificial vagina by use of a teaser ewe (Emmens and Robinson, 1962). A ewe in natural estrus secured in a stanchion at waist level may be used to train rams. After serving the ewe in estrus several times, the rams will be sufficiently accustomed to the routine to allow substitution of the hand-held artificial vagina. When a ewe in natural estrus is not available, one may be prepared by ovarectomy and treatment with estrogen subcutaneously implanted as described by Cole and Cupps (1969). The casing of the artificial vagina is filled with water and air. Temperature and pressure are important but can be readily determined for each ram by an experienced operator. The ram may refuse to ejaculate if the temperature is too hot or too cold and rams may differ considerably in their requirements for pressure. Animal breeding authorities seem to vary in their recommendations. Rodin (1940) recommends a temperature of 41^{o} - 42^{o} C and a pressure of 40 to 60 mm of Hg. Terrill (1968) states that the temperature of the inner liner at the time of service should be between 41^{o} and 44^{o} C. He does not mention the desired air pressure other than to say that the air content may be adjusted to obtain "moderate" pressure. The

literature seems to agree that rams with a good sex drive can be readily trained to the use of the hand-held artificial vagina when a ewe in estrus is used as the training mount animal. Emmens and Robinson (1962) record an instance where 54 of 57 Dorset Horn, Romney and Australian Merino rams, which had never been handled for intensive husbandry, were trained to serve the artificial vagina within two days. Foote and Trimberger (1968) state that "the experienced operator caters to the likes and dislikes of each individual male during semen collection." (p. 140)

Though a dummy can be constructed easily from wood and covered with a sheep's fleece - rams are reported to vary in their willingness to serve an artificial vagina contained within a dummy ewe. Dummy ewes are illustrated by Terrill (1968) and Foote and Trimberger (1968). Terrill states that the artificial vagina can be used with a dummy ewe, or it may be held in the hand beside a live ewe. The latter author states that live mounts have proven to be the most successful for routine semen collections. Wolberg (1967) also states that it is very difficult to train rams to mount anything other than another live sheep. This review of literature did not disclose a single instance where ram semen was collected for an extensive study, or for insemination, by the use of the dummy.

Semen collection by electrical stimulation (also known as electro-ejaculation) was first accomplished by Gunn (1936). Gunn's

original electrical ejaculation equipment made use of two separate electrodes. One was inserted into the rectum and the other was a needle pole that was inserted at about the middle of the lumbar vertebra. He used a 50 cycle alternating current at 30 volts and tried placing the electrodes in various places on the animal's body but only received ejaculation when the nerves controlling the act were electrically stimulated. He concluded that ejaculation could not be brought about by direct muscle contraction. A modification using a single rectal bipolar electrode was developed by Laploud and Cassou (1945). The use of the single bipolar electrode with gradual raising and lowering of the current seems to work very well and reduces general body reaction.

A number of workers have adapted and improved the electrical equipment used by Laploud and Cassou for use in collecting semen from rams and goats (Blackshaw, 1954; Dziuk et al., 1954; Barker, 1958; and O'Brien, 1966). They have contributed toward the development of a pistol shaped multipolar electro-ejaculator that is compact and easy to use. It is capable of giving a series of optimum electrical stimuli for ovine ejaculation when inserted via the rectum.

Inskeep and Cooke (1968) give a good review of the comparative advantages and disadvantages of electro-ejaculators as compared to the artificial vagina. The electro-ejaculator may be used on rams with no training whatsoever or physically-disabled rams. However,

they point out that some rams do not respond well to the electrical stimulus and that this is especially true when a second or third collection is needed. There is also the ever present danger of injuring the ram, unless experienced operators are doing the collection with the electro-ejaculator. These writers state that the degree of stimulation required to obtain ejaculation and the volume of the ejaculate vary considerably from day to day and there is considerable chance of contamination of the semen sample with urine. These variations and the problem of urine contamination are serious disadvantages if the semen samples are to be used for research or for insemination.

Several workers have obtained a greater volume of semen, but with lower concentration of sperm, by electrical-ejaculation than with the artificial vagina (Emmens and Robinson, 1962; Mattner and Voglmayr, 1962; Salamon and Morrant, 1963). Quinn and White (1966) found greater concentrations of sodium and potassium in both the sperm and seminal plasma obtained by electro-ejaculation. Quinn et al. (1968) found that sperm in semen obtained by electro-ejaculation had less resistance to cold shock and deep freezing than did sperm collected with the artificial vagina.

The limited number of comparisons of fertility between semen samples collected by artificial vagina and electro-ejaculation favor the artificial vagina. Salamon and Morrant (1963) found that the conception rate at first service was 16.3 percent higher when the semen

was collected by artificial vagina. This work was based on approximately 200 ewes inseminated with each kind of semen. They considered that most of this advantage was due to the fact that sperm concentration of the semen collected with artificial vagina was twice as high as that collected with electro-ejaculation.

Inskeep and Cooke (1968) concluded that "on the basis of the work reported to date one must conclude that the artificial vagina is the preferred method for collection of ram semen for artificial insemination" (p. 164). These authors also state that this will be especially true if dilution and storage, or deep freezing, are to be among the procedures being studied.

In summary of the above review it appears that the artificial vagina has the distinct advantage of furnishing a pure unadulaterated ejaculate. This would appear to facilitate objective measurements of semen characteristics. It also allows for positive identification of the entire ejaculate and makes semen research with the split ejaculate possible. The disadvantages of the artificial vagina method are: (1) it requires the additional investment of the equipment and additional labor of cleaning the equipment after each use, (2) the rams must be conditioned and trained to the use of the equipment. These disadvantages appear minor in comparison with the advantages, if the semen is to be used for research work.

Factors That Influence Viability of Ram Spermatozoa

Two types of factors should be considered: (1) those that are believed to affect the production of viable spermatozoa prior to collection from the ram, and (2) those factors that are believed to have an influence on viability *in vitro* after collection.

The complexity of the so-called "seasonal" effects on spermatogenesis in the ram are interesting. Comstock *et al.* (1943) found marked seasonal trends in spermatozoal concentration, percentage of abnormals, and glycolysis in a study of Hampshire and Shropshire rams in Minnesota. Sperm density, glycolysis, and percent normal sperm were all at minima during July through September. Shearing the rams had a beneficial effect on sperm quality. In contrast to this, Bell (1945) working in New Mexico found only slight seasonal variations in Rambouillet rams, and he found sexual desire and semen volume to be poor in the spring (March, April) and to improve in the summer.

There are several reports from the Middle East and Far East on indigenous breeds. Shukla and Bhattacharya (1953) studied Uttar Pradesh type rams in India for two consecutive years and found "reaction time," i. e., time from release to ejaculation, differed significantly between rams but there was no seasonal trend and no relationship to semen quality. There was a highly significant seasonal

variation in semen volume, pH, concentration, motility and total number of spermatozoa. There were also significant differences between month within season variations. Semen quality was said to be poorest in autumn and best in spring. Volcani (1953) made histological examination of the testes of Awassi sheep in Israel. He found active spermatogenesis during July and August - when days are long and hot - and from September to December degenerative changes of testes were apparent. He concluded that spermatogenesis was not controlled by environmental temperature but by the state of the pastures.

Emmens and Robinson (1962) stated that the main effects upon semen production in the ram include temperature, photoperiodic environment, breed, nutrition, altitude, management, and disease. They believed that interactions among these are likely. The important findings on each of these factors will be briefly reviewed.

The scrotum is a thermo-regulatory organ and one of its chief functions is to maintain the testicles at an optimum temperature for spermatogenesis. McKenzie and Phillips (1934) demonstrated that insulation applied to the scrotum of the ram in sufficient amount to raise the temperature of the testicles 2 to 2.5^{o} C above optimum (35^{o} C) would cause an increase in abnormal sperm and if continued would result in testicular damage. Buchman and Andreevskii (1940) and Gunn, Sanders, and Granger (1942) showed that prolonged daily

temperatures in excess of 90°F (32°C) with little diurnal variation, would cause seminal degeneration. Rathore (1970) made a study of acrosomal abnormality in ram spermotozoa due to heat stress. He subjected mature Merino rams to 40.5°C temperature in a hot room for 1-1/2, 2, 2-1/2, 4 or 5 days, (eight hours a day). An acrosomal anomaly characterized by (1/4-1/2) detachment of the acrosomal cap from the sperm head was seen only in the 4 and 5 day groups. Sperm with this abnormality were first seen on days 9-10 after heating, and reached a peak of 18.5 to 25 percent between days 14-18. After withdrawal of the thermo-treatment the abnormality declined and semen returned to normal by day 33-34. The author suggested that the abnormality occurring at 9-10 days occurred in the epididymis during maturation and that the continuation of the abnormality for 30-31 days indicated that some changes may also have occurred at an earlier stage.

Ortavant (1958) made a significant contribution to the understanding of the interaction between temperature and photoperiodism as it affects spermatogenesis. By injecting rams with (P^{32}) phosphate, he found that about 30 days elapsed before P^{32} labelled spermatozoa appeared in the head of the epididymis and an additional 20 days before they emerged from this organ. His tracer studies show that the rate of division of primary spermatocytes and time of subsequent maturation are relatively unaffected by the photoperiod. However,

the numbers of spermatids which survive the complete maturation process are affected. Under conditions of increasing daily illumination the failure rate is high. This would appear to be an important finding since the conditions of increasing daily illumination parallel the rise in temperatures in the spring and early summer months. In reviewing Ortavant's P^{32} tracer studies, Emmens and Robinson (1962) proposed that the effect of high temperatures in the spring and early summer months superimposed upon the basic photoperiodic effect is to destroy a high proportion of the relatively few spermatozoa which have survived the maturation process. Rathore's work would appear to support this and may be important in light of the fact that acrosomal abnormalities have long been associated with sterility in bulls (Blom, 1950 and Van Rensberg, 1956). Scrivastava, Adams and Hartree (1965) have reported that the acrosomal substance contains enzymes probably used in sperm penetration of the zona pellucida.

Concerning the influence of breed upon semen characteristics, Amir, Volcani and Genizi (1965) conducted studies in the Middle East to determine if there were seasonal changes in the sexual activity of Awassi, German Merino, Corriedale, Border-Leicester, and Dorset-Horn rams. They reported that in the breeds with less distinct breeding season (Merino, Corriedale, and Dorset Horn) the annual fluctuation in semen volume and sperm numbers was barely existent,

while in the Awassi and Border Leicester (considered by the authors to have more distinct breeding season) maximum levels of semen volume was reached during the autumn and minimum levels during the spring. McKenzie and Berliner (1937) revealed that Shropshire rams had their strongest sex drive from October to January and Hampshires had their strongest drive from August to January - though each of these breeds manifested mating desire throughout the year. El-Mikkawi et al. (1967) conducted investigations of the semen characteristics in three breeds of rams. The breeds compared were two Middle East breeds (Awassi and Ossimi) and the Merino. They found no significant differences between breeds in serving ability, semen quality per ejaculate, sperm concentration, initial motility, sperm number per ejaculate or sperm abnormalities. Emmens and Robinson (1962) state that

> ...the general picture emerges that for British breeds of sheep, maximum spermatozoa production and quality and also libido, are highest in the autumn and early winter months, and lowest in summer (p. 215).

They point out that the situation is less clear with sheep of Eastern origin in which the seasons may be reversed, and even two seasons of maximum spermatozoa production may be exhibited. These are probably true breed differences resulting from natural or assisted selection for breeding at a time for maximum chance of lamb survival. Breed differences and interactions of breed differences with

climate variables certainly exist but they need further clarification.

There can be no doubt but that nutrition exerts an influence on the quantity and quality of semen produced by the ram. Malnutrition has been known, for some time, to have a deleterious effect upon the male reproductive system. Jackson (1925) was one of the early writers who reported on this subject. His review showed that severe malnutrition might cause a complete suppression of spermatogenesis in the mammal, but that the spermatogonia would survive to renew the process when adequate nutrition was reestablished. Comstock et al. (1943) showed that high condition caused a significantly greater decrease in glycolysis and sperm density from May to September than low condition. McKenzie and Phillips (1934) found over-feeding to be deleterious to fertility in the ram. Webster (1952) showed that show-fitted animals had poor breeding records. In the area between severe malnutrition and over-fat, difficulty often arises in evaluating the literature concerning the effects upon ram fertility, because of a lack of objectivity in the reporting, i. e. we do not know what is meant by "high" and "low" planes of nutrition, or by "high" condition or "low" condition.

Concerning specific nutrients, vitamin A deficiency has been found to hurt spermatozoal production and semen quality in rams (Lindley et al., 1949; Gunn et al., 1942). Sapsford (1951) found that the deleterious effect of hot weather upon ram semen viability

characteristics could be reduced by the addition of carotene to a ration that was adequate in energy and protein. Addition of carotene to a low level diet was ineffective. This finding is interesting in view of the findings of Rehm and Kupferschmied (1966) who reported that a single injection of concentrated water-miscible vitamin ADE preparation in 25 bulls whose diet already contained sufficient vitamins, gave a significant improvement of semen density, volume and sperm motility. The cows mated to these bulls also had fewer still births than cows mated to the controls.

Altitude is known to influence fertility in the male. DeAlba (1964) tells of work by investigators in Peru that clearly showed low atmospheric pressure to cause disturbances in spermatogenesis of males of many domesticated species when transported from sea level to the high altitude of mountainous areas. The damage occurred prior to their acclimatization. DeAlba states that damage to the ovaries of females from similar treatment has not been encountered. The experience of Easley (1951) supports this. In an artificial insemination laboratory established at 12,828 feet above sea level in Peru, he obtained very poor fertility from imported Corriedale, Merino, and Karakul rams brought from a much lower altitude. He believed his failure to be due to a combination of high altitude and poor nutrition.

The literature does not yield a great deal of information on specific factors of management of rams to be used for A. I. Terrill

(1968) suggested that rams do better in small bunches and should have access to salt and water plus shade and cool quarters. He recommends an ample diet of green pasture or alfalfa hay plus some grain and that the rams be kept thrifty but not overfat. Terrill recommends that those rams that have been fitted for sale or for show should be shorn and turned out to pasture for at least several weeks before use. Webster (1952) mentioned that in New Zealand a cold southerly wind of one night rendered 18 rams infertile and this condition persisted for the remainder of the breeding season. Dun (1956) observed that flooding conditions apparently contributed to temporary infertility of rams in New South Wales, Australia. He reported seminal degeneration associated with flabbiness of the testes in 13 of 29 rams that had been exposed to flood conditions accompanied by warm humid weather and excessive mosquitoes. Before leaving the subject of management, it seems appropriate to consider the factors listed by Scott (1970) that he considers make the commerical use of A. I. impractical in the United States. These factors are: (1) Labor costs are quite high. Suitable methods for long term storage of ram semen has not been developed and the most satisfactory results have been obtained with freshly collected semen. This requires additional time for collection and dilution at time of insemination. (2) The identification of greatly superior sires has not been accomplished. A small percentage of rams in the United States have production records or progeny test

records. In ram tests accomplished to date, no individual rams have demonstrated a large degree of productive excellence. A. I. cannot be justified unless a few outstanding individuals can be identified and used on sufficient numbers of ewes to show genetic progress in a portion of the sheep population. (3) The productive rams should be progeny tested for presence of inherited defects so that these defects would not be disseminated in a large population. Also testing of a ram would reduce the number of years he could be used in an A. I. program. (4) Conception rates from a single insemination have not been high enough to produce an adequate lamb crop. A second and possibly third insemination, or use of natural service would be required.

These factors not only suggest the need for a suitable method of long term storage of ram semen, but also emphasize the necessity for better ram management as it applies to recording progeny performance and production.

Marsh (1965) has reviewed disease problems that influence fertility and viable sperm production. Any infection or febrille condition such as blowfly strike, foot-rot or an abscess will result in seminal degeneration. Arsenical dips have had the same effect. The incidence of epididymitis, orchitis, scrotal tumors, posthitis, ulcerative dermatosis, and other diseases of the ram reproductive tract have been reported upon by Marsh. Of these, epididymitis is generally

considered the most damaging to fertility. Gunn et al. (1942) found 5.5% of 9000 rams in Australia to have chronic epididymitis.

Simmons (1968) reviewed diseases affecting the reproductive capacity of the ram. He gave particular attention to epididymitis and orchitis, posthitis, and ulcerative dermotosis and mentioned that in many cases epididymitis and orchitis were found together. His review listed the following organisms as those that have been isolated from such cases: Brucella ovis, (also known as REO, or ram epididymitis organism), Pasturella pseudotuberculosis, Escherichia coli, Neisseria, Hemophilus, Corynebacterium pyogenes, Corynebacterium pseudotuberculosis, and Actinobacillus seminis. The review by Simmons shows clearly that unilateral or bilateral epididymitis or orchitis often results in semen of inferior fertility. His review also shows that present vaccines and antibiotics are not effective in controlling epididymitis as caused by all known strains of organisms and that additional research is needed on better methods of detecting which organisms are present, and in developing an efficient vaccine that will give two to three years immunity to a ram.

Certain factors affect the viability of spermatozoa after collection from the healthy and fertile ram. These include, first, the handling techniques necessary to maintain viability from the moment of collection to the time of extension. Almquist (1968) stated that:

> After collecting a good semen sample, everything possible must be done to maintain it's initial quality until the sperm are inseminated. The sample must be carefully protected against cold, light, air (oxygen), agitation, waste products of metabolism, water, harmful chemicals, and contamination (p. 118).

Temperatures above body temperature will shorten sperm life. Almquist points out that at 50°C sperm suffer irreparable loss of motility in about five minutes. White (1968) states that 10°C increase above ambient temperature will more than double the metabolic rate of sperm cells and there is a corresponding decrease in their life span.

When freshly ejaculated sperm are too quickly cooled from body temperature to a temperature near the freezing point, they suffer an irreversible loss of viability called "cold shock" (Chang and Walton, 1940; Salisbury, 1941; Easley et al., 1942; Almquist, 1968; White, 1968). The most apparent sign of cold shock is loss of motility which is not regained on warming. There is also an increase in the proportion of cells staining with dyes like eosin. Changes in cell permeability occur with leakage of potassium and proteins. It is also thought that there is a decrease in fructolysis, oxygen uptake, and fall in ATP which can no longer be resynthesized to supply energy for motility (White, 1968).

It is generally thought that sperm should be protected from strong light. Almquist (1968) tells us that short exposures to light

during normal collection, processing and insemination of semen are not harmful but prolonged exposure to high intensity sunlight and visible light from any source will result in metabolic changes that shorten sperm life. There is reliable evidence that visible light adversely affects sperm motility and that sperm viability benefits from storage in the dark (Norman and Goldberg, 1959; Foote and Gray, 1960; Norman, Goldberg and Porterfield, 1962; Vasileva-Popova, 1968). On the other hand, very brief exposure to light (less than one minute) has been observed by Hamner and Williams (1961) to stimulate oxygen uptake in rabbit, cock, and human sperm suspensions which had previously been in total darkness only.

Almquist (1968) gave a concise review of the effects of oxygen and agitation on sperm. This review shows that: (1) Sperm can use some oxygen for a short time, but this results in the production of hydrogen peroxide which is toxic to sperm; (2) The agitation of semen in the presence of air incorporates oxygen into the liquid and thereby reduces motility and the life span of sperm; (3) It is important to transport liquid semen in completely filled vials; (4) A small amount of air in the ampule above frozen diluted semen has no harmful effect during long term storage at - 196^{o}C.

Observations concerning the effect of water on sperm go back to Leeuwenhoek's work. He reported that dilution with rain water deprived the canine "animalculi" of motion (Mann, 1964). Since then

many workers have reported that water, even in small amounts, will reduce or stop sperm motility and even cause abnormalities such as coiling of the midpiece or of the tail. This damage is due to changes in osmotic pressure. A determination of the osmotic pressure in terms of the freezing point depression (Δ) has been made for the semen of several species. Mann (1964) gives the freezing point depression of ram semen as 0.58 to 0.70 degrees C. Schindler, Volcani, and Eyal (1957) published the freezing point depression of ram seminal plasma as 0.63 degrees C. Egg yolk is considered isotonic to semen by most investigators. Rothschild and Barnes (1954) found the freezing point depression of egg yolk to be 0.58 to 0.60 degrees C. Life protectors are unique components of extenders regarding their influence upon tonicity. Jones (1965) found that dimethyl sulphoxide did not contribute to the tonicity of a diluent. Mann (1964) stated that glycerol prevents denaturation changes in proteins and has a protective influence on sperm colloids that may be linked to its electrolyte and water binding capacity. Glycerol is also a potential substrate for the exogenous respiration of spermatozoa.

Koelliker (1956) was the first to report that if a drop of a fairly concentrated solution of KOH is mixed with semen on a microscopic slide, there will be a sudden outburst of activity before the spermatozoa are rendered completely motionless. This period of short-lived

stimulation is akin to a terminal convulsion that precedes the loss of higher life and is characteristic of various sperm-paralysing agents - including the addition of distilled water to the semen of mammals. It was Schlenk (1933) who named this phenomenon "Todeszuckung" or spasm of death." Mann points out that not all investigators have been able to differentiate between this effect, and prolonged inactivation. Thus, certain substances have therefore been pronounced beneficial to spermatozoa viability merely because of a transient stimulus. He also calls our attention to the fact that many substances have been declared detrimental to sperm only because they appeared to slow down cell movement and/or metabolic rate. Quite often the slowing of activity may prolong the life of spermatozoa and favor their survival.

There are many substances known to be toxic to the sperm of mammals. Spermicidal and spermiostatic agents can be placed in four main groups based upon their mode of action (Mann 1964). These are electrolytes, enzyme inhibitors, sulphydryl-binding substances and surface - active agents. Those that inhibit sperm action through hypotonic or hypertonic effects belong in the first mentioned category.

