THE FERTILIZING CAPACITY OF FROZEN RAM SEMEN USING VARIOUS DILUENTS AND ADJUVANTS

BY

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AN ABSTRACT

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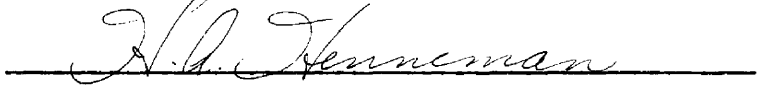
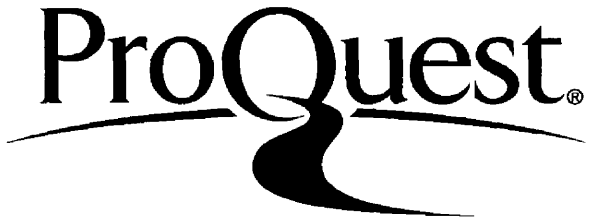
Approved 



ABSTRACT

THE FERTILIZING CAPACITY OF FROZEN RAM SEMEN

USING VARIOUS DILUENTS AND ADJUVANTS

Neal L. First

Motility determinations on six thawed frozen milk diluted ejaculates of ram semen indicated that, motility means for glycerol levels of 6, 6.5, 7, 7.5, 8 and 8.5 percent were significantly greater than the motility means for 5 or 5.5 percent glycerol.

No significant difference in sperm survival after freezing in a milk diluter occurred among added egg yolk levels of 0, 3, 6, 12 and 24 percent, although the average ram sperm survival after freezing tended to decrease as yolk levels increased. The number of sperm which survived freezing in glycerol levels of six and nine percent was highly significant over the 12 or 15 percent level. A highly significant interaction occurred between glycerol levels and egg yolk levels.

The motility of both frozen and unfrozen semen incubated for eight hours at 5° C. was significantly greater than at 39° C. No significant difference existed between the motility of thawed frozen semen or unfrozen semen after eight or 120 hour incubation at 39° C. or 5° C. Further, no significant difference occurred between thawed frozen semen equilibrated for one or 18 hours before freezing. Thawed frozen and unfrozen samples averaged 57 percent motility after 240 hours of 5° C. incubation.

None of the constituents in a milk-arabinose-glycerol diluter or a yolk-citrate-arabinose-glycerol diluter significantly depressed the

motility of ram sperm after incubation for eight hours at 39° C. Milk and yolk citrate diluted semen samples had significantly greater motility after eight hours of 39° C. incubation than undiluted ram semen.

The frozen milk diluted ejaculates of two rams were periodically examined for motility throughout 14 months of -79° C. storage. The greatest motility decline occurred during the first two months of storage with very little decline thereafter.

In 1956, two of 12 ewes lambed when cervically inseminated with 500 million sperm in one ml. of thawed frozen milk diluted ram semen. Whereas, seven of 11 lambed when cervically inseminated with 600 million sperm in .2 ml. of undiluted semen.

In 1957, three of 10 ewes lambed when cervically inseminated with 213 million sperm in one ml. of thawed frozen milk plus egg yolk diluted ram semen and six of 10 ewes lambed when cervically inseminated with 600 million sperm in .2 ml. of undiluted ram semen.

In 1958, none of 47 ewes lambed when cervically inseminated with 100 million sperm in .5 or one ml. of thawed frozen ram semen even though the average motility one week after freezing was 70 percent. Milk, yolk-citrate and a Russian-Ukranian diluter were used to dilute the semen before freezing.

An analysis of conception rates from 46 ewes cervically inseminated with unfrozen semen from eight rams indicated no significant difference among glycerolated; milk, yolk-citrate or Ukranian diluters.

Insemination of milk diluted ram semen resulted in a 46.7 percent lambing rate; whereas, the insemination of yolk-citrate or Ukranian diluted ram semen resulted in 21 and 18 percent lambing rates respectively. All was stored less than two days at 5° C.

No significant conception rate difference occurred between insemination of 100 million ram sperm in .5 or one ml. of undiluted semen.

Thirty-two ewe lambs were each cervically inseminated with 150 million sperm in one ml. of thawed frozen semen from one of two rams. No significant difference in conception rate occurred between ewes inseminated once during estrus or each 12 hours during estrus. In each case 12.5 percent of the ewes inseminated conceived.

There was no significant conception rate difference between ewes mated to a vasectomized ram before insemination and ewes which were inseminated without prior vasectomized mating. In each case, 12.5 percent of the ewes inseminated conceived.

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I. INTRODUCTION

Within a comparatively short period of time artificial insemination and the use of frozen semen has brought about a revolution in dairy cattle breeding practices. Considerable work has been carried on with bovine semen to improve methods, materials and techniques until the use of frozen semen and artificial insemination has become an efficient operation in widescale use.

Research with ram semen has been limited probably due to the lack of economic reasons for the practical use of artificial insemination. However, with the possibilities of synchronized estrus, the advent of progeny testing to locate valuable sires and the increased use of production testing it is possible that sheepmen will be taking a new look at the use of artificial insemination.

To further facilitate this possibility the perfection of a technique for freezing and storing ram semen would be of utmost importance. It would make possible the collection of semen at the time of year when it is of highest quality, extend the influence of outstanding sires, and improve conception rates for those breeders desiring early lambs.

A limited amount of research has been conducted with frozen ram semen. Enough was done to learn that the techniques used successfully for bull semen were not directly applicable to ram semen. Therefore, this study was undertaken in an attempt to find techniques which would result in frozen ram semen with high fertilizing capacity at the time of insemination.

II. OBJECTIVES

1. To develop improved techniques and materials for the low temperature storage of ram spermatozoa which will result in highly motile semen samples after freezing.

2. To fertility test the insemination of frozen ram semen and to develop insemination procedures which will improve conception from the insemination of unfrozen and frozen ram semen.

III. REVIEW OF LITERATURE

A. Storage of Frozen Ram Spermatozoa

Ram sperm were first frozen by Emmens and Blackshaw (1950) wherein semen samples were diluted one to one and frozen by the slow rate of Smith and Polge (1950). A sodium citrate phosphate buffer of pH 7.1 was the base around which diluters were formed. Diluters containing only various levels of glycerol were not successful, therefore, combinations of glycerol and sugars were tried. It was concluded that 7.5 to 10 percent glycerol and 1.25 percent arabinose, rhamnose, or xylose in the citrate phosphate buffer resulted in the greatest ram sperm survival after freezing. Some of the other substances tried as diluter constituents were: N propyl alcohol, iso propyl alcohol, ethylene glycol, monomethyl ether, trimethylene glycol, propylene glycol, hexylene glycol, erythritol, adonitol, sorbitol, dulcitol, glyceraldehyde, glucose, levulose, mannose, galactose, saccharose, trehalose, raffinose, urea and inositol.

Smirnov (1951) reported that eight of 19 ewes inseminated with ram semen which was stored at -78° C., conceived and produced 12 lambs.

Blackshaw and Emmens (1953) observed that ram semen initially diluted with 50 percent egg yolk phosphate citrate gave consistenly higher survival after freezing than without egg yolk. Equilibration with glycerol was found to be necessary. Frozen ram semen of high motility after freezing resulted in a lambing rate of only 10 percent. Glycerol and arabinose could be added to ram semen at temperatures up to 15° C. without reducing sperm survival after freezing.

White et al. (1954) observed that bull and ram spermatozoa thawed quickly after slow freezing to -79° C. in a phosphate fructose diluent, showed little respiratory or glycolytic activity and were immotile. A large number of bull and ram sperm survived freezing in a diluent containing 7.5 percent glycerol and 1.25 percent arabinose. These cells, however, had little metabolic activity and became immotile, particularly at 37° C.

Emmens and Blackshaw (1955) inseminated ewes with unfrozen and frozen semen that was undiluted as well as diluted one to four. The volume inseminated varied between .4 and .8 ml. A 54 percent conception rate was obtained from unfrozen undiluted semen; 31 percent from semen diluted with sodium citrate; 25 percent from semen diluted in 50 percent egg yolk, 7.5 percent glycerol, 1.25 percent arabinose and citrate; three percent from semen frozen in citrate, glycerol and arabinose diluter. Four percent of the ewes inseminated with non equilibrated semen frozen in an egg yolk citrate glycerol arabinose diluter conceived and five percent conceived after insemination with semen equilibrated 18 hours in the same diluter.

Blackshaw (1955b) studied the effect of several sugars on the motility revival of bull and ram semen after freezing to -79° C. The sugars were arabinose, dulcitol, galactose, fructose, mannose and sucrose. These sugars were added to the diluter conventionally used in other studies from that laboratory. The addition of arabinose or fructose resulted in the greatest survival of ram sperm.

Emmens and Blackshaw (1955) found that ram semen may be mixed with the glycerol containing diluent at temperatures up to 15° C. without harmful effects on revival after freezing. Egg yolk or lecithin addition to the Australian low temperature diluent resulted in greater sperm survival after freezing than with a lipoprotein or a milk diluter. A greater number of ram sperm revived from the frozen state when thawed at 40° C. than at 5° C.

Graca (1955) cervically inseminated 176 ewes with .2 ml. of yolk citrate diluted thawed frozen ram semen. The thawed semen was diluted 1:5 or 1:10 before insemination. The conception rate, based on non-returns, was 31.2 percent and was 16 percent higher in the 1:5 dilution group.

Szumowski et al. (1956) reported that a reconstituted powdered skimmilk diluter was superior to liquid milk or the common egg yolk diluters (Cambridge, Ukranian or Australian) for freezing ram semen. Motility of thawed sperm was 60 percent with over 70 percent survival after freezing. There was no difference in survival between samples of ram semen frozen in six to 16 percent glycerol. However, the length of glycerol equilibration did affect ram sperm survival. At least four hours of equilibration were necessary and the optimum appeared to be between six and 14 hours. No great loss of motility occurred after storage at -79° C. for four weeks. Thirty-nine ejaculates from one ram were studied.

First, et al. (1957) used 37 split ejaculates of ram spermatozoa to determine the effect of the following factors on ram sperm survival

after freezing and storage at -79° C.: (1) levels of glycerol; (2) the addition of L(-) arabinose; (3) a milk extender and (4) a yolk phosphate citrate extender containing various levels of egg yolk. The survival of frozen ram sperm in a milk diluter containing 0, 2, 4, 6, 8, 10, 12 or 15 percent glycerol was curvilinear with the greatest survival at six and eight percent. The addition of 1.25 percent arabinose significantly improved sperm survival in both milk and egg yolk-phosphate-citrate extenders. There was no significant difference between egg yolk levels of 44, 34 or 27 percent, however, the survival from all three was significantly higher than that of an extender containing 23 percent egg yolk.

Markovic (1956) observed that the after-thawing spermatozoan survival rate of ram semen diluted in yolk-arabinose before freezing was 60 percent provided that six to 14 hours were allowed for equilibration. Glucose and arabinose increased spermatozoan survival.

Galkin (1954) inseminated 500 ewes with the semen of eight rams which was diluted in yolk-glucose-citrate plus glycerine. The highest lambing rate (86 percent) was that of ewes inseminated with fresh semen. It was 69 percent when the semen was stored for two days at 0° C., 30 percent when stored for five days at 0° C., 45 percent when stored for five days at -8° C., and 43 percent when stored for 10 days at -8° C. The semen of the last two groups was thawed to 18-22° C. before using.

Morozov (1957) diluted ram semen in a hypertonic solution of glucose, yolk citrate and glycerine, placed it in waxed capsules and stored it at -20° to -20.5° C. After thawing 60 percent of the spermatozoa were motile. Spermatozoa were still viable after storage for 12 to 13 days.

Kuznetsov (1956), in reviewing Russian research, mentioned that at 0° C. storage the spermatozoa of the ram retain motility but lose fertilizing power rapidly. At 0° C. storage a gradual swelling of the thin membrane of the sperm head took place. At -79° C. storage, 40 to 50 percent of the frozen sperm regained motility after thawing. With ram semen frozen 20 days, 19.3 percent of 303 ewes inseminated conceived. In another trial, 33.5 percent of 512 ewes conceived from ram semen frozen for 30 to 50 days. In the previous work sperm were diluted 1:4 or less. In a discussion following this paper Professor Emmens stated that 15 to 20 percent of 2,000 ewes conceived after insemination with frozen semen in Australia.

Lopyrin and Loginova (1958) reported that the semen of Merino rams was diluted in an isotonic solution to which 7.5 percent glycerol was added before freezing to -78° or to -183° C; on thawing, spermatozoal motility was low and survival was poor. When a hypertonic glucose-yolk-citrate diluent plus 7.5 percent glycerine was used spermatozoan motility on thawing averaged 44 to 46 percent. Fewer spermatozoa had penetrated the cranial portion of the neck of

the uterus six to seven hours after insemination with thawed frozen semen than in ewes inseminated with fresh semen. Of 36 ewes inseminated two to three times in one estrus with thawed semen, 32 returned in heat. The four ewes conceiving produced a total of five lambs.

Hill et al. (1959) found that fewer ram sperm survived freezing in a yolk citrate extender containing 14 percent glycerol than in 3.5, 5.25 or 7 percent glycerol. They found that a slow freezing rate of 2° C per minute from 4° C. to -20° C., 4° C. per minute from -20° C. to -50° C. and 10° C. per minute from -50° C. to -79° C. resulted in greater sperm recovery after freezing than a faster rate.

B. Glycerol Requirement for Freezing Ram Semen*

First et al. (1957) have shown a curvilinear ram sperm survival after freezing in glycerol levels of 0, 2, 4, 6, 8, 10, 12 and 15 percent. However, no significant difference existed between six and eight percent. Hill et al. (1959) found greater survival after freezing yolk citrate diluted ram sperm in 3.5, 5.25 or 7 percent glycerol than from ram sperm frozen in 14 percent glycerol. There was a significant ram times glycerol interraction of semen stored for 10 days.

Emmens and Blackshaw (1950) concluded that 7.5 to 10 percent glycerol in a yolk phosphate citrate arabinose diluter resulted in the greatest ram sperm survival after freezing.

White et al. (1954), Blackshaw (1955a,b), Emmens and Blackshaw (1955), Lopyrin and Loginova (1958) have frozen ram semen in 7.5 percent glycerol.

The Russian-Ukranian diluter used by Smirnov (1951), Szumowski et al. (1956) and referred to later in this paper contained approximately 6.5 percent glycerol.

Szumowski et al. (1956) observed no difference in survival after freezing ram sperm in six to 16 percent glycerol.

Glycerol levels for freezing bull semen have been a controversial issue. Research concerning this matter was reviewed by Emmens and Blackshaw (1956). Amann and Almquist (1957) and have shown that

*All glycerol levels referred to are on a volume per volume basis.

glycerol level depends on the solid content of a skimmilk diluter. Saroff and Mixner (1955) found that the glycerol level used in a yolk citrate diluter was dependent upon the amount of egg yolk in the diluter. Kuznetsov (1956) reported that high yolk levels decreased survival and fertilizing capacity of ram semen.

Polge (1956) found that boar sperm could not tolerate ten percent glycerol in the diluting media. Polge (1951) found that glycerol adequately protected fowl sperm during freezing, however, conception did not occur unless the glycerol was slowly removed before insemination. Embryonic mortality was greatly increased when the inseminated semen contained glycerol.

C. Ram Semen Extenders

Ram semen extenders can be divided into two broad groups; (1) those which are useful for dilution and immediate insemination of fresh warm ejaculates and (2) diluters which allow cooling and storage of the ejaculate.

Anderson (1945) stated that one must consider osmotic pressure, pH, buffering capacity and toxicity when preparing a mammalian semen diluter.

Diluters Used for Immediate Insemination

Echenique et al. (1941) after studying several well-known diluting media, found that the most suitable diluent was the seminal fluid of a vasectomized ram or bull.

Physiological saline has long been used with semen that is diluted and inseminated immediately after collection. However, Milovanov (1934) mentioned that salts such as chlorides should not be used in diluting fluids. Physiological salt solution is stated to have a destructive action on the sperm lipid capsule. The K, Ca and Mg ions were also reported to have similar effects on the capsule. However, a certain amount of salt ions are necessary for normal sperm irritability. Milovanov proposed the use of colloidal diluters.

Emmens and Swyer (1948) found that the harmful effects of physiological saline dilution were due to high dilution rates rather than due to NaCl toxicity per se.

Rao and Hart (1948) found 0.9 percent sodium chloride had a stimulating effect on bull spermatozoa but that cells in highly diluted semen died within two hours after dilution.

Cheng, Casida and Barrett (1949) reported that motility of bull sperm was greater in 0.9 percent sodium chloride but that the use of sodium citrate permitted greater dilution before motility ceased.

