

**Second Edition**

**Physiology of  
Reproduction and  
Artificial Insemination  
of Cattle**

**G. W. Salisbury  
N. L. VanDemark  
J. R. Lodge**

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# Physiology of Reproduction and Artificial Insemination of Cattle

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# 16

## Extenders and Extension of Unfrozen Semen

### 16-1 INTRODUCTION

The concentration of spermatozoa in normal, freshly ejaculated bull semen is high. Considerable extension\* is necessary to provide a convenient volume to inseminate that will contain enough sperm cells to ensure maximum fertilization rates without wasting spermatozoa. Mixing spermatozoa with extender allows the addition of many ingredients that sustain and protect the spermatozoa, thereby preserving fertility until they are used for insemination. Thus the two major functions of semen extenders are to preserve the fertility of sperm cells and to increase the total volume so that the proper dose of cells for insemination can be conveniently packaged and used. A single ejaculate can therefore be used to inseminate several thousand cows.

Spermatozoa must be alive to be fertile and motility has been used to monitor viability. Motility, however, is primarily a reflection of flagellar activity, and it does not guarantee that such cells are fertile. The integrity of the genetic material in the sperm chromatin must also be preserved in order for normal embryo development to take place after fertilization. Extending media and storage conditions should preserve both functions but there is no test of semen quality prior to use that can predict how well the genetic material is maintained.

There are countless recipes for media that will preserve bull spermatozoa, several of which are described in subsequent sections. Much of the early work, particularly in Russia, has been reviewed by Anderson (1). Physiological salt solutions were found to be unsuitable for preserving sperm cells. Glucose, sulfate, and tartrate were often included in the Russian formulations, and Milovanov and Selivanova (2) reported that lecithin and peptone protected the lipid membranes of spermatozoa. None of the media, however, appreciably extended the fertile storage life of bull spermatozoa.

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\*"Extension" and "extender" are considered preferable to "dilution" and "diluter," since the material added to semen actively extends the life of the sperm cells.

# 16

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A major breakthrough in semen extenders came when Phillips (3) reported the value of hen's egg yolk. In the original formula the extender contained equal volumes of egg yolk and phosphate buffer. Salisbury et al. (4) showed that, by replacing the phosphate with sodium citrate, equal fertility, higher spermatozoan motility during prolonged storage, and improved visibility resulted. The latter of course made microscopic evaluation easier. The yolk-citrate extender, with various modifications, has been the most widely used medium for artificial insemination in cattle. A variety of extenders have been formulated for unfrozen spermatozoa (5).

Proper identification of the bull that produced each sample of semen is extremely important. Hence all containers used from semen collection to insemination must be properly labeled. Harmless vegetable dyes are added to the extender (6, 7) to facilitate the identification of breeds, or bulls where the number is limited. Different-colored straws are used for the same purpose with frozen semen. Color seems to be a convenient aid, but no data are available on its effectiveness in reducing identification errors.

### 16-2 PRINCIPLES OF SPERM PRESERVATION

**16-2.1 Temperature.** The storage time required for practical use of most semen in artificial breeding varies from a few hours to several weeks. Metabolic rate tends to be proportional to absolute temperature, so reduced temperature has been the chief means of slowing down chemical reaction and prolonging life. Spermatozoa can tolerate temperatures somewhat higher than body temperature for short periods of time, but energy requirements are high and toxic products can accumulate rapidly. Chemical inhibition of spermatozoa is helpful at ambient temperatures (see Section 16-4).

Most of the inseminations before the advent of frozen semen were performed with semen stored at about 5°C, but upon rewarming to 37°C the percentage of progressively motile cells was observed to have decreased during storage. Metabolism continues at a reduced rate at 5°C and fertility begins to decrease before day 4 (8, 9). When semen is frozen (see Chapter 17) spermatozoa may remain fertile for years, although certain characteristics of aging are evident in semen stored at -196°C (8, 10).

At the same time that temperature is decreased, changes occur in the internal and external environments of spermatozoa. The solubility of gases in the external environment increases. For example, when there is a decline in temperature from body temperature to 0°C, solubility of O<sub>2</sub> doubles and CO<sub>2</sub> triples. With air as the gas overlying the spermatozoa, this shift in solubility of gases at lower temperatures permits a relatively greater proportion of the total metabolic activity to be due to oxidative metabolism. At higher-than-body temperatures the solubility of oxygen is relatively low in relation to the rapid metabolic rate and may become self limiting under some circumstances, even at body temperature. At low temperatures the solubility of oxygen increases,

and the metabolic activity decreases several-fold, resulting in a relatively greater oxygen supply. The solubility of certain solutes decreases, and the physical form of the colloids in the semen can change as well, causing increased semen viscosity and therefore a greater physical resistance to motility.

As temperature decreases, spatial arrangements of the internal structure and outer covering of the cells may change, a contraction of protoplasm may occur and can happen at different rates depending on the chemical components of the various structures. All such factors, as well as the rates of chemical reactions, are involved in controlling the fertile life of sperm cells by temperature reduction.

**16-2.2 Energy Source.** The most obvious energy requirement of the sperm cell is for motility, but additional energy is presumably required for cell maintenance. Spermatozoa are capable of both aerobic and anaerobic metabolism. A simple source of energy such as glucose or fructose should be supplied in the extender to protect intracellular reserves and cell components. Fructose, present in the seminal plasma, will be diluted considerably by semen extension. Egg yolk contains some glucose and other compounds utilizable by bull spermatozoa.

**16-2.3 Osmotic Pressure and Electrolyte Balance.** Spermatozoa behave as osmometers and are capable of extensive changes in size depending upon the tonicity of the medium in which they exist (11, 12). Seminal plasma has an osmotic pressure of about 285 milliosmols and it is usually assumed that this represents the ideal physiological osmotic pressure. Even if this is true, however, it should not be assumed that solutions of equivalent osmotic pressure, as measured by physical tests, are isotonic with bull spermatozoa. The permeability of the cell to different substances varies. Interactions may occur between the total concentration of substances in the fluid surrounding spermatozoa, the pH of the medium, and the specific ions and nonelectrolytes present (13, 14). If the tonicity of the medium deviates considerably spermatozoa may have bent tails, swim in circles, and die. Fortunately spermatozoa are able to tolerate a moderate range of osmolarities without a reduction in fertility (15); the degree of tolerance is affected by interactions with pH and other ions present (13). Extenders developed to preserve sperm should ideally be isotonic under the conditions used, and thus should not cause morphological or functional damage.

Solutions that are similar in composition to body fluids or are naturally produced liquids, such as egg yolk and milk, appear to be compatible with sperm. Buffered egg yolk first developed for use with bull sperm in artificial insemination was hypertonic (16, 17). Subsequently the concentrations of the salt solutions used were reduced to isosmotic levels. More recently a series of zwitter ion buffers has been used (15, 18-22) and bull spermatozoa suspended in them appear to tolerate a range of osmolarities.

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**16-2.4 Buffering and pH.** Spermatozoa need protection from autotoxication due to acid products of metabolism, particularly when they are stored without refrigeration. Bull sperm motility and fertility are well preserved in egg yolk and milk extenders near neutral pH, although reduction of the pH to 6.5 may even be beneficial (23). The optimum pH probably varies with storage temperature and other components of the extender (13, 14). Saturation of bicarbonate media with  $\text{CO}_2$  causes metabolic inhibition and reduces the pH to about 6.3 (24, 25, 26). This inhibition may be due in part to a reduction in pH (23).

**16-2.5 Proper Gas Phase.** To maintain the desired gas conditions in the extending medium, the relative proportion of gas to liquid phases in sealed containers and the composition of the gas phase should be controlled (25, 27-30). As previously stated,  $\text{CO}_2$  inhibits spermatozoan metabolism, the degree of inhibition of anaerobic glycolysis being partially dependent upon  $\text{CO}_2$  levels and sulfite present. Oxygen with suitable substrate can provide the sperm cells with an efficient energy source. However, toxic products such as  $\text{H}_2\text{O}_2$  may be formed unless catalase is included (31). The effects of light are especially damaging in the presence of oxygen (31, 32, 33), and gassing with nitrogen is beneficial.

**16-2.6 Inhibition of Microorganism Growth.** Egg yolk and other extending media for bull spermatozoa provide a good environment for the growth of microorganisms, which produce products that may be harmful to spermatozoa, and which may infect the cow. Some organisms usually will be found in semen from healthy bulls even under aseptic semen collection and processing conditions. It is therefore standard practice to include antibiotics, such as penicillin and streptomycin, in all extenders used commercially (34, 35, 36). The various agents used, and their application, are discussed in Section 16-5.2.

**16-2.7 Exclusion of Toxic Materials.** Not only should extenders be formulated that will prevent the formation of toxic products during storage, but the extending media, as prepared, should be free of substances that are harmful to spermatozoa. Heavy metals, for example, are harmful to spermatozoa, but proper distillation of water insures that only nontoxic trace amounts remain. Only pure reagents should be used in formulating buffer (37). Unfortunately, cryoprotective agents such as glycerol (38, 39), required for maximum protection of spermatozoa during freezing, are somewhat toxic; and dead spermatozoa are a source of amino acid oxidase, which is harmful to surviving cells through the production of  $\text{H}_2\text{O}_2$  (40).

**16-2.8 Semen Extension and the "Dilution" Effect.** Bull semen should be mixed with enough extender so that the initial sperm concentration will be

diluted to yield an optimum number of spermatozoa for insemination in a convenient volume. Studies have shown that extensive "dilution" of the spermatozoa with simple solutions depresses motility (41) but that the addition of amino acids and macromolecules such as egg albumin or casein minimizes this effect. Rottensten et al. (42) found that heated plasma mixed in equal volumes with a citrate-glucose solution improved the survival of sperm diluted up to 1:200 and stored at 4°C. Adding 25 percent egg yolk was as beneficial as the seminal plasma. Chemical inhibition can prolong motility of bull spermatozoa highly diluted with synthetic media (43). Bovine cervical mucus also counteracts the dilution effect (44).

In the experiments of Rottensten et al. (42), egg yolk and milk in practical extenders appear almost to prevent the dilution effect. Catalase, CO<sub>2</sub>, and N<sub>2</sub> (29, 45, 46) may all help to eliminate the dilution effect.

The procedure used to yield suitable doses of spermatozoa for insemination is described in Section 16-7.

**16-2.9 Protection of Spermatozoa Against the Cold.** When mammalian sperm cells are cooled to 5°C they are subject to cold shock, which causes leakage of intracellular enzymes, potassium, lipoprotein, ATP, and other materials from the cells. Furthermore, motility is decreased and the flagellum may become bent (41). The precise mechanisms of damage are unknown, but presumably changes occur at unequal rates on the surface and internal portions of spermatozoa during cooling, and both physical and chemical damage result. Ethylenediamine tetracetic acid (EDTA) may reduce cold shock (47); and if cells are cooled slowly, particularly below 20°C (48), damage is less severe, which indicates that spermatozoa can make adaptive changes.

However, the most effective means of protecting spermatozoa against the effects of chilling is to provide them with lecithin, proteins, lipoproteins, and similar complexes found in egg yolk or milk (49-54). The importance of adding egg yolk before cooling is clearly demonstrated by experiments that show a marked beneficial effect on subsequent motility and fertility (55).

The optimum cooling rate, with regard to fertility, for semen in different extenders has not been adequately established. Extremely rapid cooling should be avoided, but otherwise considerable latitude between 1 and 4 hours is possible. There seems to be little interaction between osmotic pressure of the extenders and optimum cooling rate (56).

### 16-3 EXTENDERS FOR REFRIGERATED SEMEN

**16-3.1 Historical.** The first media used to dilute semen were primarily salt or sugar solutions designed to increase the volume of semen for immediate use rather than to conserve it (1, 41). When the value of egg yolk was discovered (3) a "preserving" extender was developed that could be coupled with the advantages of reducing metabolic activity of spermatozoa and prolonging their fertility by storage at approximately 5°C.



or insemination in a dilution" of the sperm that the addition of casein minimizes this risk in equal volumes of sperm diluted up to 1:100 as beneficial as the addition of bull spermatozoa mucus also counter-

and milk in practical use, catalase, CO<sub>2</sub>, and N<sub>2</sub>

spermatozoa for insemina-

When mammalian sperm is exposed to cold shock, which causes leakage of cellular contents, and other materials in the flagellum may be known, but presumably internal portions of the sperm may result in cold shock (47); and if damage is less severe, spermatozoa against the cell membrane, lipoproteins, and importance of adding nutrients that show a protective effect (55).

or semen in different containers, rapid cooling should be used and 4 hours is post-thaw osmotic pressure of the

1 were primarily salt solutions for immediate use. Milk was discovered (3) and replaced with the advantage of prolonging their

Semen extenders should be formulated that embody the principles of sperm preservation discussed previously. Furthermore, they should be easy to prepare, stable, and economical. Many extenders for liquid semen have been described and their development reviewed (1, 5, 20, 57, 58).

**16-3.2a Egg Yolk Phosphate.** In 1939 Phillips (3) first reported that fresh egg yolk combined with a phosphate buffer preserved the motility and fertility of refrigerated bull spermatozoa. The formula was: KH<sub>2</sub>PO<sub>4</sub>, 0.2 gram; Na<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O, 2.0 grams; glass-distilled water, 100 milliliters; hen's egg yolk, 100 milliliters. The final pH of this medium was 6.75. In field tests this extender gave good fertility (59).

**16-3.2b Egg Yolk Citrate.** In 1941 Salisbury et al. (4) reported that sodium citrate was beneficial to sperm liveability when it was added to an equal volume of egg yolk. Sodium citrate had the advantage over phosphate buffer in being a chelating agent and in dispersing the fat globules in egg yolk, thereby improving visibility when spermatozoa were examined microscopically. The fertility of bull semen in phosphate and citrate extenders appeared to be equal, as indicated by the following results:

Extension rate	Variable	Yolk-phosphate	Yolk-citrate	Reference
1:4	Services/conceptions	1.53	1.53	(4)
1:200	60-90-day % nonreturns (NR)	50.5	50.5	(60)

In these studies either 3.6 percent or 3.92 percent (w/v) of sodium citrate dihydrate (Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub> · 2H<sub>2</sub>O) was used. This buffer concentration is hypertonic for bull spermatozoa but apparently, when combined with 50 percent egg yolk, did not cause osmotic problems. Later (16) the recommended concentration of sodium citrate dihydrate was reduced to 2.9 percent (w/v).

The citrate-yolk extender especially has been modified many times with varying concentrations of sodium citrate and egg yolk and the addition of glucose, sulfanilamide, and antibiotics (61). When sulfanilamide was included in the yolk-citrate extender a highly significant increase in fertility level—about five percentage units—was obtained (60, 62). This probably explains why van Dielen (63) obtained 54.6 percent NR (nonreturn) with a yolk-citrate extender containing sulfanilamide and only 50.8 percent NR (*P* < 0.01) with the phosphate extender in an experiment involving 7,101 inseminations. Also, egg yolk was reduced to 33 percent and thus the hypotonic condition of the phosphate extender may have been deleterious (64). A nonreturn rate of 76.3 percent following insemination of 14,605 cows with sperm in a yolk-citrate-glucose extender was obtained, compared to 74.7 percent for 14,056 cows inseminated



with a yolk-phosphate extender (65). Foote and Bratton (66) also found yolk-citrate-sulfanilamide extender to be superior to yolk-phosphate. One trial (67) resulted in higher fertility for a yolk-phosphate extender than for yolk-citrate. When sulfanilamide and antibiotics were added the reverse was true, possibly because of an unfavorable antagonism between phosphate and streptomycin.

In general, yolk-citrate has been the standard against which new extenders have been compared. Salisbury and VanDemark (213) reported that glucose added to yolk-citrate increased the liveability of spermatozoa. Ohms and Willett (68) modified the 2.9 percent citrate-yolk extender by replacing part of the citrate with glucose. After one day of storage at 5°C the yolk-citrate-glucose had a 1.8 percent advantage in nonreturn rate ( $P > 0.05$ ) and after two days of storage it had a 6.0 percent advantage ( $P < 0.01$ ). The authors attribute the increase to the addition of glucose. Simultaneously the 50 percent egg yolk level of the control was reduced to about 17 percent egg yolk by volume in the yolk-citrate-glucose extender, and this change may also have contributed to the fertility level observed. Over a period of years the level of egg yolk used in the citrate-buffered extender devised by Salisbury et al. (4) has gradually been reduced to 20 to 25 percent by volume, and glucose or fructose is sometimes added.

**16-3.2c Ratio of Yolk to Buffer.** The early work was done with equal volumes of egg yolk and buffer. Using smaller amounts of egg yolk often yielded inferior results, probably because of the mitigating effect of the egg yolk on the hyperosmotic citrate solutions used and on the high concentrations of phosphate ions in the phosphate extender.

Table 16-1 shows a summary of fertility results, which clearly indicate that there was generally no fertility advantage in reducing the proportion of egg yolk and simultaneously increasing the sodium citrate but that, for convenience, the proportion could be reduced considerably. With the development of more complete buffers to combine with egg yolk, however, a reduction in the volume of egg yolk from 50 to 20 percent improved sperm survival with many buffer combinations tested (66). Fertility was also improved significantly over the standard 50 percent yolk-citrate extender (see Table 16-1).

Under most conditions where osmolarity, pH, and other characteristics of the buffer alone appear to be optimal for sperm survival, egg yolk level may be reduced from 20 to 1 percent. This is particularly true at ambient temperatures (see Section 16-4). Table 16-2 shows an example of the importance of the egg yolk level as it interacts with four other factors during an 8-day storage period. The effects of extender, molarity, and yolk level were large ( $P < 0.005$ ), accounting for a majority of the non-bull variance. Temperature had no effect, showing that with proper media bull spermatozoa can survive for 8 days at 25°C as well as at 5°C. Also pH had no effect. By far the largest interactions were extender  $\times$  temperature (CUE—Cornell University Extender—was best

(66) also found yolk-phosphate. One trial (67) than for yolk-citrate. rise was true, possibly ate and streptomycin. t which new extenders reported that glucose matozoa. Ohms and der by replacing part 5°C the yolk-citrate- > 0.05) and after two . The authors attribute he 50 percent egg yolk yolk by volume in the ave contributed to the of egg yolk used in the 4) has gradually been e or fructose is some-

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Table 16-1. Fertility results (percent nonreturns) as the proportion of egg yolk to citrate is decreased.

% egg yolk						Total inseminations	Ref.
50	33	25	20	17	12.5		
67.1		66.0				3,172	(69) <sup>a</sup>
66.7		63.9				2,929	(70) <sup>b</sup>
70.8		69.2				2,591	(71) <sup>c</sup>
73.5			72.3			11,013	(72) <sup>d</sup>
66.2			69.5			5,908	(73)
65.8				63.4		2,809	(70) <sup>b</sup>
63.1					62.4	2,484	(70) <sup>b</sup>
		74.0 <sup>e</sup>		66.2 <sup>e</sup>		2,713	(71) <sup>c</sup>
73.4 <sup>e</sup>			76.3 <sup>e</sup>			18,494	(74) <sup>e</sup>

<sup>a</sup>16-week nonreturns.

<sup>b</sup>6-month nonreturns.

<sup>c</sup>120-150-day nonreturns.

<sup>d</sup>12-week nonreturns.

<sup>e</sup> $P \leq 0.01$  differences due to chance.

at 25°C and Tris was best at 5°C), and temperature  $\times$  level of yolk (20 percent egg yolk was clearly best at 5°C and 5 percent yolk was best at 26°C). Other large and significant interactions were extender  $\times$  molarity, temperature  $\times$  molarity, and molarity  $\times$  egg yolk level.

**16-3.2d Complex Citrate-Buffered Yolk Extenders.** Foote et al. (74, 75) developed complex buffered yolk extenders based on previous work with complex buffers, and they reported beneficial effects of glycine (76) and CO<sub>2</sub> (24). They also reported on the use of Illini Variable Temperature (IVT) extender at room temperature, but it has been used as a bull-semen extender at 5°C as well. Table 16-3 shows the original composition of this extender, of CUE and CU-16, and of two modifications called 14 and 14G developed in New Zealand (77). Modifications of the IVT have been made by increasing the glucose from 0.3 to 1.2 percent and the egg yolk from 10 to 20 percent. The composition of these and similar extenders is given by Bartlett and VanDemark (25). All are gassed with CO<sub>2</sub>. The latter workers, in a limited test totaling 765 cows, found that two modified IVT extenders containing 20 percent egg yolk yielded average 60-90-day nonreturns over a 5-day use period comparable to a citrate-yolk-skim-milk control.

An extensive test (78) of a similar CO<sub>2</sub>-gassed extender with glycine and glutathione added gave results as follows:

<i>Age of semen (days)</i>	<i>CO<sub>2</sub> extender</i>		<i>Yolk-citrate</i>	
	<i>Cows bred</i>	<i>% NR</i>	<i>Cows bred</i>	<i>% NR</i>
1	724	78	279	74
2	2,293	76	646	68
3	2,272	72	506	62
4	2,005	66	50	60
5-7	1,363	64	—	—

Melrose (79) obtained a 56 percent 112-day NR rate for bull sperm in IVT used at 5°C over a period of 2 to 6 days. A 9 percent skim-milk powder-glycerol control averaged only 40 percent over this period. It is clear from these examples that semen in IVT can be used successfully for several days at 5°C. One disadvantage has been the variability in results achieved, which presumably reflects varying degrees of maintaining the proper CO<sub>2</sub> level following gassing (26).

The CUE extender (74, 75) was developed to maintain proper pH and to generate CO<sub>2</sub> through the use of bicarbonate-citric acid buffer system. This was compared with a simpler extender, CU-16 (see Table 16-3). When bull semen was extended to 10 million motile spermatozoa per milliliter and stored for 12 days at 5°C, sperm survival in CUE and CU-16 was significantly better than in the yolk-citrate control. A field trial with semen processed as in the motility trial, but with most semen used for insemination within three days of collection, gave the following results (75):

<i>Extender</i>	<i>No. 1st inseminations</i>	<i>% 60-90-day NR</i>
2.9% citrate (EYC)	6,069	73.4
CUE	6,280	76.6
CU-16	6,145	76.0

When put into routine use in the field 290,053 inseminations averaged 74.9 percent 60-90-day NR, an increase of two percentage units over a comparable period when the 2.9 percent citrate-yolk extender was used. Singleton and Josey (80) also reported that CUE and CU-16 were superior to EYC for bull sperm at 5°C.

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<i>Yolk-citrate</i>	
<i>ows bred</i>	<i>% NR</i>
279	74
646	68
506	62
50	60
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**Table 16-2.** Sperm motility as affected by storage temperature, buffer type, osmolarity, pH, and egg yolk levels. Means of fifteen ejaculates after storage for eight days.<sup>a</sup>

Buffer concentration	pH	Stored at 5°C				Stored at ambient temperature (25°C)			
		% egg yolk				% egg yolk			
		0	1	5	20	0	1	5	20
(CUE extender)									
50% <sup>b</sup>	6.50	11	27	27	43	20	20	31	38
	6.75	17	33	28	39	17	29	28	43
75%	6.50	1	54	49	51	50	57	48	45
	6.75	0	53	49	58	31	54	51	43
100%	6.50	0	47	46	46	42	55	55	41
	6.75	0	47	47	49	33	59	54	50
(Tris-yolk extender)									
0.15M	6.50	5	30	37	49	13	13	14	14
	6.75	3	31	30	50	7	12	5	15
0.20M	6.50	3	34	41	44	18	15	29	33
	6.75	2	27	43	53	15	17	25	23
0.25M	6.50	0	28	31	29	16	23	34	27
	6.75	0	35	43	43	13	25	31	16

<sup>a</sup> Foote, R. H. Unpublished data. Cornell University. 1960.

<sup>b</sup> Buffer concentration as a percentage of strength shown in Table 16-3.

Shannon (77) reported on extensive field testing of bull semen (see Table 16-4) with several of the extenders listed in Table 16-3. All extenders contained 20 percent egg yolk by volume. With the short breeding season in New Zealand, fertility is calculated on the basis of 49-day NR to insemination. The fertility results were significantly lower in the control yolk-citrate extender, which confirmed previous studies (75). Particularly striking was the difference in fertility after two days' storage. It is believed that the ability of the CUE and CUE-G extenders to maintain high fertility over a period of several days is due to the CO<sub>2</sub> liberated from the sodium bicarbonate. However, part of the beneficial effect observed previously with extenders 14, 14G, and CU-16 appears to result from an improvement in the nonelectrolyte balance through the addition of glucose and glycine.

*Table 16-3. Composition of buffered yolk extenders.<sup>a</sup>*

<i>Component</i>	<i>Citrate</i>	<i>g/100 ml of distilled water</i>				
		<i>IVT<sup>b</sup></i>	<i>CUE</i>	<i>CU-16</i>	<i>14</i>	<i>14G</i>
Sodium citrate dihydrate	2.9	2.00	1.45	1.45	2.00	2.00
Sodium bicarbonate	—	.21	.21	—	—	—
Potassium chloride	—	.04	.04	—	—	—
Citric acid monohydrate	—	—	.09	—	—	—
Glucose	—	.30	.30	1.25	.30	.30
Glycine	—	—	.94	.94	1.00	1.00
Glycerol	—	—	—	—	—	1.25
Sulfanilamide	0.6	.30	.30	.30	—	—

<sup>a</sup>Final extenders consisted of 20 parts egg yolk and 80 parts buffer to which antibiotics were added. In some studies 50% egg yolk was combined with the citrate. Adapted from references 24, 74, 75, and 77.

<sup>b</sup>Gassed with CO<sub>2</sub>.

*Table 16-4. Fertility of bull spermatozoa stored at 5°C in yolk extenders, based on 49-day nonreturns (NR)<sup>a</sup>.*

<i>Extender<sup>b</sup></i>	<i>Age of semen when used</i>					
	<i>Day of collection</i>		<i>Stored 1 day</i>		<i>Stored 2 days</i>	
	<i>No. inseminations</i>	<i>% NR</i>	<i>No. inseminations</i>	<i>% NR</i>	<i>No. inseminations</i>	<i>% NR</i>
CUE	59,091	62.8	25,309	65.2	1,190	62.4
CUE-G	30,058	63.1	13,909	64.9	2,392	62.9
14	73,993	62.8	37,758	63.5	2,681	59.2
14G	35,351	62.7	17,597	64.9	932	60.0
Citrate	98,605	60.6 <sup>b</sup>	44,610	60.8 <sup>c</sup>	2,788	51.5 <sup>c</sup>

<sup>a</sup>From Shannon. *New Zealand J. Agric. Res.* 7:357-363. 1964.

<sup>b</sup>See Table 16-3 for composition. CUE-G was CUE plus 1.25% glycerol (w/v). All extenders contained 20% egg yolk by volume.

<sup>c</sup>Significantly different from all others,  $P < 0.01$ .

extenders.<sup>a</sup>*distilled water*

CU-16	14	14G
1.45	2.00	2.00
—	—	—
—	—	—
—	—	—
1.25	.30	.30
.94	1.00	1.00
—	—	1.25
.30	—	—

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*ed**Stored 2 days*

<i>No.</i> <i>inseminations</i>	<i>%</i> <i>NR</i>
1,190	62.4
2,392	62.9
2,681	59.2
932	60.0
2,788	51.5 <sup>c</sup>

lycerol (w/v). All extenders

The CUE formula has been modified slightly by different workers but without appreciably changing fertility results. Several examples of these modifications follow.

<i>Extender</i>	<i>No.</i> <i>1st inseminations</i>	<i>% 60-90-day</i> <i>NR<sup>a</sup></i>	<i>Reference</i>
CUE	6,913	71.0	(45)
CUE + catalase, 20 µg/ml	7,615	71.5	
CUE	10,309	76.1	(45)
CUE + catalase, 20 µg/ml	10,951	74.6	
CUE	6,064	72.9	(81)
CUE + catalase, 1 µg/ml	6,243	73.2	
CUE, pH 6.80 (control)	23,727	74.5	(23)
CUE, pH 6.65	24,779	74.7	
CUE, pH 6.50	22,577	74.7	
CUE, 300 mosmols	12,797	74.0	(15)
CUE, 333 mosmols	13,966	74.0	

<sup>a</sup>None of the differences was statistically significant,  $P > 0.05$ .

Catalase has been shown to protect sperm under conditions that presumably lead to hydrogen peroxide formation (33, 41). The small amount of catalase in egg yolk, however, is apparently sufficient to protect sperm under the usual dark storage conditions at 5°C, where added catalase has only minor effects.

The pH of CUE extender may be decreased to at least 6.5 without harmful effect by increasing the citric acid component of the buffer. Other experiments with CUE at the lower pH have given actual fertility values higher than for control CUE. Pickett et al. (82) recommended the lower pH range and obtained poorer fertility with CUE as the pH approached 7.0.

Other studies (15) indicate that minor changes in osmotic pressure are not critical. These and numerous other trials with CUE or modifications (83-86) have yielded high fertility results with the extenders used over a period of a few days at 5°C.

New Zealand researchers (87) improved the citrate-buffered yolk (see Table 16-3) by gassing with nitrogen and adding caproic acid. This led to an extender called "Caprogen." Caprogen consists of 2.0 percent sodium citrate dihydrate, 0.3 percent glucose, 1.0 percent glycine, 1.25 percent glycerol, 0.0325 percent caproic acid, and 0.01 percent sulfacetamide, to which is added 20 percent egg yolk, penicillin, and streptomycin. In 1961 (88) the following fertility results were reported.



<i>Age of semen</i>	<i>14G</i>		<i>14G + caproic acid</i>	
	<i>No. inseminations</i>	<i>% 49-day NR</i>	<i>No. inseminations</i>	<i>% 49-day NR</i>
Day of collection	224,000	64.1	42,000	63.7
Day after collection	96,000	64.3	17,000	66.9

The addition of caproic acid increased NR rates on semen stored for a day at 5°C ( $P < 0.01$ ). A subsequent trial was initiated comparing caproic acid additions and nitrogen gassing. These results are summarized in Table 16-5. The caproic acid and/or gassing with nitrogen improved fertility of semen used after the first day. Thereafter Caprogen, called 14GCN, was used routinely in New Zealand for extending sperm stored at 5°C. Later (see Section 16-4) this was modified for ambient temperature use by reducing the egg yolk content. Balasov et al. (89) also reported that caproic acid was beneficial when added to a yolk-citrate-glucose extender.

Following the work of Roy and Bishop (76) there were numerous reports that yolk-glycine and yolk-glycine-glucose improved sperm survival (5, 57). However, fertility results were often disappointing. For example, Graham and Erickson (90) used a yolk-glucose-glycine extender to inseminate 3,982 cows and obtained 65.8 and 35.7 percent 60–90-day NR on the day of collection and the day after collection. These results were 7.6 and 24 percent below the heated

*Table 16-5. Fertility (% 49-day nonreturns) of bull spermatozoa stored at 5°C in extenders gassed with nitrogen and/or containing caproic acid.<sup>a</sup>*

<i>Extender<sup>b</sup></i>	<i>Age of semen when used</i>					
	<i>Day of collection</i>		<i>Stored 1 day</i>		<i>Stored 2 days</i>	
	<i>No. inseminations</i>	<i>% NR</i>	<i>No. inseminations</i>	<i>% NR</i>	<i>No. inseminations</i>	<i>% NR</i>
14G (Control)	101,982	65.2	56,687	65.4 <sup>c</sup>	2,061	62.3
14GC	99,420	65.6	52,064	66.7 <sup>d</sup>	5,854	63.5
14GN	50,637	65.8	27,163	66.2 <sup>d</sup>	2,359	61.9
14GCN	52,436	65.8	35,435	67.6 <sup>d</sup>	739	64.8

<sup>a</sup>From New Zealand Dairy Board. 48th Farm Production Rpt. 1971–1972.

<sup>b</sup>C = caproic acid; N = nitrogen gassing; CN combination is called Caprogen.

<sup>c,d</sup>Numbers with different superscripts in the same column,  $P < 0.01$ .

*4G + caproic acid*

No. inseminations	% 49-day NR
2,000	63.7
7,000	66.9

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Stored 2 days		
No. inseminations	% NR	
2,061	62.3	
5,854	63.5	
2,359	61.9	
739	64.8	

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whole milk control used simultaneously to inseminate 3,722 cows. Consequently, glycine has been little used recently, except in more complex extenders such as CUE or Caprogen.

A variety of buffered yolk preparations have been reported in the Russian literature, but the exact composition is often not given. These reports have been summarized by Turton (91). One extender extensively tested with regard to fertility was a yolk-sodium-citrate-saccharose-sodium-bicarbonate-potassium-phosphate extender. When semen was stored for up to three days at 5 to 15°C, 74.3 percent of 5,998 cows inseminated conceived. Semen stored at 0°C in a yolk-citrate-glucose control extender and used to inseminate 6,231 cows resulted in 65.0 percent conception.

**16-3.2e Tris-Buffered Egg Yolk Extenders** The development of zwitter-ion buffers of Good (92) has led to the examination of many for buffering egg yolk as extenders for bull spermatozoa (18-22). These buffers were shown by Good et al. (92) to be relatively harmless to various cell suspensions, and they provided excellent buffering capacity over a suitable pH range. In early studies (18, 19) bull sperm survival at 5°C in Tris-buffered egg yolk was equal or superior to that found for egg yolk citrate and CUE. Optimum sperm survival was obtained in 0.2 molar Tris-yolk extender, adjusted with citric acid to pH 6.75. A nomogram for preparing Tris solutions with different osmolarity and pH characteristics has been published (14).

Among the compounds that have been tested (21) using sperm motility as a criterion of suitability, are TRIS, tris (hydroxymethyl) aminomethane; TES, N-tris (hydroxymethyl) methyl-2-aminomethanesulphonic acid; MES, 2-(N-morpholino) ethanesulphonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; PIPES, piperazine-N,N'-bis(2 ethane sulfonic acid); MOPS, 2-(N-morpholino) propane sulfonic acid; and BES, N, N bis (2-hydroxyethyl)-2 aminoethane sulfonic acid. These compounds can be combined and titrated to provide buffers ranging in pH and osmolarity that are relatively innocuous to spermatozoa (20). They can be combined with egg yolk to give suitable extenders for temperatures ranging from 25 to -196°C.

Only Tris has been extensively fertility-tested with bull spermatozoa. Extensive studies were carried out before the fertility testing to establish optimum levels of Tris in combination with egg yolk and other substances (14, 18). Glycerol was included as one component because of the observed beneficial effects of glycerol at 5°C in milk extenders and the need for such a compound in extenders for freezing semen. The results of one study (see Table 16-6) showed that glycerol could be included in the initial extender, thus avoiding the time-consuming steps of adding glycerol at 5°C, as is often done with other extenders. There was no difference in times of glycerol additions, but motility of spermatozoa was lower in media with a pH of 6.25 ( $P < 0.01$ ) and when the molarity of the Tris was 0.25 ( $P < 0.01$ ).

*Table 16-6. Spermatozoan motility when glycerol was added directly to Tris-yolk extenders or after cooling to 5°C.<sup>a</sup> (Average of 20 samples stored for 8 days at 5°C.)*

<i>Glycerol added</i>	<i>Molarity of Tris</i>	<i>pH</i>		
		6.25	6.50	6.75
(% motile spermatozoa)				
Initially	.20	47	55	55
	.25	40	49	52
At 5°C	.20	47	55	53
	.25	42	47	51

<sup>a</sup>From Foote. *J. Dairy Sci.* 53:1475-1477. 1970.

The Tris extender that was selected for fertility testing (15) consisted of the following:

Tris	3.028 g
Citric acid monohydrate	1.675 g
Fructose	1.25 g
Glycerol	8.0 ml
Glass redistilled water	92.0 ml
Egg yolk (+ antibiotics)	25.0 ml

Semen was extended an average of 1:108 to give 8 million motile sperm per milliliter and the 0.2M Tris-fructose-glycerol-yolk extender, pH 6.75, was compared with CUE.

<i>Extender</i>	<i>No. 1st inseminations</i>	<i>% 60-90-day NR</i>
CUE	5,981	73.0
Tris	5,673	73.3

These results showed clearly that most of the ions in traditional buffers could be replaced by Tris. Other field trials established that glycerol was beneficial in the Tris extender (see Table 16-7). The difference, in favor of glycerol, was 2.5 percent ( $P < 0.01$ ). There was no statistically significant effect of pH, although the lower pH tends consistently to be associated with higher fertility (23). Tris and CUE gave equal fertility results. Another field trial with over

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40,000 inseminations resulted in 73.0 percent 60-90-day NR for CUE and 73.4 percent for Tris, thus confirming the equivalency of the two extenders.