The enzyme inhibitors are generally chemical compounds that render sperm inactive by inhibiting the enzymes concerned in sperm metabolism.

The sulphydryl - binding substances include the heavy metals, as well as hydrogen peroxide and ortho-iodosobenzoic acid. The toxicity of hydrogen peroxide to sperm is thought by some to be responsible for the damage resulting from oxygenation (MacLeod, 1945, Tosic and Walton, 1946, and Vandemark, Salisbury and Bratton, 1949). Semen appears in some instances to contain it's own protection against these. Mann and Leone (1953) demonstrated that ergothionine, a natural constituent of boar seminal plasma can counteract the sperm paralyzing action of cupric ions, hydrogen peroxide, and ortho-iodosobenzoic acid. MacLeod (1946) found that two physiologically occurring sulphydryl compounds in human semen, cysteine and glutathione, also protect sperm motility and glycolysis in vitro against cupric ions.

Among the surface - active substances are long-chain compounds such as detergents and soaps. Their spermicidal activity has been studied by Dunn, et al. 1942; Swayne, et al. 1952; and Gamble, 1953. Contact with these agents lead, as a rule, to irreversible loss of sperm motility and permanent damage to the cell. An interesting phenomena of detergent-treated spermatozoa (due to altered permeability) is their increased ability to oxidize certain substrates such as succinate. In studies concerning this phenomena Koefoed -Johnson and Mann (1954) noticed that ram spermatozoa treated with a detergent respired vigorously in the presence of

succinate and cytochrome but were, in fact, immotile and devoid of fructolytic activity. This experiment demonstrates that measured increase in oxygen consumption of spermatozoa does not necessarily coincide with reliable sperm viability. In this case, the increased respiration response to added succinate was the result of cellular and metabolic disorganization.

Spermatozoa collected for cryogenic preservation must be protected from the deleterious effects of all of the factors mentioned above. This can be accomplished by careful handling and by the addition of a protective media known as a diluent, dilutor or extender.

Extenders and Life Protectors

The dilution of ram semen is necessary for protection of the spermatozoa during processing and storage and it also "extends" the quantity of fluid within which the sperm are suspended - thus allowing the insemination of many ewes with a single ejaculate. Each ejaculate of ram semen contains from 0.3 to 2 ml of seminal plasma within which there are from one to five billion spermatozoa per ml (Scott, 1970).

The motility of ram semen appears to be reduced as the rate of dilution increases. Terrill (1968) states that dilutions above 1:10 are not recommended. Most workers have used dilution rates considerably lower than 1:10. Salamon and Robinson (1962a)

described the successful use of several milk diluents for ram semen and stated that dilution rates may be at least 1:4 without impairing fertilizing capacity of fresh semen - provided that the total numbers of spermatozoa are not reduced below a certain limit not yet determined in their studies but generally accepted as 50×10^6 by Emmens and Robinson (1962).

Perhaps Dott (1964) shed some light on the subject of optimum extension of ram spermatozoa. He inseminated ewes with fresh sperm that were freed from seminal plasma by a washing procedure in phosphate or bicarbonate buffers, to determine whether some factor in the seminal plasma adversely affected the fertilizing ability of ram sperm. The final theoretical dilution of the constituents of the seminal plasma was 1:2592. This was brought about in four identical cycles of dilution and centrifugation at about 300 G. The sperm density of the final suspension was the same as in the ejaculated semen. In summarizing his findings Dott stated:

> Nearly all the reports on artificial insemination of ram semen have concluded that a dilution of about 1:6 is the maximum consistent with a good conception rate. It would seem that this is not because of the dilution of some essential substance or the loss of some material from the spermatozoa, since a dilution of 1:2500 of the seminal plasma did not affect the conception rate of washed ram spermatozoa. It is probable that the critical factor in dilution is the sperm density at the external os of the cervix (p. 258).

The literature also contains references to success with high rates of extension. Tjupic (1959) reported lambing rates of 47 to 56% from vaginal insemination of 0.5 ml. or 1 ml. of ram semen diluted 1:20. He also reported as high as 70% lambing rates from 1 ml. diluted 1:10 and as high as 72% lambing rates from 0.2 ml. diluted 1:2 or 1:4. It should be noted, however, that his lambing rate of control ewes inseminated cervically with 0.1 ml. semen diluted 2 to 4 times was 75%. Dauzier et al. (1954) reported lambing rates of 50 to 83% in limited numbers of ewes using 1.0 ml. of fresh semen diluted 1:30 or 1:40. Among the studies involving high extensions is that of First et al. (1961). These workers at Michigan State University froze ram semen that was in final extension of 1:200. They reported sperm motility as high as 47% at the end of a 14 month storage period at - 79°C. This semen was not tested for fertilizing capability, however in later experiments using this same extension with frozen semen, First, Sevinge and Henneman (1961) obtained 13 ewes lambing of 148 inseminated.

High extensions appear to be the exception rather than the rule and most successful investigators, with short term storage or frozen storage of ram semen, have chosen extensions not exceeding 1:6 (Amir and Schindler, 1962; Efendieva, 1963; Lightfoot and Salamon, 1970a; Lightfoot and Salamon, 1970b; Lopyrin and Manuilov, 1966; Lunca, 1964; Nauk, 1966; Ozkoca, 1964; Salamon, 1967; Salamon and

Lightfoot, 1970; Salamon and Robinson, 1962a; Smagulov and Martynov, 1966; Ten en Bon, 1966; and Wiggan and Clark, 1967).

Ram semen has been diluted in many kinds of mediums from cow's milk to tomato juice or coconut milk. Egg yolk - citrate, egg yolk - phosphate and cow's milk have been used most extensively according to Inskeep and Cooke (1968). The yolk - citrate diluents have contained many different proportions of sodium citrate and egg yolk, along with different sugars, alcohols, and antibiotics. Although the egg yolk - citrate diluent was developed initially for bull semen, it has been used extensively, with addition of glucose, to extend ram semen in the Soviet Union, Poland and Rumania (Lunca, 1964).

When fresh or homogenized milk is used in an extender, it must be heated to 92° - 95°C and held at that temperature for ten minutes to deactivate lactenin, a nitrogenous antistreptococcal substance normally present in the albumin fraction of milk (Maule, 1962). Cow's milk in nearly all forms (whole, skim, homogenized, or reconstituted) has been used satisfactorily as a diluent of ram spermatozoa by many workers according to reviews presented by Lunca (1964), Inskeep and Cooke (1968) and Terrill (1968). Cow's milk was superior to ewe's milk as a diluent for ram spermatozoa in trials by Salamon and Robinson (1962a). The assessment of spermatozoal motility in milk diluents is difficult due to opacity of the milk. Reliable counts cannot be made with an ordinary microscope (Melrose,

1962). Zakrewska (1956) reported on the use of phase contrast microscopy to aid in overcoming this difficulty. Salisbury, Fuller, and Willett (1941) found that a sodium citrate buffer dispersed fat globules in diluents containing egg yolk to the extent that microscopic fields were clear.

The egg yolk buffer diluent developed by Lardy and Phillips (1939) was found to maintain the motility of ram semen for several days. Equal volume of fresh egg yolk and sterilized phosphate buffer (0.2 gm. KH_2PO_4 and 2.0 gm. $Na_2HPO_4 \cdot 12\ H_2O$ per 100 ml distilled H_2O) are mixed thoroughly to prepare this diluent. Moore (1949) reported that citrate or bicarbonate could be used to replace the phosphate buffer without effect on the glycolysis of ram semen and sulfathiozole could be employed satisfactorily to control microorganisms.

Schindler and Amir (1961) found that a yolk - glycine - citrate diluent maintained the motility of ram spermatozoa advantageously. These workers also found that storing ram semen (diluted 1:3) in a dialysis bag surrounded by the same diluent prevented inactivation and pH decline.

Motility and the ability to effect fertilization have been questionable when ram semen has been stored at temperatures of 0 to 12° C in the standard diluents mentioned above. Roberts and Houlihan (1961) obtained a 39% conception in ewes inseminated with semen

diluted in a standard egg yolk - citrate - glucose diluent stored for 24 hours, as compared to 63% conception in control ewes inseminated with fresh undiluted semen from the same rams. The fresh unextended or the extended semen was placed within the cervix in dosages containing approximately equal numbers of spermatozoa - i. e. not less than 50 million per insemination. The diluent was prepared according to the standard formula prescribed by the Soviet Ministry of Agriculture and was shown by Roberts and Houlihan to contain the following constituents:

Glass distilled water	100 ml.
Glucose	0.8 g.
Sodium Citrate	2.8 g.
Egg yolk	20.0 g.

Salamon (1968) found lactose and raffinose to be better components of extenders for the fast freezing of ram semen than either glucose or fructose - based upon the percent motile spermatozoa after thawing. He conducted four factorial experiments in which several factors which affect revival of ram spermatozoa after freezing were compared. The revival of spermatozoa after pellet freezing in extenders containing 15% (v/v) fresh egg yolk, 333 mM lactose, 333 mM raffinose, 355 mM glucose, and 355 mM fructose were as follows:

Egg yolk - fructose	14.8% motile spermatozoa
Egg yolk - glucose	18.9% motile spermatozoa
Egg yolk - raffinose	23.8% motile spermatozoa
Egg yolk - lactose	29.5% motile spermatozoa

The replacement of 100 mM of the sugars by equimolar amounts of sodium citrate improved revival rates. Salamon did not elaborate on his reasons for using slightly different concentrations of these four sugars in the diluents. However, he did propose that it was unlikely that this substantially affected the results. In further unpublished pellet freezing studies, using sugar diluents in varying concentrations, he found that glucose at any level yielded poorer recovery than either lactose or raffinose. He proposed that the motility differences found in his work supported the view of Nagase et al. (1964) that the sugars of higher molecular weight provide better protection to spermatozoa, during fast freezing, than do those of lower molecular weight. Jones and Martin (1965) also found lactose superior to fructose in sodium citrate buffered diluents used for deep freezing ram spermatozoa. Salamon and Robinson (1962b) found no significant difference between egg yolk - glucose - citrate and heated cow's milk as diluents when extended semen was used fresh or after storage at 10 to 15^{o}C, but highest fertility from insemination with extended semen stored from 0 to 72 hours at 5^{o}C was obtained with that semen extended in yolk - glucose - citrate. In this work Salamon and Robinson also stated that comparative studies have shown a linear decline in fertilizing capacity, with time, at the rate of about 11 - 20% per day. In their work they obtained a decrease of 14% per day and believed that this figure could be improved upon. They proposed that

critical tests with various diluents, antibiotics, and techniques of cooling might increase the effective time of storage of unfrozen semen to 48 hours without serious loss of fertilizing capacity.

Various cryoprotective agents (life-protectors) have been used to protect ram sperm during the freezing process. After the initial success of Polge, Smith and Parkes (1949), with the survival of frozen glycerol treated fowl sperm, attention turned to the freezing of semen of other species. According to Mann (1964), the main principle underlying survival of cells during the freezing and thawing process is that cooling is effected in a manner that bypasses the crystalline-phase and carries the cells straight into a sub-freezing temperature (known as the vitrification zone) where they assume the non-crystalline glass like or vitreous state. The transition on thawing from the vitreous state is equally critical and again crystallization must be avoided. When vitrification is perfect and crystallization prevented, it is proposed by Mann that colloidal changes and structural disarrangement may be prevented. He states that damage can be reduced if freezing is carried out in the presence of certain organic substances such as sucrose, glucose, fructose, glycerol, ethylene glycol, gelatin, albumin, and various gums. Glycerol has been reported to occur in the haemolymph of certain hibernating insects, and it was proposed by Salt (1961) that its presence may be

the reason for their tolerance to prolonged freezing. Glycerol has also been reported by Clegg (1962) to occur in the dormant cysts of the brine shrimp, Artemia salina.

Many substances have been tested as life protectors in the freezing of ram spermatozoa. Salamon (1967), using a basic egg yolk - citrate - lactose diluent and one step dilution, found glycerol at 5% level to give better protection than ethylene glycol (5%) or a combination of either glycerol (3%) or ethylene glycol (3%) with dimethyl sulphoxide (DMSO) (2%). Dimethyl sulphoxide at 5% level gave very poor survival. Jones (1965) also found dimethyl sulphoxide to give poor results with ram spermatozoa. He found that dimethyl sulphoxide did not contribute to the tonicity of a diluent. DMSO, or glycerol, at 5% v/v in a diluent, depressed spermatozoal activity during incubation at 5°C, but glycerol rendered a significantly greater number of the cells completely immotile after incubation at 30°C. Snedeker and Gaunya (1970) studied the effect of DMSO in combination with glycerol on the prefreeze and postfreeze motility of bovine spermatozoa extended in homogenized skim milk and egg yolk-Tris extenders. DMSO added to the milk extender did not result in a higher mean motility than was obtained with the optimum level of glycerol alone. A significant quadratic effect was found, however, in the egg yolk - Tris extender, where 1% DMSO in combination with 6% glycerol provided the highest mean motility. Horton, Greybill

and Wu (1966) were successful in freezing salmon and trout sperm using DMSO as the cryogenic protecting agent. Vermesan et al. (1966) compared 30% egg yolk, 2.88% phospholipid extract, and 2.5% polyvinyl pyrrolidone (PVP) in glucose - citrate extender for protecting ram sperm against cold shock. Sperm survival during refrigerator storage for 6-7 days showed little difference between the 30% egg yolk and the 2.88% phospholipid extract, however, was significantly higher in the 2.5% PVP extender. In two field experiments, the conception rates of 23 and 157 ewes inseminated with semen extended with the PVP containing extender were 69.6% and 83% respectively, vs. 62.0 and 81.0% for 185 and 510 ewes inseminated with extender containing the egg yolk.

Freezing, Thawing and Inseminating

Satisfactory levels of fertility have been reported with bull semen frozen in ampules (Polge and Rowson, 1952), synthetic straws (Cassou, 1964; and Jondet, 1964) and in pellet form (Nagase and Graham, 1964). Salamon (1967) has reported on the motility and fertility of ram semen frozen by the three methods: ampules, synthetic straws, and in pellet form. Ejaculates of high initial motility, collected by artificial vagina were pooled and then split into aliquots to be similarly extended and then frozen in ampules, in plastic straws and in pellet form - all at rates determined by previous workers to

result in satisfactory recovery. The diluent used for all three methods of freezing consisted of 83 mM glucose, 100 mM sodium citrate and 15% (v/v) fresh egg yolk. The semen was extended with the non-glycerol portion of extender at 30°C (1:1), cooled to 5°C in 1.5 hours and then further extended with the glycerol containing diluent and equilibrated therein for 4 hours. The final dilution rate was 1:2 and the glycerol concentration in samples frozen by all three methods was 6% (v/v). The semen frozen in ampules (1 ml) was frozen at a rate indicated by Lopatko (1962) to give 40 to 67% lambing rates. This was 0.5°C per minute from 4 to -15°C, 2.0°C per minute from -15 to -50°C, 5.0 to 10.0°C per minute from -50 to -75°C, and 100 to 200°C per minute from -75 to -196°C. Cassou-type plastic straws, containing 0.30 ml of extended semen was frozen in nitrogen vapor 2-3 cm, above the surface of liquid nitrogen. The pellets were frozen in 0.03 ml size by dropping this quantity of the extended semen onto a block of dry ice. The samples frozen by these three methods were stored in liquid nitrogen (-196°C) for two to four weeks prior to insemination use. Thawing was at 37°C. Estrus was synchronized during the natural breeding season with syncromate pessaries (Searle). The ewes were three to four year old Merinos and each had lambed the previous season. Insemination was performed at the second estrus following withdrawal of the progestogen impregnated pessaries.

Semen frozen by each method was used in both single and double inseminations during the heat period. The second insemination was performed eight hours after the first. A total of 410 ewes were inseminated. Although 60 to these (14.6%) did not return to service, only 24 of the ewes lambed (5.9%). There were no significant differences in non-return rate or in lambing rates between semen frozen by the three methods, nor did the number of inseminations per estrous period affect fertility significantly. Lambing rates and non return rates were as follows:

Ampules	Ewe inseminated	N.R.	Lambing		
single insemination	95	15-15.8%	6(93)[a]	6.5%	5.6%
double insemination	85	14-16.5%	4(84)	4.8%	
Straws					
single insemination	75	8-10.7%	3(72)	4.2%	4.4%
double insemination	65	8-12.3%	3(65)	4.6%	
Pellets					
single insemination	49	7-14.3%	3(49)	6.1%	8.9%
double insemination	41	8-19.5%	5(51)	12.2%	
Total and means	410	60-.6.6%			

[a] Number of ewes available at lambing time

In his discussion, Salamon emphasized that all three methods gave poor lambing results, despite satisfaction recovery rates at thawing and despite 90 to 150 million motile spermatozoa inseminated per

insemination. He also stated that the discrepancy between return and actual lambing rates was 9 percent and showed the doubtful value of using the non-return rate in making conclusions regarding the fertilizing capacity of frozen semen. Loginova (1962) also reported up to 40% difference between non-return and lambing rates and claimed that a high incidence of embryonic mortality occurs with frozen ram semen. Double insemination had no significant effect. Lopyrin and Loginova (1958) carried out double and triple inseminations, due to the fact that they had observed that frozen ram spermatozoa survive only six to seven hours in the female tract. They found double and triple insemination to have no significant effect, only 11% of the ewes lambed. Salamon and Lightfoot (1970) found that double insemination with a 12 hour interval between inseminations gave lambing rates of 38.8% vs. 22.6% for one insemination and 53.0% vs. 39.7% in another experiment.

Since these last quoted lambing figures are among the best obtained to date from the use of frozen ram semen, we will look at the design and results of Salamon and Lightfoot's (1970) studies in close perspective.

In a series of studies these Australian scientists undertook to study several aspects of the fertility of ram spermatozoa frozen by the pellet method. Their first study, Lightfoot and Salamon, 1970a,

was designed to examine the transport and viability of frozen spermatozoa in the genital tract of the ewe and in particular the early entry into the cervix. They conducted four studies and their important findings in Study I may be summarized as follows:

1. Very low numbers of spermatozoa were recovered from the oviducts of ewes inseminated with fresh (diluted) or frozen semen when either was used at a concentration of 0.4×10^9 motile spermatozoa per ml. High doses of oxytocin (10 to 20 I. U.) did not affect sperm transport in the ewes genital tract but significantly depressed the fertilization rate.
2. Concentrating the thawed semen, by centrifuging, prior to insemination resulted in increased numbers of spermatozoa being recovered from the fallopian tubes. Fresh semen, at the same concentration, gave higher numbers in the tubes and also an increased proportion of ova recovered with spermatozoa in the zona pellucida.
3. The number of spermatozoa recovered from the cervix 30 minutes after insemination with frozen semen was dependent upon the concentration of motile spermatozoa in the inseminate. When the concentrations were the same, fresh semen was superior to frozen semen. These results suggested that an increase in the concentration of

the thawed semen prior to insemination should result in improved fertility.

4. Uterine insemination by laparotomy at varying periods before ovulation showed that a proportion of the pellet frozen spermatozoa survived in the ewe's genital tract with retention of fertilizing ability for 18 to 35 hours.

In their second study of this series, Lightfoot and Salamon (1970b) studied the effects of method of insemination on fertilization and embryonic mortality. In an earlier study Salamon and Lightfoot (1967) obtained high fertilization rates of 88 and 93% when the cervix was by-passed and frozen semen was deposited directly into the uterus, however, the survival of zygotes was very poor. Their results of the second study may be briefly summarized as follows:

1. Two cervical inseminations at a 12 hour interval within one estrous period with frozen semen of high concentration of motile sperm (1.6×10^9 per ml) in a 0.1 ml dose resulted in fertilization and lambing rates of 58% and 50% respectively.
2. There was less embryonic mortality following either the cervical or cervical traction (deep cervical) methods of insemination than there was following uterine insemination by laparotomy. The embryonic losses for cervical, cervical traction and uterine insemination involving use of fresh

and frozen semen on 209 ewes was 13.0%, 6.3% and 47.2%, respectively. The data from all three methods of insemination of the 209 ewes with both fresh and frozen semen showed that embryonic mortality in ewes inseminated with frozen semen was not significantly higher than that observed with fresh semen.

3. The overall fertility of the frozen semen (pellet method) was 49% as compared to 70% for the fresh semen. Considering the significantly higher mortality of zygotes obtained by uterine insemination, the authors proposed that there was a possibility that surgical interference brought about a response leading to reduced survival of otherwise normal zygotes, or to a high incidence of abnormal zygotes due to anomalous fertilization. They also concluded that the cervical traction method of insemination offered no advantage over normal cervical insemination, in contrast to earlier findings of Salamon and Lightfoot (1967) and Ten En Bon (1965). Salamon and Lightfoot (1970b) later concluded that losses due to embryonic mortality were of lesser importance when using frozen semen than failure of fertilization due to impaired transport of spermatozoa.

Salamon and Lightfoot (1970) designed a third study to further

investigate the effects of insemination technique, as well as the effect of oxytocin and relaxin, with the hope of finding means to enhance the transport of thawed spermatozoa through the ewes genital tract. The important findings were:

1. Thawed semen of low motility and low concentration gave poor fertility with an overall non-return rate of 5.6% based on 269 ewes.
2. Insemination with thawed semen of low and high sperm concentrations (0.5 vs. 1.5 x 10^9 motile cells/ml) gave proportions of ewes lambing of 25.8 and 43.8% respectively.
3. There was no difference in the proportion of ewes lambing when the inseminate volume was reduced from 0.3 to 0.1 ml or to 0.05 ml of concentrated semen (1.6 x 10^9 motile spermatozoa/ml). The authors proposed that it seems likely with ewes in which semen can be deposited into the cervical os, that the use of a concentrated inseminate of even less than 0.05 ml may be practical.
4. Two inseminations gave higher fertility than a single insemination in the same heat period (38.8% vs. 22.6%) and 53.0% vs. 39.7%) in two separate experiments. The magnitude of this response seemed to vary according to time of insemination (in the day) and the concentration

and volume of the inseminate.