Emmens and Swyer (1948), Blackshaw and Emmens (1951) and Blackshaw (1953) have found that sodium chloride in isotonic diluents is non-toxic to spermatozoa except at an alkaline pH.

Blackshaw (1953b) also reported that .005 M potassium chloride partially restored the motility of ram sperm washed four times. It did not however, prevent head agglutination. Seminal plasma addition prevented sperm head agglutination. He suggested that the protective action of substances such as lecithin is a physical protection of the cell surface instead of a chemical effect.

Storage Diluents

The common storage diluents for the most part contain singly or in combination gelatin, egg yolk, milk, skimmilk and/or sugars in sodium citrate, glycine, sodium phosphate or tartrate buffer.

Anderson (1945) has reviewed diluters conventionally used.

Graziette (1942) observed that ram semen diluted with sodium potassium phosphate, sodium sulphate, glucose and ram testis serum retained motility longer on storage at 2° to 5° C. than did undiluted semen. The addition of gelatin to the diluter resulted in a rapid loss of motility.

Aslanjan (1950) inseminated 4,709 ewes with ram semen diluted in yolk citrate and stored at 0° C. for six to 168 hours. Ninety-three percent of the ewes conceived. The age of the semen prior to insemination had no effect on conception rates.

Dauzier et al. (1954) tested several diluters and found that sperm motility was preserved the longest in a diluter consisting of three parts of three percent sodium citrate solution added to one part egg yolk. Motility was not preserved as long in milk diluents and was the poorest in a salt diluent. In a breeding trial conception rate was low with the citrate yolk diluent unless it was used within two hours of collection when a rate of 66.6 percent was obtained. The highest conception (83.3 percent) was obtained after the insemination of non stored ram semen diluted in a sodium citrate, magnesium sulphate, calcium acetate diluter.

Koger (1951) has studied the motility of ram sperm diluted in several diluters at various dilutions and under 5° C. storage. The diluters studied were physiological saline, Phillips phosphate buffer and 2.5 percent sodium citrate. These diluters were tested singly and in combination with egg yolk. The effect of dilution varied with different diluents. In diluters without egg yolk, there was a negative correlation between rate of dilution and duration of sperm motility. This was apparently due to excessive metabolic activity immediately following a high rate of dilution. This response was the most pronounced in physiological saline. The

addition of egg yolk to citrate and phosphate buffers improved storage in these diluters and the detrimental effects of high dilution were largely overcome. Egg yolk apparently had no beneficial effect when added to saline or to whole semen. Although saline was the least desirable for storage it resulted in the highest motility immediately after dilution.

The most satisfactory diluter for ram semen storage contained 75 parts of 2.9 percent sodium citrate solution and 25 parts egg yolk.

When ram semen diluted in this diluter and in a phosphate egg yolk diluter, each stored from zero to five days, were used in a breeding trial, fertility dropped noticeably during the first 24 hours of storage and declined sharply with further storage. A higher percentage of pregnancies occurred from sperm diluted in seminal fluids than diluted in phosphate egg yolk. Hyaluronidase addition did not reduce the number of sperm required for a satisfactory conception rate.

Sakala (1957) studied the effect of 19 recommended diluents on semen character. The best results were obtained with a diluter composed of 25 percent yolk, sodium citrate and glucose or fructose.

In recent years several authors have investigated milk diluents.

Thacker and Almquist (1953) found that homogenized or skim-milk must be heated to at least 92° C. before it was used as a bovine semen diluter.

Thacker et al. (1954) discovered that the toxic factor in unboiled milk was in the albumin fraction and that casein and protein free milk is usable. Boyd et al. (1954) found, in vitro, that bull spermatozoa survived better in milk treated with thioglycolic acid (a sulphydryl containing compound) than in heated pasteurized milk. In essence this was confirmed by Almquist (1954), Almquist et al. (1954), Flipse et al. (1954) and by Johnson et al. (1955). These authors found heated homogenized or pasteurized milk, or one mg. per ml. of cysteine hydrochloride (another sulphydryl compount) added to pasteurized skimmed milk to be as suitable as egg yolk citrate in terms of bull sperm motility and fertility. The sulphydryl compounds lowered the oxidation reduction potential of the milk and inactivated lacterin, the anti-streptococcal and supposedly spermicidal substance present.

Istvan (1956) inseminated 535 ewes with ram semen diluted in boiled cows milk, 451 ewes with semen diluted in yolk citrate glucose and 257 ewes with undiluted ram semen. The percentages of ewes lambing after one insemination were 75.5, 63.2 and 63.5 respectively.

In the following year 83.0 percent of 2,652 ewes inseminated with milk diluted ram semen lambed and 83.1 percent of 638 ewes inseminated with undiluted semen lambed.

Dauzier (1956) reported that conception rates obtained with ram semen after storage for 12 to 24 hours at 2° to 4° C. varied

from six to 25 percent when the semen was stored undiluted, or diluted in citrate, egg yolk citrate or egg yolk phosphate diluents. When it was stored in skimmed cows milk, heated to 95° C. for ten minutes, conception rate after 12 hours storage was 51 percent. Following the addition of antibiotics, it was 65 percent. Goat semen stored in skimmilk for periods under eight hours resulted in a conception rate of 64 percent.

Fillimon et al. (1956) found that when ram semen was diluted in milk and cooled to 0° C., 80 percent of the spermatozoa were viable after storage for 48 hours and 55 percent were viable after 72 hours. Fifty-nine percent of 155 ewes inseminated conceived, as compared to 52.1 percent of 56 ewes inseminated with undiluted semen. When gluco-phosphate plus gelatin diluent was used, 73.9 percent of 23 ewes inseminated conceived versus 71.4 percent of 21 ewes inseminated with undiluted ram semen.

Mihailov (1957) reported that ram semen diluted in boiled cows milk was used to inseminate 4,700 ewes at six insemination stations. The conception rate varied between 83 and 97 percent at the different stations.

Hill et al. (1958) stored the semen of four rams for 15 days at 5° C. in reconstituted skimmilk and egg yolk sodium citrate diluters. Sperm survival was higher in the reconstituted skimmilk, however, no fertility tests were made.

Yoshioka et al. (1951) conducted experiments on the storage of semen and on the insemination of sheep and goats with stored

semen. The effects of two kinds of diluents were examined. One was prepared by mixing two parts by volume of 2 percent boric acid with one part of 1 percent sodium bicarbonate. This was added to the semen in a ratio of 1:3. The other diluent was called RH solution. It was prepared by mixing equal volumes of 0.3 percent sodium sulphamerazine and 0.2 percent homosulphamine. This was dissolved in five percent sodium citrate and the solution was added to the semen in a 1:1 ratio.

Both diluents, but especially RH solution, improved the keeping quality of the semen. Insemination with semen to which either of these diluents had been added resulted in higher conception rates than insemination with untreated semen. The percentages are as follows: untreated semen, 56.4 percent; semen treated by boric acid and sodium bicarbonate, 65.3 percent; semen treated by RH solution, 65.1 percent. The length of time for which the semen retained fertility was: untreated semen, 111-120 hours; semen treated with boric acid and sodium bicarbonate, 144 hours; semen treated with RH solution, 168 hours.

Yoshioka and Koike (1956) studied the storage and fertility properties of five semen diluters. These diluters being (1) a glucose sodium citrate diluter, (2) egg yolk sodium citrate, (3) the RH diluter previously mentioned and (4) a glucose-RH diluter containing two volumes RH and one volume of egg yolk.

The yolk-RH diluter maintained high sperm motility the longest. After 120 hours storage at 2° to 7° C., the diluters

were ranked from highest to lowest sperm motility as follows: (1) yolk-RH solution, (2) yolk citrate, (3) glucose-RH solution, (4) RH solution, (5) glucose citrate and (6) undiluted semen.

The conception rate for the first 48 hours of storage after insemination with yolk-RH diluted semen was as high as from fresh undiluted semen. Four conceptions occurred when the semen was preserved as long as 10-13 days.

No significant difference could be found, in the influence upon spermatozoa, between heated goat milk and RH solution, when both were used as the diluents of gelatinized semen.

A low electrolyte glycine buffer was studied by Ahmed (1955). Ram semen was stored at 4° C. in a standard citrate-egg yolk diluent, which served as a control, and in various glycine diluents.

Replacing citrate buffer by five percent glycine considerably improved survival over that of other concentrations of glycine and the citrate buffer control. Higher survival in five percent glycine-egg yolk buffer was obtained with a semen dilution ratio of 1:20 than with a ratio of 1:40. The addition of fructose to the glycine buffer did not improve survival. A 50 percent concentration of egg yolk in a four percent glycine buffered egg yolk diluent was reduced to 33.3 percent egg yolk without affecting either motility or survival and to 25 percent egg yolk with no effect on survival but with a slight reduction in motility.

Roy et al. (1956) found the motility and survival of egg yolk glycine diluted sperm stored at 4° C. to be greater than the sperm diluted with egg yolk citrate.

Aamdal and Hogset (1955) inseminated ewes with ram semen diluted 1:6 with three percent citrate 50 percent yolk and three percent glycine 50 percent yolk diluter. More ewes conceived from the yolk citrate diluted semen.

Kuznetsov (1956) from work done in the U.S.S.R., stated that the best diluting medium for ram semen was a three component synthetic medium composed of 90 ml. of isotonic sodium citrate plus 10 ml. isotonic glucose, fructose or glycocole and 20 ml. of egg yolk. When the quantity of egg yolk was increased the survival of spermatozoa and fertilization of ewes decreased.

Pozdnjakov (1958) found yolk glucose citrate diluent with antibiotics superior to the same diluent without antibiotics.

Dauzier et al. (1954) observed that ram sperm motility was maintained longer in a yolk citrate diluent than in yolk phosphate, yolk bicarbonate glucose, skimmilk, whole milk or salt diluents. Twenty-five and 50 percent egg yolk maintained sperm motility longer than five percent. Preservation of motility was not affected by rate of cooling or dilution. The fastest rate was 1.5° C. per minute.

The conception rate was low (15.7 percent) from 438 ewes cervically inseminated with one ml. of yolk citrate diluted semen which had been diluted 30 to 40 times. Seventy-three percent of 21

ewes conceived when inseminated with undiluted ram semen and 83 percent of natural mated control ewes conceived. A further study in which semen was diluted and inseminated within two hours after collection, resulted in the following conception data: 66.6 percent of 21 ewes bred with 33 percent yolk citrate diluted semen conceived, 53.3 percent conceived from a diluent of five percent yolk in a sodium citrate buffer, 50 percent from Milovonov diluter, 83.3 percent from a salt diluter of sodium citrate, magnesium sulphate and calcium acetate and 64.1 percent conceived after insemination with milk diluted semen.

It was suggested that egg yolk had an unfavorable action against the fertilizing ability of ram sperm and that ram sperm motility was not necessarily indicative of fertility.

D. pH, Electrolytes and Osmotic Pressure

Emmens (1947, 1948) using rabbit sperm, concluded that motility was not significantly affected by changes in the proportion of sodium chloride and glucose in buffered diluents of the same osmotic pressure, except above a pH of 9.

Blackshaw and Emmens (1951) reported that ram and bull spermatozoa exhibited optimum motility at pH 7. Hypertonic solutions were less harmful than hypotonic media, at all pH levels. The slight adverse effect of hypertonicity could be diminished by partial replacement of sodium chloride with glucose at alkaline pH levels.

Salisbury and Kinney (1957) have found that pH markedly influences aerobic fructolysis as pH increases fructolysis rate increases and more lactic acid is produced in excess of fructose utilization.

Anderson (1945), after reviewing Russian work, stated that a certain concentration of electrolytes was necessary for normal sperm irritability, but that too high concentrations over stimulated spermatozoa, which rapidly lost motility and died.

Oxygen consumption by spermatozoa was stimulated by a diluent of 0.9 percent sodium chloride. The rate of motility and the livability were higher and greater than motility and livability of sperm in a phosphate diluent, so long as the oxidation of lactic acid proceeded (Bishop and Salisbury, 1955). When aeration was

incomplete, lactic acid accumulated to toxic levels, pH of the media dropped rapidly and the cells died (Salisbury, 1957).

According to Mann (1954), appreciable quantities of potassium, magnesium and calcium occur in mammalian semen.

Winters et al. (1938) and Milovanov (1934) found calcium necessary in diluents for ram spermatozoa. However, Lardy et al. (1945), Lardy and Phillips (1943) and Blackshaw (1953a) found that calcium depressed the viability of ram and bull spermatozoa.

According to Lardy and Phillips (1943) magnesium improved the motility and glycolysis of washed bull spermatozoa.

The importance of potassium for the normal functioning of ram and bull spermatozoa has been demonstrated by Lardy and Phillips (1943), White (1953a,b,c and 1956) and Blackshaw (1953a,b).

The motility of mammalian spermatozoa was decreased by high dilution even if the diluent was an isotonic one containing a glycolysable sugar (Emmens and Swyer, 1948; Blackshaw, 1953a; and White, 1953c, 1954.). It was postulated that the dilution of salts was responsible for this reduced sperm motility after dilution.

The osmotic pressure of the semen of several species, in terms of freezing point depression, has been summarized by Mann (1954). The data expressed in centigrade temperature depression were: man .55-.58°; bull .54-.73°; ram .55-.70°; stallion .58-.62°; and boar .59-.63°.

E. Spermatozoa Death After Freezing and Thawing

The studies of Davenport (1897), as reported by Davenport (1938), Jahnel (1938), Shettles (1940), Hoagland and Pincus (1942) and Parkes (1945) illustrated that human spermatozoa were exceptional in that a small portion could survive freezing. Emmens and Blackshaw (1950) resuspended human, bull, ram and rabbit sperm in each respective seminal plasma. The human sperm survived freezing irrespective of the plasma species it was suspended in, indicating the survival property was with the cell itself. Attempts to revive frozen spermatozoa of other species met with little success until Polge, Smith and Parkes (1949) found that glycerol exerted a protective action on cells during freezing. The studies of Polge (1957), Smith and Polge (1950), Emmens and Blackshaw (1950), Szumowski (1954), Roy (1955) and Polge (1956) indicate that considerable species difference appears to exist as to the tolerance of sperm cells and red blood cells to glycerol. For most species studied the critical temperature during freezing seems to be between -15° and -20° C. (Polge 1957).

The literature suggests (Polge, 1957; Walton, 1957; Sherman, 1959; and Parkes, 1956b) that more than one factor is responsible for sperm cell death during freezing.

Luyet and Gehenio (1940) have reviewed the factors responsible for life and death of cells at low temperatures. They postulated that cells were killed during freezing by intra-cellular ice formation and subsequent protoplasmic alterations.

Luseva and Cook (1953) found that glycerol modified the rate of ice crystal growth, membrane permeability and related phenomena and partially controlled the rate of freezing.

Smith, Polge, and Smiles (1951) observed a fern like pattern of channels in semen frozen in the presence of glycerol. However, glycerol did not prevent crystal formation in the media.

The work of Sherman (1957) suggested that extra-cellular mechanical injury from ice formation on freezing and thawing did not cause sperm death.

There is some doubt as to whether crystalization is the primary factor causing sperm death during freezing.

Lovelock (1953a, b, c) found that destruction of red blood cells during freezing and thawing was caused by an increased concentration of salts which resulted from the conversion of water to ice. In the presence of glycerol the rise in electrolyte concentration below the freezing point of the fluid was sufficiently reduced to abolish this effect. Glycerol failed to exert any protective action when it was prevented from entering the blood cell by the action of copper ions.

The experiments of Lovelock and Polge (1954) have shown a relationship between the degree of sperm cell damage caused by freezing at various temperatures below zero and that caused by exposing the spermatozoa to high concentrations of salts at 0° C. The glycerol requirement for freezing sperm of various species and for freezing various cells depended on the salt concentrations of the cells normal environment.

Observations of Lovelock (1953b), (1954c) indicated that the high concentration of salt produced by ice formation did cause dispersion of lipids and liproproteins from the red cell membrane which was modified by the presence of glycerol.

Lovelock (1954b) found that red blood cells which ordinarily did not suffer from thermal shock, did succumb to thermal shock in the presence of excess salt.

There are, according to Polge (1957), apparently two major problems in freezing semen. The first problem being to freeze the semen fast enough between -15 and -25° C. such that exposure to high salt concentration is reduced. The second problem is to freeze the semen slow enough or with sufficient protection to prevent the occurrence of temperature shock.

The studies of Easley, Mayer and Bogart (1942); Walton (1951); Blackshaw (1954); and Kamschmidt et al. (1953) have illustrated that egg yolk, and particularly its lipid and lipoprotein constituents, protect smperatozoa against the damaging effects of temperature shock.

Stallion and boar sperm are the most susceptible to cold shock and ram sperm are more resistent to cold shock than bull sperm (Mayer 1955). Electron photomicrographs taken by Walton (1957b) indicated that a galecapitis and cell surface destruction took place on cold shocked sperm and sperm dying natural death.