Tris has been used successfully for storing buffalo spermatozoa (93). It has been used for titrating other zwitter-ion buffers (21). When combined with CUE (85) 4,080 inseminations resulted in 72.3 percent 60-90-day NR versus 69.7 percent for Spermasol T. When compared with Spermasol milk extender (86) the CUE-Tris was superior, declining only two percentage units in fertility over a period of four days. When semen was collected and processed only twice a week for use throughout the week, 137,074 first inseminations gave 70.2 percent 60-90-day NR. The formula for preparing one liter of the CUE-Tris extender is 7.57 grams Tris, 2.50 grams fructose, 5.53 grams citric acid, 10.88 grams sodium citrate, 1.35 grams sodium bicarbonate, 0.25 gram KCl, 2.25 grams glucose, 7.03 percent glycerol, and 2.25 grams sulfanilamide. To this is added 10 to 20 percent egg yolk and antibiotics.

It is clear that Tris types of organic buffers provide a versatile series of solutions that can be used to formulate desirable semen extenders. They are suitable for ambient temperature storage, refrigerated, or frozen semen.

**16-3.2f Other Egg Extenders.** A whole-egg extender was tested by Dunn et al. (94). Preparation of the extender was simple because the whole eggs with the chalazae removed were simply blended and added to buffer. The pH of a citrate/whole-egg medium was adjusted from about 7.9 to 6.7 by replacing sulfanilamide in the conventional 2.9 percent citrate-sulfanilamide-yolk medium with sulfasuxidine. Adjustment of the pH is essential for good sperm survival. The whole-egg formulation averaged 61.6 percent 60-90-day non-returns compared to 62.3 percent for the egg yolk control. Whole-egg extenders have not been adopted commercially.

Table 16-7. Fertility of bull spermatozoa in liquid Tris extender vs CUE.<sup>a</sup>

Treatments	Osmolarity (mosmols)	No. 1st services	% 60-90-day NR
CUE Control	333	13,966	74.0
Tris, pH 6.5	323	14,376	71.9
Tris, pH 6.75	317	13,933	71.2
Tris-gly, pH 6.5	—	13,630	74.0
Tris-gly, pH 6.75	—	12,903	73.1

<sup>a</sup>From Foote. *J. Dairy Sci.* 53:1475-1477. 1970.

Various prepared extenders incorporating egg yolk have been marketed commercially (5, 41, 95). In Japan Neoseminan is an ampuled sterilized product reported to be stable for several years. It consists of egg yolk, sodium citrate, potassium citrate, sodium phosphate, glucose, homosulfamine, sodium sulfamesadin, and chlorpromazine and is gassed with CO<sub>2</sub>. Spermasol (see Section 16-3.3e) has been used principally in Germany. Many formulations containing sodium citrate, potassium phosphate, and gelatin have been combined with either egg yolk or milk. Other extender formulations have been marketed as well (60). Although they offer convenience, any of the complete formulations containing egg yolk or similar organic material probably have limited shelf life. Large AI organizations therefore prepare their own extenders shortly before use.

**16-3.3a Milk—History and Heating.** Phillips and Lardy (96) observed that evaporated milk could maintain sperm for only 48 hours. Fresh semen gave higher fertility than semen extended and stored in yolk-phosphate or in autoclaved milk (97). Better results were obtained by Michajilov (98), who used boiled filtered milk for jack semen. A canned sterile skim milk was easy to prepare, sperm were clearly visible, and fertility was superior to that allowed by the yolk-citrate used previously (99). Later Jacquet and Cassou (100) found that the quality of the canned milk product varied and they recommended that 10 percent by weight of powdered milk plus antibiotics be added to double-distilled water and that egg yolk also be included up to 10 percent of the final volume.

Milk preparations were being investigated simultaneously by Almquist and associates, and details of proper milk handling as a bull semen extender were described (101). Milk must be heated beyond the pasteurization point to destroy lactenin, a spermicide (102). Sulfhydryl groups released by heating probably inactivate the toxic factor. Recommended procedure is to heat milk in a double boiler to 92 to 95°C for 10 minutes, cool it to 5°C, and add antibiotics. The extender is then ready for use. Other methods of inhibiting the toxic factor have been reviewed (5, 41); they include the use of thioglycolic acid or cysteine. Both contain sulfhydryl groups. Jones (103) postulated that excess cysteine might be harmful and later reported that dialysis to remove excess cysteine was beneficial (104). Heating, however, was preferable. Many laboratories in the 1950s observed that egg yolk also made heating the milk unnecessary and this was documented in the literature (105).

Fortified skim milk was found, as a semen extender, to be detrimental to spermatozoa (106). Different batches of skim milk also required different degrees of heating to inactivate the toxic factor (107). Too much heat can be harmful, possibly because of its effect on lactose (103). Others have observed variability in batches of liquid milk and dried milk. This and the different procedures used in heat treatment of milk may be responsible for some of the



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variability in the fertility results that investigators have obtained in comparing milk and egg yolk extenders.

**16-3.3b Liquid and Dried Milk Preparations.** Liquid whole milk, skim milk, cream, and various reconstituted powdered milks have been tested as bull semen extenders. The early literature has been reviewed (5, 41). Since sperm motility is difficult to assess in many milk preparations only the fertility results of studies are meaningful.

Table 16-8 summarizes fertility tests run on several milk extenders in which antibiotics were used. Most of the fertility results were based on 60-90-day nonreturns to service, but if fertility was based on other time intervals the comparisons within an experiment were made on the same basis. The first series of experiments all included egg yolk-citrate as a control. In general egg yolk-citrate and milk extenders gave comparable results. Most differences were small and appeared to be due to chance. Variations in the levels of egg yolk (20 to 50 percent), citrate (2.9 to 3.6 percent), and antibiotics, as well as the formulation and batches of milk used, also presumably contribute to the variations observed. Saacke et al. (106) called attention to the importance of milk solids and osmolarity: Although milk proteins can protect spermatozoa against moderate shifts in osmolarity (104) failure to observe the principles of sperm preservation discussed earlier in this chapter can reduce fertility.

In the studies by Almquist (109) and Melrose et al. (114) semen from bulls exhibiting low fertility appeared to benefit from being stored in milk. It is possible that the antibiotics were somehow more effective in the milk preparation as processed. Morgan et al. (119) showed that *Vibrio fetus* infection was controlled by two hours' exposure at 5°C to streptomycin in skim milk but not in egg yolk. At the time of these studies vibriosis was widespread in cattle and had not yet been eradicated from most bull studs.

Homogenized milk, homogenized cream (9 percent fat), and skim milk are capable of yielding comparable fertility results. Skim milk, however, allows spermatozoa to be seen microscopically and sperm viability can therefore easily be assessed. Moreover, skim milk powder is readily available and inexpensive. Large batches can be pretested and, if found to be satisfactory, can be used with confidence in their uniformity. The sterilized cream (115) is convenient to use because it requires no additional heating, and large batches can be secured for use over a considerable period of time. Reconstituted buttermilk (not shown in Table 16-8) can also be used (120). Spermatozoa extended in reconstituted buttermilk (10.8 grams with 89.2 milliliters of distilled water plus antibiotics) gave good results in a limited fertility test when compared with yolk-citrate extended semen.

**16-3.3c Milk Combined with Sugar, Glycine, and Glycerol.** Although the fertility of bull spermatozoa extended in milk was initially good, it often declined rapidly with storage at 5°C. This was also true of the original yolk-citrate



Table 16-8. Fertility of bull spermatozoa in heated fresh or powdered milk with sugars, glycine, or glycerol.

<i>Treatment<sup>a</sup></i>	<i>No. 1st inseminations</i>	<i>% NR</i>	<i>Reference</i>
Homogenized milk	2,931	59.9 <sup>b</sup>	(73)
Homogenized milk-sulfanilamide	4,375	64.6	
Yolk-citrate-sulfanilamide	2,892	69.5	
Homogenized milk	1,570	76.1	(108)
Homogenized milk + cornstarch	1,554	74.3	
Yolk-citrate	1,565	75.9	
Homogenized milk + cornstarch	2,852	65.3	(108)
Skim milk + cornstarch	2,873	67.8	
Yolk-citrate-sulfanilamide	2,674	66.8	
Homogenized milk	4,178	71.6	(109)
Yolk-citrate	4,222	64.2 <sup>b</sup>	
Homogenized milk	10,012	69.4	(110)
Yolk-citrate	9,927	71.2	
Homogenized milk	4,637	68.8	(111)
Yolk-citrate	5,051	69.9	
Skim milk	6,850	70.7	
Yolk-citrate	7,979	69.1	
Homogenized or skim milk	23,422	69.8	(112)
Yolk-citrate-sulfanilamide	24,269	69.6	
Skim milk	44,746	69.0	(113)
Skim milk powder, 10% w/v	48,013	69.3	
Skim milk powder, 9% w/v	5,076	68.4	(114)
Yolk-citrate	5,113	63.5 <sup>b</sup>	
Skim milk powder, 9% w/v	2,276	65.7	(114)
Skim milk powder + 12.5% yolk	2,339	68.1	
Skim milk powder, 9% w/v	1,521	66.3	(114)
Skim milk powder + 3% glycine	1,421	67.2	
Skim milk powder, 9% w/v	2,025	67.3	(114)
Skim milk (liquid)	1,949	68.9	
Skim milk	2,263	69.2	(115)
Sterilized homogenized cream	2,931	69.4	
Yolk-citrate	2,370	63.6	
Homogenized milk	25,599	71.3	(116)
Homogenized milk + 1.25% fructose	25,282	72.4	
Skim milk	14,319	61.5	(117)
Skim milk + 10% glycerol		64.5	
Skim or homogenized milk	10,845	63.7	(118)
Skim or homogenized milk + 10% glycerol	10,831	71.5 <sup>b</sup>	

<sup>a</sup>All extenders contained antibiotics (streptomycin and often penicillin) and all liquid milks were heated excepting when egg yolk was added. Egg yolk content varied from 20 to 50% in the yolk-citrate controls.

<sup>b</sup>Values that differ significantly from others within an experiment,  $P < 0.05$ .

% NR	Reference
59.9 <sup>b</sup>	(73)
64.6	
69.5	
76.1	(108)
74.3	
75.9	
65.3	(108)
67.8	
66.8	
71.6	(109)
64.2 <sup>b</sup>	
69.4	(110)
71.2	
68.8	(111)
69.9	
70.7	
69.1	
69.8	(112)
69.6	
69.0	(113)
69.3	
68.4	(114)
63.5 <sup>b</sup>	
65.7	(114)
68.1	
66.3	(114)
67.2	
67.3	(114)
68.9	
69.2	(115)
69.4	
63.6	
71.3	(116)
72.4	
61.5	(117)
64.5	
63.7	(118)
71.5 <sup>b</sup>	

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extender, and was partially responsible for the development of more complex yolk extenders created to prolong fertility of unfrozen semen. Milk is low in hexoses, although traces of glucose are present, and it contains lecithin and the fat-decomposition products glycerol and organic acids. Spermatozoa cannot utilize lactose, but they can metabolize the other substances aerobically and glucose anaerobically. No advantage was found in adding fructose to heated homogenized milk (see Table 16-8). Semen was used uniformly for four days and the decline in fertility with fructose added was 5.5 percent versus 4.6 percent for the milk extender alone (116). Thus at 5°C an additional energy source for spermatozoa appeared unnecessary.

The effect of adding glycerol to milk is usually favorable. Bratton and Foote (73) obtained, with and without glycerol, 65.8 and 64.4 percent 60-90-day NR in heated homogenized milk. Williams et al. (121) conducted a large scale experiment (14,341 inseminations) and reported that 10 percent glycerol added to heated homogenized milk increased the 60-90-day NR after 1, 2, and 3 days of storage at 4°C by 2.5, 3.5, and 4.2 percent ( $P < 0.01$ ). Almquist (118) also showed that glycerol markedly improved the fertility of bull sperm stored for several days at 5°C. On the other hand, O'Connor and Smith (117) found that glycerol helped to maintain the fertility of bull sperm only during the last two of four days of storage at 5°C. A comparison of these results follows:

Age of semen	No. 1st inseminations		Advantage of glycerol in % NR	
	O'Connor	Almquist	O'Connor	Almquist
1 day	3,682	7,106	- 3.1	+ 2.3
2 days	3,494	8,707	- 1.3	+ 7.4
3 days	3,688	3,405	+ 4.7	+ 12.2
4 days	3,455	2,458	+ 10.6	+ 19.5

No difference in fertility was found between semen from the same source extended with heated skim milk to which either 5 or 10 percent glycerol had been added (122), although 5 percent glycerol gave the best spermatozoan liveability. Glycerol added to skim milk at room temperature reduced motility and induced morphological changes in spermatozoa. Slow addition of glycerol at 5°C was recommended. In another study (79) fertility was reduced from 64.6 to 55.6 percent by including 10 percent glycerol initially in a 9 percent skim milk powder extender mixed with sperm cells at 20°C. Overall, the studies indicate that, with the proper preparation of milk and milk-glycerol extenders, initial fertility of spermatozoa in each is similar, but is better maintained for several days at 5°C with milk-glycerol extender.

**16-3.3d Milk-Egg Yolk Combinations.** After early work indicated that small volumes of egg yolk added to milk might improve sperm survival and inactivate the spermicide in milk, many combinations were tested. The early work was reviewed in the previous edition of this text (123). Table 16-9 shows several combinations of milk with yolk and glycine or glycerol. All liquid milks and most reconstituted milks used were heated to 92°C for 10 minutes, and all extenders listed in the table contained antibiotics.

The addition of glycine to milk was based on the sometimes favorable results obtained with yolk-glycine extenders (see previous sections). However, it is clear that under the conditions tested glycine did not improve fertility; in fact, when it was combined with glycerol and milk-yolk (see Table 16-9) it decidedly depressed fertility. Hence glycine has not been used as a regular constituent of milk extenders.

*Table 16-9. Fertility of bull spermatozoa in milk-yolk combinations.*

<i>Treatment</i>	<i>No. 1st inseminations</i>	<i>% NR</i>	<i>Reference</i>
Homogenized milk	3,033	73.7	(124)
50% milk-25% yolk- 25% of a 3% glycine solution	2,848	72.3	
Homogenized milk + 10% glycerol	4,850	71.4	(124)
Milk-yolk-glycine + 10% glycerol	4,078	60.9 <sup>b</sup>	
Skim milk-10% yolk + 8% glycerol	2,552	73.5	(125)
Yolk-citrate + 6.32% glycerol	2,542	74.4	
Skim milk + 5% yolk <sup>a</sup>	4,045	67.0	(126)
Skim milk-5% yolk + 10% glycerol	4,032	67.0	
Skim milk-5% yolk	8,438	76.3	(126)
Skim milk-5% yolk + 10% glycerol	7,947	77.7	
Milk-yolk	3,665	63.4 <sup>b</sup>	(127)
CU-16 (yolk base)	3,853	68.3	
9% skim milk powder, 12.5% yolk	11,524	63.3	(79)
CUE (complex buffered yolk)	11,339	64.6	
9% skim milk powder, 6% yolk	20,610	63.2	(128)
9% skim milk powder, 6% yolk, 1.5% glycerol	20,630	63.8	
9% skim milk powder, 6% yolk	16,983	63.3	(128)
9% skim milk powder, 10% yolk, 3% glycerol	16,983	62.6	

<sup>a</sup>In the first study by these authors glycerol was added at room temperature and 90-120-day % NR calculated. In the second study glycerol was added at 5°C and 30-60-day % NR calculated.

<sup>b</sup>*P* < 0.01 comparing fertility within experiments.

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ometimes favorable sections). However, improve fertility; in (see Table 16-9) it n used as a regular

yolk combina-

% NR	Reference
73.7	(124)
72.3	
71.4	(124)
60.9 <sup>b</sup>	
73.5	(125)
74.4	
67.0	(126)
67.0	
76.3	(126)
77.7	
63.4 <sup>b</sup>	(127)
68.3	
63.3	(79)
64.6	
63.2	(128)
63.8	
63.3	(128)
62.6	

ature and 90-120-day % NR  
0-day % NR calculated.

The addition of glycerol to liquid milk and egg yolk or to powdered milk with egg yolk was consistently without measurable effects. Glycerol does not appear to improve the fertility of semen stored for several days in milk-yolk extenders, in contrast with its demonstrated effect when added to milk alone (128). It is possible that egg yolk serves a similar function to that of glycerol in milk extenders, but the results of appropriate tests of that hypothesis, using the milk and milk-yolk extenders with and without glycerol, have not proved the hypothesis. It appears, however, that semen can be added directly to glycerolized milk-yolk extender (126). This could simplify processing, especially of frozen semen.

Milk-yolk extender seems comparable to CUE (79), although after 3 days of storage the 112-day nonreturn rates were 62.5 percent for CUE and 57.8 percent for milk-yolk extender. A report by Hutchinson and Cooper (129) indicated that 3 percent glycerol added to 87 parts of heated skim milk and 10 parts of egg yolk improved fertility substantially over a yolk-citrate control. A similar extender made from powdered skim milk (128) was not superior to milk-yolk when bull spermatozoa were stored for up to 3 days before insemination.

Thus the simplest satisfactory milk-yolk semen extender appears to be one made of either heated fresh or powdered skim milk (9 to 10 percent solids) with 5 to 10 percent fresh egg yolk added. Such an extender was used regularly by several AI organizations until the practice of freezing semen came into use.

**16-3.3e Other Extenders.** Various commercial preparations have been used successfully. Spermasol, for example, is an egg yolk-citrate-phosphate-gelatin product that can be combined with milk (130); Laiciphos is a powdered milk extender that gives good fertility when mixed with 10 percent fresh egg yolk (131). Tomato juice and other vegetable materials have been combined with yolk (41) and countless variations in recipes for semen extenders can be found in the literature (5, 41, 132). Examples of those tested extensively for ability to yield high fertility have been summarized in the tables. Certain extenders designed primarily for storage at ambient temperatures (see Section 16.4) have received limited use at 5°C.

Testing extenders for commercial insemination of cattle has served as a basis for developing extenders for other species. Similar extenders preserve buffalo sperm equally well (133, 134, 135).

#### 16-4 EXTENDERS FOR STORAGE AT AMBIENT TEMPERATURES

**16-4.1 Development of Room Temperature Extenders.** It is clear from the survival rates of sperm in the epididymis and the female reproductive tract that sperm cells can remain viable at body temperature for significant periods of time. Refrigerating semen at 5°C and freezing semen have greatly increased

its *in vitro* storage life. The need for unrefrigerated semen has therefore diminished, but in some areas refrigeration is not widely available or is expensive. Hence consideration will be given to techniques of preserving bull sperm at ambient temperatures.

Storing spermatozoa in nutrient media at ambient temperatures obviously makes it necessary to control bacteria. According to Foote and Bratton, a combination of sulfanilamide, penicillin, streptomycin, and polymyxin is extremely effective in preventing bacterial growth in bull semen extended with yolk-citrate and stored at 25°C (35). Under these conditions sperm survival is greatly improved and egg yolk is stabilized. Foote and Bratton conclude that "this combination of antibacterial agents gives promise of making possible the development of an extender for bovine semen which will not require refrigeration."

**16-4.2 CO<sub>2</sub> Ambient Temperature Extenders.** Biochemical control of spermatozoan metabolism is prerequisite for a good extender that can be used at room temperature. Previous sections of this chapter have referred to the Illinois work showing that CO<sub>2</sub> markedly reduced sperm metabolism. When the CO<sub>2</sub> level was properly controlled the motile and fertile life of bull sperm was prolonged (25). A successful ambient temperature extender called IVT (see Table 16-3) was developed (24). These workers reported little change in fertility when a limited number of cows were inseminated with semen stored up to seven days at ambient temperature.

IVT has been used extensively for boar semen where storage temperatures of 15°C or higher are preferable to 5°C. It has not been widely used for bull sperm, however, due to the requirements for CO<sub>2</sub> gassing and for sealing the storage vial so it will hold proper CO<sub>2</sub> levels.

CUE, a related extender (see Section 16-3) that includes glycine and does not require external gassing with CO<sub>2</sub>, has given consistently good sperm survival at 25°C. CU-16, a simplified extender, similarly preserves sperm motility. In each of these glycine may promote the desirable production of CO<sub>2</sub> as a result of sperm metabolism. Boye (136) studied temperatures of 5, 10, 15, 20, 25, and 30°C for storing bull spermatozoa in CUE and found 20°C to be the optimum. The insemination of 1,978 cows with CUE-extended semen stored for 1, 2, and 3 days gave 81, 79, and 79 percent NR on a 30-60-day basis.

Abdu (137) reported that ethylenediamine tetracetic acid (EDTA) added to a yolk-citrate-glucose medium gave good fertility when semen was stored in it at 18 to 25°C and used after 3 to 6 days. Only limited breedings were carried out.

**16-4.3 Caprogen.** By far the most extensive testing of an ambient temperature extender has been done on Caprogen in New Zealand. The initial development of this extender for 5°C storage was given in Section 16-3 (see Table



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acid (EDTA) added semen was stored in ited breedings were

an ambient tempera- l. The initial develop- tion 16-3 (see Table

16-5). Several pilot experiments indicated that semen could also be handled under the moderate ambient temperatures prevailing during the short breeding season in New Zealand. An extensive test (87) gave the following 49-day NR:

Age of semen	5°C		Ambient temperature	
	No. cows	% NR	No. cows	% NR
Day collected	268,201	65.3	44,354	66.2
Day later	172,663	66.6	29,772	68.9

Because of the good fertility achieved with semen used at ambient temperature, particularly after one day of storage ( $P < 0.05$ ), the majority of inseminations in New Zealand since 1964 have been made with semen kept at this temperature. The optimum temperature range for Caprogen is considered to be 18 to 24°C.

Egg yolk level in Caprogen has been reduced experimentally (87, 138) to as little as 2 percent by volume (see Table 16-10). Five percent egg yolk is used routinely, except for the initial premix with semen, which contains 20 percent yolk. This is consistent with other work (see Table 16-2) showing that less egg yolk is needed with higher temperatures. Furthermore, there appears to be an interaction between spermatozoa and egg yolk levels (87). With low egg yolk levels sperm numbers per insemination can be minimized. The practice for several years in New Zealand has been to extend semen to 4 or  $5 \times 10^6$  total sperm per milliliter and to use 0.5 milliliter of extended semen per insemination. Earlier work in New Zealand and elsewhere (139)

Table 16-10. Fertility of spermatozoa with different levels of egg yolk in Caprogen extender at ambient temperature.

Total no. inseminations	% egg yolk				Reference
	20	10	5	2	
	(% 49-day NR)				
9,778	67.7	65.7	68.4	—	(87)
425,456	62.8	—	66.1 <sup>a</sup>	—	(87)
16,790	—	—	64.1	66.1 <sup>b</sup>	(87)
12,512	—	—	66.9	68.2 <sup>b</sup>	(87)

<sup>a</sup>Treatments differ,  $P < 0.01$ .

<sup>b</sup>Treatments differ,  $P < 0.05$ .

established that equal numbers of sperm inseminated in 0.5 and 1.0 milliliter of extended semen resulted in similar fertility.

Caprogen contains catalase to decompose the  $H_2O_2$  that is formed as a product of sperm metabolism. This addition consistently and significantly increased fertility by 1 to 2 percent. Examples of results are shown in Section 16-6.

Finally, Caprogen can be used as a liquid extender for resuspending frozen spermatozoa. To accumulate extra semen before the start of the short breeding season New Zealand workers (140) have frozen and accumulated limited numbers of breeding units. The frozen semen is thawed, rediluted with Caprogen, and used at ambient temperature. Fertility of the rediluted semen in three extensive trials was 61.1, 61.1, and 63.3 percent NR. The corresponding regular ambient temperature control results were 62.0, 63.3, and 63.4 percent NR (140). Fertility of the semen collected from individual bulls and then frozen and rediluted was highly correlated with semen collected and used directly. Thus, with proper selection of bulls from which to freeze additional semen, overall fertility would not be appreciably affected.

**16-4.4 Coconut Milk.** Coconut milk extender (CME) is also used at ambient temperatures. The milk from coconuts had been used as a nutrient medium for the culture of plant embryos and it was combined with other ingredients in an extender for spermatozoa (141). The coconut milk is boiled for 10 minutes and filtered before use. Its formulation, as given by Norman (142), is:

Coconut milk	15 ml
Sodium citrate dihydrate	2.2 g
Sulfanilamide	0.3 g
Egg yolk	5 ml
Distilled water to	100 ml

Included per 100 milliliters of CME are 135 milligrams dihydrostreptomycin sulfate, 10 milligrams polymyxin B sulfate, 1000 units of nystatin and 15,000 units of sterile catalase. For ambient temperatures 0.5 to 1.0 percent egg yolk was suggested (32), but 5 percent egg yolk (v/v) extends the use range of refrigerated semen.

In early fertility tests in the United States CME was found to give results equivalent to skim milk-glycerol and CUE (32). Liveability tests and limited fertility data suggested that spermatozoa might be used for breeding after several days' storage in CME. Semen used after 1 to 5 days in Uganda, Kenya, El Salvador, Paraguay, India, and West Germany gave conception rates ranging from 55 to 72. Malmberg and Israelsson (143) obtained 67.7 percent 84-day NR from 5,760 services with CME used for 4 days at 15 to 25°C, and 65.5

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percent from 5,832 services with frozen-thawed semen. When semen stored in CME was used for several days (about 400 services per day) the respective 84-day NR figures for the seven days were 69, 67, 63, 61, 52, 46, and 39 percent. Fertility dropped markedly after the fourth day. CME can be used with equal success for cattle and buffalo semen (144).

Coconut palm sap was studied (145) as a possible extender that could be prepared in convenient freeze-dried form. It was less effective, however, than the coconut fluids in maintaining sperm motility.

Whether coconut fluids provide any materials unique for sperm preservation is debatable. The analytical grade reagents used in CUE provide a medium as satisfactory as the citrate-coconut milk extender for sperm survival over an 8-day period at 23 to 25°C (146). When CUE with 1 percent egg yolk was used to store 15 samples of bull semen there was no detectable decline in motility for 8 days, compared to a decline from 60 percent motile sperm to 38 percent after 8 days with 20 percent yolk-CUE. This emphasizes again the importance of using a low egg yolk level in ambient temperature extenders. The results from other studies with coconut milk (147) revealed that 5 to 15 percent of skim milk and glucose combined with citrate buffer was equal to CME in maintaining sperm motility at 16 to 27°C.

In summary, extenders that are successful at ambient temperature are generally characterized by a balanced buffer solution, low egg yolk content, catalase, and a combination of antibiotics.

#### 16-5 MICROORGANISMS IN SEMEN AND THEIR CONTROL

Microbial contamination of semen may 1) affect spermatozoa directly, 2) compete for substrate in semen extenders, and 3) infect inseminated females. The result can be lowered conception rates and increased embryonic mortality or abortion. Microorganisms can gain entry into the semen from infected bulls, during semen collection, during semen processing, or during insemination. Thus it is extremely important to use sterile equipment, sterile media, and aseptic procedures at all steps of the program. Obviously, it is important to start with a healthy bull, but this is not always possible, since unknown or undetected diseases can exist in a bull. However, organizations that comply with the American Veterinary Medicine Associations/National Association of Animal Breeder's "Code of Minimum Standards for Health of Bulls and Hygiene of Bull Studs Producing Semen for Artificial Insemination" (148) have effectively controlled known infectious diseases that might be transmissible through semen. Continuous vigilance under proper veterinary supervision is essential. It is regrettable that many custom freezing operations on farms and ranches almost totally ignore the code, thus working to the detriment of AI.

Even under the best conditions some microbial contamination can occur. Chemical treatment of semen to inhibit multiplication of these contaminants

or to kill them without damaging the sperm cells is therefore important. Comprehensive coverage of published reports on the microbiology of bull semen and means for control are available (41, 148-151). The intent of this section is to outline the development and current status of our knowledge in this area. Additional references can be found in the reviews cited.

**16-5.1 Microflora of Bull Semen.** Since Gunsalus et al. (152) called attention to the numbers of bacteria in semen several reports have appeared (34, 153-157). In general, there are usually fewer than 150,000 organisms per milliliter of semen with a range from no detectable organisms to samples with several millions per milliliter. Cleaning the sheath and underline of the bull (146) and placing a small apron on the chest of the bull and a polyurethane device at the entrance to the artificial vagina (158) effectively reduce contamination at the time of semen collection. Second ejaculates of semen have lower average bacterial counts than first ejaculates.

Bacilli, diphtheroids, micrococci, coliform organisms, streptococci, staphylococci, pseudomonas, actinomyces, protus, yeasts, viral agents, and mycoplasma have all been found in bull semen. Many of the organisms present are not pathogenic and there is therefore no direct correlation between fertility and the total number of organisms present. Most types of organisms, if present in excessive numbers, will reduce sperm survival (153, 156), but this is not always true.

One organism that appears to be associated with low fertility in bulls is *Pseudomonas aeruginosa* (152-154, 157). This organism can be transmitted through frozen semen (159). *Pseudomonas aeruginosa* and *Clostridium pyogenes* occasionally infect the seminal vesicles and then are contributed to semen in the vesicular secretion (150). Other organisms, which are contagious and can infect cows through contaminated semen, include *Brucella abortus*, *Vibrio fetus*, *Trichomonas fetus*, *Leptospira pomona*, *Mycobacterium tuberculosis*, *M. paratuberculosis* (agent of Johne's disease), *Mycoplasma bovine genitalium*, and viral agents that cause infectious bovine rhinotracheitis (IBR) and bovine viral diarrhea (BVD). International shipments of semen must come from areas free from foot and mouth disease and blue tongue, since the viruses responsible for these diseases are transmissible if present in semen.

Efforts are being made to completely eliminate such organisms from bull studs. Through strict programs of testing, selection, isolation, and treatment the major AI organizations have waged a successful battle against most of the organisms listed. Less is known about methods for preventing infection or curing animals with mycoplasma or IBR.

Simultaneously, continuous research is necessary on chemotherapeutic methods of controlling contaminants in semen. Not only can unknown organisms be present, but known organisms, previously sensitive to antibiotics, can

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develop resistance (160). Refrigeration greatly suppresses the multiplication of most such organisms, but does not necessarily stop it (161, 162). Most can survive freezing.

**16-5.2 Antibiotics and Other Antimicrobial Agents.** As pointed out in the previous section, even when rigid hygienic measures are imposed variable numbers of organisms are present in fresh bull semen. In the early commercial development of artificial insemination semen was stored at 5°C or higher. Egg yolk provided a good nutrient medium for bacterial growth. Effective agents were needed that were easy to use and would control bacterial growth, be non-toxic to spermatozoa, and increase fertility.

*Sulfanilamide.* Salisbury and coworkers had found that sulfanilamide depressed the rate of sperm metabolism, increased sperm liveability, and partially inhibited bacterial growth. Fertility tests (62) showed that 0.3 percent (w/v) sulfanilamide in a yolk-citrate extender increased the nonreturn rate about five percentage units, a value that was soon confirmed (60). Not all reports were in agreement, particularly with antibiotics in the extender (57). Subsequent studies indicated that if the extender was mixed with semen before rather than after cooling to 5°C, the beneficial effect of sulfanilamide disappeared. This beneficial effect had been interpreted earlier (62) to be primarily a metabolic one, and the fact that egg yolk during cooling also was effective supported the idea that sulfanilamide enhancement of fertility was not due to bacterial inhibition. Sulfanilamide damages spermatozoa during the freezing process (163), and should be excluded from extenders used for semen that is to be frozen. Other sulfa drugs have been tested for possible superiority over sulfanilamide (162), but none has been found that warrants adoption.

*Penicillin and Streptomycin.* These two antibiotics were among the first to be researched intensively and are still in use. They were found to be relatively harmless to sperm cells and, particularly in combination, inhibited a broad spectrum of microorganisms (57, 123). Almquist (164) first reported that 500 to 1,000 units of penicillin per milliliter of extended semen increased the fertility of low-fertility bulls. Foote and Bratton (165) obtained higher nonreturn rates when 1,000 units of penicillin per milliliter, or 1,000 micrograms of streptomycin per milliliter, or both, were added to extended semen. Sulfanilamide had no effect. These findings were substantiated by other reports (166, 167). Later most of the beneficial effect of the two antibiotics was reported to be due to streptomycin in either egg yolk or milk extender (168, 169). All American reports showed that the nonreturn rate of low-fertility bulls tended to be raised more by addition of these antibiotics to semen extenders than did that of high-fertility bulls, and that delayed returns were decreased.



An extensive trial by Campbell and Edwards (67) also showed that fertility increased when sulfanilamide, penicillin, and streptomycin were added to yolk-citrate singly or in combination. Streptomycin depressed fertility in yolk-phosphate extender, probably because of a precipitate that was formed as a result of the reaction of the streptomycin calcium chloride complex with phosphate buffer. Easterbrooks et al. (170) observed that this problem can be circumvented by using dihydrostreptomycin. Extensive fertility testing indicates that the different forms of streptomycin give equal fertility.

Penicillin usually is added at levels of 500 to 1,000 units per milliliter and streptomycin or dihydrostreptomycin at 500 to 1,000 micrograms per milliliter of extender. Levels much greater than these have been found by some to reduce sperm survival (57), but levels as high as 4,000 units of penicillin and 8,000 micrograms per milliliter of streptomycin or the dihydro form were reported to be harmless (171). Conception rates were improved with 500 to 5,000 micrograms per milliliter of dihydrostreptomycin added to a yolk-citrate-sulfanilamide extender (172).

Both penicillin and streptomycin have been widely used since about 1950. Their popularity stems from the overwhelming evidence of their beneficial effects on fertility. Even the low percentage of pregnant animals that show estrus and are again inseminated are usually protected from possible abortion. Antibiotics have not shown beneficial effects in all tests, however. The semen in these tests may have been free from pathogens, or the method used to expose the semen to antibiotics may have been ineffective. The fertility response to antibiotic treatment today would probably be less than that originally observed now that many of the organisms, such as *Vibrio fetus* (which streptomycin particularly helped to control), have been eliminated from bull studs. In the Netherlands rigid culling of bulls based on bacteriological examination has long been the rule, and antibiotic treatment of semen has had negligible effects on fertility. On the other hand, Melrose et al. (114) in England reported a 3.4 percent increase ( $P < 0.001$ ) in fertility when semen from *V. fetus*-free bulls was treated with 1,000 micrograms of streptomycin per milliliter.

*Other Antibiotics.* Many other antibiotics have been screened for effects on spermatozoa (see Table 16-11). Oxytetracycline (20  $\mu\text{g}/\text{ml}$ ), chlortetracycline (50  $\mu\text{g}/\text{ml}$ ), and chloramphenicol (500  $\mu\text{g}/\text{ml}$ ) in individual tests were ineffective in raising fertility (178). Several antibiotics such as the tetracycline series and particularly the fungicidal agents nystatin and amphotericin B are quite spermicidal. Others, such as epicillin, are relatively innocuous to sperm cells but also are ineffective against bacterial contamination. Nevertheless, an array of antibiotics exist that are effective against a variety of organisms. Since they can ordinarily be combined safely at levels that are harmless to spermatozoa when they are used singly, judiciously chosen combinations could inhibit a broad spectrum of semen contaminants. Lincomycin and spectinomycin have

Table 16-11. Nonspermicidal levels of antibiotics in bull semen extenders.<sup>a</sup>

Antibiotic	Maximum safe level	Kind of extender and temperature <sup>b</sup>	Reference
Tetracycline (Panamycin)	75 µg/ml	Krebs-Ringer, 3 hrs. at 37°C	(36)
Oxytetracycline (Terramycin)	200 µg/ml	YC, 8 days at 5°C	(173)
	<125 µg/ml	SM, 4 days at 5°C	(174)
Chlortetracycline (Aureomycin)	100 µg/ml	YC, 4 days at 5°C	(154)
	62.5 µg/ml	YC, 15 days at 5°C	(171)
	<50 µg/ml	YC, 14 days at 4.5°C	(175)
	<250 µg/ml	SM, 4 days at 5°C	(174)
Chloramphenicol (Chloromycetin)	500 µg/ml	YC, 15 days at 5°C	(171)
	1000 µg/ml	YC, SM, 12 days at 5°C	(c)
	1000 µg/ml	YCG, YTG, months at -196°C	(c)
Neomycin	500 µg/ml	SM, 4 days at 5°C	(174)
	1000 µg/ml	SM, 20 days at 5°C	(176)
Polymyxin B	2000 µg/ml	YC, 14 days at 5°C	(154)
	1000 µg/ml	SM, 4 days at 5°C	(174)
	1000 µg/ml	YCG, YTG, months at -196°C	(c)
	1000 µg/ml	YC, 30 min. at 30°C	(177)
Erythromycin	1000 µg/ml	YC, SM, 12 days at 5°C	(c)
	1000 µg/ml	YCG, YTG, months at -196°C	(c)
Kanamycin	1000 µg/ml	YC, SM, 12 days at 5°C	(c)
	1000 µg/ml	YCG, YTG, months at -196°C	(c)
Tylosin	1000 µg/ml	YC, SM, 12 days at 5°C	(c)
	250 µg/ml	YCG, YTG, months at -196°C	(c)
Thiostrepton	25 µg/ml	YC, SM, 12 days at 5°C	(c)
Nystatin	5 µg/ml	YC, SM, 12 days at 5°C	(c)
Amphotericin B	<1 µg/ml	YC, SM, 12 days at 5°C	(c)
Colymycin	<250 µg/ml	YCG, YTG, months at -196°C	(c)
Lincomycin	1000 µg/ml	YCG, YTG, months at -196°C	(c)
Spectinomycin	1000 µg/ml	YCG, YTG, months at -196°C	(c)
Epicillin	1200 µg/ml	SM, 20 days at 5°C	(176)
Ampicillin	1000 µg/ml	YCG, YTG, months at -196°C	(c)

<sup>a</sup>Tests that were conducted without egg yolk or milk in the medium generally are excluded because toxicity may be greatly different in the absence of these ingredients.