5. High does of oxytocin (5 or 10 I. U.) reduced the number of ewes lambing. Relaxin (100 to 12,500 guinea pig units) did not affect the depth to which the inseminating pipette could be inserted into the cervix.

6. Several interesting classification factors were found to be significantly related to fertility in these studies.

   a. The depth of insemination significantly increased with the increase in number of cervical papillae. These two were each found to be associated with higher lambing rates, whereas vaginal constriction (indicative of barrenness) allowed only shallow insemination.

   b. There was an association between the abundance of mucus in the vagina and the depth of insemination possible. The lambing rate was lower in those ewes in which a depth of insemination of only 1cm. or less could be achieved, if mucus was also scarce. However, lack of mucus made little difference in fertility if depths of insemination of more than 1 cm. was achieved.

The authors stated that although the number of ewes lambing in the relaxin administered groups was lower than in the controls - conclusions concerning the effect of relaxin on fertility could not be

made from this study due to the small number of ewes (17-18) included in each relaxin dosage group. They believed that more experimentation is needed before the value of the cervical traction method of insemination can be assessed properly, and they stressed that further development may lie in the critical assessment of the minimum inseminate volume that may be used without reducing fertility.

Apart from this recent report, the literature shows other recordings of high fertility being obtained with frozen ram semen. Critical assessment of many of the reports is difficult because the authors have omitted essential details.

The highest fertility to date (61 to 66%) has been reported by Mackepladze et al. (1960) and by Lopatko (1962) (41 to 67%). These reports have not been confirmed according to Salamon (1967) and Salamon and Lightfoot (1970). Lopyrin (1969), as cited by Salamon and Lightfoot (1970), reported that the high fertility rates claimed by the Soviet workers have subsequently been tested under supervision of a commission and "none" of the authors could confirm their own results and obtain a fertility rate higher than 14% after one insemination. Evidently, this was due to the replacement of fertile teaser rams by vasectomized rams. Thus, the possibility of occasional fertilization of ewes by natural mating could not be excluded. Aamdal

and Andersen (1968) have also reported 62.5% pregnancy, as determined by slaughtering or X-ray in ewes inseminated with semen frozen in straws. Good results have also been claimed by Kalev and Venkov (1961) with 55% non-return rate, and by Ten En Bon (1965) with 36 to 46%, but the last three reports cited also failed to mention the type of teaser rams that were used for detection of ewes in estrus.

Fraser (1968) reported high rates of conception in a cheviot flock inseminated with semen frozen in pellet form. This report states that the required freezing technique is fast, using a low concentration of glycerol in a simple medium with a short equilibration time. He reported 56% lambing rates in 34 ewes when inseminations were carried out over the 1st and 2nd heat periods and 80% lambing rates in 30 ewes when inseminations were carried out over 1st, 2nd and 3rd estrual periods. He used a medium consisting of 25% egg yolk, 71.5% lactose (11% solution) and 3.5% glycerol. The equilibration period was two hours. Pellets were thawed rapidly at a temperature of 45^{o}C. He found that a disposable plastic insemination rod with a beaded tip and angled neck was best for effecting deep intracervical inseminations. He recommended reduction of stress in the handling of the ewe and found a restraining crate to be helpful. In his work, it appeared that inseminations carried out between four

to eight hours after apparent onset of estrus gave satisfactory results. His report did not specify whether ewes were inseminated more than once during each estrous period, though he did state that insemination carried out between four to eight hours after apparent onset of estrus can give satisfactory results. This was an interium report and Fraser's conclusions were that no single development had led to a spectacular improvement in fertility rates but that the total effect of various findings over a period of four years had led to the success reported.

As demonstrated by these recent findings, the pellet method of freezing appears to hold promise. There are certain aspects of pellet freezing, however, that should be mentioned in this review. Lorrman (1968) reported on the possibility of mixing semen and of spreading disease organisms by storing frozen semen pellets in liquid nitrogen. When semen is frozen in pellet form it has often been common practice to store the pellets in unsealed containers in the liquid nitrogen. While direct contact with the liquid nitrogen has no apparent deleterious effect upon subsequent viability of the sperm, Lorrmann stated that bacteria and virus from certain pellets might float away "infecting" other pellets. Under practical conditions it was proved that considerable contamination occurs in event the container is used for a long time without being cleaned. An increase of bacteria and virus titer

can be observed on the pellets independent of the time factor in storage, and he stated that the possibility of transfer and spreading of infectious agents present in the semen or coming otherwise into the container should be kept in mind. His investigations also looked into the possiblity of whether this sort of transfer could apply to sperm cells - from pellet to pellet. In a series of trials, he found that spermatozoa also get transmitted. About 20 to 30% of those spermatozoa found transmitted, appeared undamaged although not living. Nevertheless these findings created doubt in his mind whether or not the identity of a calf could be guaranteed when using frozen pellets of bull semen stored in unsealed containers in liquid nitrogen. Finland has adopted the use of pellet frozen semen in their cattle breeding service and Von Koskull and Hemnell (1968) have the following comments concerning storage: Before pellets are transferred to the tubes they are rinsed in pure liquid nitrogen in order to remove possible loose particles. Pellets to be distributed to technicians are put in metal tubes made with an open slit along the full length of the tube. Pellets for long-time storage are put into aluminum tubes and sealed. Every tube contains separate freezings. The storage tubes as well as the tubes used for insemination are marked with an electric pencil, making identification safe. Every third month the storage containers are cleaned and disinfected with alcohol. No crossing of breeds has

been reported and the technicians and veterinarians report that the number of endometritis cases have gone down since the use of frozen semen.

Measurement of Spermatozoa Viability

Many methods have evolved in efforts to assess spermatozoal motility and percent alive. Emmens (1947) was one of the first to propose an index of motility based upon microscopic examination of fresh semen or spermatozoal suspension. His index, first developed and used to record the motility and viability of rabbit spermatozoa, was as follows:

Index of Motility	State of Spermatozoa
4	Full activity, but there may be up to 30 or 40% of dead cells as in the fresh ejaculate.
3-1/2	A detectable damping of activity compared with 4.
3	Sluggish, rate of motion about two-thirds that of 4.
2-1/2	Most cells progressing but many stationary with tails vibrating.
2	Most cells stationary, many with tails vibrating.
1-1/2	Many motionless, none or almost none are actively progressing.

(chart continued)

Index of Motility	State of Spermatozoa
1	Hardly any motility, only tails moving (very rarely an occasional cell in actual motion).
1/2	Only a few cells per field showing any movement.
0	Completely motionless.

Cummings (1954) found that motility ratings, as made by phase contrast microscopy, was reasonably accurate in predicting levels of fertility in bulls. He believed that phase microscopy was more accurate than ordinary bright field illumination and that more detail could be observed in the morphology and movement of spermatozoa, using phase contrast. At 100X magnifications or 200X magnifications, Cummings made motility ratings on diluted bull semen. His ratings were based on frequency of the normal movement of the spermatozoa plus an estimate of the percentage of motile sperm. He stated, regarding estimates of motile spermatozoa:

> Actual counts could not be made, because in samples of exceptionally good motility the spermatozoa moved in and out of the field so rapidly that counting was impossible (p. 11).

His most reliable motility index showed a direct relationship to breeding efficiency based on 30 - 60 and 60 - 90 day non-returns for 31,423 first services. This motility index was calculated from the microscopic motility ratings of fresh ejaculates. It was one of

six semen tests made in a study designed to determine relative values for the tests when used to evaluate breeding efficiency in bulls and also to determine the combinations of semen analyses that might have greater value in predicting relative fertility than any one test taken by itself. In this study, Cummings tested the sperm concentration, motility, respiration rate of semen, content of live and morphologically abnormal sperm and rate of impedence change with the Rothchild impedence bridge. The last named method is based on the observation that semen from species having a relatively high sperm concentration exhibit periodic changes in electrical impedence when sperm are alive and active (Rothchild, 1949). Cummings found that a fertility index made up of impedance reading, motility index, spermatozoa respiration, percent live spermatozoa and concentration had value for measuring the breeding efficiency of bull semen - based upon percent non-return of 29,810 first services.

The percentage of living spermatozoa in a fresh or extended semen sample can be estimated either by visual appraisal, actual count of a very dilute subsample, or by the vital stain method developed by Lasley, Easley and McKenzie (1942). This method made use of water soluble eosin and opal blue in minute quantities in a isotonic phosphate buffer solution. It was found that the dead spermatozoa absorb the eosin stain while the live spermatozoa do not. The

opal blue provided background color. A small drop of the stain mixture is placed on a clean glass slide. A very small amount of the semen to be tested is mixed into this with a glass stirring rod. Another clean glass slide is placed over this drop of stain and semen which then is spread into a thin film between the two slides. The slides are then drawn apart rapidly without applying pressure. One of these slides is dried by fan or breath air movement on a hot plate at 200°F and the live/dead cells subsequently counted by microscopic examination. Mayer et al. (1951) found that fast green FCF proved to be superior to all other dyes tried as a spermatozoan background stain. The combination that proved to give the best results with all semen tested (except the boar) was prepared as follows:

fast green; FCF	2 grams
eosin b (bluish)	0.8 grams
M/8 phosphate buffer pH 7.3 - 7.4	100.0 ml

Terrill (1968) stated that staining with a background stain such as opal blue or fast green appears to be useful for estimating the proportion of live spermatozoa present and also for classifying abnormal types. Other background stains used for ram semen have been reviewed by Emmens and Blackshaw (1956).

Hulet and Ercanbrack (1962) showed that rams producing semen with less than one billion spermatozoa per ml, with a pH of 7.4 or higher, with slow, sluggish motility or with more than 50% dead or

abnormal sperm, are of generally low fertility. Terrill (1968) stated that several ejaculates should be examined over a period of several weeks before a ram is judged unfit. The semen quality of some rams improves markedly with sexual use while that of others does not and may even decline. Rams often produce poor semen after a long period of sexual inactivity, and ram semen with high concentrations is usually slightly acid in reaction while that of low concentrations is basic.

Scott (1970) stated that no method has yet been devised to determine a ram's fertility with complete accuracy - except by actual breeding test. Scott also showed criteria for scoring semen quality as developed at the U. S. Sheep Experiment Station, Dubois, Idaho. These criteria were pH, motility score, concentration, percent live normal, abnormal, and percent abnormal heads. Two indexes were developed to classify ram semen. These indexes placed the most weight upon a slightly acid semen pH, high motility score, and high percent live normal spermatozoa. These characteristics seem to exist together in normal healthy ejaculates according to Terrill (1968) who describes an average ram ejaculate as consisting of slightly less than 1 cc of semen with a creamy appearance, containing from 2 to 5 billion spermatozoa of which about 90% are alive. An alkaline reaction of the semen is often associated with poor quality and low fertility. Good motility is characterized by a swirling motion so rapid that it is difficult to distinguish individual sperm. From 5 to

15% of the spermatozoa may be abnormal in morphology but higher percentages of abnormals may indicate low fertility (McKenzie and Phillips, 1934). The most common forms of abnormal sperm are tailless, misshapen or tapering heads, enlarged midpieces, and adhered, coiled or bent tails. Head abnormalities are most likely to indicate low fertility.

According to Emmens and Blackshaw (1956) there was, at that time, evidence that no single test taken alone was satisfactory for predicting fertilizing capacity. They concluded that, of the large number of tests investigated the following seemed to be of value: (1) Biochemical tests, such as rate of color loss of methylene blue or reazurin when semen is added (indicating dehydrogenase activity). (2) Measurements of impedence change frequency (indicating rapid movement of spermatozoa). (3) Estimates of the percentage of living spermatozoa, and (4) The maintenance of motility at body temperature. Bishop et al. (1954) concluded that there was a relationship between the physical activity of bull semen and fertilizing capacity of the sperm and that overall activity depended upon the number of living spermatozoa and their individual activity. Studies of oxygen consumption of semen have been made by many investigators. Bishop et al. (1954) and Cummings (1954) reached conclusions that metabolic measurements of spermatozoal activity do not add sufficient information concerning their potential fertility to warrant the

inclusion. These workers did find evidence that highly active spermatozoa are more resistant to temperature shock than less active spermatozoa.

Wiggins, Terrill and Emik (1953) studied the relationship between libido, and semen characteristics on fertility in range rams. They found that libido as measured by the frequency of ejaculation had a significant relationship to fertility. The following semen characteristics were studied:

1. Viscosity
2. pH
3. Motility score
4. Estimated percent motile sperm
5. Estimated motility count
6. Volume of semen
7. Total number of spermatozoa
8. Percentage of normal spermatozoa
9. Percentage of abnormal heads
10. Percentage of live normal spermatozoa
11. Percentage of live spermatozoa
12. Concentration

Of these twelve semen characteristics, only volume of semen, estimated motility counts, percentage of normal sperm, percentage of abnormal heads, and percentage of live normal sperm were significantly correlated with the percentage of ewes lambing from normal service. In no case was the predictive value high. Although estimated motility count (EMC) was the only motility characteristic significantly correlated with percentage of ewes lambing, the authors stated that the two components of EMC, i.e. motility score, and percent motile sperm approached significance. This study was based on prebreeding semen characteristics and subsequent breeding

performance of 1,109 rams bred to 31,473 ewes from 1936 - 1950 inclusive at the U. S. Experiment Station and Western Sheep Breeding Laboratory at Dubois, Idaho. The relationships between libido and percentage of ewes lambing, in this study, are interesting. Though the authors found a positive correlation between libido and percentage of ewes lambing, their remarks concerning these findings indicate that the bulk of the breeding was done in small panel pens to moderately sized groups of ewes (an average of 24 ewes per ram). The breeding work from which these data were taken included matings for development of inbred lines, linecrosses, test pens, and breed crosses, and the number of ewes bred to each ram varied from 5 to 65. This situation was not typical of the average breeding ratios to be found in farm or range flocks, and the authors agreed that in this work it seemed unlikely that ewes in heat would be unnoticed by the rams. In this work there was a tendency for the rams with more libido to also have higher semen scores but there were numerous exceptions and the correlations in general were not significant. Age of ram and inbreeding of the ram were not significantly correlated with percentage of ewes lambing.

Recent Findings of Interest

The effects of holding time in seminal plasma prior to extension has become a recent subject of interest, especially in the freeze preservation of bull and boar semen. In bull semen work, the conventional practice has been to reduce the holding time of semen at or near body temperature to a minimum, in order to minimize metabolic activity. Although there has not been a uniform time of holding raw semen prior to extension, the standard practice has been to evaluate the semen and make the first extension as soon as possible after collection and then cool the extended semen slowly over a one to 1 1/2 hour period to refrigerator temperature. Rajamannan, Graham and Schmehl (1971) studied the effects of holding neat (raw) bull semen at room temperature (26 ^{o}C) prior to first extension for periods up to 60 minutes, with the thought that sperm appear to undergo some beneficial effects from remaining in contact with their own seminal plasma. They worked with the semen of six bulls and held ejaculates at room temperature for 0, 10, 20, 30, 40, 50, and 60 minutes post collection, using a split sample technique. After each holding time an aliquot was extended 1:10 in a test extender containing seven percent glycerol and then cooled to refrigerator temperature over a period of one hour. It was held at refrigerator temperature for five hours and then frozen in ampules. The results showed no significant

differences between the various holding times in initial motility, pre-freeze motility, or postfreeze motility. However the field trial results, when this semen was used for breeding, showed a significant increase in fertility in favor of the held semen. The authors stated that "it was definitely achieving some form of increased vitality or protection or membrane stabilization while it was in contact with it's own plasma" (p. 6). The postfreeze motility increased from 41.7% for the control (zero minute) to 46.7% for the 60-minute held semen. The 70 day non return rates were shown only for the control and the 20 minute held semen. For one sire these were 59.75% and 69.38% respectively and for another sire, 68.99% and 73.02% respectively.

More dramatic results have been accomplished in the holding of boar semen in it's own seminal plasma prior to extension. Graham et al. (1971) conducted an experiment to study the effect of holding freshly ejaculated boar semen at room temperature of 22°C for up to four hours before extending. In previous work with boar semen the fresh semen has been extended immediately or the seminal plasma removed and the cells resuspended in an extender. Graham and his co-workers collected the sperm rich fraction into an insulated bottle and allowed it to cool to room temperature (22°C). Semen was then extended 1:5 in a test buffer at 0, 1, 2, 3, and 4 hours post collection. The extended semen was placed in a double walled water bath and

cooled to 5°C over a period of 4 hours. The cooled semen was then frozen in pellet form on dry ice and stored in liquid nitrogen. These initial studies showed that holding the raw semen gave an increase in postfreeze motility and also a reduction in glutamic oxalacetic transaminase (GOT) loss from the spermatozoa. The optimal holding time appeared to be about two hours. The authors proposed that the raw semen upon ejaculation undergoes a phenomenon of strengthening of the sperm cell wall or of the whole sperm. They saw indications that some efficient seminal plasma protein coating was taking place that seemed to be lost when egg yolk protein was added prematurely. They agreed that the egg yolk protein could coat the sperm cell but with a comparatively less efficient system, and that after an initial holding in seminal plasma egg yolk protein seems to supplement by increasing the stress resistance of the spermatozoa. Graham and his co-workers suggested that this explained the decrease in fertility and freezeability after surgical removal of seminal vesicles as described by MacPherson (1968).

It appears that Graham et al. (1971) were successful in establishing that there is a definite need for holding boar semen in the seminal plasma for a period of two hours prior to extension. Also, Rajamannan et al. (1971) showed that there was a significant increase in fertility when bull semen was held in the seminal plasma for 20 minutes prior to extension. So far as can be determined at present,

there are no such reports in the literature showing the effects per se of holding raw ram semen before processing for freezing. Perhaps the beneficial effects of holding, if such exist, have been obtained unknowingly in varying degrees due to the fact that it is routine for all workers to examine certain important semen qualities such as volume, motility, and percent live normal spermatozoa prior to starting extension and cooling to refrigerator temperature. Most workers merely report that dilution and cooling to 5°C were initiated as soon as possible after collection, but few examples can be found in the literature where the exact time of holding in the raw form is reported. As way of example: Jones and Martin (1965) stated: "Within 10 to 45 minutes of collection samples showing wave motion of 3.5 to 4 (Emmens, 1947) were diluted at 30°C to half the volume required for freezing..." (p. 414); Salamon (1967) stated: "Soon after collection the semen samples were diluted at 30°C with the non-glycerolated portion..." (p. 352); and some papers make no reference to time elapse between collection and first extension (First et al. 1961a; 1961b).

Kirton and Hafs (1965) reported that rabbit sperm could be capacitated in vitro with uterine fluid or with beta-amylase. In further work with frozen bull semen these same workers demonstrated an increase in fertility with amylase added to the extender (Kirton, Boyd and Hafs, 1968). This work was based upon the premise that

some sperm are transported to the site of fertilization within a few minutes after mating (Van DeMark and Moeller, 1951). However, there still seemed to be a decline in fertility when insemination or matings were performed near the time of ovulation (Trimberger, 1948). Kirton, Boyd and Hafs believed that this might be due to a period of sperm capacitation prerequisite to fertilization as Austin (1951) and Chang (1951) reported for rats and rabbits. With this in mind and knowing that beta amylase appeared to mimic uterine fluid in capacitation of rabbit sperm, they speculated that if bull sperm require a period of residence within the female reproductive tract before being capable of fertilization, the treatment of bull sperm with amylase could improve fertility of cattle inseminated near the time of ovulation. Although they obtained a significant increase in fertility of two to four percent when extenders contained crude amylase, they suggested an experiment designed to test fertility of cattle inseminated near the time of ovulation with extender containing amylase as a more direct test of whether the gains were due to capacitation. In a study designed to confirm or deny the findings of Kirton, Boyd and Hafs (1968), Sullivan and Elliott (1971) used alpha amylase and beta amylase in 0, 1, and 10 μg per ml in extended frozen bull semen. They also obtained significantly higher motility ratings, for both amylases, and concluded that regardless of their mode of action, both alpha amylase and beta amylase resulted in significantly improved

fertility in cattle. In a later report, Hafs and Boyd (1971) reported 5.9 percentage units increase in non return to first service for 1,734 cows when beta-glucuronidase was added to bull semen prior to freezing. These workers do not know the exact manner in which the enzymes work, but they have hypothesized that there may be enzymatic alteration of seminal plasma macromolecules which coat ejaculated sperm, and thus reduced uterine residence time is required for sperm to become capable of fertilization. Reports on freezing ram semen with added enzymes have not been found in the literature.

Acrosome morphology and how its alteration is related to the fertility, motility, and viability of the spermatozoa is becoming a subject of increasing interest. White (1969) described the acrosome as a cap-like structure that covers the anterior part of the sperm head. It is believed that the acrosome plays a vital role in fertilization since bull and boar spermatozoa with deformities of the acrosome are known to be sterile. The acrosome is thought to be made up of protein bound polysaccharide composed of mannose, galactose, fucose and hexosamine. The sperm head is also known to contain hyaluronidase and it is believed to diffuse out to break down hyaluronic acid in the cumulus oophorus cells of the ovum. An extract of acrosomes isolated from ram, bull or rabbit sperm has been reported by McLaren (1968) as effective in dissolving the zona and dispersing the corona radiata cells of rabbit ova.