The mechanism of the temperature shock protective action of substances such as egg yolk and lecithin is not known. Blackshaw and Salisbury (1957) suggested that this protection was due to a surface property which maintained the integrity and function of the cell

membrane. Their research has proven that cold shock of whole semen causes a loss of potassium and ether-alcohol extractable phosphorus from the cells into the plasma, and an uptake of sodium and calcium by the cells.

Sherman (1959) has shown that some sperm, although motile after freezing and thawing, were injured or weakened by the ordeal. This injury occurred during freezing or thawing and not as a consequence of extender toxicity.

Recent studies have proven that glycerol was metabolized by bull spermatozoa (Odell et al. 1956, White et al. 1954 and White 1957) and by ram spermatozoa, (Mann and White, 1956, 1957). Glycerol metabolism by ram and bull sperm is primarily aerobic with a sparing effect on fructolysis (Mann and White 1957 and White 1957). The rate of glycerol utilization by bull sperm (Odell et al. 1959a) varies with the type of diluent employed. Odell et al. (1959b) have shown that the sugars arabinose and fructose have a sparing effect on glycerol utilization by bull sperm. Lactic acid was not produced from arabinose metabolism whereas large quantities of lactic acid were produced by aerobic metabolism of fructose. White et al. (1954) also could find no lactic acid formed when bull and ram sperm metabolized arabinose.

The studies of Elliott et al. (1954), Emmens and Blackshaw (1950), Blackshaw (1955a), Odell and Almquist (1957), VanDemark et al. (1957) and First et al. (1957) indicated that adding various monosaccharides to diluted, glycerolated bull or ram sperm increased the percentage of spermatozoa surviving freezing to -79° C. or below.

Shaffner et al. (1941) found that levulose partially protected fowl sperm during quick freezing.

F. Equilibration Time and Temperature

Considerable discussion has centered around the necessity of equilibrating semen with glycerol for several hours before freezing. The temperature at which this equilibration should take place has also been questioned. The idea of glycerol equilibration period for bull semen began with the original work of Polge and Rowson (1952a) which indicated optimum recovery and fertility after freezing were obtained with a 15 to 20 hour glycerol equilibration. Holt (1953) and Stower (1953) reported greatest recovery with equilibration of 20 to 24 hours.

Hill et al. (1959) found a .5 hour equilibration period to be superior to an 18 hour equilibration period, in terms of ram sperm recovery after freezing in a yolk citrate extender.

Szumowski et al. (1956) found at least four hours of glycerol equilibration necessary for freezing ram semen. The optimum equilibration period was reported to be between six and 14 hours.

Graham et al. (1957) reported a 67.8 percent first service non-return rate from 1,012 cows bred with semen that was equilibrated with glycerol 12 hours before freezing, 65.2 percent non-return from 1,026 first services with semen equilibrated for eight hours and 63.4 percent non-return from 996 first services to bull semen equilibrated for eight hours. The four and 12 hour differences were significant.

Williams (1954), however, reported a higher percentage of non-return when cows were inseminated with semen equilibrated six hours as compared to that equilibrated 18 hours.

Saroff and Mixner (1955) stated that as glycerol equilibration time was increased from two to 18 hours there was a progressive increase in sperm survival on thawing. Cragle et al. (1955), using a three dimensional central composite design, found the optimum percent citrate, percent glycerol and equilibration time to be 2.9 percent, 7.6 percent and 14.9 hours respectively.

Odell and Almquist (1954) found no significant differences in bull sperm survival among glycerol equilibration periods of 0.5, 4 and 18 hours after freezing as skimmilk diluted bull semen. Odell and Hurst (1956) compared glycerol equilibration time of 0.5 and 18 hours for semen diluted in heated skimmilk and found a significant difference in favor of the shorter time.

Odell and Almquist (1957) found that in skimmilk diluent, pre-freezing glycerol equilibration periods of 30 minutes and four hours resulted in spermatozoan survival equal to or better than 18 hours. They state that equilibration time was not related to the level of glycerol present in the diluent. The stepwise addition of glycerol resulted in slightly greater post-thaw motility than if the glycerol were added in one portion at 5° C.

They found that 1.25 percent fructose added to the skimmilk diluent resulted in a significant increase in freezability of bull

spermatozoa. However, the addition of this sugar had no effect on the equilibration period or level of glycerol needed for optimum freezability.

Blackshaw (1955a) reported that equilibration of bull spermatozoa with glycerol for 18 hours before freezing does not increase survival after freezing. Blackshaw (1955b) reported that bull and ram semen may be mixed with the glycerol containing diluent at temperatures up to 15° C. without harmful effects on revival after freezing.

VanDemark et al. (1957) studied glycerol equilibration time and equilibration temperature. At an equilibration temperature of 4.5° C., little variation in motility was found after freezing and thawing, among semen equilibrated at 2, 6 and 18 hours under equilibration temperatures of 10° C. and 15.5° C. The shortest equilibration time, (two hours) was slightly more detrimental, with the differences at 15.5° C. being significant. For all temperatures considered, six hours was significantly better than two or 18 hours. Their data also supports the findings of Emmens and Blackshaw (1950), Blackshaw (1955a), Odell and Almquist (1957) and Amann et al. (1957) who reported the addition of simple sugars such as glucose rhamnose, arabinose and fructose does improve sperm survival after freezing. Emmens and Blackshaw (1950), Blackshaw (1955a) and First et al. (1957) have shown that 1.25 percent arabinose combined with glycerol before freezing results in a very marked increase in ram sperm survival with yolk phosphate citrate and milk diluters.

Odell et al. (1956b) have recently shown that fructose and arabinose have a sparing effect on the utilization of glycerol-1C^{14} by bull spermatozoa.

In summarizing the research data pertaining to equilibration time where some aspect of motility after freezing has been used as a criterium, the work of Polge and Rowson (1952a), Holt (1953), Stower (1953) and Saroff and Mixner (1955) support an equilibration period of 18 hours or more. The reports of Cragle et al. (1955), VanDemark (1957) and Szumowski et al. (1956) favor equilibration of six to 15 hours. The research of Odell and Almquist (1954) and Odell and Hurst (1956), Odell and Almquist (1957), Blackshaw (1955a) and Hill et al. (1959) indicate no more than one hour of equilibration with glycerol is necessary. In breeding trials, Graham et al. (1957), found bull semen equilibration of less than one hour the most satisfactory and Williams (1954) found six hours equilibration resulted in a higher 60 to 90 day non-return rate than 18 hours equilibration.

The studies of Polge (1957) indicate a possible explanation for the differences in equilibration results reported by different authors. Bull, ram, stallion and boar semen exhibited greater resistance to temperatures between -15 and -25° C. after 18 hours equilibration when compared with one hour equilibration. These facts suggested that differences in equilibration effects on sperm survival may not be noticed unless sperm are frozen very slowly.

G. The Storage Potential of Frozen Semen

The decrease in motility and decreased fertilizing capacity of bull sperm after prolonged frozen storage has been investigated by several workers.

Bratton et al. (1957) reported non-return rates of 73.2 percent of 1,151 first services to frozen semen stored one week and 69.8 percent for 1,094 first services to frozen semen stored 17 weeks at -79° C. This difference was not significant.

Mixner and Wiggin (1957) found that a first service non-return rate of 66.7 percent occurred with frozen bull semen stored for seven and 14 days at -79° C. After six and 12 months storage the non-return rates were 70.1 and 65.7 percent respectively.

Graham et al. (1958) found no significant differences in non-return rates from frozen semen used after one, two, three and four weeks of storage at -79° C. The first service non-return rates were 63.6 percent after one week, 62.3 percent after two weeks, 64.6 percent after three weeks and 67.1 percent after four weeks storage.

VanDemark et al. (1957) found that bull semen stored at -79° C. exhibited the following motility: 49 percent after two days, 46 percent after nine days, 40 percent after 16 days and 38 percent after 51 days storage.

Hill et al. (1959) found a significant decline in motility recovery between ram semen frozen two days and 10 days at -79° C.

Szumowski et al. (1956) observed no decline in motility of ram sperm stored four weeks at -79° C.

Ludwick (1958) observed that frozen bull semen declined more rapidly in motility after thawing than unfrozen semen of the same age. Etgen et al. (1957) and Polge and Rowson (1952b) have even observed a slight increase in percent motile or live cells with increasing storage age at -79° C. Etgen et al. (1957) explain this on the basis that there is (1) a necessary sperm adjustment period after freezing, (2) disintegration of sperm during the first weeks of storage or (3) personal bias of the evaluator.

H. Sperm Numbers Necessary for Satisfactory Conception

Kuznecov (1934) recommended the insemination of 50 million or more sperm in the cervix of the ewe. Milovanov (1934) reported that at least 500 million ram sperm were essential for vaginal insemination.

The data of Habibullin (1937) indicated that lambing percentages were higher for 1:1 and 1:2 dilutions than 1:3 dilution or undiluted semen and that 1:1 dilution resulted in the highest lambing percentage. All the above treatments were inseminated in a .1 ml. volume. He recommended the total sperm numbers inseminated should be not less than one billion.

Habibullin (1938) found that after 24 hours storage a dilution of 1:2 maintained fertilizing ability at 64 percent, whereas sperm diluted 1:1 showed a marked drop and if diluted 1:3 fertility dropped to nearly the level of undiluted sperm (36 percent).

Lukin and Eremeev (1938) estimated that to ensure 90 percent fertilization the number of undiluted ram sperm inseminated should be 1.1 billion or if diluted 506 million.

Anderson (1941) reported that when 462 ewes were inseminated with undiluted ram semen, or semen diluted 1:3, 1:8 or 1:16; conception was much lower following 1:16 dilution than it was for the other treatments (42 to 46 percent).

Keast and Morley (1949) concluded from their work that although the highest conceptions were obtained with insemination of 350 million

ram sperm in 0.1 ml. volume, reasonably satisfactory results were obtained with a concentration as low as 50 million ram sperm per 0.1 ml. insemination.

Carbonero (1955) reported the optimum dilution rate, using a physiological sub-alkaline NaCl and NaoH diluter, to be one part ram semen to five parts of diluter.

After reviewing the literature, Terrill (1952), recommended that 50 million or more sperm should be inseminated into the cervix in not more than 0.2 ml. of fluid carrier. Barretto and Mies Fihlo (1944), however, achieved moderately successful results from as low as five million sperm per insemination. Panyseva (1940) reported a direct correlation between the number of sperm inseminated and conception rate ($r = .85$). Koger (1951) found a linear relation between number of sperm inseminated and conception rate. Fifty million or more fresh sperm per insemination were required for a conception rate of more than 50 percent. A preliminary study was conducted with sperm diluted in egg yolk citrate, stored at 5° C. and used from zero through five days. The insemination of ram sperm at concentrations of 420, 105 and 26 million sperm per ml. resulted in pregnancy rates of 35.2, 26.5 and 17.1 percent respectively. When only fresh diluted semen of less than one day of age was considered, the pregnancy rates were 67, 50 and 33 percent.

In a second experiment ram semen diluted in egg yolk citrate was inseminated without storage and the following sperm concentration conception relationships were recorded:

Million Sperm/Insemination	Percent Conception Rate
420.0	62.5
210.0	65.6
105.0	50.0
52.5	46.7
35.0	33.3
17.5	21.4
8.8	25.8

A similar relationship existed between sperm numbers per insemination and conception when either physiological saline or seminal fluids were used as the diluter. However, the corresponding conception rates were all higher when sperm were diluted in ram seminal fluids. These differences were not significant.

Kuznetsov (1956), reported on work in Russia, stated that only 30 to 90 million ram sperm from a normal ejaculate penetrate the cervix and that sperm in the vagina perish within three to six hours after mating. These investigations have shown that spermatozoa leave the cervix in small portions, moving into the uterus and oviducts. The sperm in the uterus retained activity for up to 12 hours. The cervix contained seven to 12 million sperm, 15 to 20 percent of which were motile 30 to 48 hours after insemination. It was suggested that the cervix acts as a store for sperm allowing a periodic release of motile sperm throughout estrus.

I. Volume of Semen Inseminated

Smirnov et al. (1939) concluded that the insemination of 0.3 ml. of undiluted ram semen in gelatin or paper capsules resulted in conception rates equal to that of 0.1 to 0.15 ml. of undiluted semen inseminated with a syringe in the cervix. The conception rates from these treatments were between 74 and 75 percent.

Sinclair (1957), using fresh undiluted ram semen, obtained no difference in conception between cervically inseminated doses of 0.1, 0.2, 0.3 or 0.4 ml.

Malikov (1957) cervically inseminated ewes with .05 ml. of undiluted semen and with .1 and .05 ml. of semen diluted in yolk citrate or in boiled cows milk. Seventy-three percent of the ewes inseminated with .1 ml. of milk, 66 percent of those inseminated with .05 ml. milk and 66 percent and 53 percent respectively of the ewes inseminated with one ml. and .05 ml. of yolk citrate diluted ram semen conceived.

Koger (1951) has achieved 50 percent conception from vaginal inseminations of one ml. of ram semen.

J. Cervical versus Vaginal Insemination

The difference in sperm numbers necessary for vaginal insemination (Milovanov 1934) and cervical insemination (Kuznecov 1934) would suggest that cervical insemination might be more effective and efficient. Anderson (1937) considered vaginal inseminations responsible for low fertility in a group of ewes wherein cervical inseminations were attempted. Kelly et al. (1942) found no difference in lambing percentage whether semen was deposited in the cervix, vaginal folds or forynx of the vagina.

Keast and Morley (1949) reported that of 30 cases wherein semen was deposited in the vagina, because the cervix could not be located, only four ewes conceived. These four were from treatments wherein conception after cervical insemination was from 37 to 69 percent.

Koger (1951) inseminated one-half of 201 ewes in the cervix with 0.2 ml. of diluted semen. The other one-half were inseminated in the vagina with one ml. of diluted semen containing the same total number of sperm. There was no difference in the pregnancies obtained between the two methods, cervical insemination resulting in 48.5 percent pregnancies compared to 50 percent from the vaginal inseminations.

Dauzier et al. (1954) found no difference in conception rate between cervical or vaginal insemination.

Mies Filho and De Almieda Ramos (1955) after inseminating 872 ewes with fresh semen, found a significant conception difference in favor of vaginal as opposed to cervical insemination.

Dun (1955) studied the reproductive tracts of 112 slaughtered Merino ewes and concluded that an experienced inseminator would be able to deposit semen in the cervical opening in 100 percent of young maiden ewes and 70 percent of older ewes.

K. When to Inseminate the Ewe in Reference to the Beginning of Estrus and Sperm Life in the Ewe

The optimum time for insemination should depend mainly on the life of sperm in the reproductive tract of the ewe, the time of ovulation and the life of the ova.

Quinlan et al. (1932, 1933) found that in Merino sheep the majority of ram sperm inseminated were non-motile after 12 hours. Live sperm were found in the cervix of the ewe up to 48 hours after mating.

Green and Winters (1935) stated that sperm did not live more than 24 hours in the genital tract of the ewe. Kelly (1937) found ram sperm reached the threshold of infertility 34 hours after mating.

Palovceva et al. (1938) estimated the average duration of sperm survival in the ewe to be 34 to 36 hours and in some cases as much as 40 to 50 hours.

Lopyrin and Loginova (1939) found that sperm maintained motility for 32 hours in the upper portion of the genital tract of ewes in heat. The data of Green (1947) indicated that ram sperm may retain their ability to fertilize the ovum up to approximately two days after insemination.

Quinlan et al. (1932) mated ewes at intervals after the beginning of estrus and found a constant conception rate up to 30 hours, after which time a definite drop in conception occurred.

Kardymovic et al. (1935) found the optimum time to breed was 18 to 25 hours after the onset of estrus.

Warbritton et al. (1937) observed that of three insemination periods tried, the most desirable was 12 hours before ovulation. This was approximately ten to 18 hours after the onset of estrus.

Anderson (1941) reported that insemination from 12 to 30 hours after the onset of estrus resulted in the best conception.

Kelly et al. (1942) reported that there was no difference in the rate of lambing between ewes still in estrus 16 to 24 hours after insemination and those out of estrus.

Carbonerro (1955), after inseminating 8,470 ewes, concluded that the best time for insemination was 12 to 18 hours after the beginning of estrus, when the vaginal mucus was turbid.

Sinclair (1957) found no difference in conception rates among ewes inseminated, 0-20, 16-28 and 0-28 hours after the beginning of estrus. Further information was gained on this matter from another trial in which the stage of estrus was determined by the condition of the vaginal mucus. Clear mucus was associated with early estrus, clear and copious mucus with middle estrus, cloudy mucus with late estrus and creamy mucus with very late estrus. It was concluded that insemination very late in estrus, a period characterized by the presence of creamy mucus in the vagina, was likely to result in a lowered rate of conception.