<sup>b</sup>YC = yolk-citrate; SM = skim milk; YCG = yolk-citrate-glycerol; YTG = yolk-tris-glycerol.

<sup>c</sup>Foot et al. Unpublished data. Cornell University. 1963-1973.

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been combined to provide such control. This combination, along with clindamycin, is especially effective against mycoplasma (179).

Few fertility tests have been reported in recent years. The addition of polymyxin B has been reported to increase the fertility of frozen semen (180, 181); this is similar to the original report for liquid semen (165). Subsequent studies with both liquid and frozen semen have failed to demonstrate an improvement when added to extender containing penicillin and dihydrostreptomycin (see Table 16-11).

**Other Chemotherapeutic Agents.** Pyridium, phenoxethol (161), nitrofurans (36, 161), sodium azide (a respiratory poison) (182), and other agents (183) have been added to semen. These have either been relatively toxic to sperm cells at levels that are bacteriostatic, or they have been difficult to use routinely because of low solubility or instability.

**16-5.3 Control of Specific Pathogens.** Much attention has been given to the control of specific pathogens in semen (57, 148, 149, 182). Although unrecognized at the time, one of the organisms that could be spread widely by semen from infected bulls during the early expansion of artificial insemination was *Vibrio fetus*. Numerous studies (184-187) support the conclusion that dilution and streptomycin treatment of semen prevents vibriosis from being transmitted via AI by *V. fetus* carrier bulls. But Morgan et al. (119) showed that streptomycin treatment of semen extended with egg yolk was ineffective in controlling the spread of infection by AI, whereas in milk it was effective. Albertsen (182) showed that the marked effectiveness of streptomycin against *V. fetus*, *B. abortus*, and *Corynebacterium pyogenes* was greatly diminished by egg yolk. He recommended incubating the semen with antibiotic for 15 minutes at 37°C or for 60 minutes at 30°C before adding egg yolk. A modification of this incubation procedure (177, 180) with the addition of 1,000 units of polymyxin B plus 2000 micrograms of dihydrostreptomycin per milliliter of raw semen is reported to provide a more effective control of *V. fetus*. It is possible that the antibiotic neomycin would be a better choice to combine with penicillin and streptomycin because several strains of *V. fetus* are sensitive to this antibiotic (36, 174), whereas polymyxin B was less effective (174).

Similar examples could be given for other pathogens but the problems have been discussed thoroughly (148, 149). The fact is that many organisms can survive in frozen semen. Clearly the kind and quality of antibiotic, the time and temperature of exposure of organisms to the antibiotics, and the composition of the extender should be considered in formulating a satisfactory procedure for controlling microorganisms in semen.

The ultimate goal is to eliminate all harmful organisms from the bull stud and, through sanitary procedures, to minimize semen contamination. Even under the best conditions, however, an effective mixture of microbial inhibitors in the semen appears to be good insurance.

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## 16-6 ACTIVATORS, INHIBITORS, AND OTHER ADDITIVES

Early work on many types of additives has been reviewed (57), showing that activation by adjustment of pH has not given consistent results and should not be necessary in a properly buffered extender. The addition of sodium bicarbonate to Caprogen in a breeding experiment with 9,741 cows (87) resulted in 68 percent 49-day NR compared with 67.1 percent for the controls ( $P > 0.05$ ). An analog of thiamine—thiamine propyl disulfide—is reported to penetrate the cell and 10 to 60 ppm added to yolk-citrate at 4°C markedly increased spermatozoa viability (188). Adenosine increased the motility of sperm stored at 4°C in 14G (Table 16-3) extender (189). At 37°C 6 millimoles caffeine stimulated and maintained motility of bull sperm cells without loss for four hours (190). Ascorbic acid is also reported to improve fertility periodically (191). "Implementors," such as posterior pituitary extract (mostly oxytocin) and a parasympathetic nerve stimulant (carbamylcholine), have been tested with the hope of aiding sperm transport. Limited experiments in Russia (91) tend to support their value but the results are often not repeatable (192).

The tranquilizer chlorpromazine, at 200 µg/ml, could replace sulfanilamide and improve sperm motility in egg yolk extender (CU-16), but it is toxic to spermatozoa in skim milk. In extensive field trials fertility was not affected when chlorpromazine was added to egg yolk extender (75). Promazine depressed sperm motility at levels as low as 20 µm/ml. Other tranquilizers have been studied (193) and incorporated into the Japanese commercial liquid semen extender, Neoseminan (95). Carbon dioxide and reduced pH also may inhibit and preserve spermatozoa (see Sections 16-2 and 16-4). At very high sperm dilutions cyanide, a highly toxic chemical that inhibits oxidation, improved sperm survival at 35°C in a fructose-containing medium (43).

Protective agents added include the chelators EDTA (ethylenediamine tetracetic acid) and glycine (89, 194, 195). EDTA also protects against the amino acid oxidase released by dead sperm (196), but when it is added to Caprogen (87) fertility is significantly lower (4.8 percent) than with catalase (7,419 inseminations). Catalase is included routinely in ambient temperature extenders to hasten the decomposition of  $H_2O_2$ . In one report catalase alone increased sperm survival but failed to increase fertility except when combined with 8 µg/ml of  $\alpha$ -amylase (197). According to several reports from New Zealand, however, catalase increased the nonreturn rate 1 to 2 percentage units as indicated by the following 49-day NR data based upon 669,639 inseminations:

Age of semen	Caprogen	Caprogen + catalase
Day collected	63.4%	64.6%
Day later	66.0%	68.1%

In another trial (196) catalase increased the nonreturn rate 3.1 percent on 8,154 services. Total sperm numbers per insemination dose in Caprogen plus catalase with 5 percent egg yolk could be reduced, without reducing fertility, from  $2.5$  to  $1.5 \times 10^6$ .

Catalase improves sperm survival at  $5^{\circ}\text{C}$  as well. The effect is most pronounced at the higher extensions of semen (45, 198, 199). Catalase is superior to peroxidase in protecting bull spermatozoa (199). With semen used for insemination at  $5^{\circ}\text{C}$  catalase (1 to  $20 \mu\text{g}/\text{ml}$ ) added to CUE failed to improve fertility (40, 85) although there was a suggestion of a beneficial effect on fertility after 4 days' storage (45). Catalase improved fertility in heated homogenized whole milk as indicated by the following data (80).

	<i>No catalase</i>		<i>1 <math>\mu\text{g}</math> catalase/ml</i>	
	<i>CUE</i>	<i>Milk</i>	<i>CUE</i>	<i>Milk</i>
No. inseminations	6,064	5,944	6,243	5,586
% 60-90-day NR	72.9	68.0	73.2	71.6

Hyaluronidase was thought to play a role in the sperm's ability to penetrate the egg, but hyaluronidase added to semen failed to give a consistent response (57). More recently  $\alpha$ - and  $\beta$ -amylase and  $\beta$ -glucuronidase have been shown to improve sperm motility (200, 201). Fertility tests with frozen semen are described in Chapter 17. In New Zealand tests (13,082 inseminations) with semen handled at ambient temperatures  $\alpha$ -amylase alone added to Caprogen gave 63.4 percent versus 64.6 percent 49-day NR for control semen (183). The addition of nicotinamide, which as a competitor of nucleosidase in plasma reduces nicotinamide adenine dinucleotide (NAD), significantly depressed fertility 1.9 percent (87).

Hormones have been added to semen in an attempt to influence possible sperm transport in the female, sperm capacitation in the cow, and ovulation time. When 200 IU of human chorionic gonadotrophin (HCG) were added to semen to influence ovulation time fertility results were poorer (202).

	<i>No. cows</i>	<i>% 60-90-day NR</i>
Milk	2,077	64.6
Milk + HCG	1,999	62.8

The administration of female sex hormones estrogen and progesterone to extended semen has also been ineffective in altering fertility (57, 203). In conclusion we can say that catalase is the only additive that has been tested thoroughly and used frequently, particularly when semen is stored at ambient temperatures.



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## 16-7 EXTENSION PROCEDURES AND RATES

Semen should be processed in a manner that will insure maximum fertility. The final extension rate of semen should be planned so that the number of sperm present in a package convenient for handling and insemination will be sufficient but will not waste sperm cells unnecessarily.

**16-7.1 Initial Semen Extension.** It is important to mix bull semen with a preserving medium relatively soon after semen collection in order to provide spermatozoa with necessary ingredients and to protect them in various ways (Section 16-2). Semen and extender should be combined at the same temperature, usually about 30°C. The possible importance of a short incubation period at this temperature with antibiotics added to insure adequate bacterial control is discussed in Chapter 17. For semen used at ambient temperature the final extension can be made at one time.

When semen is to be cooled to approximately 5°C it is convenient to cool the extended semen in relatively small volumes and then add more cold extender to achieve the final extension rate. This can be accomplished by mixing the neat semen with 2 to 4 times its volume of extender before cooling to 5°C and then adding cold extender. Semen cooled without the benefit of extender was 6.5 percent lower in fertility (55) than semen mixed before cooling, clearly indicating the need for protection against the cold. When sperm cells become cold shocked they exhibit a decrease in motility, reduction in metabolism, loss of key components, and morphological damage.

**16-7.2 Cooling Rates.** Cooling rate undoubtedly is important (see Chapter 17), but the optimum rate for most extenders has not been tested with regard to fertility. Rapid cooling is generally undesirable. McFee and Swanson (204) found that sperm motility was considerably improved during a 10-day storage period at 5°C when sperm in a yolk-citrate-fructose extender were cooled from 32 to 5°C in 140 minutes compared to 25 minutes. An extensive fertility test with homogenized, sterilized 9 percent cream (205) gave the following results:

<i>Cooling time from 30° to 4°C</i>	<i>No. cows</i>	<i>% 60-90-day NR</i>
120 minutes	9,163	64.8
40 minutes	8,659	63.0

The slower cooling was definitely preferable ( $P < 0.02$ ).

**16-7.3 Storage Temperature.** Unfrozen semen (except for that which is stored at ambient temperature) is almost always stored at 4 to 5°C. This temperature can be maintained fairly easily with ice and has been satisfactory for

semen used to impregnate millions of cows. It may not be the optimum holding temperature, however. Swanson et al. (206) found significantly better motility and fertility in a yolk-citrate-bicarbonate-fructose extender used to store semen at 10°C rather than at 4 to 5°C. When 1,338 cows were inseminated with semen stored for 3 to 4 days at 4 to 5°C and at 10°C the 60–90-day NR figures were 67.9 and 73.5 percent respectively. Storage at 10°C has not been used commercially, however, because it is difficult to maintain this temperature under practical conditions.

**16-7.4 Final Extension Rate and Sperm Numbers.** Semen for use at 5°C is usually extended to give the desired number of sperm cells for insemination in one milliliter. Although there is little difference in fertility when 0.5 to 2.0 milliliters of extended semen is inseminated (139) the volume used most often is 1.0 milliliter. Figure 16-1 shows a summary of work by Salisbury and co-workers and Willett and others prior to 1960. In the pioneer work by Salisbury (207) fertility sometimes appeared to increase as semen was extended at higher

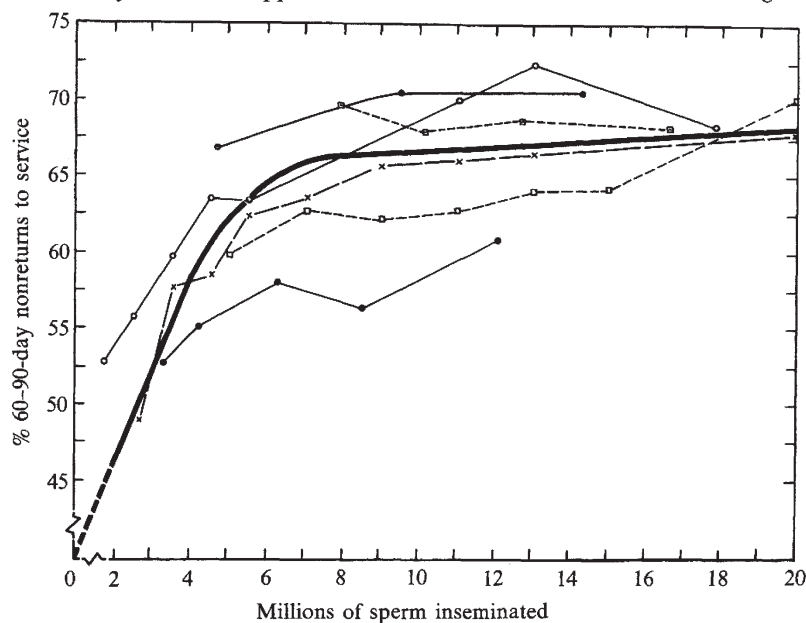
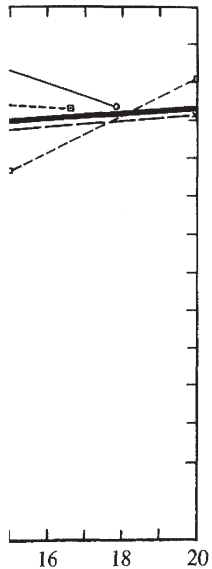


Figure 16-1. A summary of several of the investigations made to determine the minimum number of spermatozoa for optimum fertility in cattle. Most of the experiments were conducted with yolk-citrate as the diluent, with and without sulfanilamide and antibiotics. [From Salisbury and VanDemark. *Physiology of Reproduction and Artificial Insemination of Cattle*. 1st ed. W. H. Freeman and Company, San Francisco. 1961.]

the optimum holding temperature for use at 5°C. The data are based upon extending the spermatozoa at different rates regardless of initial sperm concentration. This gives a range of sperm concentrations per milliliter used for insemination. Willett and Larson (167) calculated that there was a 0.6 percent decline in nonreturn rate for each decrease of one million sperm inseminated as the numbers diminished from 12 to 4 million. Below 4 million the nonreturn rate declined 3.9 percent for each reduction of one million spermatozoa. In general, an extension rate of more than 100× was possible before too few sperm were present for maximum conception rates. Bratton et al. (208) took initial sperm cell concentration and motility into account and extended samples to 5, 10, and 15 million motile cells per milliliter. This resulted in 60–90-day NR figures of 66.7, 70.9, and 70.5 percent respectively. Therefore  $10 \times 10^6$  motile sperm were recommended for insemination.

Salisbury and co-workers extended at higher



Salisbury and co-workers extended at higher

Since 1960 the number of sperm per insemination necessary to maintain optimum fertility with liquid semen has decreased further. This has been made possible by the development of more balanced extending media. Results of several trials in this development are shown in Table 16-12. The trials at 5°C indicate that 4 million motile sperm per milliliter gave initial high fertility and that when the number per insemination was decreased to  $2 \times 10^6$  fertility changed little (109). A report by the British Milk Marketing Board showed that extremely high dilutions of semen from two bulls up to 1:3,000—giving 500,000 live sperm per milliliter—resulted in 32 and 46 percent conception. When sufficient sperm are inseminated increasing the number further does not improve the fertility of poor quality semen (210) or low-fertility bulls (15), nor does it compensate for the reduced fertility caused by poor technicians (87). (The latter effect was studied at ambient temperature.)

In ambient temperature studies (see Table 16-11)  $12.5$  to  $25 \times 10^6$  sperm per insemination originally were employed. This number was reduced drastically and fertility remained satisfactory. Reducing the proportion of egg yolk in extender from 20 percent to 5 percent facilitates extending to very low numbers of sperm. Shannon (138) observed that fertility declined 10.9 percentage units when the sperm dose in 20 percent yolk-Caprogen was reduced from  $2.5$  to  $0.5 \times 10^6$  sperm per insemination. The corresponding decline with 5 percent egg yolk was only 3.1 percentage units. This decline is probably due to a reduction in sperm numbers below the critical level, although high dilutions per se could be detrimental. Since the volume inseminated in New Zealand is 0.5 milliliter the actual semen dilution is one-half of what would be required if one milliliter were used. Nitrogen in the Caprogen extender also reduced the dilution effect (46).

The enormous impact that a single sire could have on the gene pool in a liquid semen program, if enough cows were available to inseminate with

Table 16-12. Fertility of unfrozen semen with different numbers of spermatozoa per insemination.

<i>Temperature and treatment</i>	<i>No. 1st services</i>	<i>% 60-90-day NR</i>	<i>Reference</i>
5°C, motile sperm			
CUE, $5 \times 10^6$ sperm	24,737	75.2	(5)
CUE, $10 \times 10^6$ sperm	18,933	76.1	
CUE, $4 \times 10^6$ sperm	11,065	72.7	(15)
CUE, $8 \times 10^6$ sperm	9,739	73.4	
Tris, $4 \times 10^6$ sperm	11,445	72.7	
Tris, $8 \times 10^6$ sperm	10,071	74.1	
Ambient; total sperm <sup>a</sup>			
Caprogen, $5 \times 10^6$	15,997	68.0	(196)
Caprogen, $3.75 \times 10^6$	8,574	69.3	
Caprogen, $2.5 \times 10^6$	8,922	67.0	
Caprogen, 3.75	14,110	63.6	(196)
Caprogen, 2.5	6,130	63.3	
Caprogen, 1.875	6,124	61.2 <sup>b</sup>	
Caprogen, 2.5	12,676	66.8	(87)
Caprogen, 0.5	4,114	59.5 <sup>c</sup>	

<sup>a</sup>Sperm concentration/ml is twice that given; 0.5 ml is used for insemination.

<sup>b</sup> $P < 0.05$ .

<sup>c</sup> $P < 0.01$  within experiments.

$0.5 \times 10^6$  total sperm, is obvious. About 30 billion or more sperm per week can be collected from most mature healthy Holstein sires. This number of spermatozoa is sufficient to inseminate 6,000 cows per week. In New Zealand  $2.0$  to  $2.5 \times 10^6$  total sperm are routinely used per insemination, except when frozen semen has been banked for later thawing and used as needed at ambient temperatures. In this case, the semen is thawed and reconstituted in Caprogen with 20 million sperm per insemination dose.

**16-7.5 Packaging Unfrozen Semen.** All semen should be packaged in opaque containers for transport and storage in order to prevent exposure to light (33). In moderate climates where ambient temperatures are used the semen is often wrapped in aluminum foil and placed in light-weight styrofoam containers. The containers should be closed and kept out of direct sunlight.

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For semen stored at 3 to 5°C a plastic bottle of frozen water included in a styrofoam or other insulated container will keep the cells at this temperature as long as there is ice in the water. The bottle of frozen water should be removed from the freezer, where it has been held several degrees below freezing, to allow it to warm up from freezer temperatures to 0°C before placing the ice and semen together in the container. It is safe to combine them as soon as the ice in the bottle starts to melt. If the ice bottle is not allowed to defrost it could be cold enough to freeze and damage the spermatozoa.

A similar danger is encountered in winter when the inseminating tubes may be chilled by exposure to below-freezing temperatures. Cold shock results when the diluted semen held at 5°C is moved from its container into inseminating equipment that has been chilled by exposure to freezing weather conditions.

If the temperature of the diluted semen rises during shipment, more ice or more insulation or both are needed. It is not always possible to add more ice to the package, in which case containers with better insulation are required, especially in hot weather.

**16-7.6 Warming Rate.** After extended semen has been stored at approximately 5°C the rate of warming the cells to body temperature is not critical. The temperature can be raised very rapidly from 5°C to body temperature without harming the cells.

#### 16-8 PROCESSING SEMEN FOR SEX CONTROL

Innumerable reports have appeared concerning alteration of the sex ratio (211, 212). Limited control appears to be possible, but no experimental data have been found to support claims by commercial concerns that they are able essentially to guarantee the sex of the calf produced.

#### REFERENCES

- (1) Anderson, J. *The Semen of Animals and Its Use for Artificial Insemination*. Tech. Comm. Imp. Bur. Animal Breeding and Genetics. Edinburgh. 151 pp. 1945.
- (2) Milovanov, J. K., and O. A. Selivanova. Diluters for sperm of livestock. (Trans. title). *Probl. Zivotn.* 2:75-86. 1932. Abstr. in *Animal Breeding Abstr.* 1:153-154. 1933.
- (3) Phillips, P. H. The preservation of bull semen. *J. Biol. Chem.* 130:415. 1939.
- (4) Salisbury, G. W., H. K. Fuller, and E. L. Willett. Preservation of bovine spermatozoa in yolk-citrate diluent and field results from its use. *J. Dairy Sci.* 24:905-910. 1941.



- (5) Salisbury, G. W. Recent developments with bull semen diluents. *Animal Breeding Abstr.* 25:111-123. 1957.
- (6) Almquist, J. O. The effect of certain coal-tar dyes used for semen identification on the livability and fertility of bull spermatozoa. *J. Dairy Sci.* 29:815-820. 1946.
- (7) Clamohoy, L. L., and R. H. Foote. Effects of dyes on survival of bull spermatozoa stored in yolk and milk extenders at 5 and  $-79^{\circ}\text{C}$ . *J. Dairy Sci.* 46:61-64. 1963.
- (8) Salisbury, G. W. Fertilizing ability and biological aspects of sperm storage *in vitro*. 6th Int. Cong. on Animal Reprod. Paris. II:1189-1204. 1968.
- (9) Salisbury, G. W., and F. H. Flerchinger. Aging phenomena in spermatozoa. I. Fertility and prenatal losses with use of liquid semen. *J. Dairy Sci.* 50:1675-1678. 1967.
- (10) Salisbury, G. W., and R. G. Hart. Gamete aging and its consequences. *Biol. Reprod. Supp.* 2:1-13. 1970.
- (11) Drevious, L. O., and H. Eriksson. Osmotic swelling of mammalian spermatozoa. *Expt. Cell Res.* 42:136-156. 1966.
- (12) Bredderman, P. J., and R. H. Foote. Volume of stressed bull spermatozoa and protoplasmic droplets, and the relationship of cell size to motility and fertility. *J. Animal Sci.* 28:496-501. 1969.
- (13) Salisbury, G. W. Ionic and osmotic conditions in relation to metabolic control. In *Spermatozoan Motility*. (D. W. Bishop, ed.) 59-87. AAAS Pub. No. 72. Washington, D.C. 1962.
- (14) Steinbach, J., and R. H. Foote. Osmotic pressure and pH effects on survival of frozen bovine spermatozoa. *J. Dairy Sci.* 50:205-213. 1967.
- (15) Foote, R. H. Fertility of bull semen at high extensive rates in tris-buffered extenders. *J. Dairy Sci.* 53:1475-1477. 1970.
- (16) Salisbury, G. W., C. B. Knodt, and R. W. Bratton. The freezing point depression of bull semen and its relation to the diluter problem. *J. Animal Sci.* 7:283-290. 1948.
- (17) Rothschild, Lord, and H. Barnes. Osmotic pressure of bull semen diluents. *Nature* (London) 173:636. 1954.
- (18) Davis, I. S., R. W. Bratton, and R. H. Foote. Livability of bovine spermatozoa at  $5^{\circ}\text{C}$  in tris-buffered and citrate-buffered yolk-glycerol extenders. *J. Dairy Sci.* 46:57-60. 1963.
- (19) Davis, I. S., R. W. Bratton, and R. H. Foote. Livability of bovine spermatozoa at 5,  $-25$  and  $-85^{\circ}\text{C}$  in tris-buffered and citrate-buffered yolk-glycerol extenders. *J. Dairy Sci.* 46:333-336. 1963.
- (20) Foote, R. H. Tris and other organic buffers for the conservation of semen of various species. In *Animal Reproduction and Artificial Insemination*. 99-105. Edagricole, Bologna. 1972.

diluents. *Animal Breed-*

for semen identification  
*J. Sci.* 29:815-820. 1946.

survival of bull sperma-  
*J. Dairy Sci.* 46:61-64.

ects of sperm storage in  
1204. 1968.

ena in spermatozoa. I.  
*J. Dairy Sci.* 50:1675-

its consequences. *Biol.*

mammalian spermatozoa.

d bull spermatozoa and  
to motility and fertility.

on to metabolic control.  
7. AAAS Pub. No. 72.

OH effects on survival of  
1967.

rates in tris-buffered ex-

reezing point depression  
*J. Animal Sci.* 7:283-290.

of bull semen diluents.

y of bovine spermatozoa  
erol extenders. *J. Dairy*

y of bovine spermatozoa  
ffered yolk-glycerol ex-

onservation of semen of  
*al Insemination.* 99-105.

- (21) Graham, E. F., B. G. Crabo, and K. I. Brown. Effect of some zwitter ion buffers on the freezing and storage of spermatozoa. I. *Bull. J. Dairy Sci.* 55:372-378. 1972.
- (22) Jones, R. C., and R. H. Foote. Effect of osmolality and phosphate, 'Tris,' 'Tes,' 'Mes,' and 'Hepes' hydrogen ion buffers on the motility of bull spermatozoa stored at 37 or 5°C. *Aust. J. Biol. Sci.* 25:1047-1055. 1972.
- (23) Foote, R. H. Influence of pH on survival and fertility of bull sperm. *J. Dairy Sci.* 47:807-811. 1964.
- (24) VanDemark, N. L., and U. D. Sharma. Preliminary fertility results from the preservation of bovine semen at room temperatures. *J. Dairy Sci.* 40:438-439. 1957.
- (25) Bartlett, F. D., Jr., and N. L. VanDemark. Effect of diluent composition on survival and fertility of bovine spermatozoa stored in carbonated diluents. *J. Dairy Sci.* 45:360-367. 1962.
- (26) VanDemark, N. L., K. Koyama, and J. R. Lodge. Factors affecting immobilization of bovine spermatozoa with CO<sub>2</sub> and their subsequent reactivation. *J. Dairy Sci.* 48:586-591. 1965.
- (27) Salisbury, G. W., and U. D. Sharma. Effect of surface area-to-volume ratios in storage tubes on oxygenation of diluted bull semen. *J. Dairy Sci.* 40:677-681. 1957.
- (28) VanDemark, N. L., and F. D. Bartlett, Jr. Factors affecting the maintenance of CO<sub>2</sub> levels and the survival of bovine spermatozoa in carbonated diluents. *J. Dairy Sci.* 45:375-382. 1962.
- (29) Lodge, J. R., C. N. Graves, and G. W. Salisbury. Carbon dioxide in prevention of the deleterious dilution effect on metabolism and motility of mammalian spermatozoa. *Proc. Soc. Expt. Biol. Med.* 113:824-827. 1963.
- (30) Foote, R. H. Factors prolonging survival of unfrozen bovine spermatozoa. *J. Dairy Sci.* 50:1338-1340. 1967.
- (31) Foote, R. H. Influence of light and agitation on bovine spermatozoa stored with protective agents. *J. Dairy Sci.* 50:1468-1474. 1967.
- (32) Norman, C., E. Goldberg, and I. D. Porterfield. The effect of visible radiation on the functional life-span of mammalian and avian spermatozoa. *Expt. Cell Res.* 28:69-84. 1962.
- (33) Paufler, S. K., and R. H. Foote. Influence of light on nuclear size and deoxyribonucleic acid content of stored bovine spermatozoa. *J. Dairy Sci.* 50:1475-1480. 1967.
- (34) Almquist, J. O., P. W. Prince, and J. J. Reid. Bacteriological studies of bovine semen. I. Numbers of bacteria and the relation to fertility. *J. Dairy Sci.* 32:543-548. 1949.

- (35) Foote, R. H., and R. W. Bratton. Motility of bovine spermatozoa and control of bacteria at 5 and 25°C in extenders containing sulfanilamide, penicillin, streptomycin and polymyxin. *J. Dairy Sci.* 33:842-846. 1950.
- (36) French, G. R., C. Norman, R. S. Dunbar, Jr., and I. D. Porterfield. The effects of antibiotics on *Vibrio fetus* and the metabolism of bovine spermatozoa. West Virginia Univ. Agr. Expt. Sta. Bull. 471T. 18 pp. 1962.
- (37) Foote, R. H. Water: The chief ingredient in semen extenders. *A.I. Digest.* 12(3):10. 1964.
- (38) Sherman, J. K. Questionable protection by intracellular glycerol during freezing and thawing. *J. Cell and Comp. Physiol.* 61:67-84. 1963.
- (39) Berndtson, W. E., and R. H. Foote. The freezability of spermatozoa after minimal pre-freezing exposure to glycerol or lactose. *Cryobiology* 9:57-60. 1972.
- (40) Shannon, P., and B. Curson. Toxic effect and action of dead sperm on diluted bovine semen. *J. Dairy Sci.* 55:614-620. 1972.
- (41) Mann, T. *Biochemistry of Semen and of the Male Reproductive Tract.* 493 pp. Methuen and Co. Ltd., London. 1964.
- (42) Rottensten, K., H. H. Koefoed-Johnsen, and H. Andersen. The effect of spermatozoal extracts on the viability of bovine spermatozoa. *Acta Agric. Scand.* 10:280-288. 1960.
- (43) Bredderman, P. J., and R. H. Foote. Factors stabilizing bull sperm cell volume and prolonging motility at high dilution. *Expt. Cell Res.* 66:458-464. 1971.
- (44) Mattner, P. E. The survival of spermatozoa in bovine cervical mucus and mucus fractions. *J. Reprod. Fert.* 20:193-199. 1969.
- (45) Foote, R. H., and H. O. Dunn. Motility and fertility of bull semen extended at high rates in yolk extender containing catalase. *J. Dairy Sci.* 45:1237-1241. 1962.
- (46) Shannon, P. Contribution of seminal plasma, sperm numbers, and gas phase to dilution effects of bovine spermatozoa. *J. Dairy Sci.* 48:1357-1361. 1965.
- (47) Abdu, M. S. S. The effect of EDTA and caproic acid on bull spermatozoa exposed to cold shock. *Dokl. vses. Akad. sel'-khoz. Nauk. V.I. Lenina* 6:32-34. 1968. Abstr. in *Animal Breeding Abstr.* 38:417. 1970.
- (48) Rottensten, K., and H. Andersen. The influence of the cooling rate on the viability of the semen. (Danish; English summary.) Royal Vet. and Agr. College Ster. Res. Institut., Copenhagen. Ann. Report. pp. 19-23. 1970.
- (49) Mayer, D. T., and J. F. Lasley. The factor in egg yolk affecting resistance, storage potentialities and fertilizing capacity of mammalian spermatozoa. *J. Animal Sci.* 4:261-269. 1945.
- (50) Blackshaw, A. W., and G. W. Salisbury. Factors influencing metabolic activity of bull spermatozoa. II. Cold-shock and its prevention. *J. Dairy Sci.* 40:1099-1106. 1957.

spermatozoa and control of  
nide, penicillin, strep-

Porterfield. The effects  
ine spermatozoa. West

extenders. *A.I. Digest*.

lycerol during freezing

spermatozoa after min-  
*biology* 9:57-60. 1972.

dead sperm on diluted

oductive Tract. 493 pp.

sen. The effect of sper-  
oa. *Acta Agric. Scand.*

bull sperm cell volume  
s. 66:458-464. 1971.

vical mucus and mucus

bull semen extended at  
*Sci.* 45:1237-1241. 1962.

numbers, and gas phase to  
:1357-1361. 1965.

on bull spermatozoa ex-  
t. *V.I. Lenina* 6:32-34.

he cooling rate on the  
al Vet. and Agr. College  
. 1970.

ecting resistance, storage  
rmatozoa. *J. Animal Sci.*

ncing metabolic activity  
u. *J. Dairy Sci.* 40:1099-

- (51) Choong, C. H., and R. G. Wales. The effect of cold shock on spermatozoa. *Austr. J. Biol. Sci.* 15:543-551. 1962.
- (52) Lanz, R. N., B. W. Pickett, and R. J. Komarek. Effect of lipid additives on pre- and post-freeze survival of bovine spermatozoa. *J. Dairy Sci.* 48:1692-1697. 1965.
- (53) O'Shea, T., and R. G. Wales. Effect of casein, lecithin, glycerol, and storage at 5°C on diluted ram and bull semen. *Aust. J. Biol. Sci.* 19:871-882. 1966.
- (54) Quinn, P. J., and I. G. White. The effect of cold shock and deep-freezing on the concentration of major cations in spermatozoa. *J. Reprod. Fert.* 12:263-270. 1966.
- (55) Foote, R. H., and R. W. Bratton. The fertility of bovine semen cooled with and without the addition of citrate-sulfanilamide-yolk extender. *J. Dairy Sci.* 32:856-861. 1949.
- (56) Quinn, P. J., S. Salamon, and I. G. White. Effect of osmotic pressure and temperature gradients on cold shock in ram and bull spermatozoa. *Aust. J. Biol. Sci.* 21:133-140. 1968.
- (57) Maule, J. P. (ed.). *The Semen of Animals and Artificial Insemination*. Tech. Comm. 15, Commonwealth Agr. Bureaux. Farnham Royal, England. 420 pp. 1962.
- (58) Salisbury, G. W., and W. H. Ruffener. Recent research on bovine semen extenders. In *Animal Reproduction and Artificial Insemination*. 287-298. Edagricole, Bologna. 1972.
- (59) Willett, E. L., H. K. Fuller, and G. W. Salisbury. Preservation of bovine spermatozoa in yolk phosphate diluent and field results from its use. *Cornell Vet.* 30:507-513. 1940.
- (60) Bratton, R. W., R. H. Foote, S. D. Musgrave, and N. L. VanDemark. Livability and fertility of bovine spermatozoa in different diluents. *J. Dairy Sci.* 32:604-608. 1949.
- (61) Melrose, D. R., and D. L. Stewart. The effect on fertility of changes in the composition of the normal egg-yolk citrate semen diluent. *Brit. Vet. J.* 112:536-540. 1956.
- (62) Salisbury, G. W., and C. B. Knodt. The effect of sulfanilamide in the diluent upon the fertility of bull semen. *J. Dairy Sci.* 30:361-369. 1947.
- (63) Van Dieten, S. W. J. Comparison of a citrate diluent with a phosphate diluent for bull semen. (Trans. title) *Tijdschr. Diergeneesk.* 82:194-199. 1957.
- (64) Kok, J. C. N. Inquiry into bull semen diluents used for artificial insemination of cattle in the Netherlands. (Trans. title). *Tijdschr. Diergeneesk.* 82:187-193. 1957.
- (65) Rottensten, K. Insemination results with different semen extenders. Ann. Rpt. Royal Vet. and Agric. College, Steril. Res. Inst. Copenhagen. 1961.