Saacke and Marshall (1968) reported on observations of the acrosomal cap of bovine spermatozoa. They observed fixed spermatozoa with ordinary light microscophy and electron microscophy. They also observed unfixed cells with differential interference - contrast microscophy and they stated that this method provides a new means by which observations on the acrosomes can be made on unfixed cells. The component parts of the acrosomal cap of a mammalian sperm are shown in Figure 1 (Appendix I). Saacke and Marshall studied over 800 electron micrographs of bovine sperm and proposed the following order of deterioration as the cell died: (1) loss of apical ridge, breakdown of cell membrane and swelling of the anterior acrosomal cap; (2) ruffling following by breakdown of the outer membrane of the anterior acrosomal cap with the posterior portion remaining intact at the equitorial segment; (3) loss of the ground substance in the anterior acrosomal cap. The motile cell was always characterized by an intact acrosomal cap with a distinct apical ridge. This was also the predominant type in fresh ejaculates. Immotile cells were composed of those having an acrosomal cap similar to the motile cell, as well as those in all stages of deterioration. The order of deterioration observed with differential interference-contrast microscopy on unfixed semen smears were similar to those seen by electron microscophy, i.e. (1) loss of apical ridge with slight swelling of the acrosome and appearance of the nuclear ring. After cessation of movement, this

change, once initiated, occurred quickly providing sharp transition; (2) formation of the equitorial segment with continued swelling of the acrosome; (3) loss of the anterior acrosomal cap (outer membrane) by dissolution or lift away and retention of posterior acrosomal cap or the equitorial segment. These workers stated that the alterations are either post mortem or occur immediately after cell death. They did notice some immotile spermatozoa with intact acrosomes in fresh ejaculates. They suggested that such cells may again assume motility.

Quinn, White and Cleland (1969) studied the ultra structural changes in ram spermatozoa after washing, cold shock and freezing. Electron microscopy showed that cold shock and freezing caused profound changes in the appearance of the apical ridge and corrugated section parts of the acrosomes, but no apparent changes in the smooth region. They observed considerable swelling of the acrosomes with reduction in the density of the acrosomal substance. The effects were said to be more pronounced in frozen than in cold-shocked spermatozoa. The authors stated that the most spectacular effect of cold shock and freezing is on the acrosome and that in ram spermatozoa this effect is region specific confined to the corrugated and apical regions. There was not so much a loosening of the acrosome, in their work, as there was a swelling. They were unable to observe whether the swelling itself was associated with any loss of material. They believed that the blebbing of the acrosomal membrane

would be associated with such a loss, and stated that chemical determination of the loss of orcinol-reactive material was quite definitive. Their conclusion was that despite the apparent lack of effect of cold-shocking and freezing on the plasma membrane covering the functional parts of the spermatozoa, it seems likely that the effects of these treatments depend principally on a change in membrane permeability. Such a change appears to be profound and general since it results not only in a loss of cations but also in the loss of protein from mitochondria in the mid-piece where the protein is protected by three membranes. They found no loss of proteins from the motor filaments or principle piece, but suggested that this might merely reflect the insolubility of proteins in these areas.

Quinn, White and Cleland (1969) used quite different procedures for collecting, and freezing spermatozoa in these experiments than are generally used in collecting and freezing for artificial insemination purposes. In their work the ram semen was obtained by electrical ejaculation and maintained at 20°C during transfer to the laboratory. Only samples of good initial motility were used but the semen was cold-shocked by placing directly into an ice water bath and frozen by placing in finely crushed CO_2. After 10 minutes the cold shocked samples and frozen samples were brought to 37°C for 5 minutes and the detrimental effect tests were then accomplished. No mention is made in this study of using protective slow cooling, extenders,

cryogenic life-protectors, equilibration, or rates of freezing that have given good sperm survival and motility. The effect of the cold treatment was checked on nine ejaculates by the differential staining technique using congo red-nigrosin (Quinn, Salamon and White, 1968) and by scoring motility by use of Emmen's (1947) method. The mean percentage of unstained cells and their respective motility scores are given as: Control 87%, 3.7; cold-shocked 15%, 1.1; deep freeze 1%, 0. These figures reflect low sperm survival rates and motility. The literature does not reveal work with ram semen where acrosome morphology was determined upon spermatozoa frozen by techniques used for optimum protection from cold shock and freezing damage.

Pursell and Johnson (1971) used acrosome morphology, as evaluated by phase contrast microscopy at 1560X, to evaluate frozen-thawed boar spermatozoa prior to using it for insemination. The thawed spermatozoa had 28 to 54% normal apical acrosome ridges, although the postthaw motility ranged from only 5 to 20%. Normal fertilized eggs were recovered from 5 of 11 gilts killed 48 hours after insemination. These results were obtained by suspending the sperm rich fraction of boar semen in a Beltsville extender containing 5% glycerol and cooling to 5°C over a six hour period, then recentrifuged to remove the glycerol containing extender and resuspended in the same extender without glycerol. Immediately after resuspension, the

semen was frozen on dry ice by the pellet method and pellets transferred to liquid N_2 storage.

Although glycerol has been used as a cryophylactic agent to protect spermatozoa during the freezing process, there is evidence that glycerol is contraindicted for fertility (Crabo et al, 1970; Graham and Pace, 1967; King and MacPherson, 1966; Neville, MacPherson and King, 1970; Wales and O'Shea, 1968). Graham et al (1971) made the following general summation: Results on freezing boar semen with and without glycerol indicate that semen should be frozen without glycerol since the glutamic oxalacetic transaminase release is considerably reduced. The morphological changes closely followed the GOT release. The work also showed that while the post-freeze motility of glycerolated spermatozoa was good, most of the sperm cells had lost their acrosomes. The non-glycerolated semen had lower postfreeze motility but a higher percentage of sperm with normal acrosomes. This indicates that sperm cells without motility can still maintain a morphological integrity and may have the potential to fertilize eggs while the sperm cells with good motility may be damaged and be potentially ineffective in affecting fertility. The findings of Quinn et al (1969) regarding the profound changes in the appearance of the terminal and corrugated parts of the ram spermatozoa caused by cold shock and freezing and those of Graham et al (1971) and Pursell and Johnson (1971) concerning boar spermatozoa, indicate

the need for freezing and postthaw acrosome evaluation of ram spermatozoa.

Majur et al (1971) made some interesting studies on the interactions of cooling rate, warming rate and protective additives on the survival of frozen mammalian cells. Though their work was accomplished with mouse marrow stem calls and Chinese hamster tissue culture cells, the objectives and results of the work are such that they may be of value to workers who attempt to freeze spermatozoa.

In subjecting cells to freezing, the investigator must select:

1. The final temperature to be reached.
2. The cooling rate to that temperature.
3. The storage time.
4. The warming (or thawing) rate.

The objective of Majur and his associates was to determine the consequence of varying all four of these factors over much wider ranges (than normally used) to define the maximal conditions for survival and to gain an understanding of the causes of injury and the means of preventing it. Their work was also based on the premise that, regardless of the cooling and warming rate, most nucleated mammalian cells fail to survive freezing in the absence of a protective additive. These workers proposed that the existence of an optimum cooling velocity must mean that survival is affected by at least two factors that depend oppositely on cooling rate. Two factors that have been

suggested are "solution effects" and "intracellular freezing." According to this hypothesis "solution effects" would be responsible for the low survival of cells cooled at rates lower than optimal and intracellular freezing is responsible for the low survival of cells cooled faster than optimal. "Solution effect" as used here means the change in a solution or in protoplasm resulting from the progressive removal of water during freezing as caused by dehydration, increased solute concentration, changes in pH and precipitation of solutes, or a combination of all of these. An additive, such as glycerol, could protect cells from freezing damage by decreasing:

1. the magnitude of the "solution effects".
2. the susceptibility of a cell or cellular organelle to solution effects.
3. the likelihood of intracellular freezing, and
4. the sensitivity of the cell to damage from intrafreezing.

It has been believed that additives also protect on a molar basis by reducing the electrolyte concentration in the residual unfrozen solution in and around a cell at any temperature. To protect the cell interior, the additive must therefore permeate. Most workers have used glycerol and DMSO as additives, but have used them without knowing whether these compounds really permeate the cell under study. The efforts of Majur and associates were specifically made in the hopes that the following questions would be answered.

1. Do survival curves exhibit an optimal cooling velocity which would indicate the involvement of two or more factors? Are the results consistent with the belief that the two factors are "solution effects" and "intracellular freezing"?
2. Is the optimal cooling velocity always around $1^{o}C$ per minute, or is the optimal rate dependent on the type of cell and the type and concentration of additive?
3. Is the protection conferred by an additive solely a function of its molarity and ability to permeate a cell?

They conducted many freezing studies with these two types of cells using four additives: glycerol and DMSO as examples of low molecular weight, presumably permeating additives; sucrose as an example of a low molecular weight, presumably non-permeating additive; and polyvinylprrolidone (PVP) (mol. wt. 40,000) as a high molecular weight, non-permeating additive.

Their conclusions were: (1) $1^{o}C$ per minute is not necessarily the cooling rate that yields maximum survival. Optimal rates can differ by a factor of 2000 in different cells. Even in a given type of cell the optimum can vary with the conditions of freezing. A 50 fold shift in the optimum rate of freezing for stem cells was found when the concentration of glycerol was changed only three fold. (2) There is differential cell sensitivity to slow warming. The damage

observed in cells cooled slower than optimal can be accounted for in terms of "solution effects." (3) There was a lack of clear correlation between the ability of additives to protect and either their molar concentration or their ability to permeate the cell. This suggested that protection by additives does not depend solely on their reducing the concentration of electrolyte in partially frozen solutions. Regardless of their molarity, non-permeating additives were able to provide considerable protection and this suggests that the ability of a cell to survive freezing depends more on maintaining the cell surface than on protecting the cell interior. There are a number of indications that cell surface membranes are also extremely sensitive to freezing. (4) The optimum cooling rate for rapidly thawed marrow cells ranged from 2 to 100°C per minute and that for hamster cells from 2 to 350°C per minute, depending on concentration and type of additive. The most deleterious cooling rate for hamster cells in glycerol, PVP, and sucrose was 1.5°C per minute. Sucrose protected in spite of the fact that it could not permeate. Rapidly cooled cells were damaged more by slow warming than were slowly cooled cells. (5) These results suggest that two factors are associated with cell injury; one, cells that are cooled more slowly than optimal are injured primarily by solute concentration or dehydration; two, cells cooled more rapidly than optimal are injured chiefly by intracellular freezing and by recrystallization during warming. (6) Extracellular additives

can confer protection during slow cooling. This suggests that the cell surface membrane is especially susceptible to damage from freezing.

MATERIALS AND METHODS

Selection and Training of Rams

The study was initiated in September of 1969. Fifteen ram lambs were obtained from The Eastern Oregon Experiment Station at Union, Oregon. The breeding represented within this group was straight Columbia, straight Targhee, Hampshire X Columbia, and Suffolk X Columbia. Two Cheviot X Hampshire ram lambs were also obtained from the Oregon State University flock. The birth dates of these rams fell within the period of January 6, 1969 to February 28, 1969.

During the first training sessions a ewe in natural estrus, determined by vasectomized ram, was placed in a breeding chute and the young rams were allowed to serve this female. After two days of such training, collection with the artificial vagina was initiated. The breeding chute designed and used for these studies is shown in Figure 2 (Appendix I).

Marked differences in libido and temperament were observed in the rams during their early training (Table 1). This information may be of interest because it reflects the variations found in libido and breeding behavior of 17 sexually mature crossbred ram lambs. Equal training effort was given until final selection of six rams was made

Table 1. Ram Training Response.

Ram (ear no. tag)	Libido rating	Ancestry[a]	Classification ratings[b]	
			Positive	Negative
55H	10	H X C	A, B, C, D, E, F, H	
61H	6	H X C	A	I
35T	7	Targhee	A, B, G, F, H	
3T	8	Targhee	A, F	K
71C	8	Columbia	B, G, F, H	
57H	1	H X C		J
43S	8	S X C	B, G, F, H	
59H	9	H X C	B, C, D, E, F, H	
9255	9	Ch X H	B	I
93C	5	Columbia		I
7T	8	Targhee	B, F	K
77T	9	Targhee	B, G, E, F, H	
45S	8	S X C	B, F	K
63H	0	H X C		J
41S	7	S X C		I
39S	2	S X C		I
9261	0	Ch X H		J

[a] H = Hampshire; C = Columbia; S = Suffolk; T = Targhee; Ch = Cheviot

[b] A - Served estrous ewe at first opportunity on first training day.
B - Served estrous ewe on second training day.
C - Stood for successful collection with A. V. within first week.
D - Trained readily to reliable collections with A. V.
E - Trained to serve dummy ewe within 30 days.
F - Semen of uniform good motility and normal all other respects.
G - Wild initially but responded slowly and steadily to training.
H - Selected for thesis study.
I - Wild, served estrous ewe, but would not allow collection with A. V.
J - Would not serve estrous ewe in presence of researchers.
K - Would allow collection with A. V. infrequently; sporadic behavior.

approximately 70 days after initiation of training. It was felt that five rams were needed for the desired studies. However, it was also considered wise to have one spare. This turned out to be a good decision because one ram was lost due to urinary calculi shortly afterwards.

The information in Table 1 shows that eleven of the seventeen ram lambs did not respond to training with the artificial vagina. The training experience encountered with these ram lambs differs from the findings of others, especially the instance recorded by Emmens and Robinson (1962) concerning the successful training of 54 of 57 rams to the artificial vagina in two days. These differences may have been due to the ages of the rams involved. Emmens and Robinson did not mention the ages of the rams that they were working with. Perhaps mature rams with previous breeding experience would be much easier to train to the use of the artificial vagina than would ram lambs.

Method of Semen Collection

During the early phase of training, it was believed that the rams with high libido might be trained to regular collection using a dummy ewe. In actual experience, however, only three rams would serve the dummy during the training phase. They would only do so when there was a live ewe, inaccessible to the rams, in the breeding chute

and the dummy was placed beside the chute. The use of the dummy is slower than the use of the hand-held artificial vagina. When the dummy is used the vagina must be placed in it and removed from it after each collection. Because of this and the reluctance of the rams to serve the dummy, the method was discarded. The semen for this study was collected by use of the hand-held artificial vagina with a teaser ewe in the breeding chute. After the initial training a ewe not in estrus was found to be a satisfactory teaser animal. The type of ovine artificial vagina used in this study is shown in Figure 3 (Appendix I). All rams retained the natural mounting desire and would readily mount if collection was not promptly made with the A. V. One ram was used for natural mating during the breeding season of 1970-71 and covered a normal number of ewes that subsequently lambed very satisfactorily. This indicates that rams of strong libido may be trained to the use of the artificial vagina without impairing their willingness or ability to serve ewes naturally, and may be used for both methods of breeding concurrently.

Loose soil or bedding was brushed away from the sheath and underbelly of the ram prior to collection. The sheath and prepuce were wiped dry and clean with disposable paper tissue after each collection. It was found early in this work that there was a tendency towards a benign posthitus when the sterile lubricant (used in the artificial vagina) was allowed to remain on the ram's sheath. Soil

and bedding evidently adhered to this lubricant from one collection period to the next, and this caused small scabs to form around the periphery of the prepuce. This difficulty abated after care was taken to remove all possible lubricant from the ram's sheath after collection. Kopertox (Ayerst Laboratories) was used as an effective treatment for posthitus and generally healing was complete within 7 to 10 days.

Water at 58 - 60°C was used to fill the inside casing of the artificial vagina. This was considerably above the 41° - 42°C recommended by Terrill (1968). However, due to the small size of the sheep artificial vagina, temperature of the inside liner was found to drop rapidly. When inside liner temperatures were checked, just after filling, they were around 43 to 45°C in the cold months and as high as 50°C in the warm months of the year. The rams seemed to prefer these temperatures to lower temperatures. Five artificial vaginas were normally assembled and used when regular collections were made from the five rams. The use of a separate artificial vagina for each ram at each collection period prevented the mixing of semen samples and contamination. The artificial vaginas were washed and sterilized in accordance with the recommendations of Wolberg (1966). During cold weather, a protective glove was placed over the collection sleeve and tube to prevent cold shock of spermatozoa.

Methods of Semen Evaluation

In view of the extremely concentrated nature of ram semen and due to the fact that most workers have obtained the best results with relatively low rates of extension (1:1 to 1:6) the decision was made early in this study to develop a motility index similar to the one proposed by Emmens (1947). This decision was made after many attempts to extend ram semen to a dilution where the individual motile sperm could be counted in a microscope field. As reported by Cummings (1954), accurate counts could not be made due to individual spermatozoa moving in and out of the field so rapidly. This was especially true with fresh samples of good motility. The need to develop a motility index for ram semen was considered important because motility indexes in use have been developed for the semen of bulls, or other species with semen of lower spermatozoal concentration than that of the ram. There is no standardization among these, and many researchers use a motility index used by a previous worker without regard to the species under consideration. A motility index with ratings from zero to 10 was envisioned as being practical for ram semen work (Appendix II). The spermatozoal motility was evaluated microscopically with phase contrast objectives. Semen samples on a glass slide were placed on an incubator stage maintained at 38^{o}C. Fresh semen and thawed samples of high motility were

viewed in the free drop at 100 magnifications. Samples showing no definite swirl effect (rating of 6 or below) were examined under cover glass at 430x to determine the degree and type of individual sperm movement. The cover glass and higher magnification were used at all times for rating thawed semen. The use of the cover glass facilitated observation of individual sperm movement but the swirl effect could be seen under the cover glass as well as in the free drop.

The number of live spermatozoa in the sample was determined by the use of the eosin-fast green staining technique. The motility index and percent live sperm provide two measurements for spermatozoa viability. Percent survival is a third measurement that may be obtained when percent alive is known for two different time intervals. All three of these measurements were used in this study.

The Preparation of Differential Stain Slides

The differential stain was prepared by dissolving fast green at the rate of 2% and eosin at the rate of 0.8% in the appropriate buffer (Mayer et al, 1951). Speed and precision were found to be essential in the preparation of good slides. The hot plate was warmed to 300°F. A small drop of stain was applied to the center of a glass slide. The tip of a glass stirring rod was touched to the semen and swirled briefly in this stain. A second slide was placed over it and

in a single smooth movement the stain with semen in it was spread by pulling the two slides across each other lengthwise. This motion was very rapid and left a thin film of stain on each slide. The lower slide was dried immediately on the hot plate. Blowing on the slide during the drying process was found helpful. After the semen was touched to the stain all actions were smooth and rapid. The sperm will absorb dye if they are killed prior to the drying of the slide. Finished slides were examined microscopically to determine if they were of correct density, and contained sufficient unstained sperm to be representative of the sample of semen under consideration. If not, a new slide was prepared. A photomicrograph from a differentially stained slide of ram spermatozoa is shown in Figure 7 (Appendix I).

Equipment Used in Freezing

The semen freezing containers, their capacity and methods of freezing were as follows:

1. Glass ampules (1 ml) frozen in the Linda BF-4-1 Biological Freezer unit.
2. Pellet form (.04 ml) frozen in small depressions on a block of dry ice.
3. Glass capillary tubes (.9 X 1.1 mm inside diameter and

containing .04 ml) frozen on dry ice.

4. Glass capillary tubes (.5 X .9 mm inside diameter and containing .01 ml) frozen on dry ice.

5. Cassou type plastic straws (134 mm in length with a 2.7 mm inside diameter and containing .5 ml) frozen on dry ice.

The biological freezer unit consisted of a controller and a container. It had a Varian G-11A strip chart recorder electrically associated to provide continuous temperature tracing on chart paper during freezing cycles. A 160 liter liquid nitrogen container provided the freezing medium for this unit. This equipment, as set up for use, is shown in Figure 4 (Appendix I). The 1 ml ampules, .5 ml plastic straws, and two sizes of capillary tubes are shown in Figure 5 (Appendix I).

The ampules were sealed with a Cossolli Perfektum flame ampule sealer. All ampules were marked with waterproof printers' ink for identification and stored on canes in portable frozen semen refrigerators. Plastic straws, capillary tubes, and pellets were also stored in ampules or glass tubes that could be stored on the standard refrigerator canes.

The freezing of semen in pellet form was done in a cold room at 5^{o}C. Small depressions were made in a series of rows on a dry ice block by use of a warm glass stirring rod with rounded end. The

semen was then dropped into the depressions from a calibrated capillary tube. The technique is shown in Figure 6 (Appendix I). Thawing procedures varied slightly in the studies and are discussed in the method section.

Pilot Studies

Several studies were conducted at the beginning of this work to develop an extender that would best preserve viability and fertilizing capacity of ram sperm during the freezing and thawing process.

The first of these studies was made to determine the importance of tonicity as it affected ram spermatozoa in dilution. A series of solutions was prepared by adding 5, 8, 9, 10, 11, 11.5, 12, 13, 14, and 15 g of sucrose respectively to 100 ml of distilled water. To each 100 ml solution, 0.1 ml of fresh ram semen of good motility was added. The flasks with contents were kept at refrigerator temperature for seven days. Eosin-fast green slides were then prepared of the spermatozoa from each of these solutions. The morphology of the stained spermatozoa was examined at 970x. The number of abnormal spermatozoa per 100 was counted. Three counts (for each slide) were made by each of three different workers. The results of this study are given in Table 2.

The lowest percentage of abnormal spermatozoa, for each ram, was found in the 100 ml solution containing 11 g of sucrose, the

concentration that has a freezing point depression of 0.62°C with an osmolarity equivalent to 0.179 M sodium chloride.