Concerning the time of ovulation in reference to estrus, McKenzie and Terrill (1937) found that ovulation in the ewe generally takes place near the end of estrus and that inseminations made after the end of estrus are less likely to be successful than those before the end of estrus.

Palovceva et al. (1938) reported that in the sheep fertilization occurred not earlier than two to four hours after ovulation and ovulation occurred 30 hours or more after the onset of estrus.

Lopyrin and Loginova (1939) found that the average time of ovulation was 30 to 32 hours after the onset of estrus.

Larrea (1944) reported that Corriedale ewes remained in estrus for 28 to 30 hours.

Schott and Phillips (1941) found a time interval of 20 minutes was usually sufficient for sperm to reach the upper part of the Fallopian tube after a normal service and that this was not influenced by the stage of estrus or by ovulation. Ovulation usually occurred 20 to 36 hours after the onset of estrus. The right and left ovaries were equally active. Of several breeds studied Corriedale and Dorset ewes tended to remain in estrus after ovulation.

Palovceva (1940) observed definite uterine movements associated with estrus in the ewe and that simultaneous contraction of longitudinal and relaxation of circular muscles of the uterine neck assist in opening the cervical canal.

Dauzier (1955a,b) found that uterine contraction is the essential factor in uterine migration of sperm and that spermatozoan migration in the Fallopian tube was primarily due to sperm motility.

However, the studies of VanDemark and Moeller (1951) indicated that bull sperm travel the Fallopian tube of the cow at a much greater rate than can be accounted for by their own propulsion.

Braden (1953) observed that in rabbits the tubal uterine junction acts as a barrier against sperm passage and estimated that one of every 160 sperm in the upper uterus enters the Fallopian tube. He postulated that sperm reaching the distal half of the uterus within one hour after mating may be transported by uterine activity while those reaching this location two to three hours later do so by virtue of their own motility. Noyes et al. (1958) have shown in rabbits, that only motile sperm pass the cervix. Other recent papers concerning this subject are those of Black and Asdell (1958) and Adams (1956).

The work of Chang (1951, 1955) and of Braden and Austin (1953), indicated that a sperm conditioning or capacitation period of several hours in the Fallopian tube was necessary before fertilization. Austin and Bishop (1958) suggest that capacitation involves a loosening of the acrosome from the sperm head. It was postulated that the loosened acrosome released an enzyme which enabled the sperm to pass though the zona pellucida of the ova.

L. Insemination or Mating Frequency During One Estrus

Quinlan et al. (1932) found that breeding ewes twice during one estrus resulted in a slightly greater percentage of pregnancies than breeding once. However, this difference was not significant.

Milovanov (1934) reported that results of repeated insemination have been excellent in some cases and negative in others. Milovanov (1936) as cited by Anderson (1945) reported that some Russian sheep stations had a large increase in lambing percentages when the ewes were inseminated several times. Peregan (1936) as cited by Anderson (1945), inseminated 56 Karakul ewes twice at an interval of 20 to 30 hours, this resulted in a 171 percent lambing rate whereas the average for the flock inseminated once was 115 percent.

Avramov (1937) inseminated 106 ewes twice at an interval of eight to 12 hours or 20 to 24 hours. When the interval between inseminations was 24 hours, the lambing rate of Tigai ewes was raised by 11.7 percent and of Chusbeka ewes by 14.1 percent.

Gavrilov (1937) reported that an eight hour inverval between inseminations gave somewhat better results than a 24 hour interval and suggested that the second insemination should be carried out not later than 12 hours after the first.

Kirillov (1938) as cited by Anderson (1945), concluded that all ewes should be tested for estrus twice a day, morning and evening, and only those with an estrus period of more than 24 hours should be inseminated twice (47 percent of the ewes were in estrus less than 24 hours).

Three or four inseminations did not raise the rate of lambing of ewes with estrus periods longer than $1\frac{1}{2}$ to two days.

Lopyrin and Loginova (1939) observed from multiple inseminations of 2,582 ewes that there was little difference in conception between ewes inseminated once and ewes given two or three inseminations at 16 hour intervals.

Dauzier et al. (1954) after inseminating 438 ewes found no difference between insemination once or several times during one estrus.

Anderson (1941) studied 462 ewes and reported that two to four inseminations in one estrus period gave better results than a single one. A single service was reported to give better results than a single insemination.

Larrea (1944) reported on the results of 523 single and 25 double inseminations in 1943 and 703 single and 23 double inseminations in 1944. The percent lambs born was 69 percent from one and 95 percent from two inseminations in 1943, with 74 percent from one and 114 percent from two inseminations in 1944.

Glembockii and Vasiljev (1944) inseminated Precoce ewes within the first 24 hours of estrus, with the semen of Precoce rams, and if estrus lasted more than 24 hours ewes were inseminated a second time, with the semen of Karakul rams. The percent lamb crop for 246 ewes inseminated once was 134.5 percent. The lambs born to 153 out of 190 ewes inseminated twice were of the Precoce type (80.5 percent). The lambing percent for these ewes was 135.3 percent, indicating that

80.5 percent of these ewes conceived from the first insemination. The insemination after 24 hours was effective for the remaining 37 of the 190 ewes that delivered nine Precoce and 52 Karakul x Precoce lambs for a 164.0 percent lamb crop. The nine Precoce were the result of a protracted heat and probably twins to a crossbred.

Gutierrez (1948), after analyzing the results from insemination of 38,000 ewes in Uruguay, reported that two inseminations per estrus increased conception rates by 10 percent.

Aamdal and Hogset (1955) reported that 40 days after insemination 22 of 64 ewes inseminated once with yolk citrate diluted ram semen were pregnant and four of eight ewes were pregnant after inseminating twice. None of 19 ewes became pregnant from one insemination but five of 10 were pregnant after two inseminations with ram semen diluted in yolk glycine.

The data of Lopyrin et al. (1957) from 1,000 double inseminations indicated that 80.2 percent of the ewes conceived when the two inseminations were eight hours apart and 72 percent conceived when the inseminations were 24 hours apart. The lambing percentages were 127.8 percent from the eight hour interval and 124.4 percent from the 24 hour interval.

M. Vasectomized Mating

The question of what effect a vasectomized check ram may have on conception from subsequent inseminations has been considered by Dauzier et al. (1954). They found no difference in conception when ewes were and were not mated to a vasectomized ram immediately prior to insemination.

Echenique et al. (1941) found the seminal fluids of vasectomized rams to be a more satisfactory diluent than several common diluents tested. The semen of vasectomized bulls was equally effective in diluting ram semen.

Larrea (1944) also found the most satisfactory diluent to be the ejaculate of a vasectomized ram.

Vasectomized rams have been used to induce earlier breeding in ewes going from anestrus to estrus.

Coleman (1950) found with 180 ewes that having vasectomized Merino rams running with one of two groups of ewes for three weeks before mating resulted in earlier lambing and a higher lambing percent than in the control group.

Coleman (1951a, b) used the same ewes previously reported but reversed the ewes to each treatment. Vasectomized rams were run with the experimental groups for 26 days prior to a six week mating period. During the first two weeks of lambing 59 lambs were born in the teased group versus 29 in the unteased group. The total number of lambs produced was also higher in the teased group.

Radford and Watson (1957) observed that estrus occurred approximately one week earlier in anestrus ewes run with vasectomized rams before the breeding season. In at least 80 percent of the ewes estrus was accompanied by massive desquamation of the vaginal epithelium and it was preceded by one or more periods of massive desquamation.

Pitkjanen (1958a,b) used uterine fistulas to discover that sperm reached the tubo uterine junction of the sow much more rapdily if natural mating occurred than when the sows were artificially inseminated. Evidence of hastened ovulation due to mating was presented. Ninety-five percent of the sows natural mated had ovulated 39 hours after the beginning of estrus whereas 58 percent of the unmated sows had ovulated by that time.

Marion et al. (1950) observed that sterile copulation in the cow caused ovulation to occur significantly earlier than in unmated controls. This suggested an earlier release of gonadotrophin as a result of increased oxytocin secretion. This matter was discussed by Hansel (1958) and Hansel et al. (1958).

IV. MATERIALS AND METHODS

General Procedures

Collections:

Ram semen was collected with either an artificial vagina or by means of electrical stimulation using a Production Products model 2500 stimulator. Rams were restrained on their side for electrical collection. All semen was ejaculated into graduated test tubes protected by a 39° C. water jacket for both types of collection.

When the artificial vagina was used rams were excited by either a ewe in natural estrus or a ewe in which estrus had been induced with subcutaneous injection of two to three mg. estradiol cyclopentol propionate. The rams were allowed one or two false mounts with a four to five minute restraining period before each collection. It was observed that volume of the ejaculate was increased by this excitation procedure.

Dilution:

All samples were diluted initially after collection and then again after cooling to 5° C. Split ejaculates were needed for most of the experiments, therefore, in order to prevent temperature shock, all initial dilutions were performed immediately after collection in a 35° C. room. The semen was cooled slowly to 5° C. in a refrigerator. Samples were then transferred to a 5° C. walk-in cooler where the second dilution occurred. When glycerol and arabinose were to be added, they were contained entirely in the second dilution fraction.

The second fraction was added to the initially diluted semen in three equal portions at five minute intervals. Unless otherwise

specified all samples were equilibrated approximately 14 hours before freezing.

Freezing:

All samples were frozen as a one ml. volume in "Frost T" heat-sealed plastic ampoules. Freezing was accomplished in the laboratory of the Michigan Artificial Breeders Cooperative. The samples to be frozen were suspended in a bath of ethyl alcohol at a temperature of 5° C. The temperature of the bath was further lowered by adding small pieces of dry ice to the alcohol so that it dropped 1° per minute from 5° to -10° C. then 2° per minute from -10° to -18° C., 4° per minute from -18° to -35° C. and 5° per minute from -35° to -75° C. The temperature drop was controlled by manually following a graph of this rate with a potentiometer which recorded the temperature of the alcohol bath and of the semen every 4.5 seconds.

After freezing, the samples were stored under dry ice and isopropyl alcohol in a "Head Styrofoam" semen storage chest. The storage temperature varied between -77° and -81° C.

Thawing Procedure:

All samples were thawed in 6° to 8° C. water. Inseminations were performed as soon as the semen was liquid. For motility studies all samples were held at 5° C. one hour then transferred to a 39° C. incubation room and held there for 15 minutes before microscopic examination.

Microscopic Examination Procedures:

Slides were prepared in the 39° C. incubation room by adding a small loop of semen to a drop of saline and then cover slipping.

The slides were examined on a heated microscope stage at approximately 40° C. Sperm cells were counted at 210x magnification using a Bausch and Lomb binocular phase contrast microscope.

Two slides were counted per ampoule of semen and at least two ampoules were examined for each recorded observation in the experiments reported.

The counting procedure consisted of: (1) diluting initially in the warm room with saline so that 10 to 30 cells were visable in the microscope field at 210x magnification. A total of approximately 100 cells were counted per slide. The number of cells exhibiting forward progressive motility, those motile but not progressively motile and non-motile cells were all recorded using a differential counter. The percent motile cells and the percent progressively motile cells were determined from these counts.

Motility examination of semen after collection and dilution was performed in the same manner.

Concentration determinations were obtained from the freshly collected undiluted ejaculate according to the procedure of Laing (1955). More recently the spermatocrit method of Hickman (1958) was used since it was much more rapid.

Materials and Their Preparation

Glycerol: Cevco U.S.P. grade glycerol

Glucose: Nutritional Biochemicals Anhydrous Dextrose

Arabinose: D.L. arabinose from Nutritional Biochemicals

Urea: Merck reagent grade urea

Egg Yolk: Egg yolk was obtained by washing the intact yolk in distilled water and blotting dry. The yolk membrane was ruptured and the yolk poured out. Only eggs of less than one week of age were used.

Sodium citrate: A 2.7 percent (dry formula weight) solution of sodium citrate was maintained for formulating the yolk citrate extender. The sodium citrate was of the formula $Na_3C_6H_5O_7.2H_2O$ and U.S.P. grade from Mallinckrodt Chemical.

Milk Extender: The milk extender was prepared in two parts.

Part A - The homogenized pasteurized milk used as an extender was obtained from Michigan Artificial Breeders Cooperative after it had been heated to 208° F. for ten minutes. The average solid content was 12.05 percent before heating and 12.19 percent after heating. This incidentally was a significant difference. The fat content of the milk averaged 3.4 percent before heating and 3.45 percent after heating. The milk was less than four days old when used. Five hundred oxford units penicillin and 500 micrograms of dihydrostreptomycin were added per ml.

Part B - of the milk extender consisted of 14 ml. of glycerol and 2.25 grams arabinose raised to 100 ml. volume with the milk described previously.

Egg Yolk Citrate Extender: The egg yolk citrate extender used for freezing was prepared in two parts:

Part A - which was used for the initial dilution, consisted of 25 ml. egg yolk added to 75 ml. of a 2.7 percent sodium citrate solution.

Part B - consisted of 25 ml. egg yolk, 2.25 gm. arabinose and 14 ml. of glycerol all raised to a 100 ml. volume with 2.7 percent sodium citrate.

Parts A and B were mixed in equal portions after the semen was initially added to A and cooled to 5° C. Three mixings at five minute intervals were used.

Russian-Ukranian Extender: The formula for the Russian-Ukranian yolk-glucose-urea extender was obtained from Jack Judy of Ohio State University.

Two separate dilutions were necessary. The initial dilution was made using 50 ml. distilled water, 10 ml. egg yolk, 1.4 gm. sodium citrate and 0.4 gm. glucose.

The glycerolized fraction of the diluter was as follows:

8 ml. of 5% sodium citrate solution
2 ml. of 2% urea solution
2 ml. of egg yolk
1.8 ml. of glycerol

Equal volumes of each of the two fractions were used. Glycerolization was accomplished by adding the glycerolized fraction in

three equal volumes at five minute intervals, under refrigeration at all times.

V. RESULTS AND DISCUSSION

A. The Glycerol Requirement for Freezing Ram Semen*

This study was initiated to more precisely determine the amount of glycerol required for the greatest motility of ram sperm after freezing.

Three ejaculates were collected and processed on each of two Hampshire rams according to the previously described procedure.

Data pertaining to these ejaculates is presented in Table 1.

Table 1. Ejaculates Used in Glycerol Level Experiment

Ram	Ejaculate	Volume (ml.)	Concentration (billion/ml.)	Percent Progressive Motility	Percent Motile Cells
Pope	1	1.0	3.8	60	80
	2	1.0	4.8	54	70
	3	1.0	3.4	44	80
Rayl	1	0.8	4.4	45	70
	2	1.5	4.2	33	58
	3	0.8	2.7	45	75

Each ejaculate was split into treatment groups of 5.5, 6, 6.5, 7, 7.5, 8 and 8.5 percent glycerol.

The samples were diluted 1:200 and frozen. Approximately three vials from each ejaculate were examined for motility and progressive motility, 10 months after the semen was frozen. Samples were examined

*All glycerol levels referred to here are a volume per volume basis.

according to procedures previously described. Each vial examined constituted one observation. The data may be seen in Appendix Table A.

A preliminary analysis indicated no significant interaction between ejaculates and glycerol levels as reported in Table 2.

Table 2. A Test of Interaction Between Glycerol Levels and Ejaculates

Source	df	ss	ms	F
Glycerol level x ejaculate	35	3,342.8	95.5	1.18
Error	108	8,736.8	80.9	

The mean square of interaction of glycerol level x ejaculate contains some of the main effects of glycerol level and ejaculate. However, since this mean square was not significantly larger than the error mean square, the mean square for interaction alone could not be significant. Least square equations were set up to measure the effect of glycerol levels, rams and days. This type of analysis was considered necessary because a disproportionate number of vials were available on each treatment. The procedure for setting up and solving the equations was given by Snedecor (1956 section 12.17).

The estimates of the main effect were obtained with the aid of the electronic computer 'MISTIC' using program M-13.

An analysis of the treatment effects (Table 3) indicated a significant difference among glycerol levels ($P < .05$).

Table 3. Variance Due to Glycerol Levels

Source	df	ss	ms	F
Glycerol levels	7	1,853.61	264.80	2.12*
Error	136	16,987.68	124.91	

*Significant ($P<.05$)

A student range test indicated that the motility means for glycerol levels of 6, 6.5, 7, 7.5, 8 and 8.5 percent were significantly greater than the motility means for 5 or 5.5 percent glycerol ($P<.05$). The mean motility after freezing of the 8.5 percent level was significantly greater than the mean motility of five percent glycerol ($P<.01$).

The mean percent motility for each glycerol level is shown in Table 4.