- (66) Foote, R. H., and R. W. Bratton. Survival of bovine spermatozoa stored at 5 and 25°C in extenders containing varying levels of egg yolk, glucose, glycine, glycerol, citrate and other salts. *J. Dairy Sci.* 43:1322-1329. 1960.
- (67) Campbell, R. C., and J. Edwards. The effect on conception rates of semen diluents containing citrate or phosphate buffer, with all combinations of sulfanilamide, streptomycin and penicillin. *J. Agr. Sci.* 46:44-55. 1955.
- (68) Ohms, J. I., and E. L. Willett. Fertility of bull spermatozoa in yolk-citrate partially replaced by glucose. *J. Dairy Sci.* 41:1800-1802. 1958.
- (69) Stewart, D. L., D. R. Melrose, and W. R. Wilson. The effect of reducing the quantity of egg yolk in bovine semen diluents. *Vet. Rec.* 62:617-618. 1950.
- (70) Almquist, J. O. The fertility of bovine semen in diluters containing varying amounts of egg yolk. *J. Dairy Sci.* 34:763-766. 1951.
- (71) Olds, D., L. Oliver, D. M. Seath, and M. C. Carpenter. The fertility of bull semen extended with yolk-citrate containing varying proportions of egg yolk. *J. Dairy Sci.* 34:1081-1083. 1951.
- (72) Holt, A. F. Variation in diluter composition and its effect on the fertility of bovine semen. *Vet. Rec.* 64:818. 1952.
- (73) Bratton, R. W., and R. H. Foote. Milk and yolk extenders for bovine semen. Unpublished data. Cornell University. 1953.
- (74) Foote, R. H., L. C. Gray, D. C. Young, and H. O. Dunn. Fertility of bull semen stored up to four days at 5°C in 20% egg yolk extenders. *J. Dairy Sci.* 43:1330-1334. 1960.
- (75) Foote, R. H., and L. C. Gray. Effect of promazine hydrochloride and chlorpromazine hydrochloride on the motility and fertility of bovine semen. *J. Dairy Sci.* 43:1499-1504. 1960.
- (76) Roy, A., and M. W. H. Bishop. Effect of glycine on the survival of bull spermatozoa *in vitro*. *Nature* (London) 174:746. 1954.
- (77) Shannon, P. The effect of diluents containing glycine, and glycine and glycerol, on the fertility of diluted bovine semen. *New Zealand J. Agric. Res.* 7:357-363. 1964.
- (78) Hayden, J. S., H. P. Peterson, and O. T. Stallcup. Fertility results from the use of bovine semen in a carbon dioxide extender. *J. Dairy Sci.* 43:1016-1017. 1960.
- (79) Melrose, D. R. Fertility of bull semen stored in milk-yolk, CUE, milk-glycerol and I.V.T. diluents. 4th Int. Cong. on Animal Reprod. The Hague. 4:914-918. 1961.
- (80) Singleton, E. F., and M. J. Josey. Comparison of storage motility and fertility of chilled bovine semen extended in different diluents. *Queensland J. Agric. Anim. Sci.* 26:659-664. 1969.
- (81) Hafs, H. D. Fertility of bull sperm with added catalase. *J. Dairy Sci.* 44:1529-1536. 1961.



matozoa stored at 5 and  
yolk, glucose, glycine,  
1329. 1960.

ception rates of semen  
h all combinations of  
46:44-55. 1955.

matozoa in yolk-citrate  
02. 1958.

e effect of reducing the  
c. 62:617-618. 1950.

iters containing varying

ter. The fertility of bull  
proportions of egg yolk.

effect on the fertility of

nders for bovine semen.

n. Fertility of bull semen  
rs. *J. Dairy Sci.* 43:1330-

hydrochloride and chlor-  
f bovine semen. *J. Dairy*

he survival of bull sper-

and glycine and glycerol,  
*J. Agric. Res.* 7:357-363.

tility results from the use  
*J. Sci.* 43:1016-1017. 1960.

yolk, CUE, milk-glycerol  
l. The Hague. 4:914-918.

rage motility and fertility  
its. *Queensland J. Agric.*

se. *J. Dairy Sci.* 44:1529-

- (82) Pickett, B. W., L. F. Korzun, and R. C. Martig. Comparison between milk and CUE as extenders for bull semen. *Conn. (Storrs) Agr. Expt. Sta. Bull.* 373. 1962.
- (83) Merkt, H., D. Grove, and O. Richter. Experience with Cornell University Extender. (Trans. title). *Zuchthyg.* 3:107-116. 1968.
- (84) Milk Marketing Board. Cornell University Extender (CUE) *MMB Rpt.* 12:114. 1961-62.
- (85) Paufler, S. Forward motility and fertility of bull semen diluted with Spermasol T, CUE and CUE-Tris. (Trans. title). *Zuchthyg.* 2:67-70. 1967.
- (86) Paufler, S., M. Schrodler, and R. Zappe. The fertilizing properties of a modified CUE diluent (Göttinger Verdünner). (Trans. title. German; English summary.) *Zuchthyg.* 5:73-78. 1970.
- (87) New Zealand Dairy Board. 48th Farm Prod. Rpt. 1971-1972.
- (88) New Zealand Dairy Board. 38th Farm Prod. Rpt. 1961-1962.
- (89) Balasov, N. G., N. Z. Zilijcov, M. E. Evsjukov, and J. S. Pilipavicjute. Comparisons of diluents used for preservation of cooled bull semen. *Veterinariya* 45:81-84. 1969. Abstr. in *Animal Breeding Abstr.* 37:611. 1969.
- (90) Graham, E. F., and W. E. Erickson. Unpublished results. Observations on a field study using glycine. Minnesota Agr. Expt. Sta. 1956. Used by permission.
- (91) Turton, J. D. Recent research in cattle breeding and production in the U.S.S.R.—A review. *Animal Breeding Abstr.* 37:347-391. 1969.
- (92) Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Singh. Hydrogen ion buffers for biological research. *Biochem.* 5:467-477. 1966.
- (93) Yassen, A. M. Buffalo sperm motility stored at 5°C in tris-buffered extenders. *Alexandria J. Agric. Res.* 18:147-152. 1970.
- (94) Dunn, H. O., R. W. Bratton, and C. R. Henderson. Fertility of bovine spermatozoa in buffered whole egg extenders containing penicillin, streptomycin, sulfonamides and added glucose. *J. Dairy Sci.* 36:524-530. 1953.
- (95) Nishikawa, Y. History and development of artificial insemination in the world. *Proc. 5th Int. Congress Animal Reprod., Trento.* 7:162-259. 1964.
- (96) Phillips, P. H., and H. A. Lardy. A yolk-buffer pabulum for the preservation of bull semen. *J. Dairy Sci.* 23:399-404. 1940.
- (97) Underbjerg, G. K. L., H. P. Davis, and R. E. Spangler. Effect of diluters and storage upon fecundity of bovine semen. *J. Animal Sci.* 1:149-154. 1942.
- (98) Michajilov, N. N. Sperm dilution in the milk. *Czechoslovak Vet. Mag.* Jan. 10, 1950. Abstr. in *J. Am. Vet. Med. Assoc.* 117:337. 1950.
- (99) Jacquet, J. Some biological media for the preservation of bull semen. (Trans. title). *Bull. Acad. Vet. Fr.* 24:429-432. 1951.
- (100) Jacquet, J., and R. Cassou. New work with skim milk as a basis for the dilution and preservation of bull semen. (Trans. title). *Bull. Acad. Vet. Fr.* 25:149-155. 1952.

- (101) Thacker, D. L., and J. O. Almquist. Diluters for bovine semen. I. Fertility and motility of bovine spermatozoa in boiled milk. *J. Dairy Sci.* 36:173-180. 1953.
- (102) Flipse, R. J., S. Patton, and J. O. Almquist. Diluters for bovine semen. III. Effect of lactenin and lactoperoxidase upon spermatozoan livability. *J. Dairy Sci.* 37:1205-1211. 1954.
- (103) Jones, R. C. Survival of bull and ram spermatozoa in preparations from skim-milk. *J. Dairy Sci.* 51:1288-1294. 1968.
- (104) Jones, R. C., and R. H. Foote. Nondialyzable skimmilk in diluents for ram and bull semen. *J. Dairy Sci.* 55:856-861. 1972.
- (105) Pickett, B. W., and W. A. Cowan. Technical notes. Inhibition of the spermicidal factor(s) in unheated skimmilk by the addition of egg yolk. *J. Dairy Sci.* 44:1143-1145. 1961.
- (106) Saacke, R. G., J. O. Almquist, and S. Patton. The effect of heating fortified skim-milks upon the livability of bovine spermatozoa. *J. Dairy Sci.* 38:1046-1047. 1955.
- (107) Saacke, R. G., J. O. Almquist, and R. J. Flipse. Diluters for bovine semen. VII. Effects of time and temperature of heating skim milk upon the livability of bovine spermatozoa. *J. Dairy Sci.* 39:90-96. 1956.
- (108) Almquist, J. O., R. J. Flipse, and D. L. Thacker. Diluters for bovine semen. IV. Fertility of bovine spermatozoa in heated homogenized milk and skim milk. *J. Dairy Sci.* 37:1303-1307. 1954.
- (109) Almquist, J. O. Diluters for bovine semen. V. A comparison of heated milk and egg yolk-citrate as diluters for semen from bulls of high and low fertility. *J. Dairy Sci.* 37:1308-1315. 1954.
- (110) Perkins, J. R., M. C. Carpenter, and D. M. Seath. A comparison of the fertility of bull semen diluted in egg yolk-citrate and homogenized milk. *J. Dairy Sci.* 38:155-158. 1955.
- (111) Kerruish, B. M. A field trial comparison of milk and egg-yolk citrate as semen diluters. 3rd Intern. Congr. Animal Reprod. Section 3. pp. 65-68. Cambridge. 1956.
- (112) Milk Marketing Board. Final results and observations on the use of milk as dilutor. *MMB Rpt.* 7:102-104. 1956-1957.
- (113) Milk Marketing Board. Report of use of reconstituted skim milk powder as a semen diluent. *MMB Rpt.* 9:86-87. 1958-1959.
- (114) Melrose, D. R., D. L. Stewart, and W. Bruce. Comparative fertility studies of bovine semen diluents containing powdered skim milk, fresh skim milk, glycine and egg yolk. *Vet. Rec.* 70:433-438. 1958.
- (115) Adler, H. C., and N. O. Rasbech. Investigations on skim milk and cream as semen diluents. (Trans. title). *Nord. Vet. Med.* 8:497-503. 1956.
- (116) Ehlers, M. H., and R. E. Erb. Influence of added fructose on fertility of bull sperm. *A.I. Digest.* 7(2):9. 1959.

semen. I. Fertility and  
y Sci. 36:173-180. 1953.  
bovine semen. III. Effect  
livability. J. Dairy Sci.

preparations from skim-

in diluents for ram and

bition of the spermicidal  
lk. J. Dairy Sci. 44:1143-

of heating fortified skim-  
Sci. 38:1046-1047. 1955.

s for bovine semen. VII.  
k upon the livability of

ers for bovine semen. IV.  
ed milk and skim milk.

urison of heated milk and  
and low fertility. J. Dairy

omparison of the fertility  
nized milk. J. Dairy Sci.

egg-yolk citrate as semen  
3. pp. 65-68. Cambridge.

as on the use of milk as

d skim milk powder as a

arative fertility studies of  
t, fresh skim milk, glycine

skim milk and cream as  
-503. 1956.

uctose on fertility of bull

- (117) O'Connor, L. K., and G. F. Smith. The comparative fertility of bovine semen of four ages diluted with skim milk and skim milk + glycerol. *J. Agric. Sci.* 53:354-357. 1959.
- (118) Almquist, J. O. Diluents for bovine semen. XI. Effect of glycerol on fertility and motility of spermatozoa in homogenized milk and skimmilk. *J. Dairy Sci.* 45:911-916. 1962.
- (119) Morgan, W. J. B., D. R. Melrose, and D. L. Stewart. The effect of streptomycin on the survival of *Vibrio fetus* in semen and other diluents stored at 5° and -79°C. *J. Comp. Path.* 69:257-267. 1959.
- (120) Brown, M. A., I. W. Rupel, C. E. Coppock, and N. Pati. Reconstituted buttermilk as a diluent for bovine semen. *A.I. Digest* 8(1):8-9. 1960.
- (121) Williams, J. A., R. W. Green, and F. Dombroske. Fertility of bull semen extended with glycerinated, heated, homogenized milk. *J. Dairy Sci.* 40:621. 1957.
- (122) Almquist, J. O., and E. W. Wickersham. Diluents for bovine semen. XII. Fertility and motility of spermatozoa in skimmilk with various levels of glycerol and methods of glycerolization. *J. Dairy Sci.* 45:782-787. 1962.
- (123) Salisbury, G. W., and N. L. VanDemark. *Physiology of Reproduction and Artificial Insemination of Cattle*. 1st ed. 639 pp. W. H. Freeman & Company. San Francisco. 1961.
- (124) Ehlers, M. H., and L. E. Mikota. Yolk-glycine in combination with milk and milk-glycerol as extenders for bull semen. *A.I. Digest.* 8(11):8-9. 1961.
- (125) Pickett, B. W., W. A. Cowan, A. K. Fowler, and D. G. Gosslee. A comparison of motility and fertility of bull semen extended in egg yolk-citrate-glycerol and skimmilk-egg yolk-glycerol. *A.I. Digest* 8(2):6-7. 1960.
- (126) Stower, J., and H. J. Cembrowicz. The influence of glycerol on the fertility of bovine semen diluted in skim milk-egg yolk buffer. Proc. 4th Int. Cong. on Animal Reprod. The Hague. 4:919-924. 1961.
- (127) Courot, M., R. Ortavant, and E. Richeme. A trial of the American diluent CU-16 compared with a milk-based diluent for improvement of the results of artificial insemination in bovines. (Trans. title). *Élevage et Insém.* 77:13-15. 1963.
- (128) Stewart, D. L. The effect on fertility of the addition of glycerol to skim milk egg yolk diluents for bovine semen. 5th Int. Cong. on Animal Reprod. and AI. Trento. IV:631-635. 1964.
- (129) Hutchinson, R. W., and R. J. Cooper. Field trials with glycerolized bull semen. *Vet. Rec.* 73:857-858. 1961.
- (130) Schrod, M. Spermasol-milk, a new diluent. (Trans. title). *Tierarztl. Umsch.* 16:347-349. 1961.
- (131) Jenichen, W. Experiments on preserving bull semen with the dried milk diluent "Laiciphos" at a storage temperature of 2 to 4°C. (Trans. title). *Arch. Tierz.* 7:191-211. 1964. Abstr. in *Animal Breeding Abstr.* 33:70. 1965.

- (132) Milk Marketing Board. A survey of diluents in use in the A.I. service. *MMB Rpt.* 16:119-123. 1965-1966.
- (133) Joshi, S. C., J. S. Rawat, and A. Roy. Preservation of spermatozoal viability and diluents made up of amino acids with milk and/or egg yolk base and study of biophysical and chemical characters of these diluents. *Indian J. Vet. Sci.* 38:243-252. 1968.
- (134) Yassen, A. M., and M. A. El-Kamash. Storagibility of buffalo bull sperm in skim-milk extenders. *Alexandria J. Agr. Res.* 18:7-12. 1970.
- (135) Nour El-din, A. A., M. Y. Abboud, A. A. Younes, and M. A. Omar. A diluent for preservation of buffalo semen. 7th Int. Cong. on Animal Reprod. and AI. Munich. II:1517-1520. 1972.
- (136) Boye, K. Conservation of bull semen at room temperature, with particular consideration of temperature, longevity and fertility. (Trans. title). Vet. Med. Dissertation. Tierarztl. Hochsch., Hanover. 48 pp. 1968.
- (137) Abdu, M. S. S. Conception rate of cows and heifers to semen preserved without refrigeration in a medium with EDTA (Trans. title). *Zhivotnovodstvo, Mosk.* 30:78. 1968. Abstr. in *Animal Breeding Abstr.* 36:593. 1968.
- (138) Shannon, P. The effect of egg yolk level and dose rate on conception rate of semen diluted in caprogen. 7th Int. Cong. on Animal Reprod. and AI. Munich. II:1441-1442. 1972.
- (139) Olds, D., D. M. Seath, M. C. Carpenter, and H. L. Lucas. Interrelationships between site of deposition, dosage, and number of spermatozoa in diluted semen and fertility of dairy cows inseminated artificially. *J. Dairy Sci.* 36:1031-1035. 1953.
- (140) Shannon, P. The diatube method of freezing semen for use as rediluted deep freeze. 7th Int. Cong. on Animal Reprod. and AI. Munich. II:1363-1367. 1972.
- (141) Norman, C., C. E. Johnson, I. D. Porterfield, E. Goldberg, R. S. Dunbar, Jr., and H. S. Min. Survival and fertility of bovine sperm kept at variable temperatures in coconut milk extender. *J. Agric. Sci.* 59:33-39. 1962.
- (142) Norman, C. Further studies on the preservation of mammalian sperm at variable temperatures. 5th Int. Cong. on Animal Reprod. and AI. Trento. IV:269-275. 1964.
- (143) Malmberg, G., and R. Israelsson. A comparison of breeding efficiency of semen preserved at room temperature and deep frozen semen. 7th Int. Cong. on Animal Reprod. and AI. Munich. II:1383-1387. 1972.
- (144) Norman, C., and A. S. P. Rao. Comparison of motility and fertilizing capacity of buffalo and cattle sperm preserved at ambient temperatures in coconut milk extender (CME). 7th Int. Cong. on Animal Reprod. and AI. Munich. II:1311-1315. 1972.

ae A.I. service. *MMB Rpt.*

permatozoal viability and  
egg yolk base and study of  
*Indian J. Vet. Sci.* 38:243-

buffalo bull sperm in skim-  
milk.

ed M. A. Omar. A diluent  
for Animal Reprod. and AI.

ature, with particular con-  
sistency. title). *Vet. Med. Dissem.*

semen preserved without  
egg yolk. *Zhivotnovodstvo, Mosk.*  
1968.

ate on conception rate of  
AI. Munich.

Lucas. Interrelationships  
of spermatozoa in diluted semen  
*Dairy Sci.* 36:1031-1035.

for use as rediluted deep  
freeze. *Univ. Bull.* II:1363-1367. 1972.

Edberg, R. S. Dunbar, Jr.,  
kept at variable tempera-  
ture. 1962.

mammalian sperm at vari-  
ous temperatures. *AI. Trento.* IV:269-

eeding efficiency of semen  
at 7th Int. Cong. on Animal

ity and fertilizing capacity  
at various temperatures in coconut milk  
and AI. Munich. II:1311-

- (145) Grove, D. Bull sperm diluent containing palm sap. (Trans. title). *Zuchthyg.* 4:22-27. 1969.
- (146) Foote, R. H., R. W. Bratton, C. R. Henderson, E. M. Shantz, and J. K. Pollard. Survival of bovine spermatozoa at room temperature in citrate and Cornell University and tris extenders containing whole and fractionated coconut milk. *J. Dairy Sci.* 45:1383-1391. 1962.
- (147) Moyer, R. F., and J. O. Almquist. Diluents for bovine semen. X. Livability of spermatozoa stored at ambient room temperatures in diluents containing skim-milk. *J. Dairy Sci.* 45:383-389. 1962.
- (148) Bartlett, D. E. The AVMA/NAAB Code and the USLSA Recommended Regulations. Proc. 2nd Tech. Conf. AI. pp. 37-43. NAAB. 1968.
- (149) Bartlett, D. E. A responsible health program for AI. Proc. 4th Tech. Conf. AI. pp. 49-53. NAAB. 1972.
- (150) Roberts, S. J. *Veterinary Obstetrics and Genital Diseases*. 2nd ed. Pub. by the author, Ithaca, N.Y. 1971.
- (151) MacPherson, J. W. The protection of semen from contamination. *Animal Reproduction and Artificial Insemination*. 153-157. Edagricole, Bologna. 1972.
- (152) Gunsalus, I. C., G. W. Salisbury, and E. L. Willett. The bacteriology of bull semen. *J. Dairy Sci.* 24:911-919. 1941.
- (153) Edmondson, J. E., K. L. Tallman, and H. A. Herman. A study of bacteria in bovine semen and their effect on livability of spermatozoa. *Missouri Agr. Expt. Sta. Bull.* 444. 1949.
- (154) Foote, R. H., and R. W. Bratton. Motility of spermatozoa and control of bacteria in bovine semen extenders containing sulfanilamide, polymyxin and aureomycin. *J. Dairy Sci.* 33:539-543. 1950.
- (155) Wu, S. H., P. R. Elliker, and F. F. McKenzie. Microorganisms in dairy bull semen as related to fertility. *Oregon Agr. Expt. Sta. Tech. Bull.* 24. 1952.
- (156) ———. The effect of *Escherichia coli* on the semen of bulls, rams and rabbits. *Oregon Agr. Expt. Sta. Tech. Bull.* 27. 1953.
- (157) Marinov, P., P. Hummel, and H. Böhnelt. Observations on the microbial content of bull semen and vitality of bovine spermatozoa in presence of certain microorganisms. 7th Int. Cong. on Animal Reprod. and AI. Munich. II:1569-1575. 1972.
- (158) Bonadonna, T., G. Redaelli, J. Dumitrescu, M. G. Nesti, B. N. Verma, and R. Cesarini. Studies on the bacterial and mold content of bull semen as affected by hygienic techniques at collection. 6th Int. Cong. on Animal Reprod. and AI. Paris. 2:1005-1008. 1968.
- (159) Getty, S. M., and D. J. Ellis. The experimental use of bull semen contaminated with *Pseudomonas aeruginosa* organisms. *J. Am. Vet. Med. Assoc.* 151:1688-1691. 1967.



- (160) Alford, J. A. The occurrence of bacteria resistant to penicillin, streptomycin, and sulfanilamide in diluted bull semen. *J. Dairy Sci.* 36:1097-1103. 1953.
- (161) Foote, R. H., and G. W. Salisbury. The effect of pyridium, penicillin, furacin and phenoxethol upon the livability of spermatozoa and upon the control of bacteria in diluted bull semen. *J. Dairy Sci.* 31:763-768. 1948.
- (162) Foote, R. H., and G. W. Salisbury. The effect of sulfonamides upon the livability of spermatozoa and upon the control of bacteria in diluted bull semen. *J. Dairy Sci.* 31:769-778. 1948.
- (163) Dunn, H. O., G. L. Larson, and E. L. Willett. The effects of freezing bovine spermatozoa in extenders containing antibacterial agents. *J. Dairy Sci.* 36:728-732. 1953.
- (164) Almquist, J. O. The effect of penicillin upon the fertility of semen from relatively infertile bulls. *J. Dairy Sci.* 31:681-682. 1948. *J. Dairy Sci.* 32:950-954. 1949.
- (165) Foote, R. H., and R. W. Bratton. The fertility of bovine semen in extenders containing sulfanilamide, penicillin, streptomycin and polymyxin. *J. Dairy Sci.* 33:544-547. 1950.
- (166) Almquist, J. O. A comparison of penicillin, streptomycin and sulfanilamide for improving the fertility of semen from bulls of low fertility. *J. Dairy Sci.* 34:819-822. 1951.
- (167) Willett, E. L., and G. L. Larson. Fertility of bull semen as influenced by dilution level, antibiotics, spermatozoan numbers and the interaction of these factors. *J. Dairy Sci.* 35:899-905. 1952.
- (168) Willett, E. L., and J. I. Ohms. Field trials with semen containing several combinations of antibacterial agents. *J. Dairy Sci.* 38:1360-1368. 1955.
- (169) Erb, R. E., L. E. Milota, F. H. Flerchinger, and M. H. Ehlers. Influence of antibiotics on fertility of bull semen diluted with milk. *J. Animal Sci.* 14:731-738. 1955.
- (170) Easterbrooks, H. L., P. Heller, W. N. Plastringer, and E. L. Jungherr. Comparative fertility of diluted semen treated with calcium chloride complex streptomycin or dihydrostreptomycin sulfate. *J. Dairy Sci.* 33:851-852. 1950.
- (171) Sykes, J. G., and J. P. Mixner. Toxicity to bull spermatozoa of various salts, brands and lots of penicillin, streptomycin, aureomycin and chloromycetin. *J. Dairy Sci.* 34:342-346. 1951.
- (172) Adler, H. C., and N. O. Rasbech. Investigations into the effect of antibiotics on fertility of bull semen with special reference to the employment for semen of *Vibrio fetus* transmitting bulls. 2nd Int. Cong. on Animal Reprod. and AI. Copenhagen. 3:168-174. 1952.
- (173) Stallcup, O. T., and H. K. McCartney. Toxicity to bull spermatozoa of terramycin hydrochloride and its use as an antibacterial agent in diluents. *J. Dairy Sci.* 36:293-295. 1953.

penicillin, streptomycin, and  
:1097-1103. 1953.

um, penicillin, furacin and  
pon the control of bacteria  
3.

amides upon the livability  
iluted bull semen. *J. Dairy*

effects of freezing bovine  
ents. *J. Dairy Sci.* 36:728-

ity of semen from relatively  
*airy Sci.* 32:950-954. 1949.  
ne semen in extenders con-  
polymyxin. *J. Dairy Sci.*

ycin and sulfanilamide for  
tility. *J. Dairy Sci.* 34:819-

en as influenced by dilution  
interaction of these factors.

en containing several com-  
.360-1368. 1955.

H. Ehlers. Influence of anti-  
ulic. *J. Animal Sci.* 14:731-

E. L. Jungherr. Comparative  
loride complex streptomycin  
51-852. 1950.

spermatozoa of various salts,  
eomycin and chloromycetin.

to the effect of antibiotics on  
he employment for semen of  
on *Animal Reprod.* and *AI.*

ull spermatozoa of terramycin  
ent in diluents. *J. Dairy Sci.*

- (174) DiLiello, L. R., L. J. Poelma, and J. E. Faber. Effect of antibiotics on *Vibrio fetus* and bovine spermatozoa. *Amer. J. Vet. Res.* 18:86-92. 1957.
- (175) Myers, R. M., and J. O. Almquist. A comparison of the effects of aureomycin, penicillin and streptomycin upon spermatozoan livability and control of bacteria in bovine semen. *J. Animal Sci.* 10:322-330. 1951.
- (176) Zaugg, N. L., and J. O. Almquist. Motility of spermatozoa and control of bacteria in bovine semen diluents containing penicillin and neomycin or epicillin. *J. Dairy Sci.* 56:202-206. 1973.
- (177) Sullivan, J. J., F. I. Elliott, D. E. Bartlett, D. M. Murphy, and C. D. Kuzdas. Further studies on use of polymyxin B sulfate with dihydrostreptomycin and penicillin for control of *Vibrio fetus* in a frozen semen process. *J. Dairy Sci.* 49:1569-1571. 1966.
- (178) Easterbrooks, H. L. Antibiotic treatment of diluted bull semen used in artificial insemination. *Fert. Ster.* 2:430-443. 1951.
- (179) Hamdy, A. H., and C. C. Miller. Antibiotics for bovine mycoplasmas. *J. Dairy Sci.* 54:1541-1544. 1971.
- (180) Elliott, F. I., D. M. Murphy, D. E. Bartlett, and R. A. Kubista. The use of polymyxin B sulfate with dihydrostreptomycin and penicillin for the control of *Vibrio fetus* in a frozen semen process. *A.I. Digest.* 10:10. 1962.
- (181) Hafs, H. D., L. J. Boyd, S. Cameron, and F. Dombroske. Fertility of bull semen with amylase and polymyxin. *A.I. Digest* 17:8-9, 18. 1969.
- (182) Albertsen, B. E. Bactericidal effect of antibiotics in semen diluent on pathogenic bacteria in bull semen. Thesis. Royal Vet. Agric. College, Copenhagen. 1957.
- (183) Paufler, S. Investigations concerning the effectiveness of added antibiotics on the bacterial flora in diluted bovine semen. (Trans. title). *Deutsche Tierärztl. Wochenschrift* 67:63-66. 1960.
- (184) Orthey, A. E., and H. L. Gilman. The antibacterial action of penicillin and streptomycin against *Vibrio fetus* including concentrations found in naturally infected semen. *J. Dairy Sci.* 37:416-424. 1954.
- (185) Adler, H. C. Bovine genital vibriosis: The transmission through deep frozen semen and its prevention by streptomycin treatment. *Roy. Vet. Agr. Coll. Ster. Res. Inst. Rpt.* pp. 149-154. 1966.
- (186) McEntee, K., D. E. Hughes, and H. L. Gilman. Prevention of vibriosis in inseminated heifers by treating the semen from *Vibrio*-infected bulls with penicillin, streptomycin and sulfanilamide. *Cornell Vet.* 44:395-402. 1954.
- (187) McEntee, K., H. L. Gilman, D. E. Hughes, W. C. Wagner, and H. O. Dunn. Insemination of heifers with penicillin- and dihydrostreptomycin-treated frozen semen from *Vibrio fetus* carrier bulls. *Cornell Vet.* 49:175-183. 1959.

- (188) Nishikawa, Y., T. Fujukawa, K. Utsumi, Y. Sasae, and T. Hayami. Effect of vitamin B<sub>12</sub> (thiamine) derivatives on motility and viability of bull and goat spermatozoa. 6th Int. Cong. on Animal Reprod. and AI. Paris. 2:1286-1289. 1968.
- (189) Andersen, H., and H. H. Koefoed-Johnsen. The effect of caproic acid and adenosine on the motility and livability of bull spermatozoa. (Trans. title). Ann. Rpt. Royal Vet. & Agr. College, Steril. Inst. pp. 121-131. 1965.
- (190) Garbers, D. L., N. L. First, J. J. Sullivan, and H. A. Lardy. Stimulation and maintenance of ejaculated bovine spermatozoan respiration and motility by caffeine. *Biol. Reprod.* 5:336-339. 1971.
- (191) Dimitropoulos, E. The fertility of bull sperm diluted with extenders containing alpha-amylase thiamine propyl disulfide and ascorbic acid. (Trans. title). *Ann. Med. Vet.* 114:467-480. 1970.
- (192) Milk Marketing Board. Implementors in semen diluents. *MMB Rpt.* 15:105-106. 1964-1965.
- (193) Arbeiter, K., and R. K. Rath. Effect of various tranquilizers and semen diluents on the survival of bovine spermatozoa. (Trans. title). *Zuchthyg.* 2:49-53. 1970.
- (194) Maksimov, L., Jr., and N. I. Maksimova. Liver preparations as protective components in media for bull semen. (Trans. title). *Vest. Sel'-Khoz. Nauki, Mosk.* 14:100-103. 1969. Abstr. in *Animal Breeding Abstr.* 38:84. 1970.
- (195) Popov, P. S. Storage of bull semen in a glucose-citrate diluent with EDTA and Arginine. (Trans. title). *Zhivotnovodstvo* 30:66-67. 1968. Abstr. in *Animal Breeding Abstr.* 37:66. 1969.
- (196) Shannon, P. Advances in semen dilution. *Proc. N.Z. Soc. Anim. Prod.* 28:23-31. 1968.
- (197) MacMillan, K. L. The effect of amylase, catalase, and a decapacitating preparation on fertility of bull semen diluted in ambient temperature extender. *Aust. J. Biol. Sci.* 23:691-695. 1970.
- (198) Foote, R. H. Survival of bull sperm in milk and yolk extenders with added catalase. *J. Dairy Sci.* 45:907-910. 1962.
- (199) Andersen, H., and K. Rottensten. The effect of glucose, fructose, catalase and peroxidase on the viability of bull spermatozoa. (Trans. title). Ann. Rpt. Royal Vet. Agr. College, Steril. Inst. pp. 33-51. 1961.
- (200) Kirton, K. T., L. J. Boyd, and H. D. Hafs. Fertility of bull semen with added amylase. *J. Dairy Sci.* 51:1426-1428. 1968.
- (201) Hafs, H. D., L. J. Boyd, S. Cameron, W. L. Johnson, and A. G. Hunter. Fertility of bull semen with added beta-glucuronidase. *J. Dairy Sci.* 54:420-422. 1971.
- (202) Meding, J. H. The effect of chorionic gonadotrophin on the fertility of bull semen. (Trans. title). Ann. Rpt. Royal Vet. and Agr. College, Steril. Inst. pp. 79-83. 1965.

T. Hayami. Effect of vitality of bull and goat spermatozoa. (Trans. title). *Ann. Paris*. 2:1286-1289. 1968.

Effect of caproic acid and spermatozoa. (Trans. title). *Ann.* 131. 1965.

Lardy. Stimulation and respiration and motility by

with extenders containing acid. (Trans. title). *Ann.*

exts. *MMB Rpt.* 15:105-

lizers and semen diluents *Zuchthyg.* 2:49-53. 1970.

ations as protective compounds. *Sel'-Khoz. Nauki., Mosk.* 1:84. 1970.

diluent with EDTA and 1968. Abstr. in *Animal*

*Soc. Anim. Prod.* 28:23-

a decapacitating preparation extender. *Aust. J.*

lk extenders with added

se, fructose, catalase and (s. title). *Ann. Rpt. Royal*

of bull semen with added

and A. G. Hunter. Fertility *ry Sci.* 54:420-422. 1971.

n on the fertility of bull agr. College, Steril. Inst.

- (203) Staples, R. E., W. Hansel, R. H. Foote, and H. O. Dunn. Motility and fertility of progesterone- and estrogen-treated bovine semen. *J. Dairy Sci.* 43:787-795. 1960.
- (204) McFee, A. F., and E. W. Swanson. The influence of cooling rates and storage temperatures on sperm activity. *A.I. Digest.* 8(10):12-14. 1960.
- (205) Jakobsen, F. K., and M. Jorgensen. Effect of rate of cooling on fertilizing capacity of bull semen. (Trans. title). *Ann. Rpt. Royal Vet. & Agr. College, Steril. Inst.* pp. 29-34. 1962.
- (206) Swanson, E. W., J. E. Thigpen, and L. C. Stanfill. The effect of cooling and storage temperatures upon the fertility of bull sperm. *A.I. Digest.* 10(5):10-11. 1962.
- (207) Salisbury, G. W. Fertility of bull semen diluted at 1:100. *J. Dairy Sci.* 29:695-697. 1946.
- (208) Bratton, R. W., R. H. Foote, and C. R. Henderson. The relationship between fertility and the number of spermatozoa inseminated. *J. Dairy Sci.* 37:1353-1356. 1954.
- (209) Kim, S., and K. M. Lee. Conception rates with minimum spermatozoa counts. 7th Int. Cong. on Animal Reprod. and AI. Munich. II:1449-1452. 1972.
- (210) Courot, M., R. Ortavant, and E. Richeme. Observations on the number of spermatozoa used in the artificial insemination of cattle. (Trans. title). *Élevage et Insém.* pp. 2-4. 1965.
- (211) Kiddy, C. A., and H. D. Hafs (eds.). *Sex Ratio at Birth—Prospects for Control.* Symposium. Amer. Soc. of Animal Sci. Champaign, Ill. 1971.
- (212) Beatty, R. A. Separation of X and Y Spermatozoa. *Biblio. Reprod.* 23:1-6, 127-129. 1974.
- (213) Salisbury, G. W., and N. L. VanDemark. Stimulation of livability and glycolysis by additions of glucose to the egg yolk-citrate diluent for ejaculated semen. *Am. J. Physiol.* 143:692-697. 1945.

# 17

## Principles and Techniques of Freezing Spermatozoa

### 17-1 INTRODUCTION

All life proceeds in a continuous stream, the individuals in it constituting a part of the linkage that has joined past and present life forms and will ultimately join present life forms with those of the future. The life span of each link in the chain is limited by its genetic makeup and its environment, some part of which is controlled by the metabolic rates of the individual links. Among the processes that control the metabolic rate at which spermatozoa exhaust themselves is the temperature at which their life-sustaining chemical processes proceed. These chemical processes generally take place within a measurable temperature range and vary within that range in direct response to the temperature.

Water is the primary constituent of biological fluids responsible for the internal transport of essential chemicals. Pure water freezes and forms crystals at 0°C, whereas water containing ions and other substances in solution freezes at lower temperatures depending on the concentration of these substances. As water in a solution is frozen, pure water crystals form leaving behind greater liquid concentrations of those substances in solution. This increases the osmotic pressure of the remaining solute, which can damage the cells.

British workers discovered that glycerol protected spermatozoa against some of these hazards when it was added to the extender solution in which the spermatozoa were suspended. Since then, freezing has been the preferred method of preserving spermatozoa.

Spermatozoa can now be stored in liquid nitrogen at temperatures as low as -196°C and survive with relatively high fertility after thawing. However, many of the contained spermatozoa are killed or rendered immotile by freezing and thawing. Hence, for optimum fertility larger numbers of spermatozoa are used for frozen than for unfrozen semen.



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Egg yolk and/or milk are the basic ingredients of most extenders for semen freezing. Other protective compounds similar to glycerol have been tested and found unsatisfactory in one way or another. The fertility of frozen semen depends greatly on the care exercised in the initial extension of the semen before freezing, the method of adding the glycerol, the time permitted for equilibration of the spermatozoa with the glycerol, the rate of freezing, and the stability of the temperature during storage. After storage fertility depends on the care and method of warming the extended semen before insemination. When proper procedures are used fertility rates equal to those of any other process can be maintained for extended periods.

Although many of the procedures for freezing spermatozoa are relatively simple, they must be performed correctly if the highest potential fertility for insemination is to be maintained. This chapter will acquaint the reader with the present state of the art of preserving bovine semen by freezing and will relate that to the latest research dealing with the process.

## 17-2 THEORETICAL ASPECTS OF FREEZING AND THAWING

**17-2.1 Physical and Chemical Consequences.** At least 50 percent of the sperm from an unselected population of bulls are killed or rendered immotile during freezing and thawing. The damage is caused 1) by internal ice crystal formation that affects the structure of the spermatozoa, 2) by the solute concentration that results as pure water is withdrawn from suspension media both inside and outside the cells, and 3) by the interactions of these two physical factors. This theory of cellular damage is widely accepted among cryobiologists as a working hypothesis in research in this field.