These findings prompted another pilot study designed to test the importance of solution tonicity to spermatozoa viability. A series of solutions was prepared by adding 0, 5, 8, 9, 10, 11, 11.5, 12, 13, 14, 15, and 20 g of sucrose respectively to 100 ml of distilled water. To 50 ml of each of the above solutions, 0.1 ml of fresh ram semen was added while the semen and sucrose solutions were each at room temperatures. An eosin-fast green slide was prepared from the fresh sample of semen for determination of initial percent alive. The flasks containing semen in sucrose solutions were then placed in the refrigerator and allowed to cool to 5°C. This required approximately 90 minutes. These solutions were kept at a refrigerator temperature of 5°C for four days. Eosin-fast green differential stain slides were then prepared of the spermatozoa in each solution. These slides were evaluated at 387x to determine post-storage percent alive. Three counts (on each slide) were made by two workers. The percent survival was computed for each solution. The results are shown in Table 3.

These pilot studies indicated that extenders with a molarity close to 0.179 M of NaCl should be tested against others of different molarity that have been used with success for the dilution of ram semen. With this in mind, a third pilot study was designed to

compare five different extenders in their ability to preserve the viability of ram spermatozoa at 5°C in the presence of an antibiotic. The antibiotic "Streptillin" (Trico Pharmaceutical Company) was added to all test extenders at the rate of 1000 IU of procaine penicillin G and 1.25 mg of dihydrostreptomycin sulfate equivalent per ml of extender. The five extenders tested were:

1. Non-fat dry milk. This extender was based upon extenders used by Jones (1965). It was prepared by adding 50 g of the dry milk to 400 ml of distilled water. The resulting solution contained 11.4% milk solids w/v. This was similar to the reconstituted milk diluent used by Martin (1961). Jones (1965) proposes that 9% w/v milk is isosmotic to ram spermatozoa.
2. Egg yolk-citrate extender as described by Salisbury et al (1941) for bovine semen. It was prepared by adding 2.9% sodium citrate buffer to an equal volume of egg yolk. The pH of the buffer is 6.85 and the molarity is equivalent to 0.152 M of NaCl.
3. Boric acid-sodium bicarbonate-egg yolk extender. A buffer consisting of two parts of a 2% boric acid solution and one part of a 1% sodium bicarbonate solution was described by Yoshioka et al (1951) as being very satisfactory for sheep and goat semen. The pH of this buffer

is 7.0 with a molarity equivalent to 0.251 M of NaCl. This buffer was added to egg yolk at proportions of 85% buffer to 15% egg yolk (v/v).

4. Non-fat dry milk-egg yolk. This extender was prepared by adding a 11.4% solution of non-fat dry milk to equal volume of egg yolk. This modification of the Jones extender was based upon the findings of Sikes and Merilan (1958). They reported that the addition of egg yolk to skim milk was beneficial in maintaining motility of bovine spermatozoa at storage temperatures approaching $0^{o}C$.

5. Egg yolk-citrate-lactose. This extender was based on findings of Salamon (1968) that showed lactose to give excellent results for fast freezing ram semen when used with egg yolk and citrate. The buffer was prepared by adding 2.9 g of sodium citrate, and 1 g of lactose to 100 ml of distilled water. The pH of this buffer is 7.1 and the molarity is equivalent to 0.176 M of NaCl. The extender was prepared by adding 85 ml of the citrate-lactose buffer to 15 ml of egg yolk. The quantities of lactose and sodium citrate used here differed from those used by Salamon (1968). These changes were made to bring the molarity of the extender close to 0.179 M of NaCl, the osmotic condition that was found to be most

favorable to ram spermatozoa in the two previous pilot studies.

Ten ejaculates of ram semen with excellent motility were selected for this third pilot study. Differential stain slides were made from the fresh ejaculates and they were then each split into five equal aliquots. These aliquots were extended at room temperature in the five test extenders at an extension rate of 1:3. The 50 extended samples were placed in the refrigerator in a water bath that allowed cooling to 5°C over a period of 90 minutes. The samples were kept at refrigerator temperature for seven days and then differential stain slides were prepared to determine percent survival. Five counts of 100 sperm each were made from each slide. The percent survival of each sample was obtained by comparing the seven day percent alive with that of the fresh ejaculate.

The results are shown in Table 4. Test extender number 5 gave the best survival rates and also provided a clear microscopic field for study of spermatozoa movement. The motility of individual spermatozoa was difficult to discern in test extenders No. 1 and No. 4. This was in line with the findings of Salisbury et al (1941) who reported that citrate buffer had the property of dispersing fat globules in extenders containing egg yolk.

Studies were then made to test the effects of three cyroprotective agents on the viability of ram spermatozoa extended in the

egg yolk-citrate-lactose extender. The three agents tested were glycerol, dimethylsulfoxide (DMSO) and polyvinyl pyrrolidone (PVP).

The results indicated that these substances, alone at a level of 3% (v/v) in the extender, or in combinations comprising as high as 6% (v/v) of the extender, were not toxic to ram spermatozoa stored at 5°C for periods as long as seven days.

Freezing of ram semen was then accomplished in pellet form on dry ice with these three life-protectors in the egg yolk-citrate-lactose extender. The only promising results were obtained with the extenders containing glycerol alone, or containing a combination of glycerol with DMSO or glycerol with PVP. The best survival and the best motility, however, was always found in the thawed samples containing glycerol alone in the extender. The following extender and life-protector combination therefore evolved as the best tested in pilot freezings.

Sodium citrate, dihydrate	2.4% (w/v)
Lactose	0.8% (w/v)
Egg Yolk	14.3% (v/v)
Glycerol	4.8% (v/v)

Streptillin (Trico Pharmaceutical Company) at rate equivalent to 1000 IU of procaine penicillin G and 1.25 mg of dihydrostreptomycin sulfate equivalent per ml of extender.

The citrate-lactose buffer has a pH of 7.1 and a freezing point

depression within the parameters given in the literature for ram semen, and near that found most favorable to ram spermatozoa in these pilot studies.

Table 2. The morphology of ram spermatozoa held for seven days at 5° C in different concentrations of sucrose solutions.

	Grams sucrose/ 100 ml water	Freezing point depression (° C)	Equivalent osmolarity of NaCl (g mole/l)	Percentage abnormal spermatozoa
Ram no. 59	5.0	0.28	0.080	37
	8.0	0.45	0.129	29
	9.0	0.51	0.146	24
	10.0	0.56	0.163	24
	11.0	0.62	0.179	18
	11.5	0.65	0.189	30
	12.0	0.68	0.196	29
	13.0	0.74	0.214	43
	14.0	0.80	0.233	44
	15.0	0.85	0.248	31
Ram no. 55	5.0	0.28	0.080	42
	8.0	0.45	0.129	38
	9.0	0.51	0.146	29
	10.0	0.56	0.163	35
	11.0	0.62	0.179	19
	11.5	0.65	0.189	23
	12.0	0.68	0.196	24
	13.0	0.74	0.214	26
	14.0	0.80	0.233	52
	15.0	0.85	0.248	38

Table 3. The percentage survival of ram spermatozoa held for four days at 5° C in different concentrations of sucrose solutions.

Grams sucrose / 100 ml water	Freezing point depression (° C)	Equivalent osmolarity of NaCl (g mole/l)	Survival[a] (%)
0.0	0.00	0.000	0.0
5.0	0.28	0.080	0.8
8.0	0.45	0.129	1.7
9.0	0.51	0.146	4.3
10.0	0.56	0.163	4.1
11.0	0.62	0.179	6.5
11.5	0.65	0.189	2.2
12.0	0.68	0.196	4.1
13.0	0.74	0.214	0.8
14.0	0.80	0.233	0.0
15.0	0.85	0.248	1.3
20.0	1.15	0.336	0.8

[a]Data subjected to arcsin transformation.

Table 4. The percentage survival of spermatozoa from ten ejaculates of ram semen each divided into five aliquots and held for seven days at 5° C in test extenders.

Test extender	Osmolarity	pH	Survival[a] (%)
1. Non fat dry milk	isosmotic	7.10	10.5
2. Egg yolk-citrate	0.152	6.85	14.5
3. Boric acid-sodium bicarbonate-egg yolk	0.251	7.00	13.9
4. Non fat dry milk-egg yolk	isosmotic	7.10	7.0
5. Egg yolk-citrate-lactose	0.176	7.10	21.2***

[a]Determined by differential stain technique. Arcsin transformation made.

***Significantly different from Test extender 2, $P < 0.001$.

EXPERIMENTAL RESULTS

Experiment 1. A Comparison of Freezing in Ampules, Pellets and Glass Capillary Tubes

Design and Methods

This experiment was designed and conducted to compare four methods of freezing ram semen and to compare spermatozoan viability at two postfreeze intervals. The experiment also allowed a comparison of the freezability of the semen of different rams.

The four methods of freezing were (1) in 1 ml ampules in the Linde Biological Freezing Chamber. (2) In the form of .04 ml pellets on dry ice. (3) In glass capillary tubes (0.9 X 1.1 mm ID) on dry ice, and (4) In glass capillary tubes (0.5 X 0.9 mm ID)on dry ice. The motility was determined at 3 days and at 7 days after freezing.

The egg yolk-citrate-lactose diluter was used in this experiment. The prefreeze extension rate was 1 part of semen to 3 parts of diluter. All semen collections were by artificial vagina. Split ejaculates were utilized to obtain five freezings in each container for each of the five rams. Ejaculates were collected in plastic vials which were sealed and placed in a water bath at 18^{o}C within a few minutes after collection. The fresh semen was then evaluated for motility and percent live sperm. Ejaculates with a motility rating

less than 8 were not used for freezing. The sperm concentration of the ejaculates chosen for freezing was determined with hemocytometer.

Extension of semen was made in three equal steps. The first extension of 1:1 was made with non-glycerolated extender at 18 to 20°C immediately after evaluation. The extended sample was sealed in a small plastic vial in a water bath and placed in the refrigerator. This allowed gradual cooling to 5°C over a period of three hours. The second extension was then made with extender and sample at 5°C. This second extension was made with extender that contained 4.8% glycerol (v/v) and the original semen was now in an extension of 1:2. The sample was placed back in the refrigerator and allowed to equilibrate for three more hours. The final extension was then made with sample and extender at refrigerator temperature and the extender containing 9.6% glycerol (v/v). The semen, extended 1:3, was allowed to equilibrate overnight in the refrigerator. It was then placed in freezing containers and frozen on the morning of the day after collection. The ampules were flame sealed and frozen in the Linde Biological Freezer at the slow rate shown in Figure 8 (Appendix I). The semen in pellet form and in glass capillary tubes was frozen on a dry ice block in a cold room with the air temperature at 5°C. Ampules, pellets, and glass capillary tubes were placed in liquid nitrogen storage (-196°C). The extension of 1:3 resulted in sufficient quantity of extended semen to allow the freezing of three 1 ml ampules, three

groups of eight pellets, and three each of the two different sizes of glass capillary tubes. This allowed one sample of each for thawing three days postfreezing, one sample for thawing seven days post-freezing and one for future laboratory or insemination use.

Results and Discussion

This was a 5 X 4 X 2 factorial experiment containing five rams, four methods of semen freezing, and two thawing times at which motility readings were made. The results are presented in Table 5. The data were examined by factorial analysis of variance (Steel and Torrie, 1960). When specific treatment comparisons were desired, they were obtained by use of the "Student's t" test, for paired observations.

There were highly significant differences between the four freezing methods. The semen samples frozen in ampules and in pellets were each significantly superior ($P<.001$) in postfreeze motility to those frozen in the capillary tubes. The samples frozen in ampules were also significantly better ($P<.05$) in postfreeze motility than those frozen in pellet form on dry ice. This significant difference was interesting. Salamon (1968) found no significant differences between postfreeze motility scores of ram semen frozen in ampules at a slow rate or in pellet form on dry ice. He did not freeze the ampules in a biological freezer unit. He placed the sealed ampules

(at 5°C) directly into an alcohol bath at -20°C. By adding dry ice he lowered the temperature of the bath two degrees C per minute to -50°C; then five to ten degrees per minute to -75°C; then over one hundred degrees per minute to -196°C. These are signally different rates of freezing than the controlled rates accomplished in the biological freezer in this experiment. It is possible that the carefully controlled rates provided by the biological freezer unit will give better sperm survival than will freezing in pellet form on dry ice or in ampules in an alcohol bath (where the rate of freezing is difficult to control precisely).

Spermatozoa survival was poor in the glass capillary tubes frozen on dry ice. A few sperm were observed to be moving progressively in 47 of 50 observations in the larger glass tubes but no motile sperm were observed in 36 of 50 observations of semen frozen in the small tubes. Although the paired t test accomplished on the postfreeze motility readings of these two sizes of capillary tubes showed that the semen frozen in the larger tube had significantly ($P<.001$) better postfreeze motility than that frozen in the small tube, both were inferior to the ampule and pellet form methods. The literature does not reveal experiments with the freezing of ram semen in glass capillary tubes. Parkes (1945) reported the freezing of undiluted human semen in three sizes of glass tubes and as a thin film in a platinum loop. The sizes of the glass tubing used were .15

mm I. D., .5 mm I. D., and 1.0 mm I. D. Upon thawing at 37°C no motile spermatozoa were observed in the semen frozen in thin film or in the smallest glass tube but many were motile in the semen frozen in the two larger glass tubes. He concluded in this early work that sperm do not survive when minute amounts are frozen as films or in fine capillary tubes. He also mentioned that a surface effect of some kind was indicated. Majur et al (1971), after extensive cryogenic studies, suggest that the ability of a cell to survive freezing depends more on maintaining the cell surface than on protecting the cell interior. The results obtained with the freezing of ram semen in glass capillary tubes in this experiment are in line with the findings of Parkes (1945) and Majur et al (1971).

No significant differences were found in the motility readings made after three days of storage in liquid nitrogen compared to those made after seven days. This was compatible with the findings of Szumowski, Markovic, and Cano (1956) who found no decline in the motility of ram spermatozoa stored for four weeks at -79°C. Hill, Godley, and Hurst (1959) found that frozen ram spermatozoa lost motility between two days and ten days storage. First et al (1961) also reported that the motility of ram sperm declined during the first two months of frozen storage. Unfortunately neither of the last two references stated whether the decline in motility was statistically significant. There is no complete agreement in the literature

concerning the findings on the trend of postfreeze motility of ram sperm. However, the important consideration remains the need to determine how ram sperm will maintain fertilizing capacity during frozen storage. The finding in this experiment that showed no change in motility between three days and seven days of frozen storage is therefore encouraging because motility is considered to be one of the most reliable criteria for assessing sperm fertilizing capacity.

No significant differences were found in motility readings between or among the five rams. This was in contrast to the findings of Salamon (1968) who found that the semen of five mature Merino rams differed significantly in freezability. The great differences that exist relevant to spermatogenesis in the ram and the interactions that are almost constantly present preclude the possibility of obtaining the same results with the semen of all rams at all times. Although no significant differences were found here, no inferences should be drawn concerning future similarities in the freezability of the semen of these five rams.

Experiment 2. A Comparison of Freezing at Two Controlled Rates and in Pellets on Dry Ice

Design and Methods

Various rates of freezing ram spermatozoa in ampules have been reported. The rates used in Experiment 1 were based upon

Table 5. Spermatozoan motility of ram semen frozen in 1 ml ampules in a controlled freezing chamber, in pellet form on dry ice, and in two sizes of glass capillary tubes on dry ice.

Ram identification number	Volume/ ejaculate n = 5	Fresh motility n = 5	Concentration (1×10^9/ml) n = 5	Spermatozoa motility after freezing and thawing							
				Treatment 1 Ampules n = 5		Treatment 2 Pellets n = 5		Treatment 3 Glass tubes (.9 x 1.1 mm I.D.) n = 5		Treatment 4 Glass tubes (.5 x .9 mm I.D.) n = 5	
				3 days	7 days	3 days	7 days	3 days	7 days	3 days	7 days
55	1.16	9.6	3.54	4.2	4.0	3.8	3.6	1.8	2.0	0.4	0.2
59	1.26	10.0	4.02	5.4	4.8	5.0	5.0	2.4	1.8	0.2	0.4
35	1.18	9.6	4.26	4.4	5.0	5.0	4.6	1.8	1.8	0.2	0.2
71	1.16	9.4	3.76	5.0	5.0	4.4	4.4	2.2	1.6	0.2	0.0
43	1.22	9.6	4.44	4.8	4.8	4.8	4.4	1.8	2.0	0.6	0.4
Grand $\bar{x}$ n = 25	1.19	9.6	4.00	4.76***	4.72*,***	4.60***	4.40***	2.00***a	1.84***a	0.36	0.24

*$P < 0.05$ compared to Treatment 2, 7 days.

***$P < 0.001$ compared to Treatments 3 and 4.

***a $P < 0.001$ compared to Treatment 4.

techniques given by Almquist (1968) and Mann (1964). The results of Experiment 1 indicated that these rates were satisfactory for ram semen and resulted in thawed motility indices slightly higher than pellets frozen on dry ice. Lopatko (1962) and Salamon (1967) reported a slightly different rate that gave satisfactory recovery of spermatozoa after freezing and thawing. This rate was slower to -75°C and then much faster to -196°C than the rate used in Experiment 1.

The literature review did not disclose freezing studies with ram semen frozen in ampules in a controlled freezing chamber with attached recorder and charts to show the actual freezing rates. Experiment 2 was therefore conducted to compare two controlled rates of ampule freezing with pellet freezing on dry ice. Since the rate of ampule freezing used in Experiment 1 had given satisfactory results, the decision was made to compare it with a rapid controlled rate, and with pellets frozen on dry ice.

The rapid controlled rate was obtained by doubling the values of each of the freezing steps used for ampule freezing in Experiment 1. A pilot freezing cycle was run to determine if the Linde equipment would operate satisfactorily at these rates, and if spermatozoa survival would be obtained. The equipment functioned smoothly and spermatozoa survival was encouraging at the new rapid rate. The two rates of ampule freezing used in Experiment 2 are shown in Figure 8 (Appendix I).

The egg yolk-citrate-lactose-glycerol extender and 1:3 extension rate used in Experiment 1 were selected for use in Experiment 2 because:

1. This extender and extension combination gave promising results in Experiment 1.
2. Their use would allow additional comparisons of the results between slow ampule and pellet freezings obtained in Experiment 1. It would also provide additional information on the freezability of the semen of each ram.

Semen was collected by artificial vagina. Split ejaculates were utilized to obtain five freezings of each of the three treatments for each of five rams. Only ejaculates with a fresh motility rating of eight or better were used. The evaluation, semen handling and equilibration procedures were the same as used in Experiment 1 with the following exceptions: The sperm motility and percent alive were determined by thawing one sample of each treatment seven days after freezing. Differential stain slides were prepared from the fresh semen, from the extended and equilibrated semen just prior to freezing and after freezing and thawing. The percent survival was computed by comparing the seven day postfreeze percent alive with the prefreeze percent alive.

Pellets were again frozen in groups of eight. The extension of 1:3 resulted in sufficient semen to allow the freezing of two 1 ml

ampules, for each rate of ampule freezing and two groups of eight pellets (0.4 ml) for freezing on dry ice. This allowed one sample of each treatment for thawing at seven days postfreezing and one sample for future laboratory study or insemination use.

Thawing of ampules and pellets was accomplished at 40°C in four volumes of the egg yolk-citrate-lactose-glycerol extender. Motility readings and differential stain slides were made immediately after thawing. Only one sample was thawed at a time to insure accuracy. Motility readings were made using the index shown in Appendix II. All motility data was recorded for each ampule and each group of eight pellets as they were examined under the microscope on a slide maintained at 37°C by incubator stage.

Results and Discussion

This was a 5 X 3 factorial experiment containing five rams and three methods of semen freezing. The data were examined by factorial analysis of variance. Separate analyses were performed for motility and for percent survival. Differences between specific treatment means, when required, were obtained by paired t test.

As shown in Table 6, there were significant differences between the three freezing methods. Treatment 2 was superior to treatments 1 and 3 in postfreeze motility ($P<.001$) and percent survival ($P<.01$). There were no significant differences between treatments 1 and 3 in

postfreeze motility or percent survival.

A significant ($P<0.05$) difference was found between rams in postfreeze sperm motility. This is in contrast to the findings in Experiment 1. The semen for all freezings in the first experiment were collected in the months of May through September while collections for the second experiment were made from November through March. Seasonal factors are known to influence spermatozoal viability and the extent of this influence is also known to vary among rams (Emmens and Robinson, 1962). Perhaps seasonal effects account for the fact that postfreeze motility differences existed between rams in this experiment but did not exist in Experiment 1.

Standard regression and simple correlation coefficients were computed to determine the relationship between the motility indices and percent alive measurements of the postfreeze samples of all three treatments in this experiment. The results are shown in Table 7. Significant correlations between motility and percent alive were found for all three treatments. The postfreeze motility readings obtained were therefore directly related to the number of live spermatozoa in the thawed semen.

The superiority of the rapid rate of controlled ampule freezing in comparison with a slower controlled rate and with pellet freezing was considered the most important finding in Experiment 2. Results such as these obtained with a controlled rate of freezing ram

spermatozoa in a biological freezer are not believed to have been reported upon by others to date. This finding could be of value not only because of the advantage in sperm survival but because there may also be advantages in thawing procedures that may be used with semen frozen in ampules, compared to pellets or straws. These advantages concern the temperature at which ampules may be thawed in the field. Pickett (1971) recommended that ampules containing approximately 1 ml of bovine semen be thawed in ice water at 1°C or in a water bath at 40°C. He also concluded that the difficulty of maintaining water at 40°C in the field plus the danger of cold shock when the semen is drawn into a cold catheter for insemination, eliminates thawing at 40°C. Thus, according to Pickett, thawing in ice water becomes the method of choice. Majur et al (1971) concluded that rapidly cooled cells were damaged more by slow warming than were slowly cooled cells. Spermatozoa frozen in pellet form or in plastic straws are customarily frozen at a very rapid rate in comparison with those frozen in ampules even at the rapid rate used in this experiment. Salamon (1968) found that ram semen frozen in pellets on dry ice gave higher survival rates when thawed at 45, 40 or 30°C than at 20, 15, 10 or 5°C. Aamdal and Anderson (1968) described the freezing of ram semen in plastic straws and recommended a thawing temperature of 75°C.