Table 4. Percent Ram Sperm Motility after Freezing in Various Levels of Glycerol

Glycerol level	5%	5.5%	6%	6.5%	7%	7.5%	8%	8.5%
Percent Motility after Freezing	36.05*	39.12*	41.76	45.17	42.73	42.35	45.18	47.06

*Significantly less motility than other treatments ($P<.05$)

When this experiment was initiated it was anticipated that the optimum level of glycerol would be somewhere between the extremes of five percent and 8.5 percent. However, these data indicate the optimum was at least 8.5 percent or higher. The results of First et al. (1957), Hill et al. (1959) and the experiment in this paper concerning yolk glycerol combinations, indicated that Glycerol levels of 10 to 15 percent were definitely harmful to ram sperm.

The results of Szumowski et al. (1956), however, indicate no harmful effect from glycerol levels as high as 16 percent.

Hill et al. (1959) found an interaction beteween rams and glycerol levels. No interaction was noted between rams and glycerol levels as indicated by the F value of 1.18 in Table 5 ($P<.05$). However, this is not to say that such an interaction might not be expected with more rams or different rams. Ample evidence for the reality of such treatment sire interactions have been established for frozen bull semen by White et al. (1954), Odell and Hurst (1956) and Hendrikse and Joling (1957).

Table 5. A Test of Interaction Between Rams and Glycerol Levels

Source	df	ss	ms	F
Glycerol Level x Rams	7	1,107	158.14	1.18
Error	136	18,291	134.49	

In drawing a conclusion from research to date, as to the glycerol level to be used in freezing ram semen, it would seem that satisfactory motility or survival after freezing can be obtained with levels from six to nine percent and that the precise optimum level is as yet undetermined.

B. The Addition of Egg Yolk to a Milk Diluter and its Effect on Glycerol Requirements

This study was initiated to find out if added phospholipid from egg yolk would improve temperature shock protection provided by a heated whole milk diluter and subsequently improve freezing survival. The data of Sikes and Merilan (1958) indicated that the motility of unfrozen sperm was maintained longer in a skimmilk diluter if 10 percent egg yolk were added. Hendrikse and Joling (1957) observed that the motility of bull sperm was maintained better in skimmilk with five percent egg yolk added than with 10 or 15 percent. Fourteen thousand inseminations indicated that a higher conception was obtained from the yolk plus milk diluent after storage than from milk diluent without added egg yolk.

This study was also designed to determine what combination of egg yolk and glycerol might be the most effective in terms of ram sperm survival after freezing. This was considered necessary, since Amann and Almquist (1957), reported that increased solids in a skimmilk diluter increased the glycerol need of frozen bull semen. The egg yolk addition obviously would increase the total solid content of the milk diluter. Also, Saroff and Mixner (1955) found that as the level of egg yolk in a yolk citrate extender was increased, correspondingly higher levels of glycerol were required for greater sperm survival.

An experiment was designed to study the effect of the addition of egg yolk to a milk diluter on motility and glycerol requirements

using the pooled ejaculates of two Hampshire rams. The pooled ejaculates were initially split into six egg yolk groups. The semen of group one was diluted with the milk diluter previously mentioned. Each subsequent group was diluted with the same diluter with egg yolk added. The diluter for group two contained three percent egg yolk; group three, six percent egg yolk; group four, 12 percent egg yolk and group five, 24 percent egg yolk.

All collections, handling, freezing and examination procedures were those previously mentioned except that the warm diluted semen was purposely cooled 3° C. per minute to 5° C. and the freezing rate was 3° C. per minute to -15° C. and 5° C. per minute to -75° C. These modificiations were an attempt to insure that some temperature shock might occur. At 5° C. each group was further split into four sub groups of 6, 9, 12 and 15 percent glycerol.

The final dilution rate was 1:200 and samples were equilibrated one hour before freezing.

The semen quality and concentration for each trial are reported in Table 6.

Frozen samples were thawed at 5° C., warmed and examined. Two slides per vial were used for one recorded observation. The data are expressed as percent survival one week after freezing. The data pertaining to the two trials are in Appendix Table B.

Table 6. Ejaculates Used in the Yolk Glycerol Experiment

Ram	Volume (ml.)	Concentration (billion/ml.)	Progressive Motility (%)	Motile Cells (%)
Trial I				
Pope	.8	3.2	70	80
Rayl	.7	3.0	65	80
Pooled ejaculate				
Average	.75	3.1	67	80
Trial II				
Pope	.7	2.8	53	72
Rayl	.2	3.1	47	68
Pooled ejaculate				
Average	.45	2.95	50	70

The average percent sperm survival from the combination of egg yolk and glycerol in the milk diluter are presented in Table 7.

Table 7. Average Percent Survival of Sperm Frozen in a Milk Diluter with Combinations of Egg Yolk and Glycerol

Percent Yolk	0	3	6	12	24	Average
Percent Glycerol						
6	71	65	71	55	62	65**
9	85	66	77	67	36	66**
12	49	30	25	15	7	25*
15	1	2	2	0	0	1
Average	51	41	44	34	26	

**The mean is significantly greater than the two lowest means ($P<.01$)
*The mean is significantly greater than the lowest mean($P<.05$)

The nature of the variance in this experiment is illustrated in Table 8.

Table 8. Analysis of Variance on Combinations of Yolk and Glycerol

Source	df	Sum of Squares	Mean Square	F
Total	79	82,112.8		
Yolk levels	4	5,872.7	1,468.20	1.77
Glycerol levels	3	60,998.3	20,332.80	24.45**
Yolk x Glycerol Int.	12	9,977.0	831.48	9.32**
Trials	1	1.5	1.5	0.2
Error	59	5,262.5	89.19	

**Highly significant ($P<.01$)

The F value of 24.45 indicates that a highly significant difference existed between the glycerol levels. A student range test was applied to test differences among the glycerol level means, using the highly significant interaction mean square as the error mean square. The means which were significantly different are shown in Table 7. Significantly more sperm survived freezing with glycerol levels of 6, 9 and 12 percent than survived when frozen in 15 percent glycerol ($P<.05$).

The means for either six or nine percent glycerol were significantly greater than the sperm survival at 12 or 15 percent glycerol ($P<.01$).

The apparent reduction in freezing survival of ram sperm due to high levels of glycerol support the findings of First et al. (1957)

and Hill et al. (1959). The rapid reduction in ram sperm survival with glycerol levels above 10 percent certainly need further investigation. It is recognized that bull sperm tolerate glycerol levels much higher than 10 percent even though the optimum for most bull semen diluters is between seven and 12 percent (Smith and Polge, 1950; Polge and Rowson, 1953; Emmens and Blackshaw, 1956).

Glycerol has been shown to have a damaging effect on fertility of fowl sperm (Polge, 1951) but this was prevented if the glycerol was removed by dialysis before insemination. Polge (1956) observed that boar sperm do not tolerate glycerol levels as high as ten percent.

Polge and Rowson (1952a) found that glycerol does not reduce fertility of bull semen. This has been supported by the practical field application of glycerol in freezing bull semen with subsequent successful conceptions reported in studies around the world. However, this does not mean that glycerol may not reduce fertility when added to the semen of other species.

The highly significant interaction of glycerol and egg yolk shown in Table 8 when the data of six and 15 percent glycerol are considered was the result of relatively little change in sperm survival as yolk increased. While survival definitely decreased as yolk increased when the percent glycerol was either nine or 12.

These data do not support the reports of Amann and Almquist (1957) and Saroff and Mixner (1955), wherein higher levels of solids

or of egg yolk increase the glycerol required for best freezing survival of bull sperm. It is in agreement with Hendrikse and Joling (1957) who found greater motility of bull sperm exhibited by lower levels of egg yolk as compared with 10 to 15 percent.

The analysis of variance shown in Table 8 also indicates that no significant difference occurred among egg yolk levels. The lack of significance is partially caused by the great amount of variation due to a glycerol yolk interaction.

Sikes and Merilan (1958) had shown unfrozen sperm were maintained longer by the addition of egg yolk to a skimmilk diluter.

The data of this experiment while not significant, suggest a depressing effect on ram sperm survival as egg yolk is increased in the milk diluter.

C. Frozen and Unfrozen Ram Semen Equilibrated with Glycerol for One and 18 Hours and Incubated at 39° and 5° Centigrade

This experiment was designed to answer the following questions: (1) would thawed frozen semen lose motility and die more rapidly than unfrozen when both were stored at the conventional 5° C. temperature? (2) could such a difference be expected to occur at 39° C., the approximate temperature to which semen would be exposed after insemination in the ewe? (3) was an 18 hour glycerol equilibration period necessary before freezing the semen? or (4) would the additional time involved in the 18 hour glycerol equilibration actually reduce the expected life of frozen or unfrozen sperm at either or both of the incubation temperatures?

In order that these questions might be answered, a 2x2x2 factorial experiment was utilized. It consisted of two replications of eight treatments each entailing all of the possible combinations of the following factors: fresh vs. frozen semen, one hour vs 18 hours equilibration time and incubation temperatures of 39° vs. 5° C. The semen used for each trial was obtained by pooling ejaculates of two Hampshire rams. The pooled ejaculates for trial one had a concentration of 3.7 billion cells per ml., 65 percent of these cells were progressively motile and 85 percent were motile. The semen used for trial two had a concentration of 3.3 billion cells per ml. with 63 percent of these cells progressively motile and 77 percent motile. A complete split ejaculate technique was utilized in dividing and diluting the various treatments.

The final dilution rate of all samples was 1:300. The samples were examined at 0, 2, 4, 8, 16, 32, 64, 96 and 120 hours after freezing. The zero time started immediately after the frozen samples were thawed. Frozen samples were thawed immediately after freezing. The procedure was the same for unfrozen samples except that they were held at 5° C.

The results of this experiment are shown in Appendix Table C. Figures 1 and 2 illustrate the motility decline of ram sperm of each treatment as time progressed. Semen samples incubated at 39° C. lost motility rapidly and were nearly immotile at the end of eight hours. Because the eight hour examination was the last period when sperm in all treatments exhibited motility, the eight hour examination values were used in determining differences between the treatments. Semen samples incubated at 5° C. were again analyzed after 120 hours.

The F tables for these two analysis are shown in Tables 9 and 10.

For semen incubated eight hours, the F value of 37.922 for variance due to treatments was highly significant which indicates that at least one of the main effects or interactions differed from zero. The only significant main effect was B, the effect of 39° C. incubation. The F value of 257.9 was highly significant.

The F test at 120 hours where only the 5° C. incubated samples were considered, indicated a highly significant difference between the two trials but no difference among treatments.

Figure 1. Motility Decline of Unfrozen and Frozen Ram Semen Equilibrated for One or 18 Hours at 5° C.

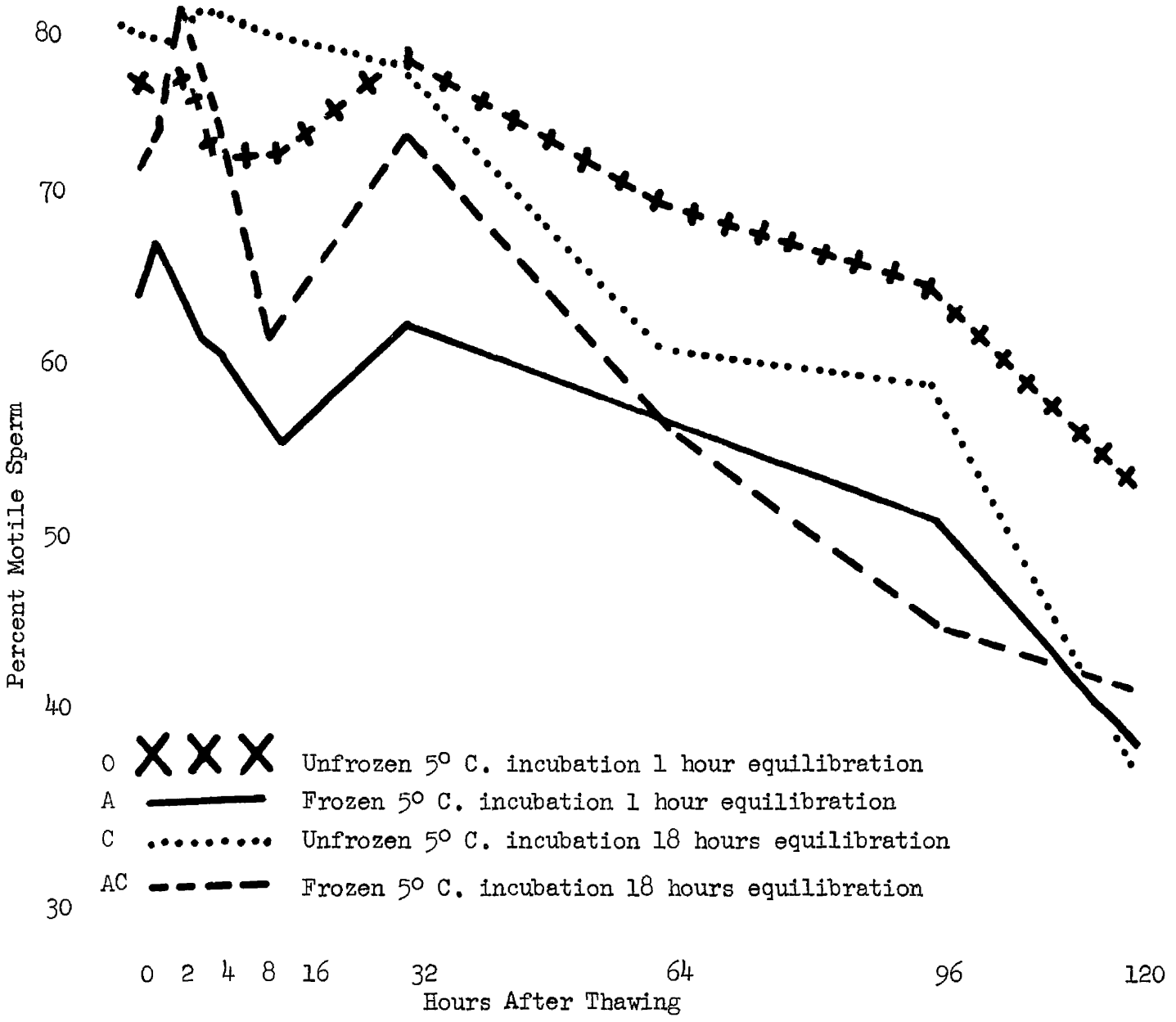

Figure 2. Motility Decline of Unfrozen and Frozen Ram Semen Equilibrated for One or 18 Hours at 39° C.