The major physical and chemical consequences of freezing are the removal of pure water from solution to form ice and the resultant increased concentration of solutes in the residual liquid. These events and their effects on the cells are influenced by the level and types of cryoprotective agents, the osmolality and pH of the extender, and the freezing rate (1, 2, 3, 4). Spermatozoa to be stored in glass ampules have generally been frozen at relatively slow rates. The range of freezing rates employed has been about 0.8 to 3°C per minute from +5 to -15°C, and 3 to 5°C per minute from -15 to -79°C. These rates result in the formation of a relatively small number of large extracellular ice crystals.

The freezing point of a solution is based on the concentration of particles it contains as solutes. A given molecular weight of an electrolyte that dissociates into ions on entering into solution will lower the freezing point more than the same proportionate weight of a molecule that does not dissociate. As a solution cools to, through, and past the point at which ice forms, a continuing series of events takes place. The rate of cooling depends on the difference in temperature between the solution and its coolant and their relative

mass. Depending on the concentration of solutes and the rate of cooling, the temperature of the solution falls below the true freezing point of water until an ice crystal forms around a "seed." At that point the heat of fusion required to form ice must be removed from the solution, so its temperature shifts upward as the process takes place. As each molecule of pure water freezes out of the solution, the concentration of particles in that which remains increases, resulting in a continuous drop in the freezing point of that solution. These inter-related changes continue until the entire solvent and its contained solutes and other contents have completed the process through the eutectic point. Due to the longer exposure of the cells, the damaging effects of high solute concentrations should be most critical with slow rates of freezing. Similarly, because cells may be exposed to damaging solute concentrations during rewarming, fast thawing should be beneficial.

Although the formation of extracellular ice crystals was once considered an important disruptive force it does not seem to be a primary cause of sperm-cell damage during freezing (6, 7). Extracellular ice is not, in fact, particularly harmful (6-9). With few exceptions (10, 11), however, intracellular ice is lethal (1, 3, 5, 6). In addition, recrystallization—the growth of large crystals at the expense of smaller ones—can occur at temperatures above  $-130^{\circ}\text{C}$  (2). Ice crystals can also form and cause cell damage as stored samples are warmed from  $-196^{\circ}\text{C}$ .

The increased solute concentrations created as water freezes appear to be a major cause of cell damage. For example, hemolysis of erythrocytes occurs at electrolyte concentrations above 0.8 M NaCl regardless of temperature (12), and high salt concentrations damage bull spermatozoa (13). During freezing the temperature at which bull sperm were damaged was found to be lower in a hypotonic extender than in an isotonic one (14). Solute concentration, the rapidity of its change, and the damaging effects on spermatozoa depend on temperature (3, 5, 8) and its rate of change. During slow freezing, the cells are exposed for prolonged periods to concentrations of solutes that are increasing relatively slowly and some cellular dehydration may occur. With rapid freezing the length of exposure to increased solute concentrations is reduced and intracellular dehydration may not occur.

The increase in osmotic pressure of the suspension medium that accompanies freezing pulls water from within the cell, causing intracellular dehydration with an attendant increase in intracellular electrolyte concentration. In contrast, rapid freezing, such as that obtained by direct immersion in liquid nitrogen with cooling rates that probably exceed  $200^{\circ}\text{C}$  per minute, results in the formation of a relatively large number of small crystals both within the spermatozoa and in the suspension fluid. Intracellular ice can form during rapid freezing because there is insufficient time for all freezable water to diffuse from the cell (4, 5). Both rapid and slow freezing cause cellular damage, although different mechanisms appear to be responsible.

the rate of cooling, the freezing point of water until the heat of fusion required for the temperature shift is upward. After freezing out of the solutes increases, resulting in a solution. These intercellular solutes and the eutectic point. Due to the high solute concentration, similarly, because cells undergo rewarming, fast

as was once considered the primary cause of sperm death, in fact, particularly intracellular ice is lethal due to large crystals at the above  $-130^{\circ}\text{C}$  (2). Ice-damaged samples are warmed

after freezing appear to be less of erythrocytes occurs with a change of temperature (12), and (13). During freezing, it was found to be lower than before. Solute concentration, spermatozoa depend on slow freezing, the cells and solutes that are increased may occur. With rapid freezing, concentrations are reduced

on medium that accompanying intracellular dehydration and electrolyte concentration. In direct immersion in liquid nitrogen at  $-196^{\circ}\text{C}$  per minute, results in ice crystals both within the cells and in the extracellular water can form during rapid freezing. Cellular damage, although

Changes in pH have been observed during freezing caused by precipitation of salts in saturated solutions (15, 16). This could influence cellular survival, although under normal conditions it seems unlikely that solutions sufficiently saturated to change pH so as to harm the spermatozoa would be produced until after their own metabolism had been greatly slowed by the drop in temperature.

The exact nature of total freeze-thaw damage is not fully understood. It has been suggested that freezing damage is a consequence of the vapor pressure differential between ice and water at temperatures below  $0^{\circ}\text{C}$ . The theory is that the greater vapor pressure of the water would cause its redistribution, leading to injury from dehydration during slow freezing, and to membrane rupture during rapid freezing (22). There is considerable evidence that cellular membranes are involved. Saacke and Almquist (17) observed loss or damage to the cellular membranes of spermatozoa frozen without glycerol, and Rapatz (18) observed damage to the sperm neck, due to intracellular ice, following rapid freezing. Pickett and Komarek (19) found that lipid was lost from spermatozoa during freezing, presumably leaching from cellular membranes. Membrane damage may also account for the increased leakage of sperm enzymes following freezing without glycerol (20). Decreased mitochondrial enzyme activity has been noted following the freezing of mouse liver (21). Such changes are presumably the results of increased solute concentrations that induce protein denaturation.

**17-2.2 Glycerol Function.** The cryoprotection afforded when glycerol is added to seminal extenders was originally attributed to changes in the size and shape of ice crystals formed (6, 9, 18)—changes that presumably reduced their mechanical destructiveness. A more popular hypothesis states that glycerol acts through a "salt-buffering" mechanism (12). Since glycerol binds water (3, 23, 24) and markedly decreases the freezing point of solutions, less ice forms in its presence at any given temperature. Consequently, the solute concentration in the residual liquid is correspondingly reduced. The damaging influences of concentrated solutes appear to depend on temperature (3, 6, 8); hence glycerol, by reducing the temperature at which such solute concentrations are attained, presumably reduces their damaging effects. In support of this concept, the optimum glycerol level of an extender increases as extender tonicity is increased (25, 26, 27). Glycerol probably dehydrates the cells (34, 35) and it forms complexes with metallic ions (33).

Although cellular damage can be caused by concentrated solutes, other unknown sources of damage presumably exist, and knowledge of the site of glycerol action is essential to a critical evaluation of both cryoprotective mechanisms and the nature of freezing damage.

It has generally been believed that glycerol enters live spermatozoa suspended in it and does so rapidly. Glycerol is oxidized by sperm cells (28, 81) and it appears in autoradiographs to concentrate in the posterior part of the

head (29). The rate of entry is not known, however; nor is it known whether glycerol buildup within the cell is necessary for protection against freezing damage (31, 35). The early view in support of an intracellular glycerol requirement was based on work with red blood cells (12), but recently has been shown not to hold in tests of bovine sperm that compare their freezability after momentary and 30-minute exposure to glycerol before freezing (35). Spermatozoal survival was greatest in one study (34) when they were frozen after only momentary exposure to glycerol rather than after 30 minutes' to 6 hours' exposure. Bull sperm cells have been successfully frozen in the presence of cryopreservatives that do not enter the cell if the freezing rate is very rapid (32). To be most effective, glycerol requires relatively slow freezing rates.

Since spermatozoa act like osmometers, swelling in hypotonic extenders as water enters the cells and shrinking in hypertonic extenders as water is withdrawn from them, it was thought that this technique could be used to determine the rate at which glycerol entered into spermatozoa. The presence of 7 percent glycerol in an isosmotic extender containing a soybean protein decreased spermatozoan volume. In two concentrations of citrate dramatically different in osmolality, in the presence or absence of egg yolk, the packed cell volume was increased by 7 percent glycerol. The change recorded was as great 5 minutes after as it was 30 minutes after the addition of glycerol and was, of course, greater in the hypoosmotic extender (30).

**17-2.3 Other Cryoprotective Agents.** Glycerol for the freezing of bull semen remains the standard of comparison for any cryoprotective agent. No substitute has yielded results as satisfactory. A number of related compounds, including ethylene glycol and propylene glycol have been investigated (36, 37, 38). Dimethyl sulfoxide (DMSO) penetrates cells rapidly, has a "salt-buffering" effect, and is most effective during slow freezing. A number of disaccharides and trisaccharides have also shown some cryopreservative effects. They do not penetrate the cell and seem most effective when used with relatively fast freezing rates (32). A number of theories have been advanced to explain the cryoprotective effects, most of which have to do with elimination of electrolyte damage (39), cell membrane permeability (40), possible preferential leakage of solutes (41), and buffering by nonelectrolytes (32).

### 17-3 EXTENDERS FOR FREEZING

**17-3.1 Basic Components of Extenders for Freezing.** The basic components of extenders for freezing bull spermatozoa are: 1) water (42) which is a solvent for seminal and extender components; 2) dissolved ionic and nonionic substances to maintain osmolality and to buffer the pH of the medium; 3) organic materials with the capacity to prevent cold shock (generally egg yolk or milk); 4) cryoprotective agents such as glycerol or DMSO; 5) simple sugars for an



or is it known whether action against freezing cellular glycerol requirement has been shown after freezing (35). Spermatozoa were frozen after 10 minutes' to 6 hours' in the presence of cryoprotectant is very rapid (32). Freezing rates.

1) hypotonic extenders and as water is withdrawn to determine presence of 7 percent protein decreased dramatically different packed cell volume was as great 5 minutes and was, of course,

freezing of bull semen agent. No substitute compounds, including investigated (36, 37, 38). as a "salt-buffering" number of disaccharides effects. They do not relatively fast freezing to explain the cryoprotection of electrolyte preferential leakage

the basic components 2) which is a solvent and nonionic sub-medium; 3) organic by egg yolk or milk); simple sugars for an

energy source (82) or di- and trisaccharides as added cryoprotectants; 6) additives such as enzymes that may improve fertility; and 7) antibiotics to control microbial growth (see Chapter 16).

*Buffers and Nonionic Substances.* A variety of buffers and nonionic substances have been used, among which are sodium citrate dihydrate (43, 44, 45), Tris (hydroxymethyl) amino methane (27, 46), monosodium glutamate (47), buffers with combinations of salt solutions (48) or carbohydrates (32, 35, 44, 49-52), and various milk preparations (25, 45, 46, 53, 54). Although a number of zwitter ionic buffers have received recent attention (55, 56), yolk-citrate remains the most widely used seminal extender. Reports of superior survival and fertility in other extenders are numerous (47, 51, 52, 54), but possible interactions with other factors in seminal processing make it impossible to generalize these findings; a satisfactory extender for one animal or one set of conditions may not be acceptable for others. Thus each laboratory must determine which extender, under its conditions, will provide maximum reproductive efficiency. It should be emphasized that almost all recommendations regarding minimum sperm numbers, the effects of long term storage, and so on, are based on results with yolk-citrate extenders. Each promising new extender or additive should be carefully evaluated before it is adopted for general use.

*Organic Materials.* Since Phillips (57) discovered that egg yolk was a valuable constituent in preserving fertility it has been used extensively in seminal extenders. VanDemark et al. (37) investigated the influence of egg yolk levels on the post-thaw motility of spermatozoa in a citrate extender; the results are shown in Figure 17-1. The highest survival was obtained with 24.2 percent egg yolk, although a second experiment showed that 16 percent egg yolk was slightly better than 24 percent. Levels of approximately 20 percent egg yolk have become standard in most extenders.

Post-thaw motility (58, 59), sugar utilization (59), lactic acid production (59), and fertility (58) were greatest when egg yolk was included in both fractions of the seminal extender—that containing no glycerol (which is added for cooling above freezing) and the remainder containing glycerol (which is added before the below-freezing cooling phase is begun).

Extenders have also been developed that consist primarily of heated skim milk and glycerol. With reconstituted skim milk the optimum combination of these components was 10 percent dry milk solids to 9.7 percent glycerol. With fresh skim milk, 10 to 13 percent glycerol was best (25, 54).

The beneficial effects of egg yolk and milk for preventing cold shock prompted research on the nature of the active constituents. It was found that egg yolk does not permanently alter the spermatozoa (60). The optimum levels of its protective constituents—lipoprotein, phospholipid, and lecithin (60-65)—in extenders range from 1 to 5 percent lipoprotein, 0.5 to 1.0 percent



phospholipid, and 0.25 to 2.0 percent lecithin (see Figure 17-2). The milk protein—casein—is the agent in milk responsible for preventing cold shock (62, 65, 66) and its optimum levels have ranged from 1 to 8 percent. The nature of cold shock prevention by these agents is not understood.

*Cryoprotective Agents.* Glycerol is the most widely used cryopreservative for bull spermatozoa. When frozen by conventional methods in glass ampules, approximately 7 percent glycerol is optimal for citrate-yolk and Tris-yolk extenders; 11 to 13 percent for fresh and reconstituted skim milk. The optimum glycerol level was lower with the pellet method of freezing (35, 51, 67). In fact, yolk-lactose and yolk-raffinose extenders without glycerol resulted in a 50.5 and 59.3 percent 60-to-90-day nonreturn (NR) on 32 and 59 first service inseminations, respectively. Partial cryoprotection (51, 67) was contributed by these sugars and they complemented glycerol when used in combination. When used individually glycerol was superior to dimethyl sulfoxide (DMSO) (68-70). Combinations of these cryopreservatives were not beneficial in homogenized and skim milk extenders. The addition of 1 percent DMSO to a yolk-Tris extender with 6 percent glycerol improved post-thaw motility. In a 7 percent glycerol-Tris-yolk extender, additions of 0.5 to 2.5 percent DMSO did not significantly improve liveability but favored the combined use of these cryopreservatives.

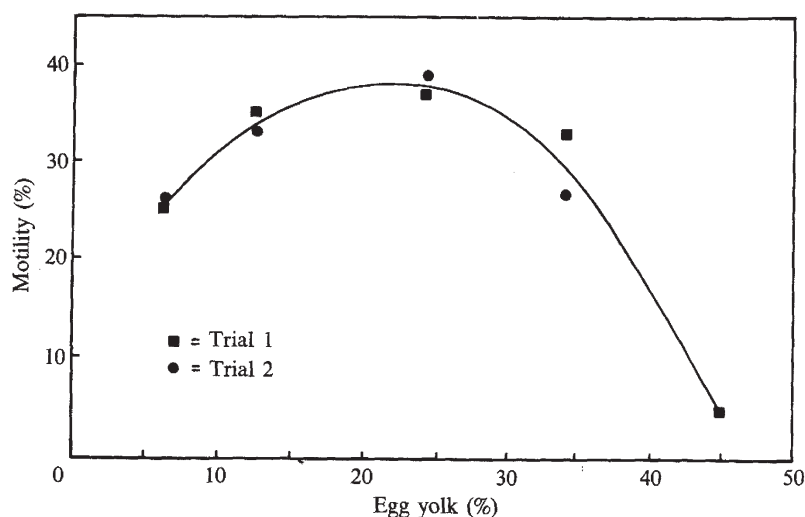
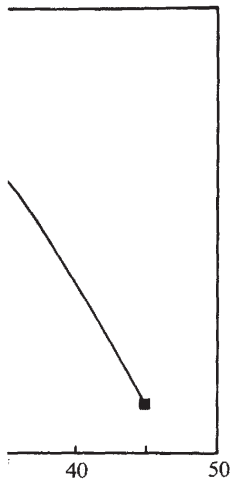


Figure 17-1. Percentage of motile spermatozoa after freezing and thawing of semen in diluents containing various levels of egg yolk. [VanDemark et al. Illinois Agr. Exp. Sta. Bull. 621. 1957.]

Figure 17-2). The milk preventing cold shock 8 percent. The nature too.

d cryopreservative for ods in glass ampules, te-yolk and Tris-yolk im milk. The optimum ng (35, 51, 67). In fact, rol resulted in a 50.5 nd 59 first service in- 7) was contributed by in combination. When xide (DMSO) (68-70). ficial in homogenized 4SO to a yolk-Tris ex- otility. In a 7 percent cent DMSO did not ned use of these cryo-



eezing and thawing of c. [VanDemark et al.

**17-3.2 Sugars.** The monosaccharides—glucose and fructose—are sugars from which spermatozoa may derive energy for their life processes; they have been studied extensively as adjuvants to extenders for freezing spermatozoa (25, 46, 54, 58, 64, 83, 84, 85). Some improvement has been observed from their addition, which suggests that the improvement resulted not only from providing immediately available substrate (54) but from providing substitutes for electrolytes in maintaining the osmotic balance of the extender. A level of 4 percent fructose was excessive in one investigation (83). It does not seem to matter whether these sugars are added to either fraction of the extender used for cooling or freezing (48), but they may affect optimum equilibration time (46, 85).

The di- and trisaccharide sugars, however, which spermatozoa are not able to metabolize, provide their cryoprotective benefits primarily as relatively large molecules that contribute to osmotic balance by acting as substitutes for electrolytes. They are not capable of passing across the sperm-cell membranes during freezing or thawing and they probably diminish the effects of solute concentration. They give best results when added to semen in the glycerol-containing fraction before freezing and they are not contained in the extender

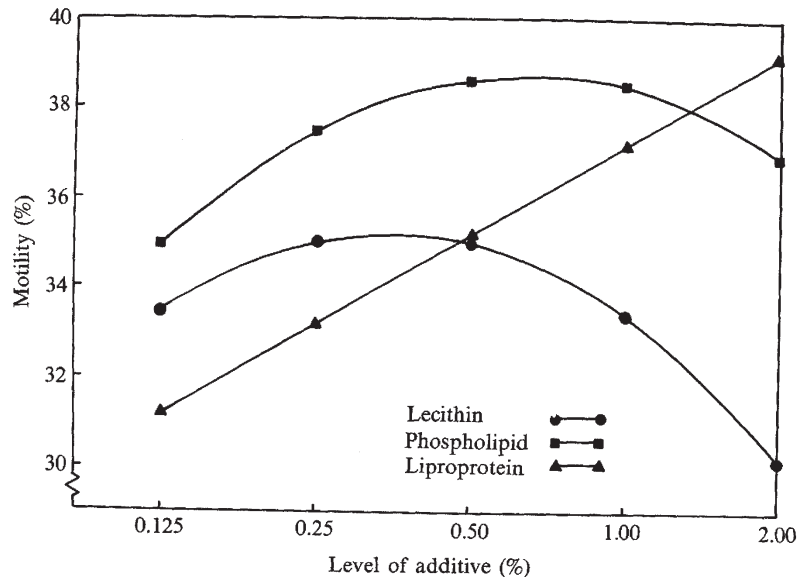


Figure 17-2. The post-thaw motility of spermatozoa extended in Tris-buffered lecithin, phospholipid, and lipoprotein extenders. [From Gebauer et al. *J. Dairy Sci.* 53:817-823, 1970.]

used for prefreeze cooling from body temperature (58). For pellet freezing, in which the rate of freezing is rapid, these sugars replace effectively the buffering salts of extenders (67) and provide cryoprotection for all (35) or part of the replaced glycerol (44) in tests based primarily on post-thaw motility. The fertility of semen frozen with sugar as the cryoprotective agent in yolk was lower than when some glycerol was included (32). The sometimes conflicting results reported in the literature involve differences in equilibration times, rates of freezing, and the kind of seminal containers used in freezing. The protection afforded by the large sugar molecules is different from that provided by glycerol and the two are not interchangeable. The data indicate that at this stage in our understanding glycerol is indispensable and sugars are sometimes complementary and useful.

**17-3.3 Osmolality.** Osmolality—the number of particles suspended as solutes in a solution—influences not only the osmotic pressure of a solution but the point at which the solvent freezes. Thus the lowered freezing point of a solution reflects its osmolal concentration of particles. An osmolal concentration of a solute in water depresses its freezing point  $1.86^{\circ}\text{C}$ . This is usually expressed as milliosmols; one milliosmol depresses the freezing point  $0.00186^{\circ}\text{C}$ . The freezing point depression of bull semen is approximately  $-0.55^{\circ}\text{C}$ , equivalent to a concentration of about 300 milliosmols, which increases as the spermatozoa metabolize nutrients (71, 73). Thus evidence indicates that it is better to err on the side of hypotonic than hypertonic extenders (77). Results of a number of studies show that seminal extenders that are isosmotic with semen preserve the life of spermatozoa best when stored above or below freezing temperatures (14, 27, 71, 74, 75). Figure 17-3 illustrates the results of freezing semen in yolk extender made up of varying levels of sodium citrate. Clearly the effects of osmolality on spermatozoan liveability and ultimate fertility interact with a wide range of other variables, including the hydrogen ion concentration measured by pH (27, 76).

**17-3.4 pH.** The optimal pH for a seminal extender is influenced by its nature and by the ions it contains. The optimal pH for a phosphate buffer is 7.5 (76), and that for Tris and citrate extender is 6.5 to 7.0 (27) (see Table 17-1). The optimal pH appears to differ, however, at different stages of the semen processing procedure. Steinbach and Foote (27) noted that the least damage before freezing occurred in an extender at pH 6.5, but the smallest loss of spermatozoan motility during freezing was observed in an extender with a pH of 7.5. This was consistent with earlier findings of Pickett et al. (79). With unfrozen spermatozoa a decrease of 0.1 pH unit in a CUE extender increased the NR figures by 2.58 percent over the pH range shown in Figure 17-4. Freezing injury may be reduced in the presence of lower concentrations of hydrogen ions than are optimal for storage in the unfrozen state, and in Tris buffer high

For pellet freezing, effectively the buffer (35) or part of the haw motility. The agent in yolk was netimes conflicting bration times, rates eezing. The protec- n that provided by ndicate that at this gars are sometimes

suspended as solutes f a solution but the g point of a solution . concentration of a usually expressed as 00186°C. The freez- 55°C, equivalent to as the spermatozoa it is better to err on sults of a number of with semen preserve eezing temperatures eezing semen in yolk Clearly the effects of tility interact with a i concentration mea-

luenced by its nature ate buffer is 7.5 (76), see Table 17-1). The of the semen process- least damage before allest loss of sperma- der with a pH of 7.5. . (79). With unfrozen der increased the NR Figure 17-4. Freezing itations of hydrogen nd in Tris buffer high

pH was more toxic at low than at high osmolality of the buffer (27, 76). The pH may change during storage but the change bears little relationship to post-thaw motility (76, 80).

**17-3.5 Enzymes.** Spermatozoan capacitation (see Section 4-6.1), which occurs naturally in the uterus preparatory to fertilization, may occur at least partially *in vitro* by incubation with enzymes hydrolyzing long-chain carbohydrates (86). One of these enzymes is amylase, which is available commercially in either the alpha ( $\alpha$ ) or beta ( $\beta$ ) forms. Most of these discoveries, and tests of the fertility of semen treated with amylase, have been made by Hafs

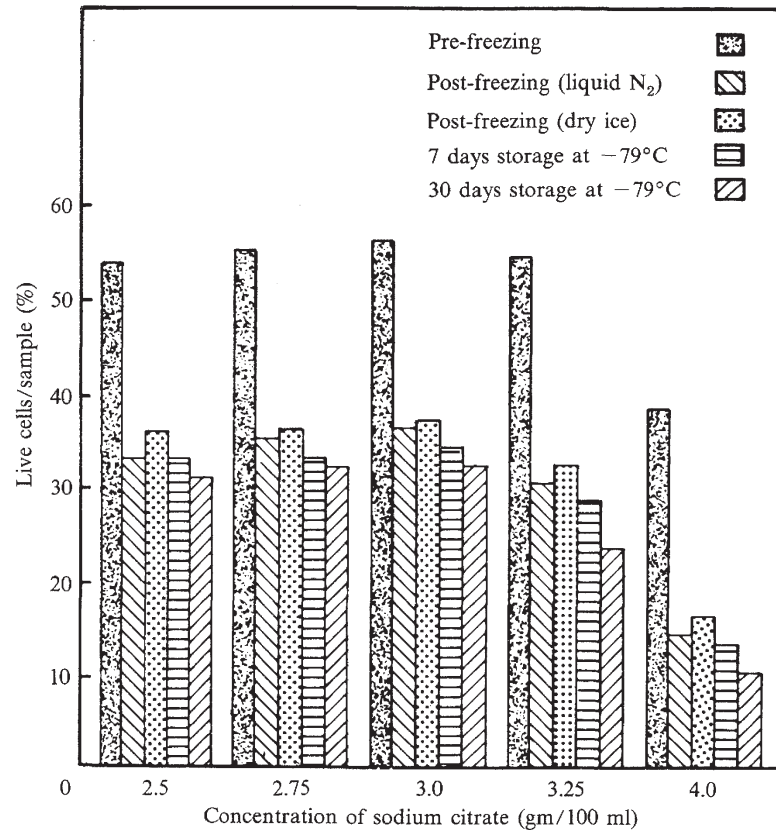


Figure 17-3. The influence of sodium citrate concentration upon the percentage of live spermatozoa at various intervals during processing and storage. [From Rao et al. *Fert. and Ster.* 19:129-136, 1968.]

*Table 17-1.* The influence of processing techniques and pH on fertility of bovine spermatozoa frozen in Tris and citrate extenders.<sup>a</sup>

<i>pH</i>	<i>Item</i>	<i>Tris</i> <sup>b</sup>			<i>Total or mean</i>
		I	II	III	
6.5	1st services	1,363	1,491	1,083	3,947
	% NR	74	73	74	74
6.75	1st services	1,320	1,499	1,165	3,984
	% NR	73	72	72	72
		<i>Citrate</i> <sup>c</sup>			
		I	II	III	
6.5	1st services	2,615	2,454	2,464	7,533
	% NR	70	72	69	70
7.0	1st services	2,516	2,478	2,438	7,432
	% NR	70	70	69	70

<sup>a</sup>From Foote. *J. Dairy Sci.* 53:1478-1482. 1970(78).<sup>b</sup> I. All samples glycerolized upon cooling and frozen 6 hours later.

II. Extenders contained glycerol initially.

III. Extenders contained glycerol initially, and samples were frozen 3 hours after cooling.

<sup>c</sup> I. Four-time glycerolization immediately after cooling.

II. One-time glycerolization immediately after cooling.

III. One-time glycerolization 5 hours after cooling (1 hour before freezing).

and his associates at the Michigan Agricultural Experiment Station. Additions of from 1 to 20 micrograms of crude  $\alpha$ - or  $\beta$ -amylase generally increased fertility (87, 88, 89), although contrary observations have been reported (228, 230) (see Table 17-2). The optimum levels of amylase additions have not yet been established and may be different for the two forms of the enzyme (90). The improvements recorded have been attributed to increasing availability of metabolic substrates (88, 89). They are most likely due to the shortening of the time necessary for capacitation to occur in the uterus, especially when inseminated in cows bred too late in estrus for normal capacitation to have occurred before ovulation (88, 91).

Beta glucuronidase, another of these enzymes, has resulted in important fertility increases when added to stored semen. The addition of 10, 30, or 150 Fishman Units of  $\beta$ -glucuronidase per milliliter of extender increased fertility



ies and pH on  
rate extenders.<sup>a</sup>

III	Total or mean
1,083	3,947
74	74
1.165	3,984
72	72

III	
2,464	7,533
69	70
2,438	7,432
69	70

3 hours after cooling.

reezing).

ment Station. Additions generally increased fertility have been reported (228). Additions have not yet shown the enzyme (90). Increasing availability of the enzyme to the shortening of the freezing process, especially when insemination to have occurred

s resulted in important addition of 10, 30, or 150 units of enzyme under increased fertility

of semen in 1.0-milliliter ampules by 0.3, 1.2, and 5.9 percentage points, respectively (90) (see Table 17-3). The fertility response was almost linear, with 150 units being higher than either 10 or 30 units. O'Hagan et al. (229) compared 0 or 150 units of  $\beta$ -glucuronidase, with and without 10 micrograms of  $\beta$ -amylase per milliliter of semen packaged in 0.5-milliliter straws. The fertility responses with amylase were 1.9 and 1.8 percentage points above the controls at 28 and 112 days respectively, compared to 0.3 and 2.0 for  $\beta$ -glucuronidase. When both enzymes were added, fertility was improved 2.0 and 4.1 percentage points over the controls at 28 and 112 days after insemination, respectively. At 112 days there was a significant increase in fertility from the addition of  $\beta$ -amylase ( $P < 0.1$ ),  $\beta$ -glucuronidase ( $P < 0.05$ ) or both enzymes ( $P < 0.001$ ).

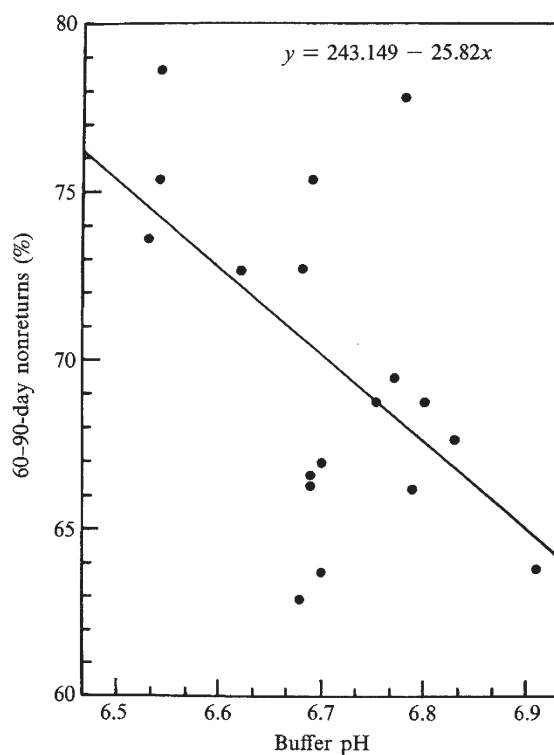


Figure 17-4. The effect of buffer pH on the fertility of liquid semen. [From Pickett et al. Conn. (Storrs) Agr. Exp. Sta. Bull. 373. 1962.]

Although the fertility response appears to be more favorably consistent to the addition of  $\beta$ -amylase than to the addition of  $\beta$ -glucuronidase, it has been postulated (229) that each additive contributes by hydrolyzing complex polysaccharides on the sperm-cell membrane. Contradictory fertility results have been obtained by others, however, (229, 246), so that final conclusions about the use of these enzymes, their amounts, and the best mode of action await further evidence.

Table 17-2. The effect of amylase on percent nonreturn<sup>a</sup> using frozen semen.

Level of amylase	Type of amylase		Ref.
	Alpha	Beta	
( $\mu\text{g/ml}$ )			
0	68.1(4,294) <sup>b</sup>		(88)
1	69.0(3,807)	71.1(4,684)	
10	72.1(4,770)	70.7(4,336)	
0	67.1(1,414)		(87)
1 <sup>c</sup>	68.6(1,932)		
10	69.4(4,623)		
100	65.0(3,619)		
0	72.0(3,401)		(90)
10	74.3(1,504)		
20	74.2(2,057)		
0	72.9(307)		(228)
10	69.8(318)	70.2(323)	
0	73.4(6,152)		(89)
1	75.3(11,492)	75.5(11,329)	
10	75.1(12,084)	75.2(12,237)	
0 <sup>d</sup>	63.1(2,000) <sup>e</sup>		(229)
10 <sup>d</sup>		64.9(2,000) <sup>e</sup>	

<sup>a</sup>60-90-day, except d.

<sup>b</sup>Number of first services in parentheses.

<sup>c</sup>Highly purified, equal to about 10  $\mu\text{g}$  crude amylase.

<sup>d</sup>112-day.

<sup>e</sup>Approximate.

Table 17-3. The effect of enzymes on percent nonreturn using frozen semen.

Level of enzyme		Inseminations	% NR	Ref.
$\beta$ -glucuronidase	Amylase			
( $\mu$ /ml)	( $\mu$ g/ml)			
0	0	3,401	72.0 <sup>a</sup>	(90)
10	0	1,961	72.3	
30	0	1,912	73.2	
150	0	1,734	77.9	
0	0	2,000 <sup>c</sup>	63.1 <sup>b</sup>	(229)
150	0	2,000	65.1	
0	10	2,000	64.9	
150	10	2,000	67.2	
0	10	4,651	77.8 <sup>a</sup>	(246)
30	0	4,500	77.2	
30	10	4,337	76.1	

<sup>a</sup>60-90-day.<sup>b</sup>112-day.<sup>c</sup>Approximate.

#### 17-4 PREPARATION FOR FREEZING

**17-4.1 Initial Extension Ratio.** It is common practice to partially extend semen soon after collection to a constant spermatozoal number or to a standard volume for cooling to 5°C. After cooling, sufficient additional extender generally is added to provide twice the final number of sperm desired for insemination. The partially diluted semen is then glycerolated by the addition of an equal volume of extender that contains twice the final desired concentration of glycerol. The authors are aware of only one investigation designed to determine the influence of the initial extension rate on subsequent survival during freezing.

Benson et al. (92) evaluated the pre- and post-freeze motility of spermatozoa subjected to initial extensions to 1,000, 250, 40, or  $20 \times 10^6$  spermatozoa per milliliter in either yolk-citrate or yolk-Tris extender. The semen was then cooled in four hours to 5°C, at which time all samples were extended to a final concentration of  $20 \times 10^6$  spermatozoa per milliliter. The highest pre-freeze

and immediate post-freeze motility occurred at an extension to  $250 \times 10^6$  and a trend toward lower motilities at higher extension rates was observed. A slightly greater rate may be possible with yolk-Tris extender.

In a subsequent experiment these authors (92) compared the motility of spermatozoa in similar extension rates after 0, 4, 8, and 12 weeks of storage at  $-196^\circ\text{C}$ . No pre-freeze dilution effect was observed, but statistically significant differences were found favoring lower extension rates in immediate post-thaw motility and motility after four weeks of storage. Samples stored for longer than four weeks were not influenced appreciably by the initial extension ratio.

**17-4.2 Incubation.** The fertility of bull semen that is used immediately after collection is not as high as that of semen that has been kept for a period of time (see Chapter 19) (93, 94). At 4 to  $5^\circ\text{C}$  the period is a day or less, then fertility declines (95, 96, 97, 98). However, further evidence that fertility improves after storage at  $5^\circ\text{C}$  has been provided by studies on equilibration and spermatozoal maturation. Post-thaw motility and/or fertility was improved by increasing the interval at  $5^\circ\text{C}$  between collection and freezing (74, 75, 78, 99-103). Furthermore, it has been shown that four hours' cooling time was significantly superior ( $P < 0.05$ ) to one hour (92). The changes in spermatozoa that occur during equilibration may correspond to those responsible for the increase in fertility of liquid semen observed from the day of collection to the following day.

Elliott et al. (104) showed that although post-freeze motility was somewhat depressed, fertility was improved after mixing antibiotics with neat semen, followed by dilution and incubation at  $30^\circ\text{C}$  for 30 minutes before cooling. An improvement ( $P < 0.01$ ) in fertility of two low-fertility bulls was recently reported as a result of incubation at  $26^\circ\text{C}$  for 20 minutes (106) irrespective of antibiotics. Thus it is possible that the improvement in fertility shown in these studies (104, 105, 106) resulted from cellular maturation and not entirely from the additional time provided for antibiotics to reduce the number and pathogenicity of organisms (107).

**17-4.3 Cold Shock.** Cold shock occurs when bovine spermatozoa are subjected to sudden cooling. The magnitude of the effect depends on the terminal temperature, the rate of temperature reduction, the extension ratio, and the length of time that spermatozoa are exposed to the terminal temperature (66, 115). The consequences of cold shock are irreversible loss of motility, increase in the number of spermatozoa that are permeable to vital stains, and significant reductions in respiratory activity and glycolysis (66, 115-119, 124-128).

Blackshaw and Salisbury (117) have shown a significant uptake of sodium and calcium and a loss of potassium and lipid phosphorus in cold-shocked spermatozoa. Quinn and White (120) also reported an increase in sodium and

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icant uptake of sodium horus in cold-shocked increase in sodium and

calcium and a decrease in potassium and magnesium. It was further reported that dilution accentuated the cation movement of both cold-shocked and deep-frozen spermatozoa (120).

Other consequences of cold shock include coiled tails (121) and loss of lipid (19) and enzymes into the seminal plasma (122) (though a significant portion of glucose phosphate isomerase and lactic acid dehydrogenase are from protoplasmic droplets).

Epididymal spermatozoa are more resistant to cold shock than ejaculated sperm (123), and the loss of cold shock resistance seems to occur in the ampulla during ejaculation (124). Sperm in second ejaculates are more resistant to cold shock than sperm from first ejaculates (119), but this is probably true only when the interval between collections is longer than two days (125). Resistance to cold shock varies greatly among spermatozoa and is inherent in the individual spermatozoon (119, 123).