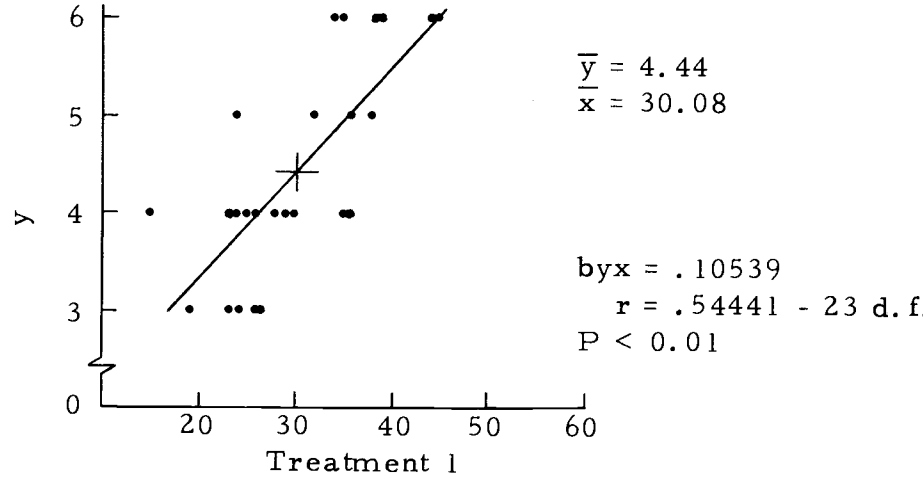
The freezing of ram semen in ampules at an optimum controlled

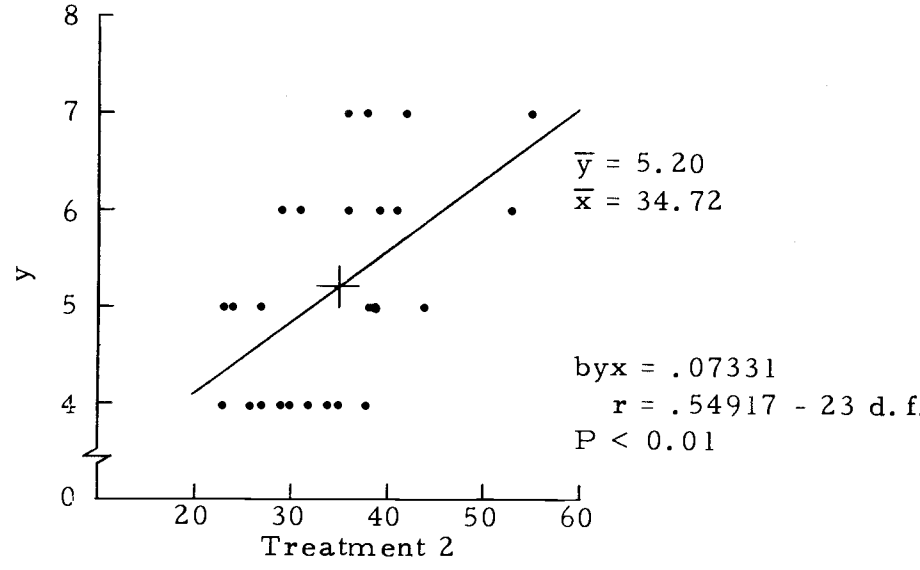
Table 6. Comparative motility and percent survival of ram spermatozoa frozen at two different speeds in a controlled freezing chamber and in pellet form on dry ice.

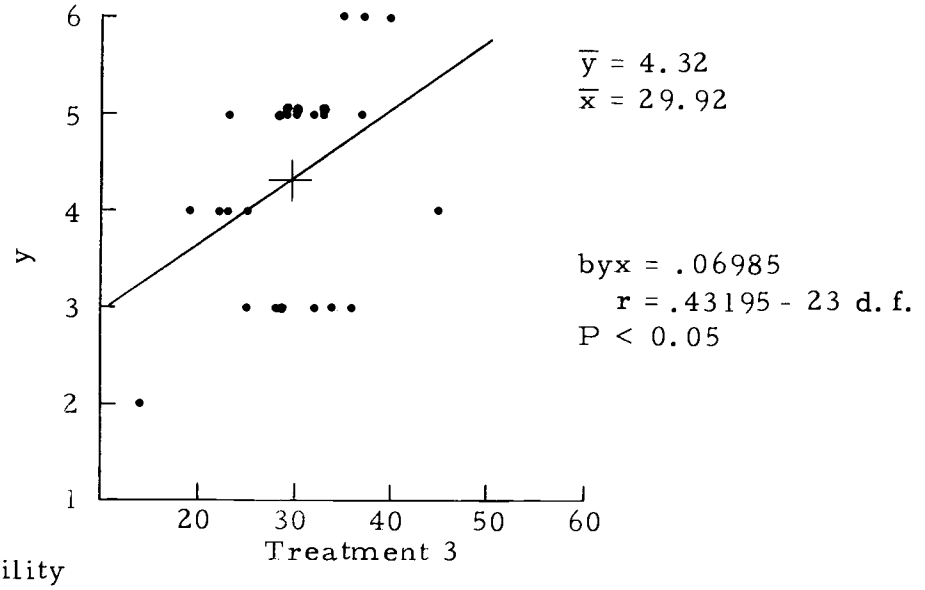
Ram identification number	Volume/ ejaculate (n = 5)	Fresh motility (n = 5)	Concentration (1×10^9/ml) (n = 5)	Live sperm		Treatment 1 Slow rate controlled freezing in ampules (n = 5)		Treatment 2 Rapid rate controlled freezing in ampules (n = 5)		Treatment 3 Pellet form on dry ice (n = 5)	
				Fresh % (n = 5)	Prefreeze % (n = 5)	thawed motility	thawed survival (%)	thawed motility	thawed survival (%)	thawed motility	thawed survival (%)
55	1.13	9.2	3.90	69.6	64.6	4.2	51.9	4.8	53.9	4.4	42.9
59	1.14	9.8	3.82	81.0	76.0	5.0	44.4	6.2	55.8	5.0	41.7
35	0.91	9.0	4.66	72.8	66.4	4.8	40.1	5.4	42.2	4.0	39.6
71	1.40	9.8	4.68	75.4	70.8	3.8	37.1	4.4	40.2	3.8	38.4
43	1.20	9.0	5.66	72.6	75.2	4.4	39.5	5.2	40.7	4.4	41.0
Grand $\bar{x}$ n = 25	1.14	9.4	4.54	74.3	70.6	4.4	43.5	5.20***	49.9***	4.3	42.5

***$P < 0.001$ compared to Treatment 1 or 3.

Table 7. Correlation and regression of motility and percent alive (Exp. 2).







y = motility
x = percent alive

rate in a Biological Freezing unit may hold promise for increased viability and decreased damage from cold shock at thawing time.

Experiment 3. The Effect of Added Enzymes on Thawed Viability

Design and Methods

One of the important problems in the area of artificial insemination, as it applies to sheep, is the low birth rate resulting from the use of frozen semen. Many workers report satisfactory motility and percent survival of ram spermatozoa after freezing but no worker has obtained lambing rates from frozen semen that compare favorably with natural mating.

Improvement in this situation may not come until many problems in the freezing of ram spermatozoa are solved. One area that appears to hold promise is the use of enzymes added to extenders as reported by Kirton, Boyd and Hafs (1971). Working with bovine spermatozoa they found significant increases in fertility, thought to be due to enzyme ability to mimic sperm capacitation and thus reduce the *in vivo* residence time required for spermatozoa to accomplish fertilization.

This could be an important factor with frozen ram spermatozoa. Before this can be determined, preliminary freezings of ram semen with enzymes in the extender must be made to test the effect of

enzymes upon postfreeze spermatozoan viability.

Experiment 3 was conducted for this purpose, using the three enzymes reported to be effective with bovine semen i.e. alpha amylase, beta amylase, and beta glucuronidase. These enzymes were added to the egg yolk-citrate-lactose-glycerol extender at the rate of 10 μg per ml of extender. The work of Sullivan and Elliott (1971) was used as a basis in arriving at the amount of enzyme to add to the extender. The antibiotic content of the extender was kept identical to that used in Experiments 1 and 2.

Three methods of freezing were selected for comparison to determine whether the enzyme content of extender would interact with rate of freezing. The three methods selected were: (1) Freezing in 1 ml glass ampules at the controlled rapid rate used in Experiment 2. (2) Freezing in 0.5 ml Cassou type plastic straws on dry ice. (3) Freezing in 0.04 ml pellets on dry ice.

A change was made in the periods of time allowed for equilibration with glycerolated extender in this experiment. A new equilibration procedure was found to give slightly better results than the three hour intervals that elapsed between the second and third steps of extension in experiments 1 and 2. The new procedure adopted for experiment 3 was as follows:

1. Extension 1:1 was made with semen and non-glycerolated extender at 18°C immediately after motility evaluation.

The extended semen (in 18°C water bath) was placed in refrigerator at 5°C.

2. Extension made with glycerolated extender and 1:1 extended semen at 5°C eleven hours after first extension. Semen now at 1:2 extension.

3. Extension was made with glycerolated extender and 1:2 extended semen at 5°C one hour later, twelve hours after first extension.

4. Semen in final extension (1:3) was now placed back in 5°C storage for one more hour and then into freezing containers. The ampules were sealed and freezing accomplished in the Linde controlled freezing chamber or on dry ice in a cold room.

This experiment had four treatments:

1. A control, frozen in extender containing no enzyme.

2. Semen frozen in extender containing alpha amylase.

3. Semen frozen in extender containing beta amylase.

4. Semen frozen in extender containing beta glucuronidase.

Each sample of semen was divided into four equal aliquots prior to first extension and after extension as indicated above each aliquot was frozen in 1 ml ampules, in plastic straws and in pellet form. No less than two ampules, two straws, or two groups of eight 0.04 pellets were considered sufficient for determination of postfreeze

viability. With the 1:3 extension, the quantitative requirement for each initial semen sample was slightly in excess of 4 ml. This necessitated the use of pooled samples. Collections were made from all five rams and those samples of excellent motility (9 or 10) were pooled for each freezing in this experiment. Sufficient freezings were made to give five observations for each of the four treatments. Thawings were made after the samples had been in liquid nitrogen storage for seven days.

Thawing of ampules and pellets was made in three volumes of the extender at 40°C. Thawing of the plastic straws was at 75°C for 12 seconds according to the method of Aamdal and Anderson (1968). One change was made in their procedure. They recommended that the plastic straws be thawed by holding for 12 seconds in water at 75°C. Instead of risking damage to spermatozoa by water, the straws in this experiment were thawed for 12 seconds in extender at 75°C. The motility readings and differential slides were made immediately after thawing. Only one ampule, straw, or group of 8 pellets were thawed at a time.

Results and Discussion

This was a 4 X 3 factorial experiment containing four enzyme treatments and three methods of freezing. All semen samples were of pooled semen divided into four equal aliquots.

The results are presented in Table 8. Separate factorial analyses were accomplished for motility and percent survival. When specific treatment comparisons were desired, they were obtained by paired t test.

There were no significant differences found in motility or in percent alive between or among the control and the three enzyme treatments. The three enzyme treatments were each slightly higher in percent survival than was the control. These differences were small and did not approach significance at the 0.05 level. The semen frozen in alpha amylase had the highest motility and also the highest percent alive. The results of this experiment indicated that these enzymes may be added to ram semen extenders without significantly affecting the postfreeze motility or percent survival of spermatozoa. This may be an important finding in view of the increases in fertility that have been obtained by addition of these enzymes to bovine semen prior to freezing. No significant interactions existed between the enzyme treatments and the freezing methods.

There were significant differences between freezing methods in motility and also in percent survival. There were no significant differences between the ampule freezings and the Cassou type plastic straw freezings. The plastic straw method was found significantly superior ($P<.001$) in motility and percent survival to the pellet method. This is in contrast to the findings of Salamon (1968) who

obtained significantly better motility from ram semen frozen in pellet form on dry ice than from that frozen in synthetic straws on dry ice. In his work the synthetic straws were thawed at 37°C in contrast to the 75°C for 12 seconds used here. This may have accounted for the difference. Aamdal and Andersen (1968) found about 17% more unstained spermatozoa in ram semen frozen in straws and thawed at 75°C for 12 seconds than at 35°C. The Cassou type plastic straws frozen on dry ice and thawed in extender (or other isotomic medium) at 75°C for 12 seconds may hold promise as a means of freezing and storing ram semen. This method would have an economic advantage over the controlled ampule freezing method due to the lesser requirement for expensive equipment and exact procedures. On the other hand, the straw freezing method may not be as practical or successful in the field due to the critical temperature requirement for thawing and the expected greater susceptibility of rapidly frozen spermatozoa to cold shock when compared to those frozen slower and thawed at ice water temperature.

The results obtained with the ampules in this experiment were not as good as those obtained with ampules frozen at the rapid rate in Experiment 2. The same was true for the pellet method. Three factors differed from the conditions prevailing during Experiment 2. These factors were the equilibration procedure prior to freezing, the addition of enzymes to the extender, and the time of year that

the semen was collected. The new equilibration procedure was given a thorough testing in several enzyme freezings made between Experiments 2 and 3. It gave better results in each case than the old method; so it is not believed to be the cause of the differences. The addition of enzymes can not be considered a causative factor because the control treatment, in Experiment 3, had a lower percent survival than did any of the treatments containing enzymes. The season of collection may be a factor to consider in the difference. In Experiment 2, all semen was collected during November, December, January, February or March. In Experiment 3, all semen was collected during June, July, and August. Salisbury (1968) found that at 39 to 40° north latitude, bulls (not females) were responsible for the seasonal variations in fertility. His work showed that the fertility obtained with semen collected in the six months of May through October was significantly lower than that obtained with semen collected during the other months. These results were confirmed by Sullivan and Elliott (1968) with bulls housed at approximately 43° north latitude. They found that the 60 to 90 day non-return rate for semen collected in the fall and winter was significantly higher than for semen collected in the spring and summer. This was surprising because these bulls were maintained in a temperature controlled environment. If such differences are true for bulls then perhaps the same is true for rams and the differences may be more

complicated due to scrotal wool covering and the seasonal breeding pattern of sheep. Though no comparable work pertaining specifically to seasonal variation in fertility of frozen ram semen is known to exist, the work of McKenzie and Phillips (1934) and of Rathore (1970) as well as the reports by Emmens and Robinson (1962) support the premise that seasonal differences exist and that ram spermatozoa maturing in the fall and winter months may be more viable than those maturing in the spring and summer. Seasonal effects may have played some role in the difference observed between ampule and pellet freezings in Experiments 2 and 3. This is recognized as a fertile field for additional work. The determination needs to be made regarding freezability-fertility advantages of ram semen produced in the fall and winter months compared to that produced in the spring and summer.

Experiment 4. The Effect of Holding in Seminal Plasma Prior to First Extension

Design and Methods

Graham and his co-workers (Graham et al. 1971), Rajamannan, Graham, and Schmehl (1971) reported beneficial effects by holding boar and bovine semen in seminal plasma for short periods of time at room temperature prior to extension. Experiment 4 was conducted to study the effect of "holding time" in seminal plasma on

Table 8. Comparative motility and percent survival of ram spermatozoa frozen with enzymes in extender and frozen in ampules, pellet form, and plastic straws.

Treatment	Fresh (n = 5)		Preefreeze (n = 5)		Postfreeze motility and survival						Grand $\bar{x}$ (n = 15)	
					In 1 ml ampules (n = 5)		In pellet form (N = 5)		In plastic straws (N = 5)			
	motility	percent alive	motility	percent alive	motility	percent survival	motility	percent survival	motility	percent survival	motility	percent survival
Control	9.6	83.0	8.2	80.2	5.2	38.4	4.2	34.2	5.2	44.4	4.9	39.0
Alpha amylase	9.6	83.0	8.0	76.4	5.4	47.0	4.2	36.8	5.2	44.8	4.9	42.8
Beta amylase	9.6	83.0	8.8	78.4	4.6	42.6	4.0	33.6	5.4	49.0	4.7	41.7
Beta glucuronidase	9.6	83.0	8.6	77.4	4.6	40.2	4.6	40.2	5.0	46.2	4.7	42.2
Grand $\bar{x}$ (n = 20)			8.40	78.10	4.95	42.05	4.25	36.16	5.20***	46.66***		

***$P < 0.001$ compared to Pellets.

the freezing of ram spermatozoa.

Semen samples were evaluated for motility within five minutes after collection. Those having a motility of 8 or better were divided into two equal aliquots. One aliquot was immediately extended 1:1 with egg yolk-citrate-lactose extender (without glycerol) and placed in a water bath at 18°C. The other aliquot was extended 1:1 at the same time with ram seminal plasma and placed in a water bath at 18°C.[1/] These two aliquots were then placed in refrigerator storage in a water bath that allowed gradual cooling to 5°C over a 3 hour period. At the end of four hours the refrigerated semen was extended 1:3 with glycerolated egg yolk-citrate-lactose extender to give equal amounts of all extender components in each aliquot. The extended aliquots were kept in 5°C storage for one more hour and then frozen on dry ice in 0.5 ml Cassou type plastic straws.

The technique of freezing semen in plastic straws on dry ice was fast and allowed several straws in each sample to be frozen within seconds of each other. The semen in this experiment was frozen at five hours post collection with the treatment aliquots being held in seminal plasma for four hours prior to extension while the control

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[1/] The seminal plasma was obtained by centrifugation of fresh ram semen at 5210 G for 20 minutes. This was collected and centrifuged the previous day and kept at refrigerator temperature until warming to 18°C for extension use.

aliquots were not.[2/]

The straws were thawed seven days after freezing, by being held in the extender at 75°C for 12 seconds. Motility was determined immediately by the index method described previously. Differential stain slides were also prepared.

Results and Discussion

The paired t test was used to compare differences between control and treatment motility and percent survival. The results are shown in Table 9.

The extension of semen with seminal plasma appeared to depress prefreeze motility of the treatment aliquots and a significant ($P< 0.05$) difference was found in favor of the control. This was accompanied, however, by a slight drop in spermatozoa survival in the control prior to freezing. Thawed motility and survival rates were slightly in favor of the spermatozoa aged in seminal plasma. In no instances did these values approach significance.

No similar work with ram semen is known to be in the literature so these findings can not be compared to others obtained with ram semen. They do compare favorably with the findings on bovine

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[2/] A five hour post-collection time for freezing was selected due to a report in the literature of better results being obtained in sheep A.I. with fresh semen not exceeding five hours of age (Salamon and Robinson, 1962b).

semen motility by Rajamannan, Graham, and Schmehl (1971). Their work showed no significant differences in initial motility, prefreeze motility, or postfreeze motility as a result of holding bovine spermatozoa in contact with their own seminal plasma for periods up to 60 minutes prior to extension. The field trial results with this semen, however, showed a significant increase in fertility in favor of the semen "held" in it's own plasma.

Graham et al (1971) obtained a significant increase in the prefreeze and postfreeze motility of boar semen by holding it in the raw state up to four hours prior to extension. Studies in this area are continuing by Graham and his co-workers. They report using the "holding" procedure in connection with other techniques in continuing efforts to improve the fertility of frozen swine semen.

The slight increase in postfreeze motility and percent alive obtained here is encouraging. In view of this and the findings of Rajamannan, Graham and Schmehl concerning the increase in fertility of "held" bovine semen (without significant increases in motility) the technique holds promise as one of the tools that may be further refined to increase the fertility of frozen ram semen.

Table 9. A comparison of freezing results obtained with ram semen aged in seminal plasma for four hours prior to extension versus control with extension started immediately after collection.

Fresh ejaculates (n = 10)		Treatments	Motility		Percent alive		Percent survival	
Motility	Percent alive		Pre-freeze	Post-freeze	Pre-freeze	Post-freeze	Pre-freeze[a]	Post-freeze[b]
9.1	71.9	Control	8.0*	3.1	62.3	28.9	89.3	47.6
		Aged in seminal plasma	7.3	3.5	65.1	30.3	91.5	48.1

[a]Based upon comparison between prefreeze percent alive with fresh percent alive. Arcsin transformation used.

[b]Based upon comparison between thawed percent alive with prefreeze percent alive. Arcsin transformation used.

*$P < 0.05$.

Experiment 5. Determination of Acrosomal Damage by Glycerolated Extender

Design and Methods

The findings of Graham et al (1971) and Pursell and Johnson (1971) regarding acrosome morphology of boar spermatozoa frozen in non-glycerolated extender indicate the need for similar investigations with ram spermatozoa. This experiment was conducted to study the comparative damage to acrosome morphology of ram spermatozoa frozen in the presence of glycerol and in extender not containing glycerol.

The glycerol and antibiotic content of the egg yolk-citrate lactose extender was the same as used previously in these experiments (except as noted later). The final extension prior to freezing was 1:3. Equilibration differed from the previous experiments. These differences will be discussed in detail.