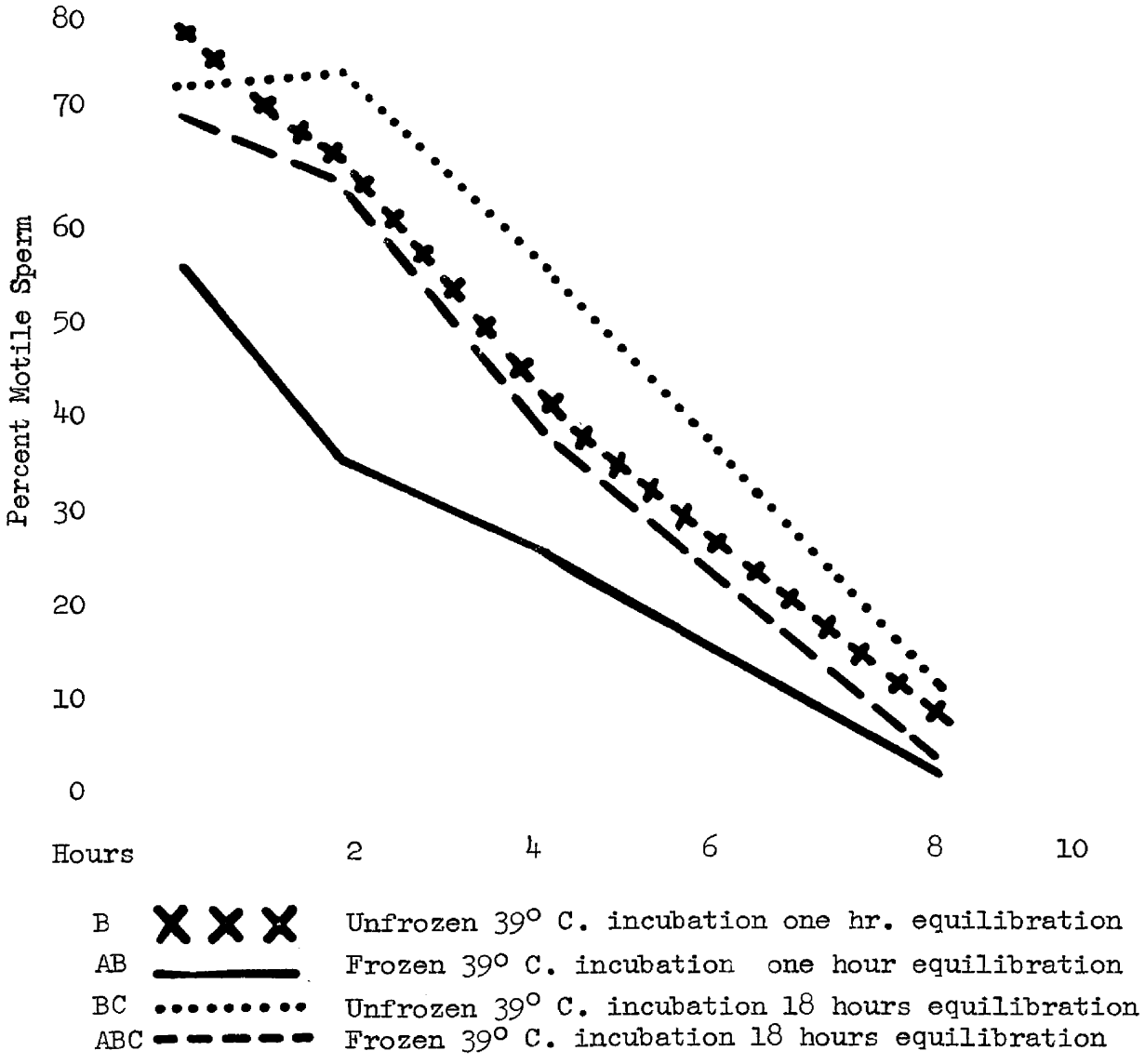

Table 9. Variance in the Motility of Frozen and Unfrozen Ram Semen Incubated Eight Hours at 39° and 5° C. and Equilibrated with Glycerol for One or 18 Hours

Source		df	ss	ms	F
Total		15	18,110.9		
Replicate		1	52.52	52.52	0.79
Treatment		7	17,594.42	2,513.48	37.92**
A	1		162.56	162.56	2.45
B	1		17,095.56	17,095.56	257.93**
AB	1		45.56	45.56	0.69
C	1		150.06	150.06	2.26
AC	1		14.06	14.06	0.21
BC	1		126.56	126.56	1.91
ABC	1		0.06	0.06	0.00
Error		7	463.96	66.28	

**Highly significant ($P<.01$)

Table 10. Variance in the Motility of Frozen and Unfrozen Ram Semen Incubated 120 Hours at 5° C. and Equilibrated with Glycerol for One or 18 Hours

Source		df	ss	ms	F
Total		7	4,982.0		
Trials		1	4,512.5	4,512.5	56.760**
Treatments		3	231	77.0	0.968
A	1		180.5	180.5	2.27
C	1		0.5	0.5	0.1
AC	1		50.0	50.0	0.63
Error		3	238.5	79.5	

**Highly significant ($P<.01$)

It can, therefore, be concluded that on the basis of this experiment significantly more frozen and unfrozen sperm remained motile after eight hours storage at 5° C. than at 39° C.

There was no significant difference in motility between thawed frozen semen and unfrozen semen of the same age after both were stored at 39° C. or 5° C. for eight hours.

No interactions occurred between equilibration time, eight hour storage temperatures and unfrozen versus frozen semen.

The lack of significance due to the main effect A at 120 hours indicates that there was no significant difference between the motilities of thawed frozen semen and unfrozen semen from the same ejaculate. There was no significant difference between glycerol equilibration periods of one or 18 hours before freezing as indicated by the AC interaction effect and main effect A. It can be noted from the data that a motility of 58 percent was maintained by one hour equilibrated frozen samples of trial two which had been thawed for 240 hours. Frozen and unfrozen samples equilibrated for 18 hours each had an average of 57 percent motile cells after 240 hours incubation at 5° C.

From these data it would seem that frozen ram semen could not be expected to lose motility more rapidly after thawing than unfrozen semen. This fact appears to be true at the 39° C., simulated uterine temperature, as well as at 5° C. storage. It was evident that ram sperm lost motility more rapidly at 39o C. than at 5° C.

The studies of Anderson (1941), Kelly (1942), Carbonerro (1955) and Sinclair (1957) indicated that the majority of ewes bred 12 hours after the beginning of estrus conceived. The studies of McKenzie and Terrill (1937), Polovceva et al. (1938) and Lopyrin and Loginova (1939) indicated that ovulation occurred approximately 30 hours after the onset of estrus. It seems evident that ram sperm must live longer than eight hours in the female tract.

The fact that a high percent of motile cells remained in frozen and unfrozen semen after 10 days of 5° C. storage with a glycerolated milk diluter suggested that long time storage of unfrozen ram sperm may be possible.

The data suggest that one hour glycerol equilibration was as satisfactory as 18 hour equilibration. This is in agreement with the ram semen data of Hill et al. (1959) and the bull semen equilibration data of Odell and Almquist (1957), Blackshaw (1955a) and Graham et al. (1957). The ram semen data of Szumowski et al. (1956) and the bull semen data of Polge (1952a), Stower (1953), and Saroff and Mixner (1955) do not agree with these results.

D. Extender Toxicity at 39° C.

After observing the rapid motility decline of ram sperm at 39° C., the question arose as to a possible extender toxicity at 39° C. which was not evident at 5° C. The experiment designed to test the possibility of such an extender toxicity at warm temperatures consisted of one ejaculate each from three Hampshire rams, split equally among eight treatments with incubation at 5° or 39° C. Motility determinations were made at zero and eight hours after the beginning of incubation.

Data pertaining to the quality of the three ejaculates are presented in Table 11.

Table 11. Ejaculates Used in Studying Extender Toxicity at 39° C.

Ram	Volume (ml.)	Concentration (billion/ml.)	Progressive Motility (%)	Motile Cells (%)
Pope	.8	2.8	75	86
Rayl	.6	2.2	78	90
Pope lamb	.6	2.0	85	92
Average	.67	2.5	79	89

Collection, cooling and handling procedures were those previously described.

Each ejaculate was initially split three ways. The three groups being ram semen diluted with milk, yolk citrate and undiluted.

At 5° C., second dilution fractions were added to the milk diluted semen and the treatments were: milk only, milk plus 7 percent glycerol, milk plus 14 percent glycerol, milk plus 1.25 percent arabinose and milk plus 7 percent glycerol and 1.25 percent arabinose. The yolk citrate diluted semen was split into two treatment groups; yolk citrate and yolk citrate plus 7 percent glycerol and 1.25 percent arabinose.

Semen samples from each treatment were then divided and incubated at 5° or 39° C. Zero time for all diluted samples was the beginning of incubation. Zero time for the undiluted samples was determined when they entered 39° C. incubation immediately after collection.

All samples were diluted 1:250. Two slides on two vials of each ejaculate for each treatment were examined at zero hours and again after eight hours incubation at the two temperatures. The average of the values for the two vials was used as an observation.

The data pertaining to this experiment are presented in Appendix Table D.

The criterium used to test differences in this experiment was percent eight hour survival.

An analysis of the variance in these data is shown in Table 12.

Table 12. Variance in the Extender Toxicity Study

Source	df	ss	ms	F
Total	47	55,117		
Treatments	7	22,906	3,272.3	4.02*
Conditions (5° versus 39° C.)	1	2,552	2,552.0	3.13
Treatment x conditions	7	3,606	515.0	0.63
Error	32	26,053	814.2	

*Highly significant ($P < .01$)

Table 13 presents the average eight hour percent survival for each treatment and incubation temperature.

The F value for treatments of 4.02 indicates that a highly significant difference occurred among treatment means. A student range test was used to determine the significance of the difference between the means.

Table 13. Percent Eight Hour Survival of Ram Sperm Diluted in Constituents of Two Diluters and Incubated at 5° C. or 39° C.

Treatment	Incubation conditions		
	39° C.	5° C.	Average
Undiluted ejaculate	28	0	14.0
Milk	47	62	54.5
Milk plus 7% glycerol	42	66	54.0
Milk plus 14% glycerol	31	59	45.0
Milk plus 1.25% arabinose	39	53	46.0
Milk plus 7% glycerol plus 1.25% arabinose	31	62	46.5
Yolk citrate	68	88	78.0
Yolk citrate plus 7% glycerol plus 1.25% arabinose	85	99	92.0
Average	46.4	61.1	

The student range test of treatment means shown in Table 14, indicate that significantly more diluted sperm survived incubation for eight hours than did undiluted sperm in the incubated undiluted ejaculate ($P<.05$). The greatest survival following incubation was achieved by ram sperm diluted in yolk citrate plus glycerol and arabinose.

Table 14. Student Range Test of Treatment Means

Treatment	Undiluted Ejaculate	Milk plus 14% Glycerol	Milk plus 1.25% Arabinose	Milk plus 7% Glycerol plus 1.25% Arabinose	Milk plus 7% Glycerol	Milk	Yolk Citrate	Yolk Citrate plus 7% Glycerol plus 1.25% Arabinose
Mean (% survival)	14	45	46	46	54	54	78	92

*_____Significantly greater than the mean of undiluted ejaculate (P<.05)

**- - - - -Highly significantly greater than the mean of undiluted ejaculate (P<.01)

Both of the egg yolk citrate diluted treatments appeared to result in greater survival than the milk diluted treatments, however, the differences were not significant.

The mean survival for each of the two yolk treatments was highly significantly greater than the mean survival of the undiluted ejaculate.

Sperm agglutination was observed in previous studies as the slides for motility determination dried and aged. It was thought that perhaps a constituent of the diluter or the examining fluid was responsible for this agglutination. Agglutination was observed, in this study, to occur in all treatments and regardless of whether the slide dilutions were made with ringers solution, 2.7 percent sodium citrate or physiological saline. This suggested that the agglutination was independent of the fluid in which the sperm were suspended.

On the basis of this data it would seem that no one constituent of the two diluters was damaging to ram sperm at 39° C.

E. Storage Potential of Frozen Ram Semen

In this study an attempt was made to determine the rate at which frozen stored ram sperm lost motility after storage at -79° C.

One ejaculate each from two Hampshire rams was diluted 1:200 in a milk diluter. Data pertaining to the ejaculates are shown in Table 15.

Table 15. Ejaculates Used to Determine the Storage Potential of Frozen Ram Semen

Ram	Volume (ml.)	Concentration (billion/ml.)	Progressive Motility (%)	Motile Cells (%)
Pope	1	3.1	62	82
Rayl	0.4	2.4	35	84

Four vials of semen per ram were periodically thawed and examined for motility over a 14 month period. The interval between examinations increased somewhat as time progressed, in order that the study might be extended over a longer period. The results of this experiment are shown in Appendix Table E. Each recorded observation is the average of four vials examined.

The combined data from both rams are presented in Figure 3.

The motility of ram sperm declined rapidly during the first two months of frozen storage after which time little motility decline occurred. This is in agreement with the data of Hill et al. (1959) wherein frozen ram sperm rapidly lost motility between two days and

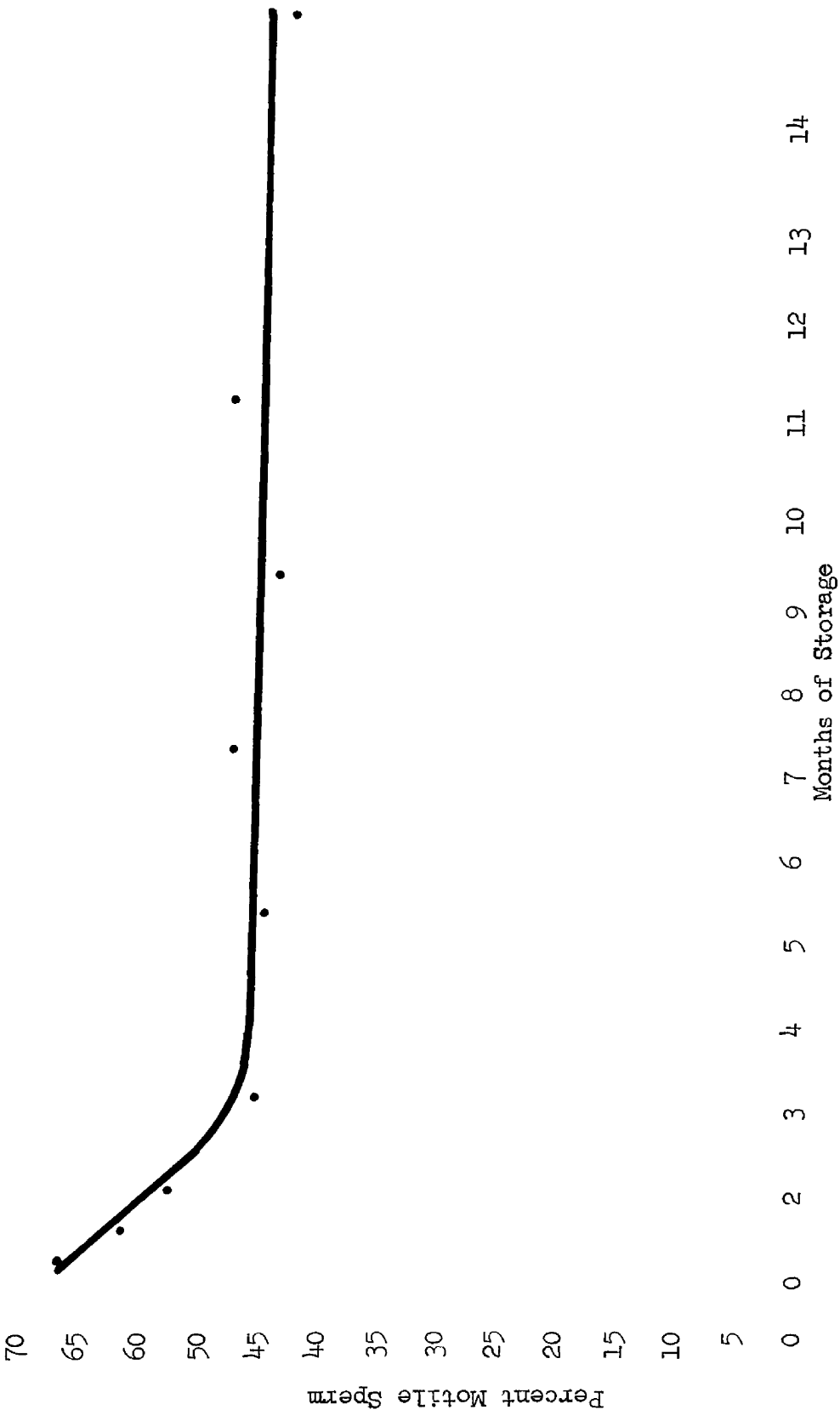

Figure 3. Ram Sperm Motility Decline at -79° C. Storage

ten days storage. The results also agree with those of Vandemark et al. (1957) who found a progressive decline in frozen bull sperm motility during the first 51 days of storage.

The data of this study do not agree with the increase in motility of stored bull semen reported by Etgen et al. (1957) and Polge and Rowson (1952b), Szumowski et al. (1956) found no decline in motility of ram sperm stored for four weeks at -79° C.

The studies of Bratton et al. (1957), Mixner and Wiggins (1957) and Graham et al. (1958) have not demonstrated a decline in first service non-return rate of frozen bull semen when inseminations were performed with semen frozen for various storage intervals.

It would seem that further investigation is needed to determine the relationship between motility and fertility.

F. Breeding Trials

1956 Breeding Trials:

Twelve Hampshire ewes were each cervically inseminated with one ml. of frozen semen from a Hampshire ram. Eleven Hampshire control ewes were each inseminated with .2 ml. of fresh undiluted semen from the same ram. The samples were collected with an artificial vagina.

The semen frozen had an average concentration of three billion sperm before dilution, 64 percent of which were motile before freezing and 15 percent after freezing. The semen was diluted 1:6 with heated homogenized milk diluter.

Ewes were checked for estrus with an aproned ram, twice per day at 12 hour intervals. Ewes in estrus were inseminated 12 hours after estrus was observed. The semen was thawed at 5° C. and allowed to warm to room temperature. Cervical inseminations were performed with the aid of a head lamp, glass speculum and a narrow tipped one ml. pipette. The ewes were rechecked for return to estrus with the aproned ram.

Data pertaining to the quality of semen inseminated and the results of this experiment are presented in Table 16.