Cold shock can be prevented by cooling semen slowly in the presence of protective agents. The deleterious effects of cold shock are reduced by skim milk, casein, various milk extracts, and to some extent by bovine albumin, egg albumin, lecithin, egg yolk, and egg yolk extracts (116, 117, 118, 124). The protective mechanism must be one that stabilizes the cellular membrane thus reducing permeability. Dramatic changes take place in membrane permeability due to cold shock but more subtle changes may occur as well (126).

**17-4.4 Cooling.** It has generally been recommended that cooling of semen be commenced immediately after collection and extension, although, as stated above, an incubation period for raw or neat semen may be beneficial. In any event, semen should be added to an extender containing ingredients that will prevent cold shock and it should then be cooled slowly for maximum liveability of the cells.

VanDemark (108) and Foote and Bratton (109) have shown that the fertility of liquid semen was improved significantly ( $P < 0.05$ ) by adding yolk-containing diluent to the semen before cooling. Despite this practice, rate of cooling can significantly affect fertility. Erickson (110) mixed semen and skim-milk extender at  $32.2^\circ\text{C}$  and then cooled split-samples to  $3.3^\circ\text{C}$  in either 110 minutes or four hours. Table 17-4 shows the fertility results of that study. The fertility of the samples cooled slowly (4 hours) was significantly ( $P < 0.01$ ) higher than those cooled rapidly (1.8 hours).

In 1958, the National Association of Animal Breeders (NAAB) (111) found in a survey that, of the semen processors responding, 15 percent cooled semen in less than 1 hour; 52 percent in 1 to 1.5 hours; 10 percent in 1.5 to 2 hours; and only 23 percent allowed 2 or more hours for semen to cool.

Earlier laboratory experiments showed clearly that, for above-freezing storage, slow cooling was superior to rapid cooling in preserving motility and the capacity of sperm to metabolize substrates (112, 113, 114). An interaction was shown between temperature and cooling rate, indicating that a slower



*Table 17-4.* Effect on fertility, expressed as 30–40-day nonreturns,<sup>a</sup> of two rates of cooling bull semen in heated skim milk plus antibiotics.

<i>Variable</i>	<i>First services</i>	<i>% NR</i>
Skim milk + antibiotics (cooled rapidly)	1,772	74.5
Skim milk + antibiotics (cooled slowly)	1,817	80.6
Skim milk + antibiotics + 10% glycerol (cooled slowly)	1,759	81.5

<sup>a</sup>From Erickson (110). Unpublished results (1958) used by permission.

cooling rate was required for storage at 1°C than at 5°C (112). Similar results have been obtained (48, 92) for frozen semen. Post-thaw motility was maintained significantly longer in semen that was cooled 4 hours before freezing than in semen cooled only 1 hour (92). Similarly, use of live-dead staining techniques have shown that longer cooling times preserve the life of spermatozoa better than faster ones.

To our knowledge, no fertility studies have been conducted with frozen semen using cooling rate as a variable. The desire to complete semen processing and freezing on the day of collection seems to have resulted in a cooling rate of convenience rather than one based on sound experimental data. Although sufficient data are lacking for specific and precise recommendations, there is considerable danger in rushing the cooling process.

Figure 17-5 shows the equipment used in the laboratory at Colorado State University to obtain a slow (4 hours) cooling rate. It consists of a 600-milliliter nalgene beaker and a circular piece of masonite, 4½ inches in diameter, to which is glued a piece of cork ¾ inch thick and 3¼ inches in diameter. A hole 1 inch in diameter is drilled through the masonite and cork. Tubes of several sizes are fitted with rubber rings to suspend the tube in the 600-milliliter beaker filled with 38°C water. Tube size varies to permit selection of a tube most suited for the volume of semen and extender.

A typical cooling rate, measured in the center of the test tube with a copper-constantin thermocouple, is presented in Figure 17-6. The rate of temperature reduction can be accelerated at any point along the curve by removing the tube and placing it in a test tube rack in the cold room.

Every AI unit should own and use a suitable, direct-reading instrument for measuring temperature with wire thermocouples (see Figure 17-7). The expense is minimal and could prevent some disastrous results. By placing the small thermocouple in the center of the diluted semen and conducting a few simple trial-and-error experiments, the desired cooling rate can be established for any set of conditions. Furthermore, the temperatures of the refrigerators,

3-day nonreturns,<sup>a</sup>  
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First services	% NR
1,772	74.5
1,817	80.6
1,759	81.5

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#### 17-4.5 Glycerol Addition.

*Temperature of Glycerol Addition.* Polge (127) reported that spermatozoan revival after freezing was greater when glycerol was added at 5°C than when it was added at 20°C. Miller and VanDemark (43) obtained a slightly higher percentage of motile spermatozoa when glycerol was added at 4.5°C than at 10°C and both of these were superior to addition at 15.5°C ( $P < 0.01$ ). Others have concluded that spermatozoa were damaged upon exposure to glycerol at temperatures above 5°C (53, 128, 129) and found that the damage to



Figure 17-5. Equipment that will permit slow cooling of semen.

spermatozoa exposed to glycerol at 32°C was more pronounced as the length of glycerol exposure at this temperature increased (130).

Other investigators (76, 131, 132) have not observed a depression in post-thaw motility following glycerolization at temperatures above 5°C. In one investigation there were no differences in 75-day NRs for spermatozoa glycerolated at 5, 10, 20, or 35°C (132).

The damaging effects of exposure to glycerol at higher temperatures are at least partially reversible upon subsequent equilibration at 5°C (43, 130). It is likely that survival would be most severely depressed in samples glycerolated at higher temperatures when equilibration time was also shortened.

Loss of the enzyme glutamic-oxalacetic-transaminase (GOT) was greater from bull spermatozoa glycerolated in yolk-citrate at 37°C than at 5°C. The rate of release was no different after 4 hours of equilibration at those temperatures (133). With a Tris-yolk extender and 3 hours' equilibration there was no depression of fertility by addition of the glycerol at 35°C (78).

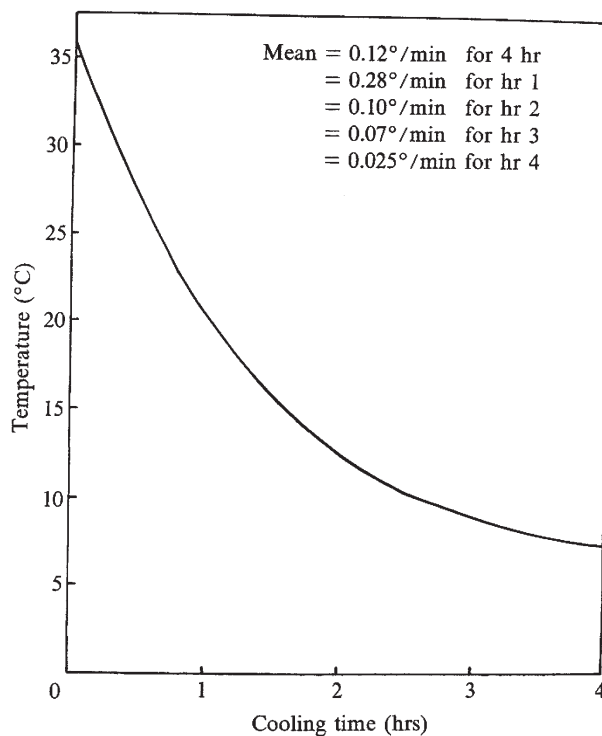


Figure 17-6. A typical semen cooling rate at the center of the test tube depicted in Figure 17-5.

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*Rate of Glycerol Addition.* Post-thaw motilities of 38 and 51 percent were observed when glycerol was added in 1 or in 3 steps at 20-minute intervals (53), respectively. In a subsequent experiment no significant differences were found between 4 and 16 equally spaced glycerol additions during a 1- or 2-hour period. An advantage of stepwise addition of glycerol to bull semen was observed, but no more than 4 additions were necessary.

In contrast, no differences were found in the post-thaw motility of spermatozoa glycerolated at 5°C in 5 equal additions at 6- or 12-minute intervals, in 3 equal additions at 10-minute intervals, in a single addition (134) or an experiment with glycerol addition in 1, 3, or 5 equal portions (43). The fertility of spermatozoa frozen after glycerolization at 5°C in either 1 or 4 steps immediately after cooling was compared with 1 step 5 hours after cooling (1 hour

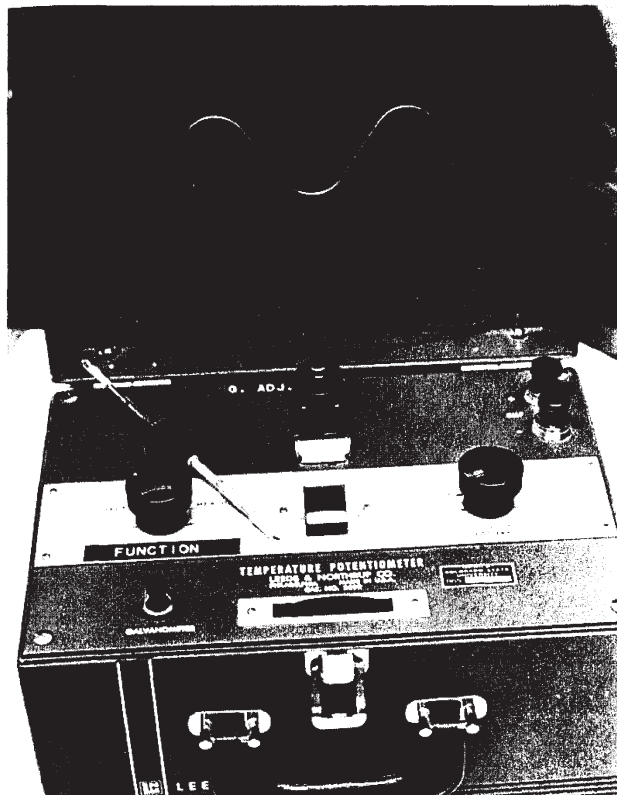


Figure 17-7. A potentiometer capable of measuring temperature by means of wire thermocouples.

before freezing). Fertility was not significantly influenced by the method of glycerol addition; it was similar for semen frozen in straws when samples were glycerolated either slowly, over 2 hours, or quickly (135).

Under some conditions the stepwise addition of glycerol to bull semen may enhance post-thaw motility; the advantage has generally been small, however, and may not be reflected in increased fertility (78, 135).

It has also been reported (136) that the liveability of spermatozoa in liquid semen was enhanced by adding glycerol stepwise in 20, 30, and 50 percent portions at 10-minute intervals, as compared to addition in a single step. Drop-wise glycerolization has been employed successfully for both liquid (136) and frozen semen programs.

Shannon in New Zealand (137) has described a novel approach to the bulk freezing of semen that may be applicable to the seasonal breeding of beef cattle in the United States (146). Semen was initially diluted about 1:1 for cooling to 5°C, followed by dilution to a concentration of  $600 \times 10^6$  sperm/ml without glycerol. The extended semen was then placed in dialysis tubing about 5 inches long and 0.25 inches in diameter, and suspended in extender that contained glycerol. This allowed the exchange of glycerol and metabolic end-products. Following a period of equilibration, the material in the dialysis sack was frozen. By freezing semen in bulk at a high sperm concentration, efficient utilization of labor and storage space was achieved. This technique was well suited to the seasonal breeding of dairy cattle in New Zealand.

**17-4.6 Equilibration.** Investigators (35, 74, 75, 99-103, 138) have demonstrated that post-thaw motility and fertility of bovine spermatozoa are both enhanced by extending the interval between adding the glycerol and beginning freezing. This stage of semen processing has been called the "glycerol equilibration" period. The results of several fertility trials designed to determine the optimal glycerol equilibration period have been summarized in Table 17-5. These studies clearly established the beneficial influence on the fertility of bull spermatozoa of an equilibration period of several hours.

Experiments have been conducted in which equilibration times have been varied at 5°C both in the presence of glycerol and without glycerol (53, 78, 101, 139, 140). The goal has been to determine whether it is spermatozoan-glycerol interaction or a process of sperm-cell maturation. A number of investigators (35, 75, 78, 138) have found evidence that lengthy glycerol equilibration *per se* is not as essential as the time in storage at 5°C before freezing. Jondet's (141) experiments using straws as containers for frozen semen suggest the same conclusion.

It is clear that additional fertility trials with the different methods of freezing are desirable, but on the basis of existing information 4 to 18 hours of storage at 5°C before freezing are recommended; and it appears that glycerol can be added at any time during this period without affecting fertility.



Table 17-5. The influence of glycerol equilibration on non-return rates.

<i>Equilibration time</i>	<i>No. services</i>	<i>% NR</i>	<i>Ref.</i>
0.5 hr	449	56.2 <sup>a</sup>	(99)
18 hr	450	63.9	
30-50 min	334	71.8 <sup>b</sup>	(100)
overnight	330	78.1	
0.5-1.0 hr	864	54.4 <sup>c</sup>	(101)
18 hr	864	61.4	
4 hr	996	63.4 <sup>d</sup>	(102)
8 hr	1,026	65.2	
12 hr	1,012	67.8	
2 hr	2,256	66.5 <sup>a</sup>	(103)
18 hr	2,176	71.3	

<sup>a</sup>30-day NR.

<sup>d</sup>75-day NR.

<sup>b</sup>31-day NR.

<sup>e</sup>60-90-day NR.

<sup>c</sup>3-month NR.

**17-4.7 Sperm Numbers.** The demand for semen from genetically superior sires has made it necessary that a minimum number of frozen sperm cells be established for maximum fertility of each insemination (142-154). Fertility is the single most important consideration in AI and no fewer sperm should be used than are necessary. One problem in recommending a minimum number is that new extenders and new methods of processing, freezing, and packaging make continual reevaluations necessary.

Table 17-6 shows the influence that the number of spermatozoa per insemination has on the fertility of semen frozen in 1.0-milliliter ampules. Since only one study (231) resulted in statistically significant differences, the lowest number of spermatozoa could logically be selected in all except that study. In all except one of the seven studies, however, mean fertility was higher with the highest number. Thus for maximum reproductive efficiency, the higher numbers of spermatozoa presented in Table 17-6 should be used.

Experiments illustrated in Figures 17-8 and 17-9 (151, 186) show that the number of spermatozoa per insemination is somewhat higher for the bull with

lower than average fertility. Thus the fertility level of each bull in a breeding operation must be considered before the absolute number of spermatozoa for each insemination can be established.

Table 17-7 summarizes the results of experiments on the number of spermatozoa necessary in extended semen that is to be packaged and distributed in straws (135, 147, 148, 149, 153, 189, 232-234, 235). These studies have also shown differences among bulls. In another study (153) the fertility difference between "good" and "poor" inseminators decreased as sperm numbers increased.

**17-4.8 Packaging: Ampules, Straws, Pellets.** At first, bull semen was packaged for freezing in glass ampules. Then a process for freezing extended semen in straws was developed, and more recently semen has been frozen in pellets. Satisfactory conception rates have been reported with all three systems (247, 248).

Danish investigators developed the idea of packaging and freezing extended semen in straws and were also responsible for much of the other earlier work. Soda straws were used in the first studies. French workers Cassou (156) and Jondet (157) have refined the technique. The French straws are plastic containers 113 millimeters long. They vary in diameter from 2.0 millimeters (0.25 milliliter) to 4.2 millimeters (1.2 milliliters), with an intermediate diameter of 2.8 millimeters (0.5 milliliter). More recently, groups in Canada, West Germany, and the United States have developed systems using straws of various sizes (see Figure 17-10). Macpherson and Penner (155) have reviewed the history of this technique and have described a modified method developed in Canada.

In the French and Canadian techniques, one end of the straw is plugged with a powder of polyvinyl alcohol that forms a seal when it comes into contact with an aqueous solution, the semen. The other end is ultrasonically or electrostatically sealed after filling. West German and U.S. straws are sealed with nonabsorbable plugs (see Figure 17-10).

The system has now been automated and its popularity is likely to increase. The straw system requires less cold storage space: Field units will accommodate at least twice as many straws as ampules; and central storage units will hold three to five times as many (158). There might also be some advantage with regard to fertility (see Table 17-8) (247, 248).

The technique of freezing semen in pellets was originally developed by Japanese workers (44, 163) and has not been widely adopted by the AI industry in the United States or Western Europe. The system has not been automated, and permanent labeling of the pellets has proven difficult. Furthermore, there is the possibility of contamination (248) by bacteria in the process. To freeze semen by the pellet method, small depressions are made in a block of solid CO<sub>2</sub> to accommodate the desired volume of semen. Volumes of 0.05 to 0.2 milliliter are placed in the depressions on the dry ice (see Figure 7-11), where the semen is allowed to remain for about 10 minutes before transfer to liquid nitrogen storage.

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Table 17-6. The influence of number of spermatozoa per insemination on the fertility of semen frozen in 1.0-milliliter ampules.

Spermatozoa (10 <sup>6</sup> )	Inseminations	% NR	Ref.
13.3 <sup>a</sup>	490	61.0 <sup>b</sup>	(84)
20.0	490	63.3	
30.0	489	61.5	
20.0 <sup>c</sup>	5,607	71.7 <sup>d</sup>	(146)
30.0	5,638	72.2	
16.0 <sup>c</sup>	1,977	72.8 <sup>d</sup>	(78)
24.0	2,103	74.6	
16.0 <sup>c</sup>	5,916	73.1 <sup>d</sup>	(78)
24.0	5,831	73.2	
12.0 <sup>c</sup>	13,939	70.4 <sup>d</sup>	(78)
24.0	13,060	71.6	
12.0 <sup>a</sup>	— <sup>e</sup>	70.6 <sup>d</sup>	(151)
24.0	—	72.0	
35.0	—	73.1	
10.0 <sup>c</sup>	1,576	61.1 <sup>d</sup>	(231)
15.0	1,488	61.5	
20.0	1,539	69.5	

<sup>a</sup>Total spermatozoa.

<sup>b</sup>3-month.

<sup>c</sup>Motile spermatozoa before freezing.

<sup>d</sup>60-90-day.

<sup>e</sup>20,815 cows were utilized for the experiment.

*Table 17-7. Influence of number of spermatozoa per insemination on the fertility of semen frozen in straws.*

<i>Spermatozoa (10<sup>6</sup>)</i>	<i>Inseminations</i>	<i>% NR</i>	<i>Ref.</i>
10 <sup>a</sup>	557	61.6	(232)
20	527	61.9	
30	565	65.7	
5 <sup>a</sup>	624	55.6	(232)
10	622	58.7	
20	638	61.0	
10 <sup>a</sup>	565	79.5	(147)
20	527	78.6	
30	557	78.6	
5 <sup>a</sup>	532	76.5	(147)
10	533	74.5	
20	550	77.3	
20 <sup>a</sup>	2,665	58.4	(233)
30	2,888	60.2	
8 <sup>b</sup>	2,648	69.7	(149)
12	2,551	73.3	
.375 <sup>c</sup>	805	49.9	(148)
.75	816	57.4	
3	765	65.8	
14	836	71.8	
5 <sup>a</sup>	993	74.0	(234)
10	998	79.5	
15	990	76.7	
20	995	79.2	

<sup>a</sup>Total spermatozoa.

<sup>b</sup>Motile spermatozoa before freezing.

<sup>c</sup>Live spermatozoa after thawing.

Spermatozoan concentrations in the pellets are about 10 times higher per milliliter of extended semen than in ampules, and the pellets therefore use far less storage space. Pellets are thawed rapidly in enough warm extender (163) to provide the right volume and the proper number of spermatozoa for each insemination.

The choice of packaging system should depend upon conception rate, economics, and convenience, in that order.

## 17-5 FREEZING AND THAWING

**17-5.1 Freezing Rates.** In early studies, ampules of semen were frozen in a dry ice-alcohol bath. Cooling rates of 0.8 to 3°C per minute from 5 to -15°C, and 3 to 5°C per minute from -15 to -79°C, were maintained by controlling the rate at which dry ice was added to the bath. Slow freezing rates were considered essential until Luyet and Keane (164) froze semen by transferring

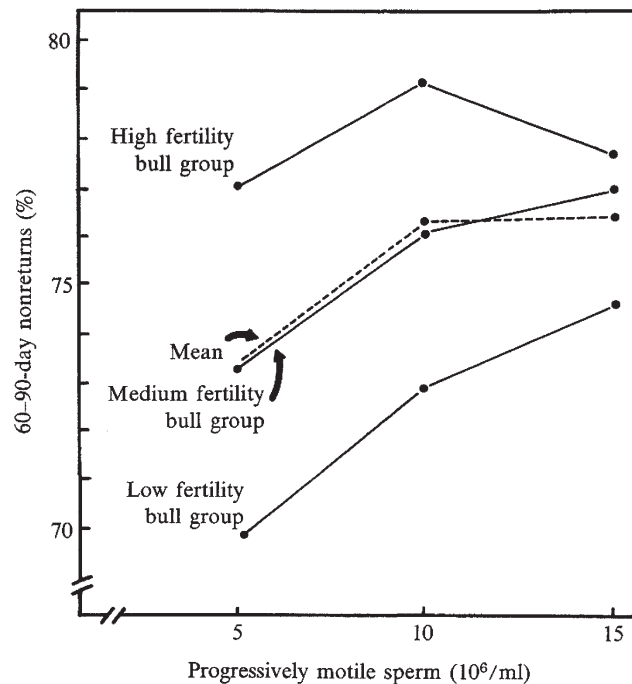
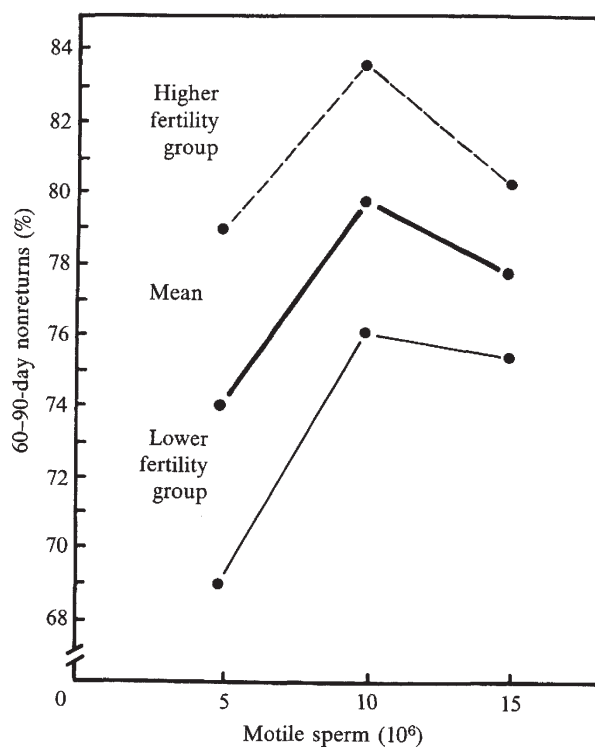


Figure 17-8. Nonreturn rate as affected by motile spermatozoan concentrations and fertility level of Holstein bulls. [From Sullivan. Proc. 3rd NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 36-43. 1970.]



ampoules abruptly from  $0^{\circ}\text{C}$  to an alcohol bath at temperatures from  $-20$  to  $-50^{\circ}\text{C}$ , where it was held for five minutes and then transferred abruptly to liquid nitrogen. When the intermediate temperature was  $-20^{\circ}\text{C}$  virtually none of the cells survived freezing. In contrast, an intermediate temperature of  $-27^{\circ}\text{C}$  provided a high rate of survival. Thus the existence was established of a critical temperature range in which rapid cooling was detrimental to spermatozoan survival. This finding was supported by a number of investigations in which conventional freezing rates were employed (165, 166) and was consistent with the report (167) of an optimal cooling rate of 4 to  $20^{\circ}\text{C}$  per minute between  $-10$  and  $-30^{\circ}\text{C}$  (see Figure 17-12).

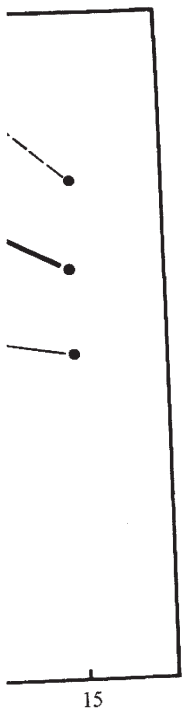
In contrast, other workers (168) found that semen could be transferred directly to the bath at  $-20$  and  $-40^{\circ}\text{C}$  for subsequent freezing at the normal



*Figure 17-9.* Nonreturn rate of dairy cows as affected by motile spermatozoa concentration and fertility level of semen from Angus and Hereford bulls. [From Sullivan, Proc. 3rd Tech. Conf. Artif. Insem. and Reprod. NAAB, pp. 36-43. 1970.]

temperatures from  $-20^{\circ}\text{C}$  in transferred abruptly to  $-20^{\circ}\text{C}$  virtually immediate temperature of existence was established was detrimental to sperm. A number of investigations (55, 166) and was consistent of 4 to  $20^{\circ}\text{C}$  per minute

can be transferred at freezing at the normal



tested by motile spermatozoa from Hereford bulls. [From Reprod. NAAB. pp. 36-43.]

rate. It was apparent that spermatozoa could be cooled rapidly between  $5^{\circ}\text{C}$  and  $-40^{\circ}\text{C}$ . This was supported by the successful freezing of spermatozoa in pellets (32, 163), films (169), and by direct placement into liquid nitrogen (32). With these techniques spermatozoa are cooled rapidly from  $5^{\circ}\text{C}$  to  $-79^{\circ}$  or  $-196^{\circ}\text{C}$  (see Figure 17-13) (170). Spermatozoa may be frozen successfully using a variety of rates and methods.

The inconvenience of manually regulating the cooling rate has led to the evolution of programmed instrumentation techniques and then simplification to the point where the semen is now frozen in the nitrogen vapor of a liquid nitrogen container (171-174). These techniques are simple and convenient and they eliminate the need for costly automation. The freezing rate in such a system depends upon the ampule's position within the nitrogen vapor. Typical freezing curves for ampules frozen in liquid nitrogen vapor are presented in Figure 17-14. The comparable survival rates of spermatozoa in ampules that occupy different positions within the vapor supports the contention that spermatozoa can withstand a wide range of freezing rates.

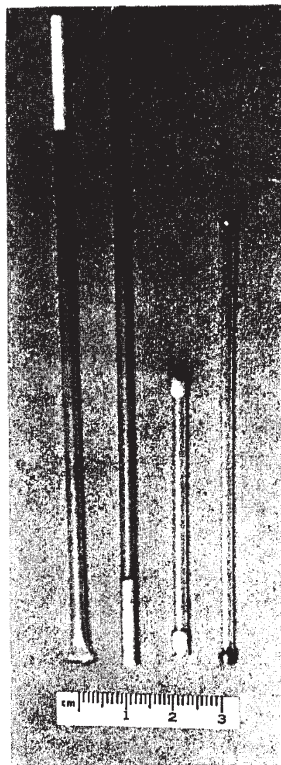


Figure 17-10. Various types and sizes of straws currently in use. From left to right: Canadian, French, U.S., and German straws.

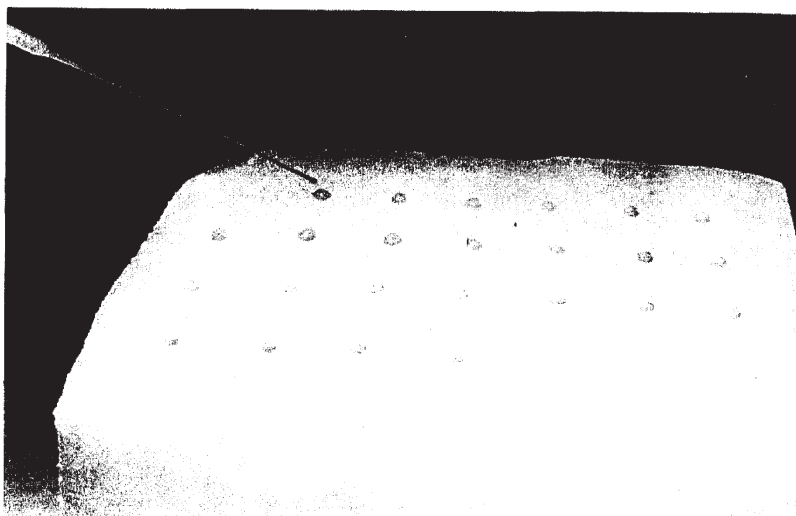


Figure 17-11. Semen being frozen by the pellet method.

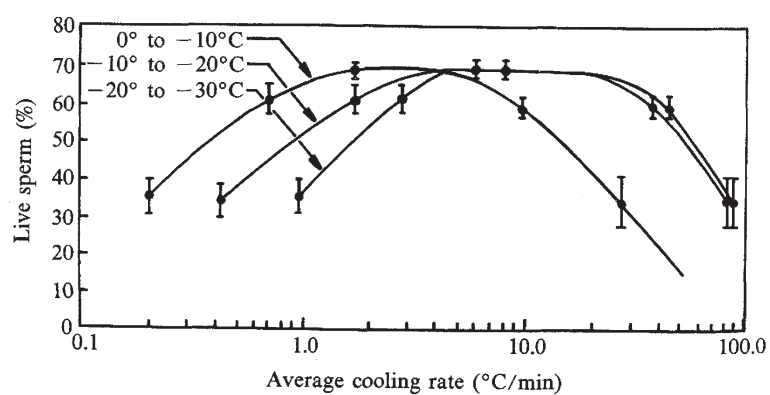


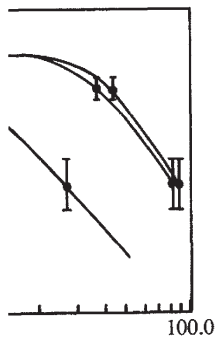
Figure 17-12. The optimum range of cooling rates between  $-10^{\circ}$  and  $-30^{\circ}\text{C}$ .  
[From Bruemmer et al. *J. Cell and Comp. Physiol.* 62:113-118. 1963.]

If the current popularity of the straw method of packaging continues, the use of vapor freezing methods will become more extensive.

**17-5.2 Storage Time and Temperature.** The cost of storing material at temperatures below freezing increases as the temperature decreases. Thus for economical reasons it is necessary to determine the temperature that will provide extended storage, minimum expense, and minimum deterioration in spermatozoan quality. VanDemark et al. (37) have shown (see Figure 17-15) that storage temperature has a dramatic effect on sperm motility after storage.

A number of detailed experiments have been conducted to elucidate the details of the effects of storage temperature and duration on the survival and fertility of frozen bull spermatozoa. The results show not only the effects of the main factors already discussed but important interactions among these factors. In general the data indicate that the lower the storage temperature the longer motility and fertility can be maintained. To ensure this, however, it is

et method.



en  $-10^{\circ}$  and  $-30^{\circ}\text{C}$ .  
3-118. 1963.]

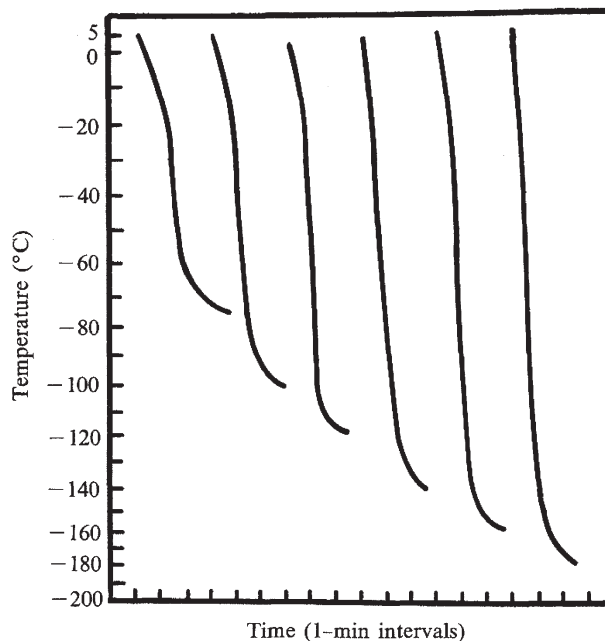


Figure 17-13. Cooling rates at the center of 0.3-milliliter pellets. Curves from left to right represent freezing on dry ice ( $-79^{\circ}\text{C}$ ), and on a stainless steel plate at  $-79^{\circ}$ ,  $-100^{\circ}$ ,  $-110^{\circ}$ ,  $-140^{\circ}$ ,  $-160^{\circ}$ , and  $-180^{\circ}\text{C}$ , respectively. [From Salamon. *Aust. J. Biol. Sci.* 23:459-468. 1970.]

necessary to use the procedures, timing sequence, and other techniques that are optimum for that lower holding temperature. In one experiment (175) using split ejaculate samples, the motility of the spermatozoa in semen frozen to  $-79^{\circ}\text{C}$  declined more rapidly during incubation after thawing than the spermatozoa in semen that had not been frozen. When a thrice-repeated freezing-thawing stress was applied to semen when it was first frozen to either  $-79^{\circ}\text{C}$  or  $-196^{\circ}\text{C}$  no differences were observed in the resistance of the contained spermatozoa to this stress at either temperature. When the semen was stored for 6 months at these temperatures, however, the spermatozoa kept at  $-196^{\circ}\text{C}$  withstood the repeated stress better than those kept at  $-79^{\circ}\text{C}$  (176). Similarly, when semen was exposed to ambient room temperature for short intervals after 0 to 120 days' storage, spermatozoa in liquid nitrogen were more resistant than those from the same ejaculate frozen in dry ice/alcohol (177). Greater deterioration in motility and metabolic activity of spermatozoa occurs at  $-79^{\circ}\text{C}$  storage than at  $-196^{\circ}\text{C}$  regardless of the storage interval (178, 179, 180).

Semen transferred from  $-196^{\circ}\text{C}$  survived better on subsequent storage at  $-79^{\circ}\text{C}$  after holding at  $-196^{\circ}\text{C}$  for 7 or 10 days rather than for 3 days (182).

Investigations involving temperatures of  $-79$ ,  $-92$ ,  $-96$ , and  $-196^{\circ}\text{C}$  show an advantage for temperatures below  $-79^{\circ}\text{C}$  (80, 181) for spermatozoan survival and motility recovery. Spermatozoa in milk extenders, however, appear to require a lower holding temperature than yolk-citrate extender for optimum motility recovery after several months of storage (80).

Some investigators have reported a gradual improvement in the post-thaw recovery of motility after 3 weeks' storage at  $-196^{\circ}\text{C}$  (179) and for as long as 24 weeks at  $-96^{\circ}\text{C}$ , suggesting that, over time, spermatozoa make a significant adjustment to the conditions of storage (181). More recent studies (183, 184) show that fertility increases for a time immediately after freezing. A similar increase has been observed at the beginning of storage at temperatures above freezing. Whether the fertility of frozen semen in commercial AI will increase with storage depends largely on the procedures used in processing and freezing. Fertility is not likely to increase if, for example, the semen has been subjected to a relatively long incubation or equilibration after cooling immediately upon collection and before freezing.

Studies on the effects of temperature on rates of chemical change and the effects of storage indicate that fertility will be maintained longer at the lowest practical storage temperature ( $-196^{\circ}\text{C}$ ) than at higher temperatures. Considering the expected temperature control of metabolic turnover from body temperature to  $-196^{\circ}$ , no system of semen storage has produced results comparable to those in which rabbit spermatozoa in the ligated epididymis were held in the scrotum at a few degrees below body temperature (see Section 8-7.4). Based on a temperature coefficient of 2, which would halve the metabolic activity each drop of  $10^{\circ}\text{C}$  the 38-day period found by Hammond and



other techniques that one experiment (175) spermatozoa in semen frozen after thawing than the hen a thrice-repeated as first frozen to either resistance of the con-

When the semen was spermatozoa kept at kept at  $-79^{\circ}\text{C}$  (176), temperature for short liquid nitrogen were more dry ice/alcohol (177), of spermatozoa occurs storage interval (178,

on subsequent storage rather than for 3 days

2,  $-96^{\circ}\text{C}$ , and  $-196^{\circ}\text{C}$  (181) for spermatozoan tenders, however, ap-k-citrate extender for age (80) movement in the post- $-96^{\circ}\text{C}$  (179) and for as spermatozoa make a ). More recent studies diately after freezing. of storage at temper- men in commercial AI res used in processing ample, the semen has ibration after cooling

chemical change and the d longer at the lowest r temperatures. Con- turnover from body produced results com- ated epididymis were perature (see Section ould halve the meta- and by Hammond and

Asdell (251) for fertility maintenance in the ligated rabbit epididymis would be comparable to a hundred years or so at  $-196^{\circ}\text{C}$ . Theoretically, optimum fertility in cattle spermatozoa can be maintained far longer at  $-196^{\circ}\text{C}$  than it has ever been.