The semen was evaluated at room temperature one hour after collection. Large, or multiple/single ram ejaculates containing in excess of 2 ml and with a motility of 9 or 10 were selected for use. Each of the selected ejaculates was divided into two equal aliquots and each aliquot was extended 1:1 with non-glycerolated extender at room temperature. These samples were placed in a water bath at 18°C and thence into the refrigerator. After 5-1/2 hours, one

aliquot was extended 1:3 with glycerolated extender and placed back in the refrigerator for an additional six hours. The other aliquot was extended 1:1 and kept at refrigerator temperature of 5°C for 10-1/2 hours before further extension to 1:3 with glycerolated extender. It was then placed back in the refrigerator for an additional hour. This procedure gave two equal aliquots. One was exposed to glycerol for 6 hours and the other was exposed to glycerol for 1 hour. Each of these aliquots was then washed three times with non-glycerolated extender. This was accomplished by three 10 minute cycles of centrifugation at 770 G's in a refrigerated centrifuge.

At the end of the third washing, the two aliquots were each divided into equal portions. One-half of each was now re-extended (1:3) with glycerolated extender and the other portion re-extended with non-glycerolated extender. This resulted in four aliquots of the initial ejaculate. Each of the four had been treated differently as follows:

1. Exposed to glycerol in extender for one hour but not containing glycerol in final extended form. Refer to as Treatment 1 - 0.
2. Exposed to glycerol in extender for one hour and containing glycerol in final extended form. Refer to as Treatment 1 - G.
3. Exposed to glycerol in extender for six hours but not

containing glycerol in final extended form. Refer to as Treatment 6 - 0.

4. Exposed to glycerol in extender for six hours and containing glycerol in final extended form. Refer to as Treatment 6 - G.

The amount of glycerol in final extender, in 1 and 3 above, was 4.8% (v/v) - identical to that amount used in the initial extender prior to washing.

The four aliquots were placed in 1 ml glass ampules, sealed, and frozen in the Linde controlled freezer at the rapid rate shown in Figure 8 (Appendix I). Sufficient freezings were made to obtain ten separate samples of semen frozen with glycerol in the extender and semen frozen without glycerol in the extender, or five each of the treatments shown above.

The ampules were thawed in three volumes of non-glycerolated extender at 40°C. Motility readings and differential stain slides were made immediately after thawing. Acrosome morphology was examined at 1600 X with a Zeiss Photomicroscope using a Neofluar 100/1.30 oil immersion phase contrast objective. Sodium fluoride solution (0.3%) was used to inhibit motility for microphotography of the acrosome. Slides were prepared by placing 10 to 12 µl of the thawed sperm suspensions under a 22 mm^2 cover slip. Three counts were made of abnormal acrosomes per 100 acrosomes observed.

The characteristics considered to be abnormal were damaged or missing apical ridges and/or deformed, loose or missing acrosomal caps. Figure 1 (Appendix I) illustrates acrosome morphology.

Results and Discussion

The results of this experiment are shown in Table 10. The treatment comparisons desired were performed by paired t test. All samples of semen frozen without glycerol in the final extender exhibited zero motility. There were no exceptions to this, regardless of exposure time to glycerol in the extender prior to washing. An interesting phenomena was associated with this finding: the differential slides made from these samples gave substantial counts of unstained spermatozoa. This indicated that these cells were not "dead" as defined by the eosin-fast green staining technique. A comparison of the percent survival computed on the basis of these cells being alive, though immotile, showed no significant differences in unstained spermatozoa between treatments 1 - 0 and 6 - 0.

The motility indices and the percent survival of treatments 1 - G and 6 - G were excellent. 6 - G was slightly superior to the best freezings of Experiment 2 or 3. Centrifugation as used in these washings had no apparent ill effects.

The percent survival was significantly better ($P<.001$) for treatments 1 - G and 6 - G than for 1 - 0 and 6 - 0. This indicates

that the exposure to glycerol during freezing significantly benefitted sperm survival.

When the percent abnormal acrosome data for treatments 1 - G and 6 - G were pooled and compared with the corresponding data from treatments 1 - 0 and 6 - 0 the difference was highly significant ($P < 0.001$). The semen frozen in glycerolated extender had a significantly higher percent of abnormal acrosomes. Ram spermatozoa with normal and abnormal acrosomes, as observed in this experiment, are shown in Figures 10 and 11 (Appendix I).

There was no significant difference between the percent abnormal acrosomes for that semen exposed to glycerol for one hour prior to washing and that exposed for six hours. However, these differences approached significance.

It is suggested that under the conditions of this experiment the presence of glycerol in the freezing media:

1. was necessary to obtain motility of thawed spermatozoa.
2. resulted in a significant increase in percent survival of spermatozoa - based upon differential stain count.
3. resulted in a significant increase in percent damaged acrosomes - based upon count with the phase contrast microscope at 1600X.

Consideration of these three findings initiated a comparison of percent live normal spermatozoa (those cells not absorbing stain

and with apparent normal acrosomes). These data were obtained by comparison of percent normal acrosomes with percent survival. When subjected to paired t test analysis the pooled data from 1 - G and 6 - G were highly significant in favor of survival of live spermatozoa with normal acrosomes. Due to the absence of motility in treatments 1 - 0 and 6 - 0, the necessity for this type of comparison may be questioned. Graham et al (1971), working with boar spermatozoa, proposed that sperm cells frozen without glycerol but alive and without motility could still maintain morphological integrity and might have the potential to fertilize eggs while sperm cells with good postfreeze motility frozen in glycerol extender might be damaged and ineffective in fertilization.

There is another factor that should also be considered. Quinn, White, and Cleland (1969) found that acrosomal damage in ram spermatozoa was confined to the corrugated and apical regions of the acrosome. Their work was done with electron microscopy and perhaps phase contrast microscopy does not provide the critical acrosome evaluation that is necessary to identify region-specific damage. The experience obtained in this study supported this view. Only the most apparent damage was discernable with the phase contrast microscope. Interference contrast microscopy should hold promise in this work and may prove to be essential.

The results of this experiment are believed to have shown that

the presence of glycerol in the freezing medium results in significant acrosomal damage to ram spermatozoa but that the presence of glycerol is necessary to obtain motility and may possibly result in an increased percent survival of spermatozoa with normal acrosomes. Acrosome morphology studies appear to hold promise as a means of evaluating the potential fertilizing capacity of frozen ram spermatozoa.

Fertility Tests

Design and Methods of the Insemination of Ewes with Frozen Semen

Five ewes were available for insemination during natural estrus. A vasectomized ram, with painted brisket, was placed with these ewes to detect estrus. The ewes were checked each morning and evening. When a ewe was found to be marked by the vasectomized ram, she was placed in a separate pen with another vasectomized ram. If she stood for natural mating she was considered to be in estrus and was inseminated within 30 minutes and again 12 hours later.

Using this procedure, all five of the ewes were detected in estrus and inseminated during one estrous cycle. Two of the ewes cycled at a later date and were inseminated again at 12 hour intervals. These inseminations were made in the late Fall and early

Table 10. Comparative motility, survival, and abnormal acrosomes of ram spermatozoa frozen after four different glycerol treatments.

Treatment[a]	Fresh (n = 5)		Prefreeze (n = 5)		Thawed (n = 5)		Percent survival[b] (n = 5)	Percent abnormal acrosomes (n = 5)	Percent live normal survival[c] (n = 5)	Grand $\bar{x}$ for percent live normal survival (n = 10)
	motility	percent alive	motility	percent alive	motility	percent alive				
1 - 0	9.25	67	7.25	65.5	0	17.8	27.1	11.5	23.2	
1 - G	9.25	67	7.75	60.0	5.0	25.6*	42.6	23.6**	31.4*	24.1
6 - 0	9.25	67	7.50	63.0	0	18.4	29.5	14.1	25.0	33.1**
6 - G	9.25	67	8.00	60.3	5.4	30.7	50.9	29.5*	34.8	
Grand $\bar{x}$ for abnormal acrosomes:			1 + 6 without glycerol (n = 10)					12.8		
			1 + 6 with glycerol (n = 10)					26.6***		

[a] As described on p. 120 and 121.

[b] Based on comparison of thawed percent alive with prefreeze percent alive. Arcsin transformation used.

[c] Based on comparison of percent normal acrosomes with percent survival. Arcsin transformation used.

*P < 0.05

**P < 0.01

***P < 0.001

Winter months of 1970.

Inseminations were made with semen frozen in ampules and in the form of pellets that had been in storage at -196°C for 90 to 120 days. The ampules and pellets were thawed in four volumes of additional extender at 40°C as in Experiments 1 and 2. Only those thawed samples showing motility readings of five or higher were used for insemination. In nearly all instances these samples had the same motility found during the initial thawings made several months previously.

Each ewe was inseminated with 1 ml of the thawed inseminate. The number of live spermatozoa per insemination ranged from 152 million to 322 million in the ten first cycle inseminations, and 24 to 36 million in the four inseminations accomplished during the second cycles.

The ewes were placed in an inseminating stanchion that allowed elevation of their rear quarters. A speculum and headlamp were used to locate the cervix. Small plastic bovine inseminating pipettes were used, and an effort was made to deposit the 1 ml inseminate into the cervix or through the cervix into the uterus. A record was kept of the location of each insemination. Twelve inseminations were made in the anterior portion of the cervix, one insemination was mid-cervical and one was through the cervix into the uterus. A great deal of difficulty was experienced in making penetrations beyond the

os of the cervix. Penetrations into the mid-cervical region were often accompanied with difficulty in expelling the inseminate inside the female reproductive tract. The interior cervical ridges apparently compressed against the opening of the pipette. Rotating the pipette and/or an easing of the forward pressure was found to allow expulsion of the inseminate. After insemination, these ewes were allowed to go full term.

In June 1971 eleven ewes were inseminated with frozen semen after synchronization of estrus by hormone treatment. They were given estrogen followed by progestogen similar to the recommendations of Foote (1968):

1st day - 2.5 mg of estradiol in corn oil injected intramuscularly.

4th day - 25 mg progestogen impregnated pessaries (Syncromate, Searle) were inserted intravaginally and allowed to remain through day 16.

19th day - 500 IUs PMS injected intramuscularly.

35th day - 750 IUs PMS injected intramuscularly.

On the evening of the 36th day, each ewe was inseminated with 0.5 ml to 1 ml of semen containing no less than 150 million motile spermatozoa and each ewe was inseminated again 24 hours later. The semen used for these inseminations was frozen in ampules at the controlled rapid rate used in Experiment 2 and stored at -196^{o}C for

approximately 30 days. The extender contained 10 micrograms of alpha amylase per ml of extended semen. Only semen with a thawed motility better than 4 was used in this study.

The techniques developed for inseminating the five ewes during natural estrus were followed in this study with some modifications. Specially constructed inseminating pipettes were made for ovine use by chamfering and tapering the ends of plastic bovine pipettes until they were approximately 3 mm in diameter at the modified end. The semen used for insemination was thawed out in ice water prior to opening of the sealed ampule. When thawed in this manner the semen showed excellent motility equal to or slightly better than samples of the same semen thawed at 40°C. However, when semen was frozen in pellet form and thawed in extender at ice water temperature the thawed motility was always zero. Other pellet samples, from the same semen, thawed at 40°C showed sperm motility equal to the ampules thawed in ice water. In this second fertility test, the volume of extended semen varied per insemination from 0.5 ml to 1 ml. depending upon the concentration of the initial ejaculate and the percent live spermatozoa after freezing and thawing.

Difficulty was also experienced in the insemination of this second group of ewes. The modified inseminating pipettes were considered only a slight improvement. The same difficulty was experienced in expelling the inseminate into the cervix.

Eight weeks after insemination the ewes were slaughtered and their reproductive tracts were examined for signs of ovulation and implantation sites.

Results and Discussion

The inseminations with frozen semen did not result in the production of live lambs from the five ewes inseminated in natural estrus and examined at term. Neither was there site of implantation among the 11 ewes inseminated following the synchronization of estrus.

The reasons for this can only be conjectured upon. The procedures used in this study were very similar to those used with some success by other investigators. A small number of ewes were inseminated in each of these studies and perhaps this should be considered against the percentage fertility figures reported in the literature. The dilution rate of the extended semen was probably an important cause of failure with the five ewes inseminated during natural estrus. The number of live spermatozoa per insemination was compatible with that recommended by others. However, the dilution rate of 1:19 used per inseminate in this study may be much too high. Salamon and Lightfoot (1970) reported that thirteen of twenty-one ewes lambed after two cervical inseminations of 0.1 ml of frozen semen containing 1.5×10^9 motile spermatozoa per ml. The semen used by the Australian workers was centrifuged after thawing and the

supernatant discarded to achieve the concentration of motile spermatozoa desired. This concentration approached the live spermatozoal concentration of freshly ejaculated ram semen, and was much more concentrated than the inseminate semen used with the five ewes reported here.

Several factors may have contributed to the failure of conception among the eleven ewes in the second test. The factor that is most suspect is the low fertility often associated with synchronized estrus following the use of synthetic progestogens. The repeat PMS treatment used with these animals was developed by Hulet and Foote (1967) to improve synchronized fertility. Even though this method is used, the level of fertility following natural mating is often below that obtained from ewes mating during the breeding season. Foote (1968) stated that treatment with PMS following pretreatment with progestogen, or estrogen and progestogen, usually results in a higher incidence of ewes ovulating than ewes showing estrus but that most ewes showing estrus also ovulate. A vasectomized ram was placed with the eleven ewes in this thesis study on the 36th and 37th day. He marked only four of them. Inskeep and Cooke (1968) report that sperm transport is considerably reduced at synchronized estrus, and that a lesser degree of success can be expected when diluted semen and smaller numbers of sperm are used for AI in animals in which estrus and ovulation has been controlled.

The dilution effect that is believed to have been a factor in the

failure with the first five ewes may also have been a factor with the eleven ewes in the second trial. Though the semen used with the eleven ewes was not subjected to further dilution at thawing, it was still at the freezing dilution of 1:3, and so the dilution of the inseminate used was 5 to 10 times greater than that reported to give success by Salamon and Lightfoot (1970). This factor may have had an important bearing upon the negative results obtained in these fertility tests.

Viability of Spermatozoa Held in Extended Storage at -196°C

During this study the frozen semen samples not thawed for data or insemination purposes were kept in liquid nitrogen storage. Near the end of the study there were eight such samples that had been kept at -196°C for more than 18 months and that were represented in the early records with seven day postfreeze motility readings and percent survival data. Four of these were in ampules and four were in pellet form. These samples provided an opportunity to study the effect of extended storage upon motility and percent survival.

The samples were thawed at 40°C using the same procedures as used for the seven day postfreeze thaw. The results are shown in Table 11. There was no change in motility from that observed at seven days postfreeze. The changes in percent survival approached

zero and when subjected to paired t test were not significant. There are aspects of these changes that merit discussion. In six of the eight samples the percent survival increased while in extended storage. In two samples there was a decrease. In one instance (ampule No. 99) there was a considerable loss in percent survival. A review of inventory records shows that the cane upon which this ampule was stored was transferred twice from its original position in the liquid nitrogen refrigerator. The sample was therefore exposed to atmospheric temperatures for several seconds upon two occasions. The exposure of frozen semen to elevated temperatures at any time during storage may be detrimental to motility and fertilizing capacity (Pickett, 1971). Pickett recommended that if semen transfers, or inventories are made, the frozen ampules should not be exposed to elevated temperatures for more than 10 seconds. This is recognized as a very critical factor in the storage of frozen semen. In each instance (in this study of extended storage) the two samples that suffered decreases in spermatozoa percent survival were transferred twice.

Concerning the increase in percent survival noted in six of the eight samples; Salisbury (1968) and Salisbury and Hart (1970) reported that the fertility of bovine semen stored in liquid nitrogen increased sharply from one to two months of storage and then more slowly up to 11 months. Pickett (1971) found no effect on length of

Table 11. The motility and percent survival of ram spermatozoa held in liquid nitrogen storage beyond 18 months.

Sample identity	Motility		Percent alive			Percent survival		Time in storage (months)	Change in motility	Change in survival	Number of transfers during storage
	Postfreeze		Fresh	Postfreeze		Postfreeze					
	7-day	End of storage		7-day	End of storage	7-day	End of storage				
Ampule 117	7	7	68	28	36	41.2	52.9	19.3	0	+11.7	1
Ampule 50	4	5	76	21	26	27.6	34.2	18.8	+1	+ 6.6	1
Ampule 99	5	4	67	28	16	41.7	23.8	18.3	-1	-17.9	2
Ampule 129	3	3	79	17	16	21.5	20.2	21.8	0	- 1.3	2
$\bar{x}$	4.8	4.8				32.7[a]	32.3[a]	19.5	0	- 0.4[a]	
pellets 67	5	4	79	23	25	29.1	31.6	18.8	-1	+ 2.5	1
pellets 26	3	3	80	22	24	27.5	30.0	18.3	0	+ 2.5	1
pellets 143	3	4	54	20	21	37.0	38.8	19.5	+1	+ 1.8	1
pellets 29	2	2	76	5	6	6.5	7.8	18.8	0	+ 1.3	1
$\bar{x}$	3.3	3.3				23.5[a]	25.8[a]	18.9	0	+ 2.3[a]	
Grand $\bar{x}$ (n = 8)	4.0	4.0				28.1[a]	29.0[a]	19.2	0	0.9[a]	

[a]Based on arcsin transformation.

storage on the fertility of frozen bovine semen stored in glass ampules up to two years in liquid nitrogen. Pickett et al (1961) found that the motility of frozen bovine semen stored for extended periods decreased with increasing storage time. First et al (1961) found that the motility of ram semen declined rapidly during the first two months of storage at -79°C but changed little thereafter during a storage period of 14 months. Szumowski, Markovic, and Cano (1956) found no decline in the motility of ram sperm stored for four weeks at -79°C.

Semen frozen in pellet form was stored in open ampules throughout this study. When the pellets were removed for thawing some pellet containers were found to harbor loose particles of debris from the liquid nitrogen. This observation points out the need for a reliable pellet container that may be sealed if pellet freezing of ram semen is to become an important method of preservation. This may be a serious disadvantage of pellet freezing, as ampules and plastic straws are each comparatively easy to seal for liquid nitrogen storage of semen.

Ram semen was found in this study to maintain seven day post-freeze motility and sperm survival while in storage at -196°C for periods up to 21.8 months.

SUMMARY AND CONCLUSIONS

The broad objective of this study was to examine the methods of freezing ram semen employed by others and to expand upon them towards procedures that would allow cryogenic preservation with retention of satisfactory fertilizing capacity.

Pilot studies were conducted to develop a satisfactory extender and life protector combination. Freezing experiments were performed to compare freezing methods and to test the effects of added enzymes as well as the effect of holding in seminal plasma prior to freezing on postfreeze motility and survival of spermatozoa. The question of whether glycerol in the freezing medium is a causative factor in acrosomal damage was also examined. Fertility tests were made with frozen semen by inseminating ewes in natural estrus and at induced estrus. The viability of ram spermatozoa held in liquid nitrogen storage for 18 to 21.8 months was investigated.

The results obtained under the conditions of these experiments warrant the following conclusions:

1. Ram semen may be diluted 1:3 in an isotonic extender containing glycerol and frozen in 1 ml ampules in a controlled freezing chamber, in pellet form on dry ice, and in plastic straws on dry ice with satisfactory retention of spermatozoa motility and percent survival

2. The freezing of ram semen in ampules at an optimum controlled rate holds promise for better viability with less damage from cold shock at thawing time compared to the faster rates of freezing - as in pellets or in straws on dry ice. A rapid controlled rate of freezing was found to give significantly higher spermatozoa motility and percent survival than a slow controlled rate-or freezing in pellet form on dry ice.

3. Alpha amylase, beta amylase, and beta glucuronidase may be added to an egg yolk-citrate-lactose-glycerol extender at the rate of 10 micrograms/ml without decreasing the cryogenic properties of the extender for ram spermatozoa.

4. Ram semen frozen on dry ice in plastic straws and thawed in an isotonic medium at 75°C for 12 seconds may be significantly superior, in motility and percent survival, to that frozen in pellet form on dry ice and thawed at 40°C.

5. Aging of ram spermatozoa in seminal plasma for four hours prior to extension appears to exert no significant effect upon sperm motility or percent survival.

6. The presence of glycerol in the freezing medium appears to cause significant increases in the percentage of abnormal acrosomes present in thawed ram semen as

determined by phase contrast microscopy. The presence of glycerol is necessary to obtain sperm motility and may also result in an increased percentage survival of spermatozoa with normal acrosomes as determined by phase contrast microscopy.

7. The motility and percent survival of ram spermatozoa, stored in ampule or in pellet form may be expected to change very little in liquid nitrogen storage for periods as long as 21.8 months provided that stored samples are not exposed to atmospheric temperatures prior to thawing time.