Table 16. 1956 Breeding Trial

	Motil. Before Insem. (%)	Total Sperm /Insem. (B.)	No. Mot. Sperm (B.)	Motile sp/.3ml. in cerv. (B.)	No. Ewes	1st ser. % non-return	% conceiving 1st service	% Lamb Crop
Frozen	15	.500	.075	.025	12	17	17	33
Unfrozen	80	.600	.480	.480	11	91	64	108

1957 Breeding Trial:

Ten Hampshire ewes were each cervically inseminated with one ml. of frozen semen from the same Hampshire ram that was used in 1956. Ten other Hampshire ewes were each cervically inseminated with .2 ml. of fresh undiluted semen from the same ram. The frozen semen had an average concentration of 3.2 billion sperm per ml. before freezing with 80 percent motile cells. The semen was diluted 1:15 with milk diluter which contained in addition, two percent egg yolk. After freezing, 38 percent of the sperm were motile. Ewes were checked for estrus and the semen was handled in the manner described for 1956, except that each ewe was inseminated every 12 hours during estrus. The semen quality at insemination and results of this experiment are presented in Table 17.

Table 17. 1957 Breeding Trial:

	Motil. Before Insem. (%)	Total Sperm /Insem. (B.)	No. Mot. Sperm (B.)	Motile sp/.3ml. in cerv. (B.)	No. Ewes	1st ser. % non-return	% conceiving 1st service	% Lamb Crop
Frozen	38	.213	.081	.024	10	60	30	50
Undiluted	80	.600	.480	.480	10	80	60	80

1958 Breeding Trials:

These experiments were designed to investigate the practical possibilities of inseminating ewes with ram semen frozen in egg yolk citrate, milk or yolk citrate glucose urea diluters and to ascertain the relative effectiveness of these diluters for insemination of unfrozen ram semen. The studies also attempted to answer several questions regarding insemination techniques and breeding practices.

Experiment A

Procedure: Experiment A was a factorial experiment utilizing eight rams and 96 white-faced western ewes. Ewes were allotted to each sire after balancing for weight and previous breeding history. Within a sire group 12 ewes were randomly allocated to each of the following 12 treatments: (1) frozen ram semen diluted in yolk citrate and inseminated as 0.5 ml. containing 100 million sperm; (2) a like treatment but with the 100 million sperm insemination in one ml. of extender; (3) ram semen frozen in milk and inseminated as 0.5 ml.; (4) ram semen frozen in milk and inseminated as one ml.; (5) ram semen frozen in the Russian egg yolk citrate glucose urea diluter and inseminated as 100 million sperm in 0.5 ml. of extender; (6) ram semen frozen in the same Russian diluter but inseminated as 100 million sperm in one ml. of extender. A total of six unfrozen treatments identical to the previous six frozen treatments were used. The one ml. volume was used in accordance with Koger (1951) so that an effective insemination might occur even if cervical insemination

were not possible. It was postulated that if cervical insemination of a small volume was a better procedure, then 0.5 ml. at twice the concentration of one ml. should be the most effective.

Unfrozen semen was glycerolated with seven percent glycerol plus 1.25 percent arabinose and handled in all ways except freezing exactly like its frozen counterpart.

Eight rams were used for replications. These were seven Hampshire ram lambs and one Shropshire ram lamb from various Michigan breeders. They were subjected to a progeny test which involved mating to the ewes in this study.

The frozen semen was collected, frozen and stored two weeks before the actual insemination began.

Ejaculate volume, concentration, progressive motility and motile cells before freezing as well as the average progressive motility and motile cells one week after freezing are shown for these eight rams in Table 18.

Semen was collected from the rams every second day, diluted, examined and stored in vials at 5° C. This allowed ewes to be inseminated with 4, 8, 20, 32 or 44 hour old semen. Data pertaining to the ejaculates of unfrozen semen used may be seen in Table 19.

In order to correlate semen age with conception from semen diluted in each diluter, the semen age was recorded on the ewes breeding card after insemination.

Table 18. Frozen Ram Semen Used in Experiment A

Ram	Collected (ml.)	Concentration (B./ml.)	Before Freezing		1 Week After Freezing	
			Progressive Motility (%)	Motile Cells (%)	Progressive Motility (%)	Motile Cells (%)
Vesey	1.0	3.03	78	86	70	75
Hyde	0.8	2.48	61	88	57	66
Shrop	1.2	4.55	70	94	51	65
I.S.C.	1.5	3.93	77	93	60	76
Mrock	0.5	2.77	75	84	70	75
Stahl	1.1	5.10	68	80	40	60
MSU 307	0.8	3.00	88	94	59	76
Travis II	1.0	6.41	80	87	59	64

Table 19. Unfrozen Ram Semen Used in Experiment A

Ram	Volume (ml.)	Concentration (billion/ml.)	Progressive Motility (%)	Motile Cells (%)
Vesey	1.07	4.19	70	87
Hyde	0.97	3.68	73	87
Shrop	1.09	4.71	76	90
I.S.C.	1.57	6.43	67	82
Mrock	0.81	3.56	73	85
Stahl	0.90	4.29	72	83
MSU 307	0.66	3.57	69	86
Travis II	0.85	5.28	70	84

Other data on the ewes breeding card included the ewes ear tag number, state of semen to be used (frozen or unfrozen), volume containing 100 million sperm, diluter to be used, a record of when in estrus, when bred and date returned to service. The presence or absence of cervical mucus was also recorded for correlation with conception.

All critical motility examinations were performed 30 hours after collection and dilution.

The ewes were checked for estrus at 12 hour intervals, morning and evening of each day, by exposing them in groups of 10 to 15 to aproned rams and sorting the ewes observed mounted by the ram. The ewes were inseminated 12 hours after heat was observed. Ewes were checked for estrus each of three days before insemination began to insure that the ewes bred the first day would be recently in estrus.

Ewes to be inseminated were held in a crate and the rear quarters elevated with a sling. Nearly all of the inseminations were a combination of cervical and vaginal. The cervix was filled with 0.2 to 0.3 ml. of semen and the remaining volume allowed to bathe the vaginal end of the cervix. A spreading metal speculum and head lamp were used to locate the cervix and insemination was via a two ml. syringe attached to glass tubing with a narrow drawn tip. Frozen semen was thawed immediately before insemination. Unfrozen semen and frozen semen were at approximately 5° C. when inseminated.

Three of the 96 ewes were not included in the data since two of them lambed during the inseminations from prior service and one exhibited no estrus.

None of 47 ewes inseminated with frozen semen lambed, even though first service non-return data suggested that 15 percent should lamb.

When the three diluters were compared on the basis of average motile cells four months after freezing, thawed semen diluted in milk averaged 62 percent motility with a standard deviation of 8; 68 percent of the sperm diluted in yolk citrate were motile with a standard deviation of 8, and 68 percent of the sperm diluted in Russian diluter were motile with a standard deviation of 7.

First service non-return data indicated that 35 percent of 46 ewes inseminated with unfrozen semen conceived. Actually 13 of 46 or 28.3 percent of these ewes did lamb from one insemination with the various treatments. Two of seven ewes inseminated with 0.5 ml. of yolk citrate diluted semen conceived and one of seven ewes insemination with one ml. yolk citrate diluted semen conceived for a total of three of 14 ewes conceiving (21.4 percent) from the insemination of yolk citrate diluted ram sperm. Seven of 15 ewes conceived (46.7 percent) when inseminated with milk diluted semen. These seven contained three of seven inseminated with 0.5 ml. and four of seven inseminated with one ml. Three of 17 ewes (17.7

percent) inseminated with semen diluted in the Russian diluter conceived and all three were a part of nine ewes inseminated with 0.5 ml. of semen.

Eight of 24 ewes or 33.3 percent inseminated with 100 million sperm in 0.5 ml. of unfrozen semen conceived as compared with five of 22 or 22.7 percent of the ewes inseminated with 100 million sperm in one ml.

Table 20. Ewes Conceiving From Insemination With Unfrozen Semen

Ram	Yolk Citrate		Milk		Russian		Total
	.5 ml.	1 ml.	.5 ml.	1 ml.	.5 ml.	1 ml.	
Hyde	X				X		2
Stahl							0
Mrock	X		X				2
Travis					X		1
Vesey				X	X		2
MSU 307			X	X			2
I.S.C.				X			1
Shrop.		X	X	X			3
Total	2	1	3	4	3	0	13

X the ewes lambed from insemination

Table 21 illustrates the variance in this experiment.

Table 21. Analysis of Variance Among Ewes Conceiving from Unfrozen Semen

Source	df	ss	ms	F
Total	45	9.33		
Diluters	2	0.75	.375	1.86
Volume	1	0.12	.120	0.60
Error	42	8.46	.201	

The F value of 0.60 indicated no significant differences existed between volumes inseminated ($P<.05$). The F value of 1.86 indicated no significant difference existed among the three diluters ($P<.05$) even though the percent ewes conceiving from milk diluted semen was twice that from the insemination of ram sperm diluted in yolk citrate or Russian diluter.

It appears that there were too few observations to accurately determine whether the three diluters responded differently after different lengths of storage. Data concerning this matter, however, are summarized in Table 22.

Cervical mucus was present in the vagina of four of the 13 ewes conceiving after insemination with unfrozen semen. Seventy-six percent of the 93 ewes inseminated in this experiment had cervical mucus present at insemination.

Table 22. Conception After Insemination of Ram Semen of Various Ages and Diluted in Three Different Diluters

Diluter	Age of Diluted Semen at Insemination				
	4 hrs.	8 hrs.	20 hrs.	32 hrs.	44 hrs.
Yolk citrate					
ewes conceiving	0	1	1	0	1
ewes inseminated	0	4	5	0	5
Milk					
ewes conceiving	1	2	1	1	2
ewes inseminated	1	3	4	3	3
Russian					
ewes conceiving	0	1	1	0	1
ewes inseminated	2	4	5	1	4
Total ewes conceiving	1	4	3	1	4
Total ewes inseminated	3	11	14	4	13

Experiment B

The B experiment in 1958 was conducted concurrently with Experiment A. The frozen semen of two mature Hampshire rams was used to inseminate 32 California ewe lambs in a factorial experiment consisting of two rams, eight treatments and two replications. This experiment was designed to determine (1) the effect of breeding a ewe once during an estrus with thawed frozen ram semen as compared to every 12 hours during estrus

and (2) to find what effect a vasectomized mating immediately before insemination might have on conception. Inseminations were also split equally between three week and seven month stored semen of each ram although sperm number differences existed between the two storage groups.

All collections, dilutions and semen examinations were by the previously described procedures. The semen was diluted with the heated homogenized milk extender.

Data pertaining to the ejaculates used are presented in Table 23.

All ewe lambs in this experiment were checked for estrus and inseminated in the same manner as the ewes in Experiment A.

Four of the 32 ewes (12.5 percent) lambed as a result of one insemination with frozen ram semen. Table 24 illustrates the number of ewes conceiving from each ram and each treatment.

Three of the 16 ewes bred to the Pope ram conceived whereas one of 16 ewes bred to the Rayl ram conceived from the insemination of frozen semen.

There was no difference in conception rate between ewes bred once or at every 12 hours during one estrus. Two of 16 ewes or (12.5 percent) conceived in each case. There was no significant difference between ewes mated to a vasectomized ram before breeding and ewes which were not. Again 12.5 percent conceived in each case.

Table 23. The Ram Semen Used in Experiment B

Ram	Ejaculate volume (ml.)	Concentration (B/ml.)	No. sperm/ml. after dilution (billion)	Before Freezing		Before Insemination	
				Progressive Motility (%)	Motile Cells (%)	Progressive Motility (%)	Motile Cells (%)
7 month stored							
Pope	1.0	3.3	.165	55	75	30	35
Rayl	0.8	5.0	.250	60	85	30	36
3 week stored							
Pope	0.8	4.5	.150	63	75	48	52
Rayl	0.9	4.4	.150	70	87	66	69

Table 24. Ewes Conceiving From Frozen Ram Semen Insemination in Experiment B

Ram	Stores 7 Months				Stored 3 Weeks			
	Bred once		Every 12 hours		Bred once		Every 12 hours	
	Apron	Vasectomized	Apron	Vasectomized	Apron	Vasectomized	Apron	Vasectomized
Pope		X	X			X		
Rayl			X					

X denotes conception

Twenty percent (3/15) of the ewes inseminated with semen stored seven months conceived and 5.8 percent (1/17) conceived with semen from the same rams stored for three weeks. The concentration difference biasing the data in favor of seven month stored semen should again be noted from Table 23. Because of the difference in concentration no definite conclusion can be drawn regarding semen storage and conception.

Seventy-five percent of the ewe lambs conceiving had cervical mucus in the vagina at insemination. Cervical mucus was present in the vagina of ninety-seven percent of all the ewe lambs.

Discussion of Breeding Trials

The fact that in Experiment A none of the ewes bred with frozen semen diluted with milk, yolk citrate or Russian-Ukranian diluter conceived prevents any conclusions regarding their suitability as frozen semen extenders. Where they have been reported in the literature the following success has been achieved.

Smirnov (1951) achieved 42 percent conception with the Ukranian diluter. Kuznetsov (1956) reported 19.3 percent of 303 ewes and 33.5 percent of 512 ewes conceived after insemination with ram semen diluted in the Ukranian diluter. Graca (1955) obtained 31.2 percent conception from 176 ewes inseminated with yolk citrate diluted semen. Emmens and Blackshaw (1955) reported five percent conception using a yolk citrate arabinose diluter and Emmens (in Kuznetsov 1956) mentioned that 15 to 20 percent of 2,000 ewes conceived after insemination with frozen ram semen, presumably diluted in the Australian yolk citrate arabinose diluter.

The results presented in this manuscript indicate some ewes conceived after insemination with thawed frozen semen diluted in a heated homogenized milk diluter containing arabinose. Conceptions of 17 percent in 1956; 30 percent in 1957 and 12.5 percent in the B experiment of 1958, compare favorably with the conceptions or non-returns reported by other authors for other diluters. It should be noted that sperm were diluted less than 1:10 and usually less than 1:4 in the studies mentioned except in the 1957 and 1958 data of this manuscript where dilutions ranged from 1:15 to 1:30.

The three diluters have been compared as far as fertility of unfrozen semen is concerned. Twenty-one percent of the ewes bred with semen diluted in yolk citrate arabinose diluter, 47 percent of the ewes inseminated with milk diluted semen and 18 percent of those inseminated with Ukranian diluter lambed. These differences were not significant, however, as indicated by the analysis of variance, Table 21. It should be recalled that all three diluters contained glycerol and were diluters that had been used for freezing semen.

Yolk citrate and milk have been reported as successful diluters for unfrozen semen by several authors. Aslanjan (1950) obtained 92 percent conception from ram semen diluted and stored up to seven days in yolk citrate. Dauzier et al. (1954) found conceptions were low from yolk citrate diluted semen unless the semen was used soon after collection.

The results of experiment A agree with Istvan (1956) and Dauzier (1956) who achieved higher conception rates with milk diluents than yolk citrate. Fillimon et al. (1956) and Mihailov (1957) have also found milk to be a satisfactory ram semen diluter, with Mihailov (1957) reporting conception rates of 85 to 97 percent on 4,700 ewes.

The particularly low conception rate from both egg yolk diluents are of the magnitude reported by Dauzier (1956) for stored yolk diluted ram semen. However, this experiment was

not designed to test the fertility inhibitory effect of egg yolk on stored ram semen postulated by Dauzier (1956). Hendrikse and Joling (1957) have observed that head agglutination of bull sperm occurred more frequently in yolk citrate diluted semen than in milk diluted semen. No particular agglutination differences were observed between any of the diluters studied here. Although, occasionally some agglutination was noticed with each diluter.

The conception persistency from the milk diluter as observed from the limited data of Table 22 and the fact that 58 percent of the milk diluted sperm survived 5° C. storage for 10 days, suggest that the milk diluter used here may be an excellent diluter for the storage of unfrozen ram semen over several days.

The 1958 experiment A has presented a real problem. Why didn't the ewes inseminated with frozen semen conceive? Table 25 is a summary of the four breeding trials. The frozen semen used in experiment A was higher in motility than any other frozen semen used and yet some ewes conceived from frozen semen in each of the other three experiments. Insemination conditions were slightly different in 1958 than the previous years, however, ewe lambs from experiment B bred concurrently with ewes of experiment A conceived. Were too few sperm or too few motile sperm inseminated? Table 25 indicates that fewer sperm were inseminated in experiment A than any of the other studies, however, experiment A employed slightly more motile sperm per insemination than did inseminations of seven month stored semen from the Pope ram in experiment B. Twenty-nine percent of the ewes bred with the seven

Table 25. Summary of Breeding Trials

	Motil. Before Insem. (%)	Total Sperm /Insem. (m.)	No. Mot. Sperm (m.)	Motile sp/.3 ml. in cervix (m.)	No. Ewes	1st service % non-return	% conc. 1st service	% Lamb Crop
1956								
Pope - Frozen	15	500	75	25	12	17	17	33
- Undiluted	80	600	480	480	11	91	64	108
1957								
Pope - Frozen	38	213	81	24	10	60	30	50
- Undiluted	80	600	480	480	10	80	60	80
1958 - Expt. A								
Av. 8 rams - frozen								
	70	100	70	21/1 ml.	24	17	0	0
				42/.5 ml.	25	13		
Av. 8 rams - unfrozen								
	85	100	85	25.5/1 ml.	22	27	23	32
	85	100	85	51.0/.5 ml.	24	42	33	50
1958 - Expt. B								
Pope - 3 wks. - Frozen								
	52	150	78	23.4	9	0	11	11
7 mos. - Frozen								
	35	165	58	17.4	7	43	29	29
Rayl - 3 wks. - Frozen								
	69	150	104	31.2	8	25	0	0
7 mos. - Frozen								
	36	250	81	27.0	8	13	13	13

month stored semen of the Pope ram conceived. The motility of frozen and unfrozen semen in experiment A are higher than any of the other diluted ejaculates studied. Perhaps these data support the observation of Dauzier et al. (1954) that motility of ram semen has little relationship to fertility.