Mixner (154) has shown, with a limited number of services (626 in the 12 years), that bull spermatozoa remain fertile when stored at  $-79^{\circ}\text{C}$  for 8 years and at  $-196^{\circ}\text{C}$  after that for up to 12 years. After performing similar long term storage tests with a total of 1310 inseminations Stewart (185) has reported a conception rate of 58 percent after 9 years. This represents an average yearly decrease of 0.8 percent NR. The report by Salisbury (183, 186) for bull semen stored in glass ampules at  $-79$  to  $-88^{\circ}\text{C}$  refers to all semen used by one AI

Table 17-8. Effect on fertility of packaging semen in straws or ampules.

Packaging system				
Straws		Ampules		Ref.
% NR	Inseminations	% NR	Inseminations	
67.2	—	64.3	—	(236)
73.0	78	73.9	69	(237)
70.4	1,462	67.7	1,909	(161)
70.7	888	73.8	725	(238)
63.7	—	57.9	—	(239)
71.1	305	69.5	240	(159)
72.0	150	67.8	158	(240)
67.7	176,293	69.1	161,151	(241)
67.5	7,380	67.6	7,188	(160)
61.1	3,915	65.5	3,847	(160)
69.0	—	67.5	—	(242)
71.0	599	67.0	625	(243)
74.3	354	73.2	381	(243)
71.5	5,544	70.3	5,637	(243)
73.2 <sup>a</sup>	395 <sup>b</sup>	54.3 <sup>a</sup>	383 <sup>b</sup>	(244)
70.7	1,407	72.7	1,448	(245)
74.9	7,592	72.8	6,714	(162)

<sup>a</sup>Percent pregnant by rectal palpation.

<sup>b</sup>First and repeat services.

organization for inseminations during 1962 and for all semen collected in 1962 and used either in that year or in 1963. A total of 216,222 first service inseminations were involved. For a major organization at that time it was a very precise study of fertility expectation and of seasonal influences on fertility; but it was a less precise estimate of the potential duration of fertility for selected semen. Fertility peaked after 2 months of storage at  $-79^{\circ}\text{C}$ , and stayed at a maximum for about 4 months, after which it decreased at the rate of 5 to 6 percent NR a month. When semen is transferred from liquid nitrogen to dry ice-alcohol the ability of the spermatozoa to recover motility is depressed (178, 179, 182). This ability lasts longer in liquid nitrogen (80, 176, 177, 178, 180, 182). Direct comparisons of fertility after storage at the two temperatures for less than 6 months are slightly in favor of the lower temperature (145, 182, 185) (see Table 17-9), but no long term comparisons have been made of fertility duration using samples of the same semen stored at the two temperatures. Salisbury's study (183) dealt with the fertility level found for the same AI unit after a shift to semen freezing and storage under liquid nitrogen by

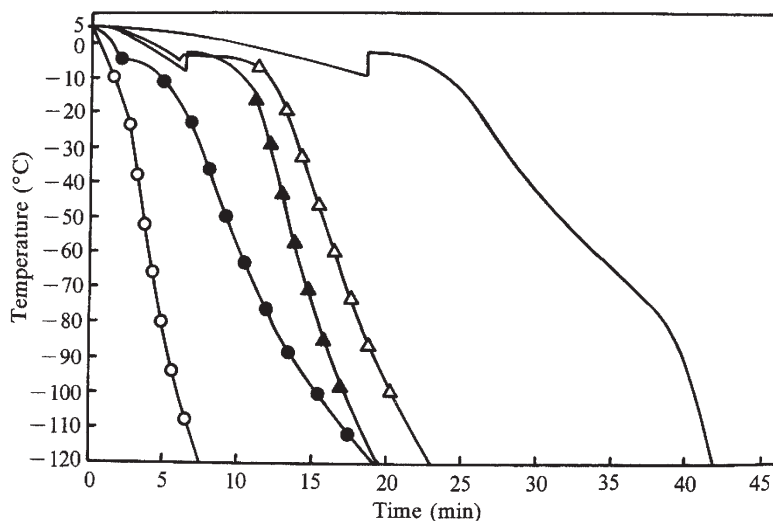
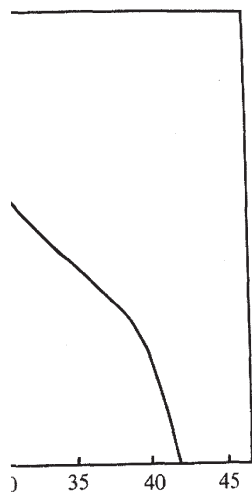


Figure 17-14. Representative intra-ampule cooling rates for semen frozen in an automatic freezer and in liquid nitrogen vapor. The various lines represent the following: —, control (automatic freezer);  $\Delta$ , top ampule in center of cardboard box (vapor frozen);  $\blacktriangle$ , third ampule from top in center of box (vapor frozen);  $\bullet$ , third ampule from top in outside corner of box (vapor frozen); and  $\circ$ , bottom ampule in center of box (vapor frozen). [From Clegg et al. Univ. of Conn. (Storrs) Agr. Exp. Sta. Res. Rep. 5. 1965.]

semen collected in 1962, 222 first service inseminations at that time it was a very strong influence on fertility: motility of fertility for selected semen stored at  $-79^{\circ}\text{C}$ , and stayed at a level of 5 to 6 percent NR on 9,053 first services in liquid nitrogen to dry storage. Motility is depressed over time (80, 176, 177, 178, 179) at the two temperatures over temperature (145, 146). Comparisons have been made of fertility at the two temperatures and level found for the same semen under liquid nitrogen by



ates for semen frozen in the various lines represent top ampule in center of box in center of box (vapor frozen); and from Clegg et al. Univ. of

the inseminator technicians with weekly shipments out of central storage. The results for 196,448 first service inseminations show an increase in fertility for the first 4 months of storage at  $-196^{\circ}\text{C}$ , after which fertility peaked for the next 8 months and then decreased during the second year of storage. Clegg and Pickett (187) studied 21,488 inseminations with semen stored at  $-196^{\circ}\text{C}$ . They found no decrease in fertility over 2 years of storage. Foote (191), who obtained the same results, also found no evidence of important seasonal variations in fertility such as those that had been shown in the Illinois studies (183, 186). Fertility may last longer under liquid nitrogen if the storage conditions, including the actual semen temperature, are kept constant.

In studying semen stored in straws at  $-196^{\circ}\text{C}$  Cassou (189) found no differences in fertility between 1 and 3 months (69.8 percent NR on 9,053 first services); between 4 and 6 months (68.4 percent NR on 11,472 first services); or between 7 and 66 months (69.1 percent NR on 83,843 first services). Ström (188) made a similar study of semen frozen in pellets and held in liquid nitrogen. A total of 63,863 cows were inseminated. He found no evidence of a change in fertility over a mean storage period of 44 weeks.

The latest data available on the questions raised by these studies are reported in a recently completed statistical analysis at the Illinois Agricultural Experiment Station (252). This analysis was performed on data provided by

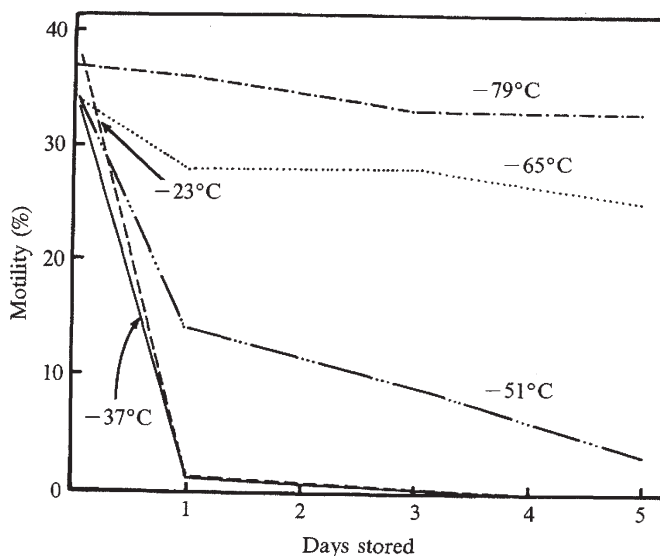


Figure 17-15. The effect of storage temperature upon post-thaw motility. [VanDemark et al. Illinois Agr. Exp. Sta. Bull. 621. 1957.]

*Table 17-9. Effect of storage temperature on nonreturn rate.*

<i>Storage temperature</i>					
<i>−79°C</i>		<i>−196°C</i>		<i>Difference</i>	<i>Ref.</i>
<i>First services</i>	<i>% NR</i>	<i>First services</i>	<i>% NR</i>		
463	69.3	495	71.9	2.6	(182) <sup>a</sup>
1,300	58.4	1,251	60.1	1.7	(185) <sup>b</sup>
4,791	68.4	4,951	71.1	2.7 <sup>d</sup>	(144) <sup>c</sup>

<sup>a</sup>75-day NR.<sup>b</sup>112-day NR.<sup>c</sup>60–90-day NR.<sup>d</sup>*P* < 0.01.

the Michigan Animal Breeders Cooperative on 555,449 first service inseminations made with semen stored in ampules at  $-196^{\circ}\text{C}$  from 1965 to 1970. Some of the semen was used 5 years after collection. The day of seminal collection and the day of insemination were recorded for all inseminations. The data show the fertility responses to length of storage were significantly different among ejaculates both from different bulls and from the same bull. Fertility increased with time after collection and freezing (the use of semen on 5th to 15th day after freezing tended to yield the lowest 180-day NR rate). In one separate analysis of 97,586 inseminations using semen from 123 bulls maximum fertility (180-day NR) occurred after 810 days or about 2.2 years of storage. In a 2-way analysis of variance of 108,311 inseminations using semen from 133 bulls maximum fertility occurred when semen had been stored 1260 days or about 3.4 years. In neither case was there a statistically significant decrease in fertility with time up to 1260 days, though the variability did increase greatly. In each case, however, these samples were selected to test, as well as possible, the duration of fertility under nitrogen storage. When a regression analysis was calculated, using semen one year old or less and involving 83,198 inseminations from the semen of 122 bulls, the maximum fertility occurred for semen that was 255 days old. This fertility level stabilized for a time and then declined. These analyses indicate that, with proper selection of bulls and ejaculates and proper handling, some semen can maintain optimum fertility for relatively long periods—up to 3.4 years in one study.

**17-5.3 Handling Techniques.** Until recently, most cows in commercial AI programs were bred by full-time, closely supervised technicians. The sale of semen directly to the herd owner, with insemination to be performed by him

return rate.

ifference	Ref.
2.6	(182) <sup>a</sup>
1.7	(185) <sup>b</sup>
2.7 <sup>a</sup>	(144) <sup>c</sup>

first service insemination 1965 to 1970. Some of seminal collection seminators. The data significantly different the same bull. Fertility use of semen on 5th to 1-day NR rate). In one from 123 bulls maximum about 2.2 years of seminators using semen n had been stored 1260 statistically significant h the variability did in- s were selected to test. trogen storage. When a year old or less and in- 22 bulls, the maximum s fertility level stabilized ; that, with proper selec- : semen can maintain op- years in one study.

cows in commercial AI technicians. The sale of to be performed by him

or by a farm employee, has posed an entirely new set of problems. This is particularly true with the AI of beef cattle, in which an inseminator often breeds a large number of animals on a seasonal basis during a short period of time. This often requires an annual relearning of skills, often with little or no supervision. It is therefore important for the inseminator to review the proper methods of semen handling periodically throughout the year (190). Many of the fertility problems associated with the use of frozen semen are due to improper handling or deposition of the semen.

In many cases, the first mistake comes when the semen is received at the farm or ranch and must be transferred from one storage unit to another. This should take place out of the wind and sunlight. Wind increases the rate at which the temperature of the ampule rises by removing the "layer of cold" that surrounds the ampule. The effect of sunlight on frozen semen is not known, but it is not likely to be beneficial and it could actually be harmful.

Frozen semen is further exposed to elevated temperatures when the semen is removed from storage tanks, when they are opened as inventories are taken, etc. The amount the temperature rises is determined by:

1. length of time exposed,
2. ambient temperature,
3. air circulation,
4. intensity of solar radiation,
5. nitrogen level in the tank, and
6. the height to which the canister is raised above the liquid nitrogen level.

When semen is transferred the exchange should be made quickly into tanks previously filled with nitrogen. It is essential that semen is handled properly, which involves careful temperature control more than anything else, in order to prevent fertility reduction. It is reasonably clear from the work reported by Sullivan (150) (see Figure 17-16), that, as sperm numbers are reduced to obtain maximum use of each superior bull, the storage temperature, exposure times, and so on will become more critical. It has been shown that exposing frozen semen in the neck of a liquid nitrogen tank generated no significant decrease in motility unless the exposure was relatively long (192). The fertility of fluid semen, however, decreases much more rapidly than motility (79) and the same is probably true of frozen semen. A fairly high correlation has been shown (177) between fertility and loss of motility due to exposure of ampules of frozen semen to ambient temperatures. Thus it is extremely important to minimize the number and times of exposures (see Figure 17-17). It is much more harmful to expose ampules of semen to elevated temperatures uninterruptedly for one minute than to expose them for two minutes in 10-second periods, particularly if the ampules are returned to  $-196^{\circ}\text{C}$  between the 10-second exposures.



**17-5.4 Thawing.** It is difficult, if not impossible, to separate the effects of thawing from those of freezing. Theoretically, the faster a specimen is frozen the more rapidly it should be thawed for optimal survival (193). Furthermore, the results obtained from thawing studies may be influenced by other events (43, 128, 167, 193, 194). Interrelationships have been shown between thawing and freezing rates (167) and extenders (128) and other factors, such as glycerol concentrations, may contribute to some of the discrepancies in the literature (194).

Rate of thawing significantly affects the survival of spermatozoa that have been frozen (43, 128, 134, 159, 167, 194). The rate at which thawing occurs depends on the size and shape of the container (ampule, pellet, straw) in which the semen is stored, the composition of the container (plastic, glass), the thawing medium (water, air), the temperature imposed, and the type of thawing system (195).

*Thawing in Glass Ampules.* It has been reported that thawing bovine semen in ampules in water maintained at 38 to 40°C resulted in superior sperm survival

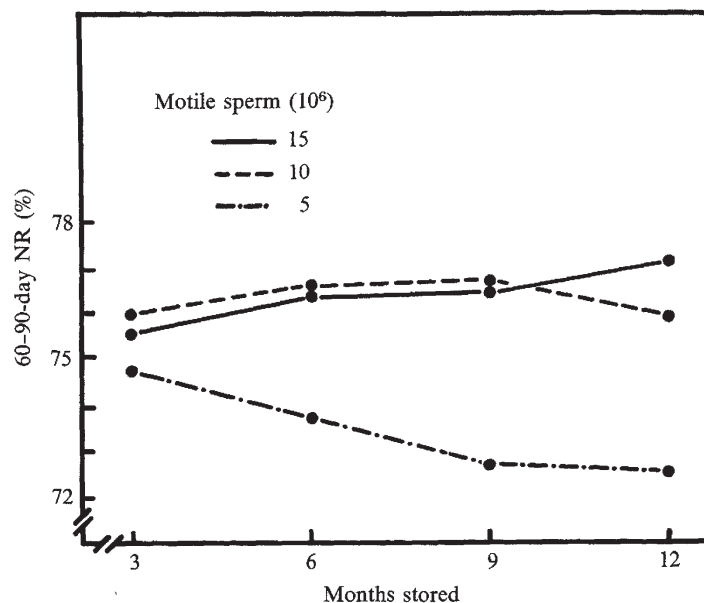
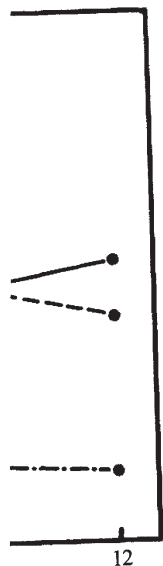


Figure 17-16. The influence of sperm numbers on the fertility of semen stored for up to one year. [Sullivan. Proc. 3rd NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 36-43. 1970.]

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as compared to thawing at lower temperatures (128, 167). Miller and Van-Demark (43), however, using 5-milliliter plastic ampules containing 1 milliliter of extended semen, observed significantly greater motility in samples thawed at 5°C than in those thawed at 38°C; others (134, 194, 196, 197) found no difference in survival between spermatozoa water-thawed at 38 to 40°C and those thawed at 5°C. In general, thawing in air or water at intermediate temperatures of 15 to 22°C resulted in decreased survival (134, 142, 194, 198). It is exceedingly difficult to explain why an intermediate thawing rate is inferior to a slower rate, since it has been postulated (127, 128) that thawing time between -15 and -30°C should be as rapid as possible. Although this may be expected on a theoretical basis, the critical range for freezing may not correspond to a critical range for thawing.

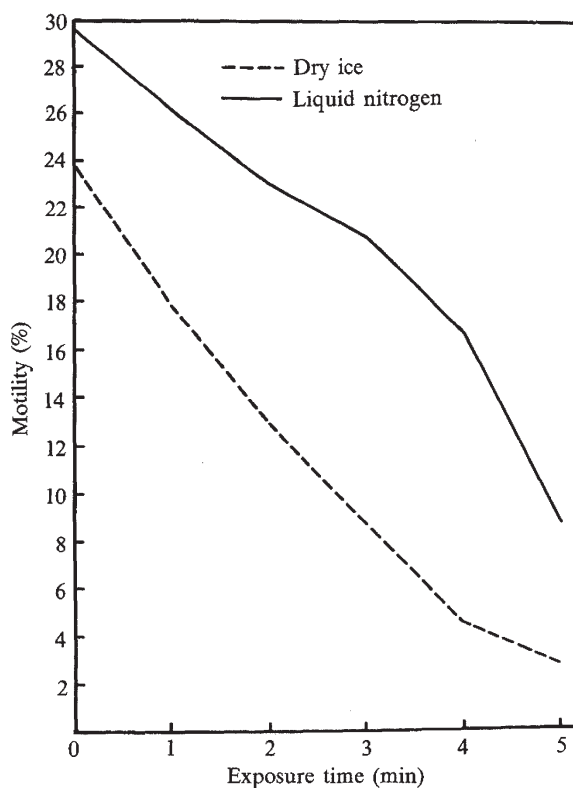


Figure 17-17. The effect of exposing ampules of frozen semen to ambient temperature on percent motility. [From Pickett. *A.I. Digest* 19(2):8-23. 1971.]

The end fertility level is the ultimate test of superiority of any semen handling method. Unfortunately such data on thawing rates are limited. Arnott (142) conducted two studies comparing semen thawed in water at 15 and 30°C, and at 4 and 15°C. A significant difference in 60-90-day percent NR was found in favor of 15°C in the first study, and a 2.0 percent NR difference in favor of thawing at 4°C in the latter study. These latter results agree with those of Pickett et al. (194), who found a difference of 1.26 percentage points in NR between 1 and 15°C thawing in favor of the colder temperature. Dunn et al. (199) observed no difference in NR figures between semen thawed at 40°C and semen thawed at 5°C. In contrast, a limited field trial (198) showed that fast thawing at 40°C and slow thawing at 5°C were both better than thawing at an intermediate temperature of 20°C. The differences were not statistically significant.

Although there is some disagreement on practical methods for thawing, it must be concluded from these data on rates of thawing for ampules containing 1 milliliter of semen, that thawing in water at 40°C or in ice water are the only methods that should be considered. The difficulty of keeping water at 40°C in the field, and the possibility of later cold shock if the ampules are held more than a few seconds at that temperature, eliminate 40°C. Thus thawing in ice water becomes the method of choice.

Current recommendations are to place at least a pint of water in an insulated container constructed so that ampules cannot freeze to the ice cubes or to each other (see Figure 17-18). If ampules freeze together or to ice cubes the rate of thawing could be altered. A typical thawing curve is presented in Figure 17-19. Although it is not known whether a critical temperature range analogous to that during freezing affects thawing survival, it is at this point that the rate of rewarming begins to change (see Figure 17-19). Thus thawing procedure could be critical.

Eight to 10 minutes should be allowed for the ampule to thaw and it should not be disturbed during that time. This recommendation differs from that of Boyd and Hafs (200). They compared motility and fertility of spermatozoa in ampules from which the "ice coat" was removed (fast thawing) to that of ampules allowed to thaw undisturbed in ice water (slow thawing). There were no statistically significant differences in motility or fertility between the methods. The results of two field trials, however, favored the slow thawing technique by 0.6 and 2.0 percentage points in NR, respectively.

From the results presented in Figure 17-20, it is recommended that the semen be used as soon as possible after thawing; if it is not used within one hour it should be discarded. This point is not universally agreed upon, however (201).

*Thawing in Straws.* The principles involved in thawing semen stored in straws should be similar to those that apply to semen stored in ampules. However,

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the heat transfer characteristics of a long, slender plastic container require different thawing techniques. Aamdal and Andersen (159) used a staining technique to evaluate the results of thawing semen in straws. Thawing in water at 4°C, or in water or air at 20°C, was inferior to thawing in water at 35°C; and thawing at 35°C for 30 seconds was inferior to thawing at 75°C for 12 seconds.

French investigators (141, 148, 189) who were responsible for many of the techniques employed with the straw, thaw them in water at 35°C. In contrast, Canadian workers (155) thaw the 0.5-milliliter straw by "rubbing them back

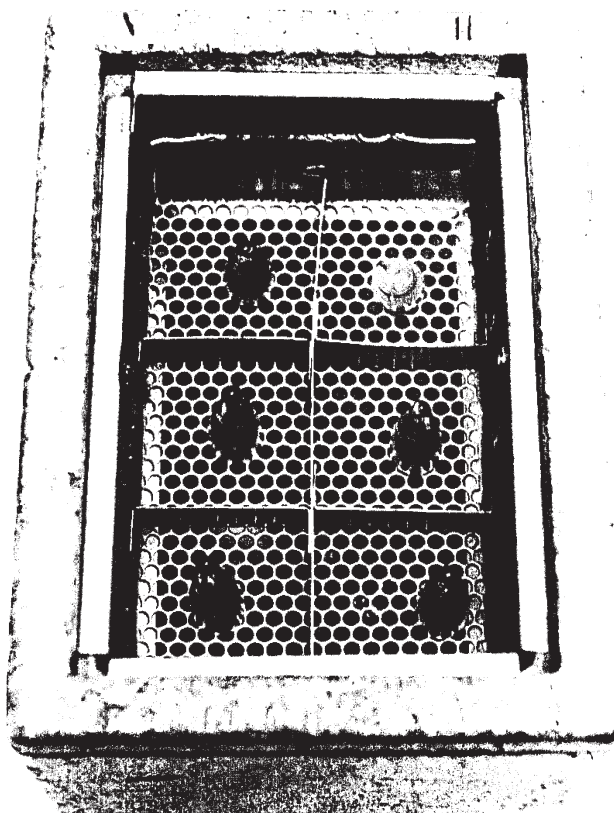


Figure 17-18. A thawing container designed to prevent ampules from freezing together or to ice cubes.

and forth between the palms of the hands.” In a study by Bean (149) 16,000 to 21,000 first service inseminations per treatment were performed with semen in 0.5-milliliter straws thawed in ice water, warm water (30 to 35°C), or air. The 60–90-day NR rates were 71.1, 72.7, and 72.8 percent for thawing in ice water, warm water, and air, respectively. No statistical analyses were reported, but air thawing by wrapping the straw in a paper towel and placing it in the outside pocket of a technician’s coveralls was adopted.

Robbins et al. (202) thawed semen in straws by plunging them into water baths maintained at temperatures of 5, 20, 35, or 75°C. In general, the more rapid thawing rates resulted in better motility and retention of the acrosomal cap (249). These results (202) disagree with the fertility results obtained by Bean (149). Although the ultimate test of a procedure is fertility (141), the results obtained by Robbins et al. (202) conform to the theoretical expectation—the faster semen is frozen the faster it should be thawed.

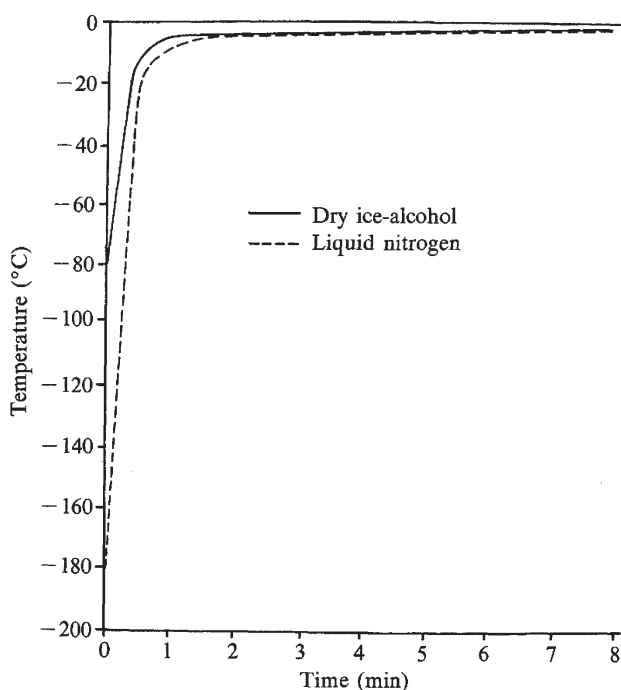
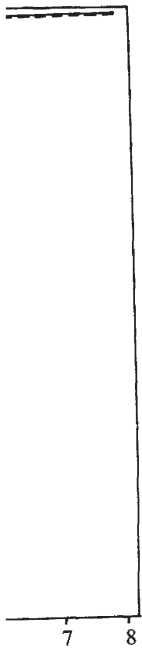


Figure 17-19. Typical thawing curve from  $-79^{\circ}$  and  $-196^{\circ}\text{C}$  for 1.0-milliliter glass ampules of bovine semen in ice water. Thawing rate measured by copper-constantan thermocouple located in the center of the ampule. [From Pickett. *A.I. Digest* 19(2):8–23. 1971.]



by Bean (149) 16,000 performed with semen (30 to 35°C), or air. nt for thawing in ice alyses were reported, and placing it in the

ging them into water In general, the more tion of the acrosomal / results obtained by is fertility (141), the : theoretical expecta- thawed.



96°C for 1.0-milliliter : measured by copper- mpule. [From Pickett.

#### 17-6 VARIATION IN SEMEN QUALITY

The individual bulls producing the semen are a major source of variation in all frozen semen experiments. Among the factors that can contribute to this effect are genetics, age, health, frequency of collection, and degree of sexual preparation (213, 218). Dairy and beef bulls vary in the quality of semen they produce and in the processing techniques needed for optimum fertility (219). Within types the heritability estimates of fertility in bulls vary (203, 204). However, the genetic variance of male fertility is relatively small and the selection pressure necessary to improve inherent breeding efficiency would be very high and could be made at the expense of other economically important traits (205). The major emphasis in commercial AI should therefore probably be directed to methods of obtaining and maintaining semen of greatest possible quality from available animals.

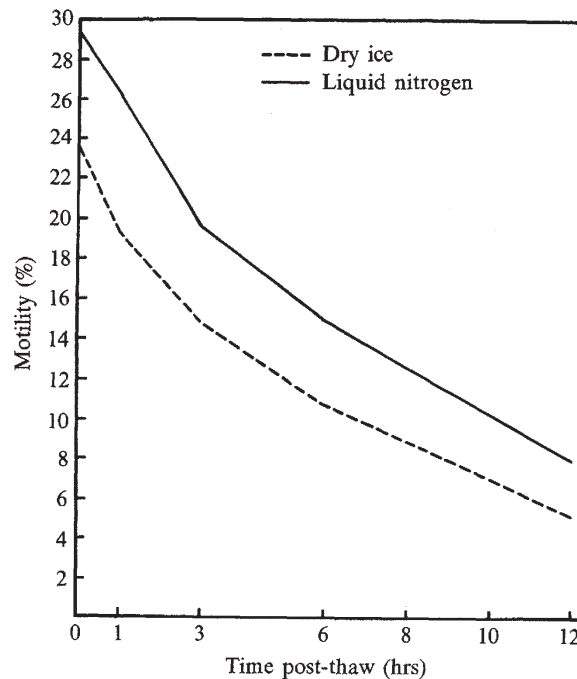


Figure 17-20. The effect of storage time at 5°C after thawing on percent motility of bovine spermatozoa. [From Pickett. *A.I. Digest* 19(2):8-23. 1971.]

The quality and fertility of semen produced declines as a bull ages (206, 207). Theoretically, semen can be collected from bulls at the height of their fertility and then frozen and stored for subsequent use. However, economic considerations and the delays inherent in evaluating and adopting innovations in semen processing and handling may preclude such policies (208).

The ability of semen to be frozen successfully, as indicated by post-thaw motility of several successive ejaculates, is often higher in the second ejaculate than in the first (210) and increases up to the fourth (209), especially if the semen has not been collected frequently on a regular schedule. For collection after 21 to 30 days of sexual rest the fertility of several consecutive ejaculates were not significantly different statistically, though the means favored the second (211). Short intervals between collection periods tend to eliminate the differences in freezability between two consecutive ejaculates (125). Proper sexual preparation has the same effect (212). The differences among ejaculates of the same bull are due to differences in the age distribution of individual sperm cells within ejaculates; this in turn affects the mean chemical composition (71, 72, 214, 215, 217) and resistance to cold shock (216). It is likely that some bulls are by-passed for AI because their semen freezes poorly under routine procedures that could be improved by generating special processing, freezing, and thawing procedures.

The number of cows to be bred each month of the year influences the amount of semen to be collected, processed, and frozen. The breeding of beef cows is confined to a relatively short season, usually in early spring or summer, depending on weather conditions and latitude. Dairy cows are bred more regularly throughout the year. Bulls proven to be genetically exceptional will undoubtedly be collected as frequently as possible, regardless of season. However, young bulls and bulls from which only a limited number of semen units are required should be collected during the time of the year when the semen is most fertile.

Salisbury (183) has presented evidence that at approximately 39° to 40° north latitude the observed seasonal variation in fertility is due primarily to an effect on the bulls of the seasonal differences in climate. The semen used for this study was frozen and stored at -79° to -88°C. The fertility of semen collected from May through October was significantly lower than that of semen collected during the other 6 months (see Table 17-10). These observations of decreased fertility in the summer months have been corroborated (191). In addition, Sullivan and Elliott (220) have found that semen collected in the fall and winter from bulls housed in a standard temperature-controlled environment and stored at -196°C was significantly higher in fertility (76.3 vs 74.2 percent, 60-90-day NR) than semen collected in the spring and summer (see Figure 17-21).

Custom freezing of semen is highly seasonal. Too often an owner will not present a bull for freezing of the semen until shortly before it is needed. This

is a bull ages (206, the height of their However, economic (opting innovations icies (208).

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is not good. Several ejaculates often must be collected before semen is obtained that is suitable for freezing. Waiting until the semen is needed could thus result in costly delays in the breeding schedule or in having to use a bull of less genetic potential.

From the results of these studies (183, 191, 220) it is recommended, economics and genetics notwithstanding, that semen should be collected for freezing during the fall and winter.

*Table 17-10.* The decrease in fertility (mean difference in 167-day nonreturns) between 1 and 2 months' storage and > 6 months' storage at about  $-79^{\circ}\text{C}$  for the months in the year of insemination (cow response) and of semen collection (bull response).<sup>a</sup>

Month	Cow response	Bull response
	Decrease after > 6 mo. storage	Decrease after > 6 mo. storage
	(%)	(%)
January	13.8	7.0
February	13.8	11.8
March	16.6	8.4
April	13.5	11.5
May	12.8	16.8 <sup>b</sup>
June	13.2	16.7 <sup>b</sup>
July	11.3	20.9 <sup>b</sup>
August	14.4	14.2 <sup>b</sup>
September	7.3	21.4 <sup>b</sup>
October	13.1	15.7 <sup>b</sup>
November	14.6	12.8
December	14.7	8.9
Total insemin.	174,307	176,432
Mean decrease	13.3	13.3

<sup>a</sup>From Salisbury. 6th Inter. Congr. Anim. Reprod. and Artif. Insem. Paris. II:1189-1204. 1968 (183).

<sup>b</sup>P = 0.01 that difference from mean is not due to chance.

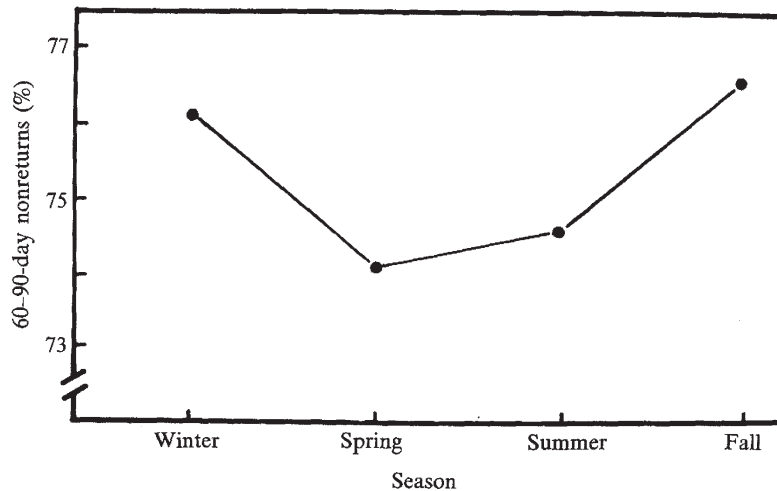
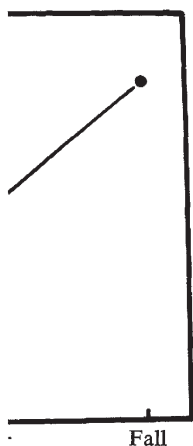


Figure 17-21. The effect of season on 60- to 90-day % NR [From Sullivan and Elliott. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. I:329-332. 1968.]

#### 17-7 FREEZE-DRYING

The long term storage and subsequent shipment of spermatozoa at  $-196^{\circ}\text{C}$  is costly and often inconvenient. If semen could be dried, placed in small packages, and stored at room temperature and still retain its fertility monumental savings could be realized. Attempts to recover motile spermatozoa following freeze-drying have met with variable success (9, 221-227, 238, 250). Meryman and Kafig (221) performed a single successful insemination with freeze-dried bull sperm, but they were unable to repeat this success (222). More recently, Graham et al. (250) have reported another successful insemination with freeze-dried sperm. It is believed that earlier attempts to freeze-dry semen failed because of the presence of glycerol in the extending medium. It is also true that the studies reporting successful recovery of motility upon reconstitution have been under conditions in which the semen samples have not been taken to a steady state of dryness (0.5 to 2.5 percent moisture) but have a higher moisture content. Very little success has been reported after the freeze-dried semen has been stored for a while; this may be related to the fact that when sperm cells are taken to a steady state of dryness motility cannot be recovered upon reconstitution with water. Reliable procedures for preserving bull spermatozoa by freeze-drying are not currently available, and much time and research are still necessary before the procedure will be of practical use.



From Sullivan and  
Paris. I:329-332.

spermatozoa at  $-196^{\circ}\text{C}$  and, placed in small vials, its fertility mononucleated spermatozoa (221-227, 238, 250). Successful insemination with spermatozoa (222). More successful insemination to freeze-dry semen in a dry medium. It is also possible upon reconstitution (moisture) but have attributed after the freeze-drying to the fact that fertility cannot be restored for preserving spermatozoa, and much time can be of practical use.

The basic principles and techniques set forth in this chapter are intended to provide guidelines for obtaining maximum reproductive efficiency in a frozen semen program. Recommendations have been made according to available research data. While these techniques should provide optimal results under most circumstances, an awareness of the special needs of spermatozoa from individual bulls, and the flexibility required to meet these needs, should accompany every AI program.

## REFERENCES

- (1) Mazur, P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J. Gen. Physiol.* 47:347-369. 1963.
- (2) Meryman, H. T. Mechanics of freezing in living cells and tissues. *Science* 124:515-521. 1956.
- (3) ———. Preservation of living cells. *Fed. Proc.* 22:81-89. 1963.
- (4) ———. Absence of unfrozen freezable water in rapidly frozen red cells. *Cryobiology* 7:252-255. 1971.
- (5) Leibo, S. P., J. Farrant, P. Mazur, M. G. Hanna, Jr., and L. H. Smith. Effects of freezing on marrow stem cell suspensions: Interactions of cooling and warming rates in the presence of PVP, sucrose, or glycerol. *Cryobiology* 6:315-332. 1970.
- (6) Smith, A. U., C. Polge, and J. Smiles. IX. Microscopic observation of living cells during freezing and thawing. *J. Roy. Microsc. Soc.* 71:186-195. 1951.
- (7) Smith, A. U., and J. Smiles. XI. Microscopic studies of mammalian tissue during cooling to and rewarming from  $-79^{\circ}\text{C}$ . *J. Roy. Microsc. Soc.* 73:134-139. 1953.
- (8) Farrant, J. Mechanism of cell damage during freezing and thawing and its prevention. *Nature* 205:1284-1287. 1965.
- (9) Sherman, J. K. Freezing and freeze-drying of bull spermatozoa. *Amer. J. Physiol.* 190:281-286. 1957.
- (10) Mazur, P., and J. J. Schmidt. Interactions of cooling velocity, temperature, and warming velocity on the survival of frozen and thawed yeast. *Cryobiology* 5:1-17. 1968.
- (11) Sherman, J. K. Survival of higher animal cells after the formation and dissolution of intracellular ice. *Anat. Rec.* 144:171-189. 1962.
- (12) Lovelock, J. E. The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. *Biochim. Biophys. Acta.* 11:28-36. 1953.