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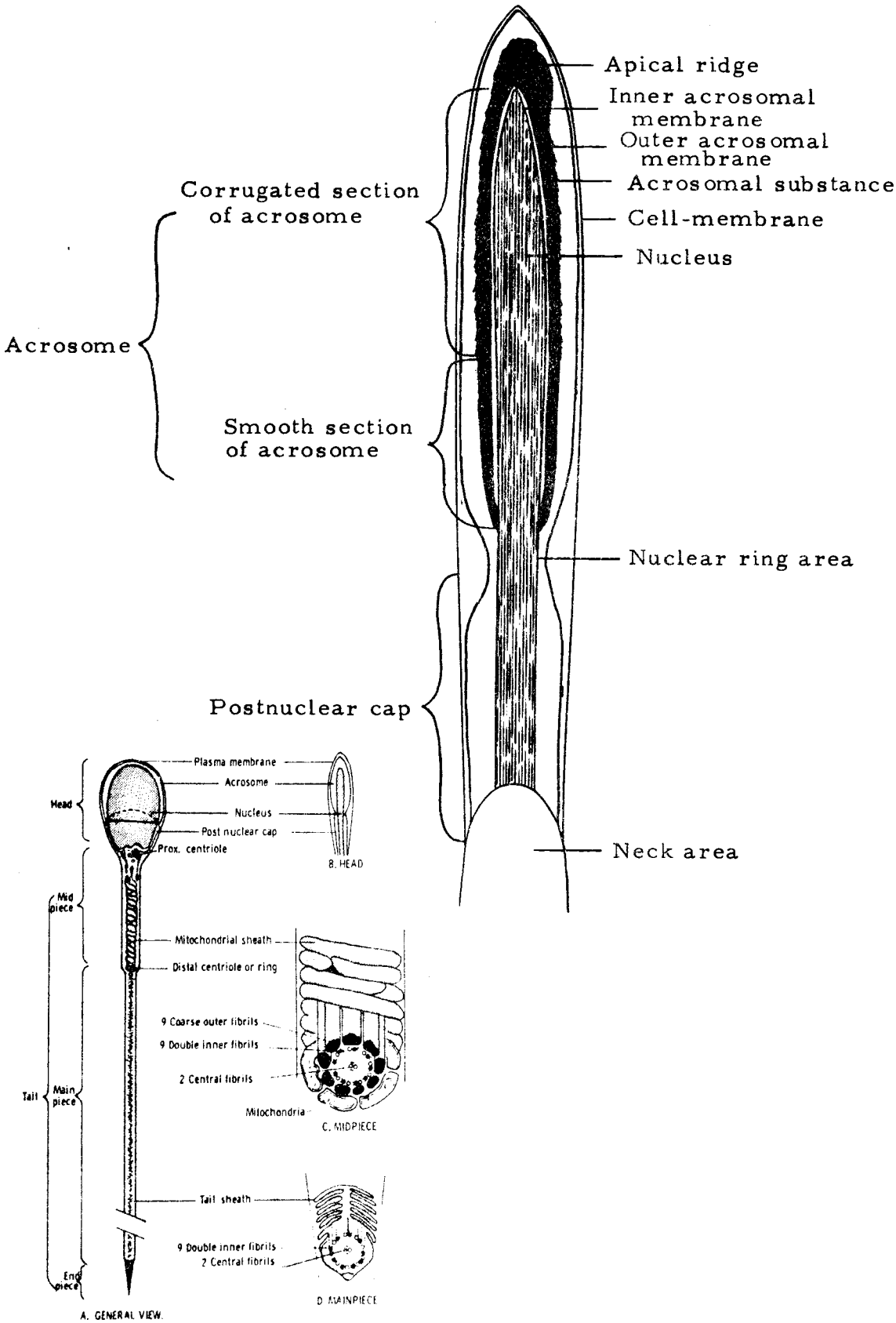
APPENDICES

Figure 1. Schematic drawing of the acrosomal complex of a ram spermatozoa (sagittal view). Adapted from White (1968).

Insert: Diagram illustrating the tentative structure of a typical ungulate sperm. Adapted from Wu (1966).


Apical ridge
Inner acrosomal membrane
Outer acrosomal membrane
Acrosomal substance
Cell-membrane
Nucleus
Corrugated section of acrosome
Acrosome
Smooth section of acrosome
Nuclear ring area
Postnuclear cap
Neck area
Plasma membrane
Acrosome
Nucleus
Post nuclear cap
Prox. centriole
Head
B. HEAD
Mid piece
Mitochondrial sheath
Distal centriole or ring
9 Coarse outer fibrils
9 Double inner fibrils
2 Central fibrils
Mitochondria
C. MIDPIECE
Tail
Main piece
Tail sheath
9 Double inner fibrils
2 Central fibrils
End piece
D. MAINPIECE
A. GENERAL VIEW.

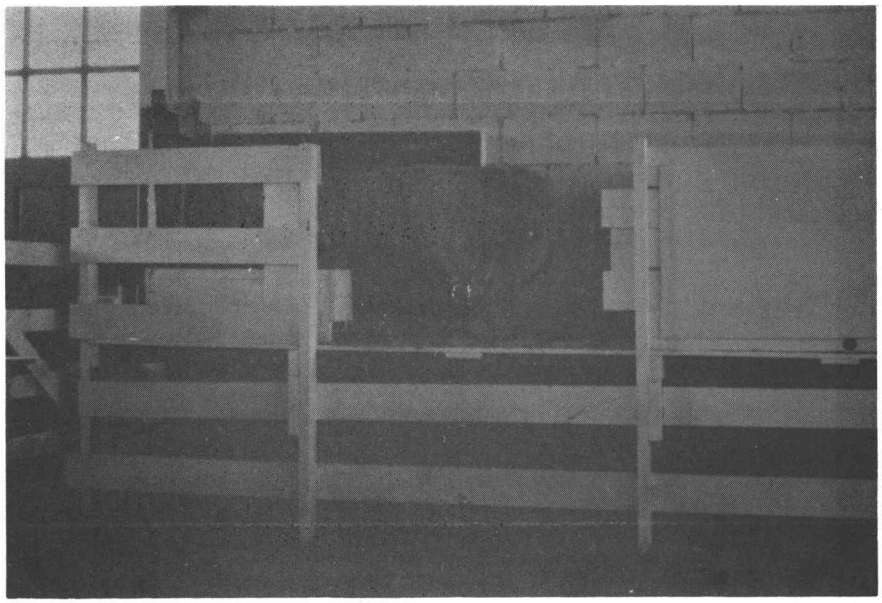


Figure 2. Breeding chute used to facilitate collection of semen from rams with the artificial vagina. A dummy ewe is also shown in the chute.

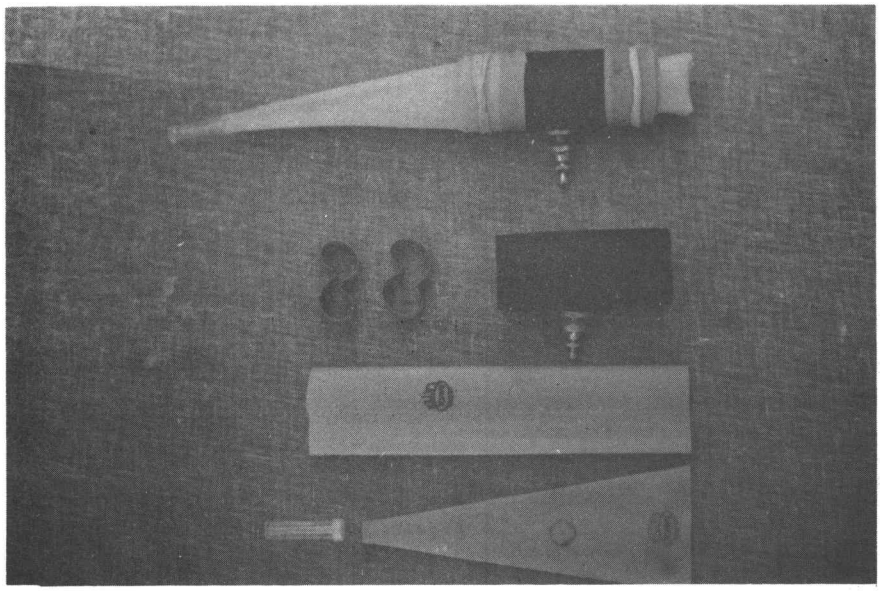


Figure 3. Ram artificial vagina, assembled and unassembled.

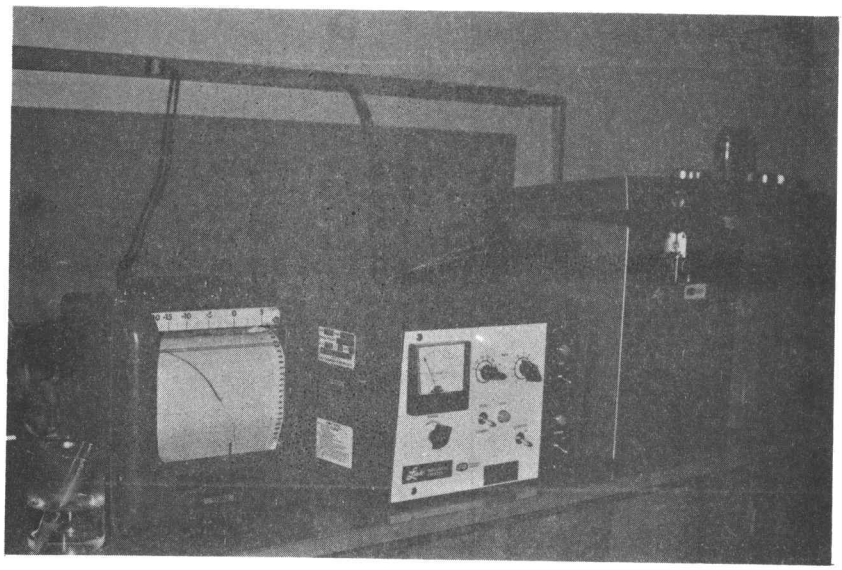


Figure 4. The Linde Biological Freezer unit and Varian Chart Recorder used for the controlled freezing of semen in ampules.

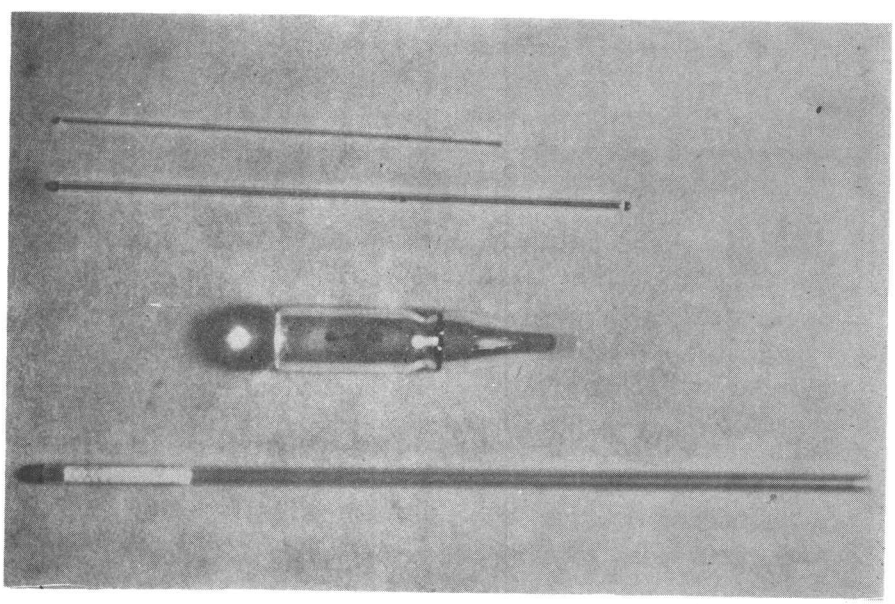


Figure 5. The glass capillary tubes, glass ampule and Cassou type plastic straw used as containers for the freezing of semen.

Figure 6. Freezing of ram semen in pellet form on dry ice.

Figure 7. Photomicrograph of ram spermatozoa on a differential stain slide. The sperm that were alive remain clear while those that were dead absorb the stain. Magnification is 135X.

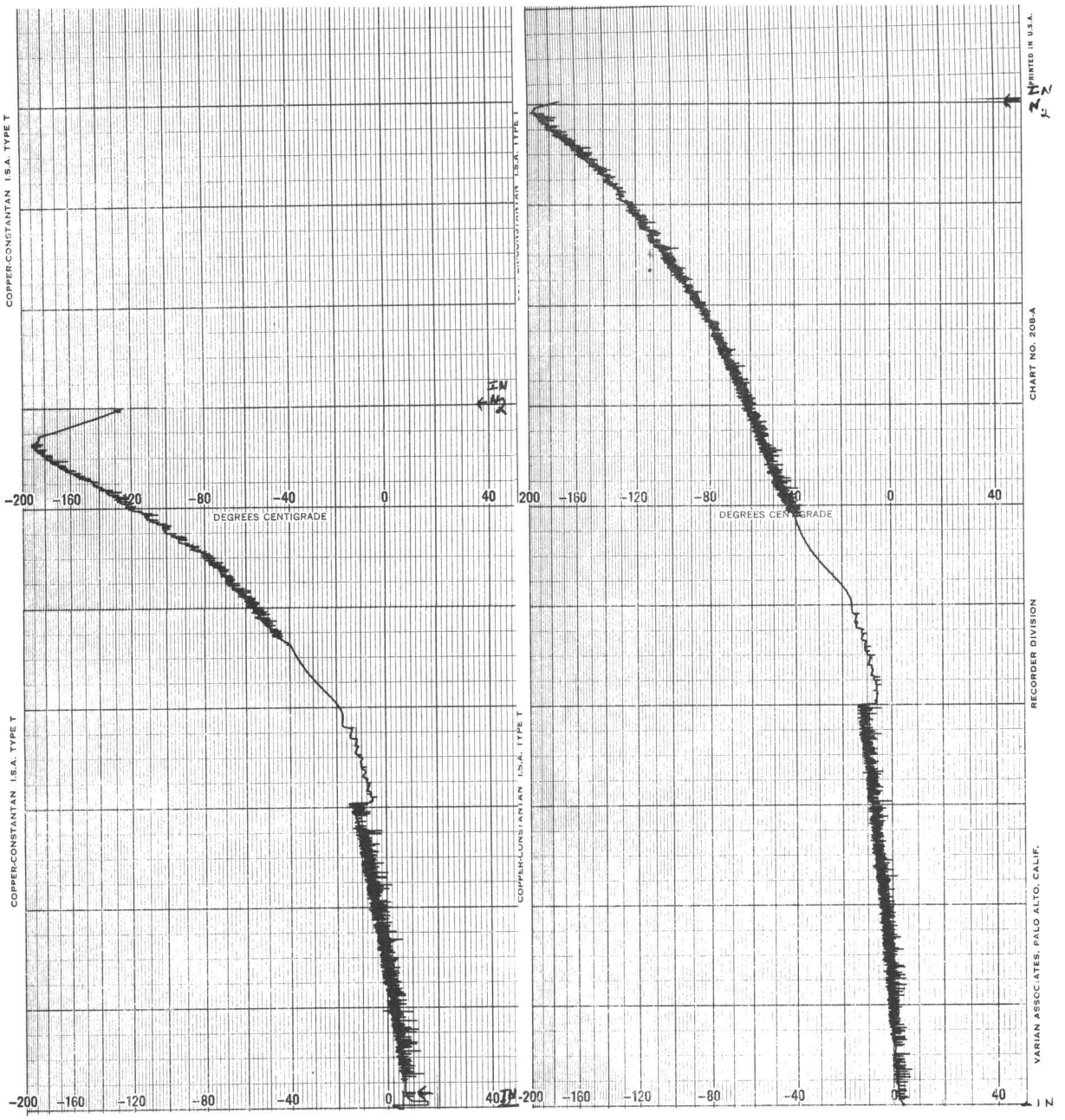


Figure 8. Chart recorder tracings of the two rates of controlled freezing used in this study.

Rapid rate	Slow rate
2 degrees per minuts to -15°C	1 degree per minuts to -15°C
6 degrees per minute to -40°C	3 degrees per minute to -40°C
10 degrees per minute to -80°C	5 degrees per minute to -80°C
20 degrees per minute to -196°C	10 degrees per minuts to -196°C

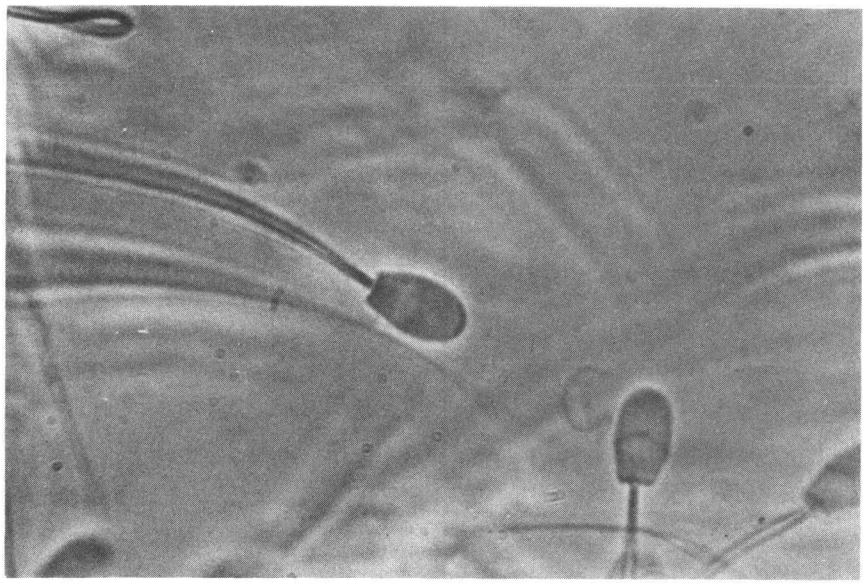


Figure 9. Phase contrast photomicrograph of unstained ram spermatozoa with normal acrosome. Magnification is 1600X.

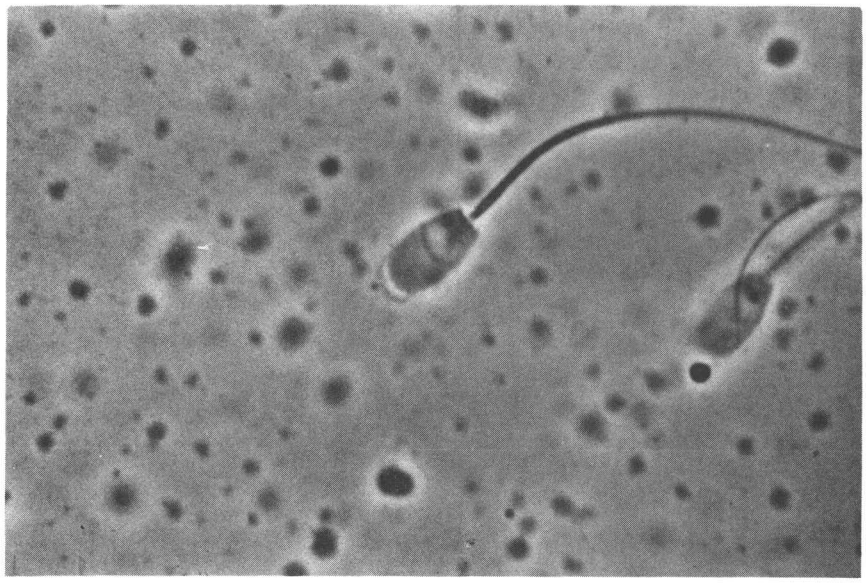


Figure 10. Phase contrast photomicrograph of unstained ram spermatozoa with abnormal acrosome. Magnification is 1600X.

APPENDIX II

RAM SEMEN MOTILITY INDEX DEVELOPED IN THIS STUDY

10 - Exceptional rapid swirl with great turbulence throughout and many "flares." No evidence of rafting or drifting of dead sperm. Characteristic only of fresh semen of superior motility with more than 85% live sperm. "Flares" throughout field.

9 - Rapid swirl with good turbulence throughout sample. Some "flares" evident though scattered; only a few in evidence in field at any one time. Slight drifting of dead sperm may be discerned, as focus is changed.

8 - Good swirl effect with continuous swirl, however, there is little evidence of "flares" except single isolated "flares," generally toward edge of liquid. Rafting and drifting of dead sperm is evident in many locations in the field. As focus is changed, dead sperm may be seen drifting to some extent across and throughout field. There may be slight rafting of these dead cells.

7 - Slight steady swirl effect with no cessation of mass movement, and a decided slowing of semen mass due to sperm movement. No "flares" are to be seen and rafting and drifting dead sperm are seen everywhere in field. The mass movement sensation is slow here, but still steady.

6 - Very slow swirl, or perhaps only a hint of the swirl effect. This is sometimes seen as a pulsating effect that is not strong enough to start the continuous steady swirling of the entire mass. The movement sensation barely exists, or exists only spasmodically or very sluggish. Dead sperm are generally much in evidence throughout field. Much rafting of dead cells.

5 - No swirl, however, sperm may be seen in motion throughout field. This motion may be slow, may be circling, or may be fluttering (especially in thawed samples) yet there are sperm in motion everywhere that one looks in field. Most sperm are in straight ahead motion, perhaps a few circling, however the majority are progressive. Dead sperm are much in evidence; however, they still do not completely obliterate motion of live sperm throughout field, as focus is changed.

4 - Sperm not in motion throughout field. There are blank or rafted spaces; however, sperm are seen to be in motion almost everywhere in field. Most sperm have straight ahead progressive movement, some slow straight ahead motion and some very fast straight ahead motion--also many sperm seen to be circling and fluttering in thawed samples, or moving very slowly with only tail flutter in unfrozen samples.

3 - Some sperm show fast progressive movement in extended thawed samples; however, most sperm have only circling, flutter, tail-vibrations or very slow straight ahead movement. In unextended, or unfrozen semen, there are many places in field where only dead sperm may be seen rafting or drifting, i.e., without apparent motility. Many spaces in the field devoid of motile sperm.

2 - Very few sperm to be seen with progressive straight ahead movement. Those in motion, for the most part, have only tail flutter, or move in circles, or very slow ahead. Very few sperm showing movement.

1 - No sperm with progressive straight ahead movement. Those that move have only tail flutter, or spasmodic slow circling movement. Just an odd sperm here and there in motion.

0 - No sperm motion observed. All sperm completely immotile.

Ratings of 5 through 10 were found characteristic of fresh raw ejaculates in this study and

Ratings of 0 through 7 were found in thawed, extended, or refrigerator stored semen that had been in storage several days.

Extension beyond 1:20 complicates accurate estimation of swirl; however, it is possible to observe swirl in ram semen extended even more than this. This depends upon initial motility and concentration. It is very important that extension and concentration be near the same in samples being examined consecutively for motility--to allow comparison.

This index was developed using phase contrast objectives.