The data suggest that the insemination of more sperm in the 1958 experiment A might have increased conception.

The 100 million total number of sperm inseminated was initially considered sufficient since Kuznecov (1934), Keast and Morley (1949), Koger (1951) and Terrill (1952) had reported satisfactory conception rates from insemination of 50 million sperm. If half the sperm died during freezing 50 million of the 100 million would remain as motile sperm. The 50 million minimum has been determined by dilution and insemination immediately after collection. The minimum number of stored or frozen sperm may be considerably greater.

The sperm numbers reported necessary for high conception rates seem to vary according to the conditions of each study in the literature. Milovanov (1934), Habibullin (1937), Lukin and Eremeev (1938), Anderson (1941) and Carbonerro (1955) found at least 500 million sperm per insemination necessary for satisfactory conception.

The results from insemination of unfrozen semen in experiment A indicate no difference between insemination of equal sperm numbers in one ml. or .5 ml. of fluid. It was expected that cervical insemination of .5 ml. might result in a higher conception rate than with

one ml., because of the extra concentration per unit volume and especially since Kuznetsov (1956) reported only 30 to 90 million sperm from a normal ejaculate penetrate the cervix and that sperm in the vagina perish within three to six hours.

The matter of whether cervical or vaginal inseminations should be used seems to be in question, for if the one ml. insemination is as effective as .5 ml. and only .3 ml. of either are sufficient to fill the cervix, then it appears that sperm from the vagina must have entered the cervix and aided conception.

From the literature, Anderson (1937) and Keast and Morley (1939) suggested that vaginal insemination lowered fertility as compared to cervical. It must be noted, however, that neither of these authors purposely inseminated ewes in the vagina. Kelly et al. (1942), Koger (1951), and Dauzier et al. (1954) found no difference in conception rates between cervical and vaginal inseminations. Mies Filho and DeAlmedia Ramos (1955) after inseminating 872 ewes, have even found a significant difference in favor of vaginal inseminations.

These data and the literature reviewed suggest that vaginal insemination of the ewe is as satisfactory as cervical insemination.

It appears that number of sperm inseminated is a more important consideration than volume. Malikov (1957) reported 81 percent conception from inseminations of 0.1 ml. of milk diluted semen and 66 percent from as little as 0.05 ml. While, Koger (1951), achieved satisfactory conception by inseminating one ml. in the vagina.

In experiment A, each ewe was exposed to one of the eight rams for approximately five estrous cycles after insemination. Ten of 93 ewes inseminated in experiment A failed to conceive even after this prolonged exposure to a ram. All 10 ewes conceived the previous year. Nine of the 10 had been bred with frozen semen in experiment A. Seven of the nine ewes returned to estrus the first service after insemination but did not conceive from natural service. One other ewe aborted in late pregnancy. Four of the nine barren ewes had been inseminated with thawed frozen milk diluted semen, four with yolk citrate diluted semen and one with ram semen diluted in Ukranian diluter.

Sixty-two of the 80 ewes not conceiving from insemination conceived from the first natural service. If we remove the ten ewes that did not conceive, 62 of 70 or 88.8 percent conceived from one natural service. The remaining eight ewes conceived from the second natural service.

In experiment B, the ewes were exposed to a ram for approximately three estrous cycles after insemination. Five of the 28 ewes not conceiving after insemination failed to conceive from at least three natural services. All five had been bred with frozen semen. Four of the five did not return in estrus the first cycle after insemination. Of all 15 barren ewes, cervical mucus was present in some and absent in others at insemination.

It could be expected that the ewe lambs of experiment B due to their youth might not all conceive and that irregular cycles might occur.

However, it is difficult to explain why nine of the ten barren ewes in experiment A were ewes previously inseminated with frozen semen rather than unfrozen. The numbers are too few to show whether or not this is more than a matter of chance, however, the data do suggest fertilization in the barren ewes may have been followed by a very early abortion or that a protein antagonism may have been induced in the uterus.

In analyzing these two experiments the data have been expressed in terms of numbers of ewes inseminated even though it is apparent that some of these ewes would not conceive even to natural service during the 1958 breeding season.

Carbonerro (1955) concluded the best time for insemination was when the vaginal mucus was turbid. Sinclair (1957) concluded conception was greatest when the mucus was clear or cloudy and that conception decreased when the mucus was very thick and creamy.

The criterium of cervical mucus in this experiment was based on the presence of, or absence of much mucus in the vagina, this corresponds with the periods of lower cervical mucus viscosity or approximately with the clear and cloudy stage mentioned by Sinclair (1957). It is assumed that cervical mucus would be thick and not noticeable during late estrus.

In experiment A, 33 percent of the ewes conceiving had cervical mucus whereas 76 percent of all the ewes bred had cervical mucus present when bred. This suggests that ewes bred late in estrus with short lived sperm conceived.

In experiment B, 75 percent of the ewe lambs conceiving had cervical mucus in the vagina at one or more inseminations. Cervical mucus was present in the vagina of 97 percent of all the ewe lambs. It should be remembered that half of the ewe lambs in the B experiment were inseminated every 12 hours during estrus, therefore, cervical mucus would be expected to occur in more of these ewes. From the data it is impossible to tell from which insemination the multiple inseminated ewes conceived. However, there was no difference between the number of ewes conceiving after one insemination or after several inseminations during one estrus. In each case 12.5 percent of the

ewes bred conceived. There was no real conception rate difference between 1956 and 1957 control ewes even though the 1957 ewes were inseminated at 12 hour intervals throughout estrus. Quinlan et al. (1932), Peregon (1936) as cited by Anderson (1945), Avramov (1937), Anderson (1941), Larrea (1944), Glembockii and Vasiljev (1944), Gutierrez (1948) and Aamdal and Hogset (1955) increased conception slightly by inseminating twice during one estrus instead of inseminating once.

Kirillov (1938) concluded that ewes should be tested for estrus twice a day and only those with an estrus period of more than 24 hours should be inseminated twice. Dauzier et al. (1954) and Lopyrin and Loginova (1939) could find no difference in conception between ewes inseminated once or several times during one estrus. Gavrilov (1937) and Lopyrin et al. (1957) indicate that more ewes conceive from double inseminations at eight hour intervals than at 16 or 24 hour intervals.

It would appear from the literature reviewed that two inseminations during one estrus are advantageous and that the interval should be less than 16 hours and nearer eight hours.

One-half of the ewes in experiment B were mated to a vasectomized ram before insemination. It was thought that the vasectomized mating might increase uterine motility, hasten ovulation and increase conception. Marion et al. (1950) observed that sterile mating in the

cow caused ovulation to occur earlier than in unmated controls. Hansel (1957) has reviewed this matter concerning various species. For this discussion it would suffice to mention that Coleman (1950) and (1951a) also Radford and Watson (1957) found the presence of a ram hastened the onset of the estrous cycle in anestrous ewes.

In the present experiment there was no difference between the number of ewes conceiving after vasectomized mating and ewes without such a mating prior to insemination. In each case 12.5 percent of the ewes conceived.

VI. SUMMARY AND CONCLUSIONS

Four laboratory experiments were employed to study ways and means of increasing ram sperm survival after freezing. Three ejaculates each, from two rams were used in an attempt to precisely determine the glycerol requirement for freezing ram semen in a heated homogenized milk diluter. Motility means for glycerol levels of 6, 6.5, 7, 7.5, 8 and 8.5 percent were significantly greater than the motility means for 5 or 5.5 percent glycerol.

The pooled ejaculates of two rams in two trials were used to study the effect of several levels of egg yolk added to a milk diluter on ram sperm survival after freezing and glycerol requirement for freezing. No significant difference in sperm survival after freezing in a milk diluter occurred among added egg yolk levels of 0, 3, 6, 12 and 24 percent, although the average ram sperm survival after freezing tended to decrease as yolk levels increased. The number of sperm which survived freezing in glycerol levels of six and nine percent was highly significant over the 12 or 15 percent level. A highly significant interaction occurred between glycerol levels and egg yolk levels.

The pooled ejaculates of two rams in two trials were factorialy used to study the effect of incubating unfrozen and frozen ram semen at 39° C. or 5° C. and equilibration with glycerol for one or 18 hours on motility. The motility of both frozen and unfrozen semen incubated for eight hours at 5° C. was significantly greater than at 39° C. No significant difference existed between the motility

of thawed frozen semen or unfrozen semen after either eight or 120 hours incubation at 39° C. or 5° C. Further, no significant difference occurred between thawed frozen semen equilibrated for one or 18 hours before freezing. Thawed frozen and unfrozen samples averaged 57 percent motility after 240 hours of 5° C. incubation.

A short study involving one ejaculate each from three rams indicated that none of the constituents in a milk-arabinose-glycerol diluter or a yolk-citrate-arabinose-glycerol diluter significantly depressed the motility of ram sperm after incubation for eight hours at 39° C. Milk and yolk citrate diluted semen samples had significantly greater motility after eight hours of 39° C. incubation than undiluted ram semen.

The frozen milk diluted ejaculates of two rams were periodically examined for motility throughout 14 months of -79° C. storage. The greatest motility decline occurred during the first two months of storage with very little decline thereafter.

Four breeding trials were used to determine the fertilizing ability of frozen ram semen and also study insemination procedures and diluters pertaining to frozen and unfrozen ram semen. In 1956, two of 12 ewes lambed when cervically inseminated with 500 million sperm in one ml. of thawed frozen milk diluted ram semen. Whereas, seven of 11 ewes lambed when cervically inseminated with 600 million sperm in .2 ml. of undiluted semen.

In 1957, three of 10 ewes lambed when cervically inseminated with 213 million sperm in one ml. of thawed frozen milk plus egg yolk diluted

ram semen. Six of 10 ewes lambed when cervically inseminated with 600 million sperm in .2 ml. of undiluted ram semen.

In 1958, none of 47 ewes cervically inseminated with 100 million sperm in .5 or one ml. of thawed ram semen lambed even though the average motility one week after freezing was 70 percent. Milk, yolk-citrate and a Russian-Ukranian diluter were used to dilute the semen before freezing. The combined data of all four breeding trials suggested that the lack of conception in these 47 ewes was due to insufficient sperm numbers for the insemination of frozen ram semen.

An analysis of conception rates from 46 ewes cervically inseminated with unfrozen semen from eight rams indicated no significant difference among glycerolate; milk, yolk-citrate or Ukranian diluters. Insemination of milk diluted ram semen resulted in a 46.7 percent lambing rate; whereas, the insemination of yolk-citrate or Ukranian diluted ram semen resulted in 21 and 18 percent lambing rates respectively. All was stored less than two days at 5° C.

No significant conception rate difference occurred between insemination of 100 million ram sperm in .5 or one ml. of undiluted semen.

Thirty-two ewe lambs were each cervically inseminated with 150 million sperm in one ml. of thawed frozen semen from one of two rams. No significant difference in conception rate occurred between ewes inseminated once during estrus or each 12 hours during estrus. In each case 12.5 percent of the ewes inseminated conceived.

There was no significant conception rate difference between ewes mated to a vasectomized ram before insemination and ewes which were

inseminated without prior vasectomized mating. In each case, 12.5 percent of the ewes inseminated conceived.

The results of the above described breeding trials suggested that the lack of conception in 47 ewes was due to insufficient sperm numbers.

The following conclusions were drawn from the data: Ram sperm will survive freezing equally well within glycerol level limits of six to 8.5 percent. Ram sperm survival after freezing in a heated homogenized milk diluent is not improved by the addition of egg yolk.

Ram sperm survive freezing equally well after glycerol equilibration periods of one or 18 hours.

Thawed frozen ram semen does not lose motility more rapidly after incubation at 5° C. or 39° C. than does unfrozen ram semen.

Ram sperm can be stored for long period at -79° C. with no great loss of motility and some stored ram sperm are fertile.

Vasectomized mating before artificial insemination of the ewe is not necessary.

From limited data conception rate is as high from one insemination during estrus as from several inseminations.

These data have demonstrated that ram sperm can achieve excellent survival after freezing in terms of motility and that some frozen ram sperm are capable of causing conception. Further investigation may indicate a reduced fertilizing capacity of apparently highly motile

previously frozen ram sperm, a need for improved activation of frozen ram semen before insemination or the necessity for increased sperm numbers when frozen ram semen is to be inseminated.

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VII. APPENDIX

Appendix A

Percent Motile Ram Sperm After Freezing in Various Levels of Glycerol

Ram	Ejaculate	Percent Glycerol 5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
Pope	1	29	32	41	41	27	36	36	39
	2	42	50	63	54	49	54	55	57
	3	39	45	48	48	42	58	56	55
Rayl	1	42	40	35	41	41	44	44	50
	2	24	23	26	24	36	32	39	31
	3	41	42	43	54	51	26	43	49

Appendix B

Percent Survival of Ram Sperm Frozen in a Milk Diluter with Combinations of Egg Yolk and Glycerol

Percent Egg Yolk	0	3	6	12	24
Percent Glycerol					
Trial I					
6	73	49	81	78	79
	84	71	50	51	83
9	80	58	70	74	54
	81	56	61	58	59
12	50	28	18	11	0
	35	19	33	14	3
15	4	1	3	0	0
	1	1	3	0	0
Trial II					
6	50	66	93	51	34
	76	74	61	39	53
9	89	74	97	77	9
	90	74	81	59	21
12	44	33	27	16	13
	66	40	21	20	10
15	0	4	0	0	0
	0	0	0	0	0

Appendix C

Percent Motility of Frozen and Unfrozen Ram Semen Incubated Eight Hours at 39° and 5° C. and Equilibrated With Glycerol for One or 18 Hours

	One Hour Equilibration				18 Hours Equilibration			
Trial I	5° C.		39° C.		5° C.		39° C.	
Treatment Hours	Unfrozen	Frozen	Unfrozen	Frozen	Unfrozen	Frozen	Unfrozen	Frozen
0	81	60	81	57	88	75	81	81
2	81	69	65	20	85	81	83	75
4	78	60	15	12	85	72	48	26
8	77	56	2	2	88	79	0	0.5
16	70	54	0	0	80	68	0	0
32	76	62	0	0	87	76	0	0
64	68	56	0	0	61	58	0	0
96	65	39	0	0	50	39	0	0
120	33	19	0	0	25	10	0	0
Trial II								
0	73	69	81	58	75	68	73	63
2	70	65	72	50	73	67	62	57
4	76	63	66	35	79	71	70	53
8	68	66	17	7	77	70	16	13
16	75	58	0	0	79	57	0	0
32	81	65	0	0	66	72	0	0
64	71	57	0	0				
96	65	60	0	0	68	53	0	0
120	72	58	0	0	69	76	0	0
197	72	58			60	57		
240		58			60	54		

Appendix D

Percent Eight Hour Survival of Ram Sperm Diluted in Constituents of Two Diluters and Incubated at 5° or 39° C.

Treatment	Ram	Incubated at 39° C. (%)	Incubated at 5° C. (%)
Undiluted Ejaculate	1	46	0
	2	0	0
	3	39	0
Milk	1	17	5
	2	66	92
	3	58	88
Milk plus 7% Glycerol	1	0	27
	2	78	64
	3	47	106
Milk plus 14% Glycerol	1	0	34
	2	58	55
	3	36	87
Milk plus 1.25% arabinose	1	0	45
	2	85	47
	3	33	68
Milk plus 7% Glycerol plus 1.25% Arabinose	1	0	35
	2	43	63
	3	49	88
Yolk Citrate	1	74	80
	2	33	84
	3	97	100
Yolk Citrate plus 7% Glycerol plus 1.25% Arabinose	1	79	104
	2	86	96
	3	91	97

Appendix E

Percent Motility of Ram Semen Frozen for Various Lengths of Time

Time After Freezing	Rams	
	Pope	Rayl
1 hour	67	69
2 weeks	62	62
1 month	57	61
3 months	52	50
4 "	47	52
6 "	52	56
8 "	42	52
10 "	49	54
14 months	43	50