- (13) Lovelock, J. E., and C. Polge. The immobilization of spermatozoa by freezing and thawing and the protective action of glycerol. *Biochem. J.* 58:618-622. 1954.
- (14) Yassen, A. M., and R. H. Foote. Freezability of bovine spermatozoa in Tris-buffered yolk extenders containing different levels of Tris, sodium, potassium, and calcium ions. *J. Dairy Sci.* 50:887-892. 1967.
- (15) van den Berg, L., and F. S. Soliman. Composition and pH changes during freezing of solutions containing calcium and magnesium phosphate. *Cryobiology* 6:10-14. 1969.
- (16) ———. Effect of glycerol and dimethyl sulfoxide on changes in composition and pH of buffer salt solutions during freezing. *Cryobiology* 6:93-97. 1969.
- (17) Saacke, R. G., and J. O. Almquist. Ultrastructure of bovine spermatozoa freeze-thawed with and without glycerol. *J. Dairy Sci.* 45:674. 1962.
- (18) Rapatz, G. L. What happens when semen is frozen. Proc. First NAAB Tech. Conf. Artif. Insem. and Bovine Reprod. pp. 45-56. 1966.
- (19) Pickett, B. W., and R. J. Komarek. Effect of cold shock and freezing on loss of lipid from spermatozoa. *J. Dairy Sci.* 50:753-757. 1967.
- (20) Graham, E. F., and M. M. Pace. Some biochemical changes in spermatozoa due to freezing. *Cryobiology* 4:75-84. 1967.
- (21) Fishbein, W. N., and R. E. Stowell. Studies on the mechanism of freezing damage to mouse liver using a mitochondrial enzyme assay. I. Temporal localization of the injury phase during slow freezing. *Cryobiology* 4:283-289. 1968.
- (22) Litvan, G. G. Mechanism of cryoinjury in biological systems. *Cryobiology* 9:182-191. 1972.
- (23) Farrant, J. Pharmacological actions and toxicity of dimethyl sulphoxide and other compounds which protect smooth muscle during freezing and thawing. *J. Pharm. Pharmacol.* 16:472-483. 1964.
- (24) Stein, W. D. Spontaneous and enzyme-induced dimer formation and its role in membrane permeability. II. The mechanism of movement of glycerol across the human erythrocyte membrane. *Biochim. Biophys. Acta.* 59:47-65. 1962.
- (25) Amann, R. P., and J. O. Almquist. Freezing of bovine semen. II. Effect of milk solids level, glycerol level, and fructose on freezability of bull spermatozoa in reconstituted and fresh skim-milk diluents. *J. Dairy Sci.* 40:1542-1549. 1957.
- (26) Saroff, J., and J. P. Mixner. The relationship of egg yolk and glycerol content of diluters and glycerol equilibration time to survival of bull spermatozoa after low temperature freezing. *J. Dairy Sci.* 38:292-297. 1955.
- (27) Steinbach, J., and R. H. Foote. Osmotic pressure and pH effects on survival of frozen bovine spermatozoa. *J. Dairy Sci.* 50:205-213. 1967.
- (28) White, I. G. Metabolism of glycerol and similar compounds by bull spermatozoa. *Amer. J. Physiol.* 189:307-310. 1957.



spermatozoa by freezing  
58:618-622. 1954.  
spermatozoa in Tris-  
dium, potassium.

I changes during  
thawing. *Cryobiology*

as in composition  
6:93-97. 1969.

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-289. 1968.

systems. *Cryobiology*

ethyl sulphoxide and  
freezing and thawing.

nation and its role  
of glycerol across  
59:47-65. 1962.

n. II. Effect of milk  
spermatozoa in  
10:1542-1549. 1957.  
and glycerol content  
of spermatozoa after

effects on survival  
1967.

of bull spermatozoa.

- (29) Pickett, B. W., and C. P. Merilan. Localization of glycerol-1-C<sup>14</sup> in bovine spermatozoa by an autoradiographic technique. *Missouri Agr. Exp. Sta. Res. Bull.* 644. 1957.
- (30) Berndtson, W. E., and R. H. Foote. Bovine sperm cell volume at various intervals after addition of glycerol at 5°C. *Cryobiology* 9:29-33. 1972.
- (31) Sherman, J. K. Questionable protection by intracellular glycerol during freezing and thawing. *J. Cell. and Comp. Physiol.* 61:67-84. 1963.
- (32) Nagase, H., S. Yamashita, and S. Irie. Protective effects of sugars against freezing injury of bull spermatozoa. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. II:1111-1113. 1968.
- (33) Lohmann, W., C. F. Fowler, A. J. Moss, Jr., and W. H. Perkins. Some remarks about the effect of glycerol on cells during freezing and thawing: Electron-spin resonance investigations concerning this effect. *Experientia* 20:290-293. 1964.
- (34) Berndtson, W. E., and R. H. Foote. The survival of frozen bovine spermatozoa following minimum exposure to glycerol. *Cryobiology* 5:398-402. 1969.
- (35) ———. The freezability of spermatozoa after minimal pre-freezing exposure to glycerol or lactose. *Cryobiology* 9:57-60. 1972.
- (36) Sherman, J. K. Low temperature research on spermatozoa and eggs. *Cryobiology* 1:103-129. 1964.
- (37) VanDemark, N. L., W. J. Miller, W. C. Kinney, Jr., C. Rodriguez, and M. E. Friedman. Preservation of bull semen at sub-zero temperatures. *Illinois Agr. Exp. Sta. Bull.* 621. 1957.
- (38) Smith, A. U. Effects of low temperature on living cells and tissues. *Biological applications of freezing and drying.* (R. J. C. Harris, ed.) 1-62. Academic Press, New York. 1954.
- (39) Persidsky, M., and V. Richards. Mode of protection with polyvinylpyrrolidone in freezing of bone marrow. *Nature* 196:585-586. 1962.
- (40) Mazur, P., J. Farrant, S. P. Leibo, and E. H. Y. Chu. Survival of hamster tissue culture cells after freezing and thawing: Interactions between protective solutes and cooling and warming rates. *Cryobiology* 6:1-9. 1969.
- (41) Meryman, H. T. Cryoprotective agents. *Cryobiology* 8:173-183. 1971.
- (42) Foote, R. H. Water: The chief ingredient in semen extenders. *A.I. Digest.* 12(3):8. 1964.
- (43) Miller, W. J., and N. L. VanDemark. The influence of glycerol level, various temperature aspects and certain other factors on the survival of bull spermatozoa at sub-zero temperatures. *J. Dairy Sci.* 37:45-51. 1954.
- (44) Nagase, H., T. Niwa, S. Yamashita, and S. Irie. Deep freezing bull semen in concentrated pellet form. II. Protective action of sugars. 5th Inter. Congr. Animal Reprod. and Artif. Insem. Trento. IV: 498-502. 1964.

- (45) O'Dell, G. D., and V. Hurst. The effect of glycerol equilibration time on the freezing of bovine spermatozoa in egg yolk-sodium citrate and skimmilk semen extenders. *J. Dairy Sci.* 39:1156-1160. 1956.
- (46) Steinbach, J., and R. H. Foote. Post-thaw survival of bovine spermatozoa frozen by different methods in buffered-yolk and skimmilk extenders. *J. Dairy Sci.* 47:909-915. 1964.
- (47) Roussel, J. D., M. A. Pinero, and T. E. Patrick. Freezability and fertility of bovine semen frozen with L.S.U. extender No. 1. *A.I. Digest.* 19(3):14-15. 1971.
- (48) Martin, I. C. A. Effects of rate of cooling to 5°C, storage at 5°C, glycerol concentration, sodium chloride, fructose and glycine on the revival of deep-frozen bull spermatozoa. *J. Agr. Sci.* 64:425-432. 1965.
- (49) Blackshaw, A. W. The effect of equilibration and the addition of various sugars on the revival of spermatozoa from -79°C. *Aust. Vet. J.* 31:124-128. 1955.
- (50) Cragle, R. G., R. M. Myers, R. K. Waugh, J. S. Hunter, and R. L. Anderson. The effects of various levels of sodium citrate, glycerol, and equilibration time on survival of bovine spermatozoa after storage at -79°C. *J. Dairy Sci.* 38:508-514. 1955.
- (51) Nagase, H., E. F. Graham, and T. Niwa. Pelleted semen: the effect of glycerol level and composition of thawing solution on fertility of bovine spermatozoa. 5th Inter. Congr. Animal Reprod. and Artif. Insem. Trento. IV:404-409. 1964.
- (52) Rajamannan, A. H. J., E. F. Graham, and F. Smith. The effect of a new extender incorporating dried egg yolk, on the fertility of bovine spermatozoa. 5th Inter. Congr. Animal Reprod. and Artif. Insem. Trento. IV:392-397. 1964.
- (53) Choong, C. H., and R. G. Wales. The effects of glycerol addition and equilibration on the revival of bull spermatozoa frozen in reconstituted skim milk. *Res. Vet. Sci.* 5:228-236. 1964.
- (54) O'Dell, W. T., and J. O. Almquist. Freezing bovine semen. I. Techniques for freezing bovine spermatozoa in milk diluents. *J. Dairy Sci.* 40:1534-1541. 1957.
- (55) Graham, E. F., B. G. Crabo, and K. I. Brown. Effect of some zwitter ion buffers on the freezing and storage of spermatozoa. I. Bull. *J. Dairy Sci.* 55:372-378. 1972.
- (56) Jones, R. C., and R. H. Foote. Effect of osmolality and phosphate, 'Tris', 'TES', 'MES', and 'HEPES' hydrogen ion buffers on the motility of bull spermatozoa at 37 or 5°C. *Aust. J. Biol. Sci.* 25:1047-1055. 1972.
- (57) Phillips, P. H. Preservation of bull semen. *J. Biol. Chem.* 130: 415. 1939.
- (58) Hafs, H. D., and F. I. Elliott. The effect of methods of adding egg yolk and monosaccharides on the survival of frozen bull spermatozoa. *J. Dairy Sci.* 38:811-815. 1955.
- (59) Sullivan, J. J., and J. P. Mixner. Effects of method of egg yolk addition and of glycerol equilibration time upon post-thawing motility and metabolic activity of frozen bull semen. *J. Dairy Sci.* 46:463-467. 1963.

tion time on the  
skim milk semen

spermatozoa frozen  
extenders. *J. Dairy Sci.*

fertility of bovine  
4-15. 1971.

-5°C. glycerol con-  
centration of deep-frozen

effect of various sugars  
31:124-128. 1955.

1 R. L. Anderson.  
equilibration time on  
*Dairy Sci.* 38:508

effect of glycerol  
on bovine spermatozoa.  
IV:404-409. 1964.

effect of a new extender  
on spermatozoa. 5th Inter.  
97. 1964.

equilibration and equilibra-  
tion of skim milk. *Rev.*

1. I. Techniques for  
10:1534-1541. 1957.

effect of zwitter ion buffers  
*Dairy Sci.* 55:372

effect of phosphate, 'Tris', 'TES'.  
on bull spermatozoa

130: 415. 1939.

effect of adding egg yolk and  
*J. Dairy Sci.* 38:811-

effect of yolk addition and of  
on metabolic activity

- (60) Kampschmidt, R. F., D. T. Mayer, and H. A. Herman. Lipid and lipoprotein constituents of egg yolk in the resistance and storage of bull spermatozoa. *J. Dairy Sci.* 36:733-742. 1953.
- (61) Bialy, G., T. M. Ludwick, E. A. Hess, and F. Ely. Influence of lipoprotein on the freezing of bovine spermatozoa. *J. Dairy Sci.* 40:1189-1192. 1957.
- (62) Gebauer, M. R., B. W. Pickett, R. J. Komarek, and W. S. Gaunya. Motility of bovine spermatozoa extended in "defined" diluents. *J. Dairy Sci.* 53:817-823. 1970.
- (63) Lanz, R. N., B. W. Pickett, and R. J. Komarek. Effect of lipid additives on pre- and post-freeze survival of bovine spermatozoa. *J. Dairy Sci.* 48:1692-1697. 1965.
- (64) Martin, I. C. A. Effects of lecithin, egg-yolk, fructose and period of storage at 5°C on bull spermatozoa deep-frozen to -79°C. *J. Reprod. Fert.* 6:441-449. 1963.
- (65) O'Shea, T., and R. G. Wales. Effect of casein, lecithin, glycerol, and storage at 5°C on diluted ram and bull semen. *Aust. J. Biol. Sci.* 19:871-882. 1966.
- (66) Choong, C. H., and R. G. Wales. The effect of cold shock on spermatozoa. *Aust. J. Biol. Sci.* 15:543-551. 1962.
- (67) Nagase, H., and E. F. Graham. Pelleted semen: Comparison of different extenders and processes on fertility of bovine spermatozoa. 5th Inter. Congr. Animal Reprod. and Artif. Insem. Trento. IV:387-391. 1964.
- (68) Page, R. D. Dimethyl sulfoxide as a freezing additive for bovine semen. M.S. Thesis. University of Connecticut. Storrs. 1968.
- (69) Page, R. D., M. R. Gebauer, W. H. Snedeker, and W. S. Gaunya. Dimethyl sulfoxide as a freezing additive for bovine semen. *J. Dairy Sci.* 51:949-950. 1968.
- (70) Snedeker, W. H., and W. S. Gaunya. Dimethyl sulfoxide as a cryoprotective agent for freezing bovine semen. *J. Animal Sci.* 30:953-956. 1970.
- (71) Salisbury, G. W., C. B. Knodt, and R. W. Bratton. The freezing point depression of bull semen and its relation to the diluter problem. *J. Animal Sci.* 7:283-290. 1948.
- (72) Seidel, G. E., Jr., and R. H. Foote. Influence of semen collection techniques on composition of bull seminal plasma. *J. Dairy Sci.* 52:1080-1084. 1969.
- (73) Rao, P. S., J. D. Sikes, and C. P. Merilan. Freezing-point depression and viability of bovine sperm during freezing and storage. *Fert. Ster.* 19:129-136. 1968.
- (74) Martin, I. C. A. Revival of bull spermatozoa after deep-freezing to -79°C following equilibration in diluents of various tonicities. *J. Reprod. Fert.* 6:433-439. 1963.
- (75) Martin, I. C. A. The influence of diluents and processing time after ejaculation on the revival of deep-frozen bull spermatozoa. *J. Agr. Sci.* 61:55-63. 1963.

- (76) Blackshaw, A. W. The effects of pH, tonicity and temperature of glycerolisation on the revival of ram and bull spermatozoa after freezing to  $-79^{\circ}\text{C}$ . *Aust. Vet. J.* 36:376-379. 1960.
- (77) Yassen, A. M., and R. H. Foote. Relationship of ion concentration to the critical temperatures during freezing of bull spermatozoa. *J. Dairy Sci.* 50:893-895. 1967.
- (78) Foote, R. H. Influence of extender, extension rate, and glycerolating technique on fertility of frozen bull semen. *J. Dairy Sci.* 53:1478-1482. 1970.
- (79) Pickett, B. W., L. F. Korzun, and R. C. Martig. Comparison between milk and CUE as extenders for bull semen. Conn. (Storrs) Agr. Exp. Sta. Bull. 373. 1962.
- (80) Bean, B. H., B. W. Pickett, and R. C. Martig. Influence of freezing methods, extenders and storage temperatures on motility and pH of frozen bovine semen. *J. Dairy Sci.* 46:145-149. 1963.
- (81) O'Dell, W. T., J. O. Almquist, and R. J. Flipse. Metabolism of bovine semen. V. Effect of various diluents on the uptake and metabolic utilization of glycerol- $1\text{-C}^{14}$  by bovine spermatozoa. *J. Dairy Sci.* 42:83-88. 1959.
- (82) Mann, T. Fructose content and fructolysis in semen. Practical application in the evaluation of semen quality. *J. Agr. Sci.* 38:323-331. 1948.
- (83) Curtis, P. G., A. D. Forteach, and C. Polge. Survival of bull sperm frozen in milk diluents containing varying concentrations of glycerol and fructose. 4th Inter. Congr. Animal Reprod. and Artif. Insem. The Hague. IV:952-956. 1961.
- (84) Martin, I., and C. W. Emmens. Factors affecting the fertility and other characteristics of deep-frozen bull semen. *J. Endocrin.* 17:449-455. 1958.
- (85) Martin, I. C. A., and C. W. Emmens. Effects of time of equilibration and addition of fructose on the survival and fertility of bull spermatozoa deep-frozen to  $-79^{\circ}\text{C}$ . *J. Reprod. Fert.* 2:404-410. 1961.
- (86) Kirton, K. T., and H. D. Hafs. Sperm capacitation by uterine fluid or beta-amylase *in vitro*. *Science* 150:618-619. 1965.
- (87) Hafs, H. D., L. J. Boyd, S. Cameron, and F. Dombroske. Fertility of bull semen with amylase and polymixin. *A.I. Digest.* 17(11):8. 1969.
- (88) Kirton, K. T., L. J. Boyd, and H. D. Hafs. Fertility of bull semen with added amylase. *J. Dairy Sci.* 51:1426-1428. 1968.
- (89) Sullivan, J. J., and F. I. Elliott. Fertility of frozen bull semen with added amylase. *A.I. Digest.* 19(3):10-11. 1971.
- (90) Hafs, H. D., L. J. Boyd, S. Cameron, W. L. Johnson, and A. G. Hunter. Fertility of bull semen with added beta-glucuronidase. *J. Dairy Sci.* 54:420-422. 1971.
- (91) Hafs, H. D. Capacitation of spermatozoa and fertility of bull sperm with added amylase. Proc. Second NAAB. Tech. Conf. Artif. Insem. and Reprod. pp. 23-26. 1968.

temperature of glycerolisation  
ing to  $-79^{\circ}\text{C}$ . *Aust. Vet. J.*

concentration to the critical  
*airy Sci.* 50:893-895. 1967.  
and glycerolating technique  
78-1482. 1970.

comparison between milk and  
. *Exp. Sta. Bull.* 373. 1962.  
ence of freezing methods,  
H of frozen bovine semen.

metabolism of bovine semen.  
colic utilization of glycerol-  
3. 1959.

Practical application in the  
.. 1948.

of bull sperm frozen in milk  
col and fructose. 4th Inter.  
ue. IV:952-956. 1961.

of fertility and other charac-  
449-455. 1958.

of equilibration and addi-  
spermatozoa deep-frozen to

n by uterine fluid or beta-

oske. Fertility of bull semen  
1969.

y of bull semen with added

1 semen with added amylase.

, and A. G. Hunter. Fertility  
*Dairy Sci.* 54:420-422. 1971.

ity of bull sperm with added  
*Insem. and Reprod.* pp. 23

- (92) Benson, R. W., T. J. Sexton, B. W. Pickett, J. J. Lucas, and M. R. Gebauer. Influence of processing techniques and dilution rates on survival of frozen bovine spermatozoa. *Conn. (Storrs) Agr. Exp. Sta. Res. Rep.* 28. 1968.
- (93) Salisbury, G. W., and F. H. Flerchinger. Aging phenomena in spermatozoa. I. Fertility and prenatal losses with use of liquid semen. *J. Dairy Sci.* 50:1675-1678. 1967.
- (94) Almquist, J. O., R. J. Flipse, and D. L. Thacker. Diluters for bovine semen. IV. Fertility of bovine spermatozoa in heated homogenized milk and skim milk. *J. Dairy Sci.* 37:1303-1307. 1954.
- (95) Almquist, J. O. Diluents for bovine semen. XI. Effect of glycerol on fertility and motility of spermatozoa in homogenized milk and skim milk. *J. Dairy Sci.* 45:911-916. 1962.
- (96) Almquist, J. O. Efficient, low cost results using milk-glycerol diluent. *A.I. Digest.* 7(8):11. 1959.
- (97) Foote, R. H., L. C. Gray, D. C. Young, and H. O. Dunn. Fertility of bull semen stored up to four days at  $5^{\circ}\text{C}$  in 20% egg yolk extenders. *J. Dairy Sci.* 43:1330-1334. 1960.
- (98) Pickett, B. W., W. A. Cowan, A. K. Fowler, and D. G. Gosslee. A comparison of motility and fertility of bull semen extended in egg yolk-citrate-glycerol and skim milk-egg yolk-glycerol. *A.I. Digest.* 8(2):6. 1960.
- (99) Blackshaw, A. W., C. W. Emmens, I. Martin, and J. Heyting. The preparation of deep frozen semen. *A.I. Digest.* 5(1):6-8. 1957.
- (100) Emmens, C. W., and I. Martin. The controlled introduction of deep-freeze techniques into artificial insemination. 3rd Inter. Congr. Animal Reprod. and Artif. Insem. Cambridge. III:18-21. 1956.
- (101) ———. The effects of equilibration period and sugar content of the diluent on the survival and fertility of bull spermatozoa deep-frozen to  $-79^{\circ}\text{C}$ . 4th Inter. Congr. Animal Reprod. and Artif. Insem. The Hague. IV:964-968. 1961.
- (102) Graham, E. F., W. E. Erickson, and N. D. Bayley. Effect of glycerol equilibration on frozen bovine spermatozoa. *J. Dairy Sci.* 40:510-515. 1957.
- (103) Martig, R. C., and J. O. Almquist. Effect of glycerol equilibration time on fertility of bull spermatozoa frozen in homogenized milk-fructose diluent. *A.I. Digest.* 14(5):8-9. 1966.
- (104) Elliott, F. I., D. M. Murphy, D. E. Bartlett, and R. A. Kubista. The use of polymyxin B sulfate with dihydrostreptomycin and penicillin for the control of *Vibrio fetus* in a frozen semen process. *A.I. Digest.* 10(2):10-13. 1962.
- (105) Norman, C., J. L. McCoy, H. A. Wilson, I. D. Porterfield, and E. Goldberg. Elimination of *Vibrio fetus* from suspensions of bovine sperm stored at low temperatures. *Amer. J. Vet. Res.* 23:1019-1022. 1962.



- (106) Rajamannan, A. H. J., E. F. Graham, and M. K. L. Schmehl. Effects of holding on bovine semen. *A.I. Digest*. 19(4):6. 1971.
- (107) Sullivan, J. J., F. I. Elliott, D. E. Bartlett, D. M. Murphy, and C. D. Kuzdas. Further studies on use of polymyxin B sulfate with dihydrostreptomycin and penicillin for control of *Vibrio fetus* in a frozen semen process. *J. Dairy Sci.* 49:1569-1571. 1966.
- (108) VanDemark, N. L. Extension of semen before and after cooling and its effect on fertility. Illinois Agr. Exp. Sta. Ann. Proj. Rep. 1950.
- (109) Foote, R. H., and R. W. Bratton. The fertility of bovine semen cooled with and without the addition of citrate-sulfanilamide-yolk extender. *J. Dairy Sci.* 32:856-861. 1949.
- (110) Erickson, W. E. East Central Breeders Assn. Coop., P.O. Box 191, Waupun. Wisconsin 53963. Personal communication.
- (111) Report of the semen preservation committee. Proc. 11th Ann. Conv. NAAB. pp. 91-95. 1958.
- (112) Willett, E. L., and G. W. Salisbury. The effect of various diluters, cooling rate, temperature of storage, and some other factors, on the livability of spermatozoa in stored samples of bull semen. Cornell Univ. Agr. Exp. Sta. Mem. 249. 1942.
- (113) McFee, A. F., and E. W. Swanson. The influence of cooling rates and storage temperatures on sperm activity. *A.I. Digest*. 8(10):12-14. 1960.
- (114) McFee, A. F. Effects of various cooling methods on spermatozoan livability. *J. Dairy Sci.* 42:931. 1959.
- (115) Mayer, D. T. The chemistry and certain aspects of the metabolic activities of mammalian spermatozoa. Michigan State Univ. Cent. Sympos. Rep. Reprod. and Infert. pp. 45-53. 1955.
- (116) Blackshaw, A. W. The prevention of temperature shock of bull and ram semen. *Aust. J. Biol. Sci.* 7:573-582. 1954.
- (117) Blackshaw, A. W., and G. W. Salisbury. Factors influencing metabolic activity of bull spermatozoa. II. Cold-shock and its prevention. *J. Dairy Sci.* 40:1099-1106. 1957.
- (118) Quinn, P. J., S. Salamon, and I. G. White. Effect of osmotic pressure and temperature gradients on cold shock in ram and bull spermatozoa. *Aust. J. Biol. Sci.* 21:133-140. 1968.
- (119) Wales, R. G., and I. G. White. The susceptibility of spermatozoa to temperature shock. *J. Endocrin.* 19:211-220. 1959.
- (120) Quinn, P. J., and I. G. White. The effect of cold shock and deepfreezing on the concentration of major cations in spermatozoa. *J. Reprod. Fert.* 12:263-270. 1966.
- (121) Salisbury, G. W., E. L. Willett, and J. Seligman. The effect of the method of making semen smears upon the number of morphologically abnormal spermatozoa. *J. Animal Sci.* 1:206-212. 1942.



umehl. Effects of holding

phy, and C. D. Kuzdas.  
lihydrostreptomycin and  
en process. *J. Dairy Sci.*

ter cooling and its effect  
50.

e semen cooled with and  
der. *J. Dairy Sci.* 32:856-

P.O. Box 191, Waupun.

11th Ann. Conv. NAAB.

ous diluters, cooling rate.  
livability of spermatozoa  
Exp. Sta. Mem. 249. 1942.

cooling rates and storage  
-14. 1960.

1 spermatozoan livability.

he metabolic activities of  
it. Sympos. Rep. Reprod.

ck of bull and ram semen.

ncing metabolic activity of  
n. *J. Dairy Sci.* 40:1099-

osmotic pressure and tem-  
matozoa. *Aust. J. Biol. Sci.*

permatozoa to temperature

sk and deepfreezing on the  
*rod. Fert.* 12:263-270. 1966.

he effect of the method of  
biologically abnormal sper-

- (122) Harrison, R. A. P., and I. G. White. Glycolytic enzymes in the spermatozoa and cytoplasmic droplets of bull, boar and ram, and their leakage after shock. *J. Reprod. Fert.* 30:105-115. 1972.
- (123) Lasley, J. F., and D. T. Mayer. A variable physiological factor necessary for the survival of bull spermatozoa. *J. Animal Sci.* 3:129-135. 1944.
- (124) Bialy, G., and V. R. Smith. Cold shock of epididymal spermatozoa. *J. Dairy Sci.* 42:2002. 1959.
- (125) Seidel, G. E., Jr., and R. H. Foote. Influence of semen collection interval and tactile stimuli on semen quality and sperm output in bulls. *J. Dairy Sci.* 52:1074-1079. 1969.
- (126) Mann, T., and C. Lutwak-Mann. Biochemical changes underlying the phenomenon of cold-shock in spermatozoa. *Arch. Sci. Biol.* 39:578-588. 1955.
- (127) Polge, C. The storage of bull semen at low temperatures. *Vet. Rec.* 65:557-559. 1953.
- (128) Blackshaw, A. W. Factors affecting the revival of bull and ram spermatozoa after freezing to  $-79^{\circ}\text{C}$ . *Aust. Vet. J.* 31:238-241. 1955.
- (129) Dunn, H. O., and H. D. Hafs. Extenders and techniques for freezing bovine spermatozoa. *J. Dairy Sci.* 36:577. 1953.
- (130) Clegg, E. D., R. J. Komarek, and B. W. Pickett. Preliminary investigations on glycerol entry into bovine spermatozoa. *J. Dairy Sci.* 48:1709-1711. 1965.
- (131) Ehlers, M. H., J. Rice, J. L. Albright, and R. E. Erb. Equilibration length as related to glycerolating and freezing procedures for semen extended in heated, homogenized milk. *A.I. Digest.* 7(7):10-12. 1959.
- (132) Graham, E. F., D. W. Vogt, and G. R. Fisher. Effect of method of glycerol addition on the fertility of frozen bovine spermatozoa. *J. Dairy Sci.* 41:1553-1556. 1958.
- (133) Crabo, B. G., R. E. Bower, K. I. Brown, E. F. Graham, and M. M. Pace. Extracellular glutamic-oxaloacetic transaminase as a measure of membrane injury in spermatozoa during treatment. *Current Problems in Fertility.* (A. Ingelman-Sundberg and N.-O. Lunell, eds.). 33-38. Plenum Press, New York. 1971.
- (134) O'Dell, W. T., and J. O. Almquist. Techniques for freezing bull spermatozoa in heated milk and preliminary breeding results. *J. Dairy Sci.* 37:652. 1954.
- (135) Hunter, W. K. Glycerolisation and freezing techniques with bull semen. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. II: 1053-1056. 1968.
- (136) Wickersham, E. W., and J. O. Almquist. Use of egg yolk in skim milk-glycerol diluents to improve bovine sperm livability and simplify glycerolation. *J. Dairy Sci.* 42:931. 1959.
- (137) Shannon, P. Artificial Breeding Centre, New Zealand Dairy Production and Marketing Board, Newstead, Hamilton. Personal communication. 1971.

- (138) Berndtson, W. E. Glycerol as a factor in the cryoprotection of bull spermatozoa. Ph.D. Thesis. Cornell University, Ithaca, N.Y. 1971.
- (139) Milk Marketing Board. A preliminary study of deep frozen semen technique at Northampton. Report of the Breed. and Prod. Organization. 6:74-79. 1956.
- (140) Yassen, A. M. Effect of electrolyte concentration, method of glycerolation and aging period on the freezability of bull semen extended with Tris-buffered egg-yolk extender. Ph.D. Thesis. Cornell University, Ithaca, N.Y. 1966.
- (141) Jondet, R. Survival rate and fertilizing ability of frozen bull spermatozoa following 8 and 1 minute exposure to glycerol. 7th Inter. Congr. Animal Reprod. and Artif. Insem. Munich. II: 1371-1382. 1972.
- (142) Arnott, W. J. Problems of artificial breeding of cattle. *Aust. Vet. J.* 37:140-145. 1961.
- (143) Erickson, W. E., and E. F. Graham. Factors affecting the fertility of frozen bovine spermatozoa. *J. Dairy Sci.* 42:520-528. 1959.
- (144) Goffaux, M. Effet du nombre de spermatozoïdes mobiles utilisés par insémination, sur le pouvoir fécondant du sperme congelé de taureaux. *Ann. Zootech.* 14:409-415. 1965.
- (145) Pickett, B. W., R. C. Martig, and W. A. Cowan. Preservation of bovine spermatozoa at -79 and -196°C. *J. Dairy Sci.* 44:2089-2096. 1961.
- (146) Pickett, B. W., R. C. Hall, Jr., J. J. Lucas, and E. W. Gibson. Influence of sperm number on fertility of frozen bovine semen. *J. Dairy Sci.* 47:916-919. 1964.
- (147) Stewart, D. L., and G. H. Bennett. The minimum number of spermatozoa per frozen semen insemination compatible with normal fertility in cattle. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. II:1167-1169. 1968.
- (148) Jondet, R. Contribution to the assessment of the minimal number of frozen spermatozoa necessary to obtain fertilization in the cow. 7th Inter. Congr. Animal Reprod. and Artif. Insem. Munich. II:1443-1448. 1972.
- (149) Bean, B. Laboratory procedures and field trial results with straws. Proc. 4th NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 77-81. 1972.
- (150) Sullivan, J. J. Sperm numbers required for optimum breeding efficiency in cattle. Proc. 3rd NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 36-43. 1970.
- (151) Hafs, H. D., L. J. Boyd, and S. Cameron. Fertility of cattle inseminated with 12, 24 or 35 million sperm in 0.5 or 0.9 milliliter. *J. Dairy Sci.* 53:1693-1696. 1970.
- (152) Cassou, R. La miniaturisation des paillettes. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. II:1009-1012. 1968.
- (153) Milk Marketing Board. Report of the Breed. and Prod. Organization 18:134-135. 1967-68.
- (154) Mixner, J. P. Fertility of bull semen frozen for twelve years. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. II:1095-1098. 1968.

on of bull spermatozoa.

rozen semen technique at  
ization. 6:74-79. 1956.

od of glycerolation and  
l with Tris-buffered egg-  
a, N.Y. 1966.

bull spermatozoa follow-  
igr. Animal Reprod. and

e. *Aust. Vet. J.* 37:140-

ig the fertility of frozen

iles utilisés par insémina-  
taureaux. *Ann. Zootech.*

servation of bovine sper-  
-2096. 1961.

ibson. Influence of sperm  
ry *Sci.* 47:916-919. 1964.

umber of spermatozoa per  
ertility in cattle. 6th Inter.  
167-1169. 1968.

inimal number of frozen  
v. 7th Inter. Congr. Animal  
72.

ilts with straws. Proc. 4th  
7-81. 1972.

reeding efficiency in cattle.  
rod. pp. 36-43. 1970.

cattle inseminated with 12.  
ry *Sci.* 53:1693-1696. 1970.  
er. Congr. Animal Reprod.

Prod. Organization 18:134-

live years. 6th Inter. Congr.  
98. 1968.

- (155) Macpherson, J. W., and P. Penner. A modified straw technique for frozen semen. *A.I. Digest.* 20(2):6-8. 1972.
- (156) Cassou, R. The evolution of the mini-straw. 61 L'Aigle, France. Personal communication.
- (157) Jondet, R. Congelation rapide du sperme de taureau conditionne en paillettes. 5th Inter. Congr. Animal Reprod. and Artif. Insem. Trento. IV:463-468. 1964.
- (158) Carpenter, B. The "Straw" sperm package. *A.I. Digest.* 19(4):8. 1972.
- (159) Aamdal, J., and K. Andersen. Fast thawing of bull semen frozen in straws. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. II:973-976. 1968.
- (160) Hahn, R., B. Cassou, and K. Eibl. Comparative studies of semen freezing techniques using French straws and ampoules (Summary). *Deutsche Tierärztliche Wochenschrift.* 76:346-350. 1969.
- (161) Macpherson, J. W., and G. J. King. Plastic straws for frozen semen. (A field evaluation). *Can. J. Comp. Med. and Vet. Sci.* 30:109-111. 1966.
- (162) Kellgren, H. Select Sires. 1224 Alton-Darby Road, Columbus, Ohio 43228. Personal communication. February 1973.
- (163) Nagase, H., and T. Niwa. Deep freezing bull semen in concentrated pellet form. I. Factors affecting survival of spermatozoa. 5th Inter. Congr. Animal Reprod. and Artif. Insem. Trento. IV:410-415. 1964.
- (164) Luyet, B., and J. Keane, Jr. A critical temperature range apparently characterized by sensitivity of bull semen to high freezing velocity. *Biodynamica* 7:281-292. 1955.
- (165) Larson, G. L., and E. F. Graham. Effects of rapid freezing on progressive motility of semen. *A.I. Digest.* 7(6):6-7. 1959.
- (166) O'Dell, W. T., J. O. Almquist, and L. A. Marsh. Freezing bovine semen. III. Effect of freezing rate on bovine spermatozoa frozen and stored at -79°C. *J. Dairy Sci.* 41:79-89. 1958.
- (167) Bruemmer, J. H., R. W. Eddy, and W. J. Duryea. Temperature control in the low temperature preservation of spermatozoa. *J. Cell. and Comp. Physiol.* 62:113-118. 1963.
- (168) Kennelly, J. J., R. S. Hoyt, R. H. Foote, and R. W. Bratton. Survival rates of rapidly frozen bovine spermatozoa. *J. Dairy Sci.* 43:1140-1146. 1960.
- (169) Rajamannan, A. H. J., and E. F. Graham. Film freezing of semen. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. II:1141-1143. 1968.
- (170) Salamon, S. The survival of ram spermatozoa following pellet freezing below -79°C. *Aust. J. Biol. Sci.* 23:459-468. 1970.
- (171) Clegg, E. D., B. W. Pickett, and E. W. Gibson. Nonmechanical nitrogen vapor freezing of bull semen. Conn. (Storrs) Agr. Exp. Sta. Res. Rep. 5. 1965.
- (172) Rajamannan, A. H. J. Vapour freezing of bull semen. *A.I. Digest.* 14(2):8. 1966.

- (173) Roussel, J. D., H. C. Kellgren, and T. E. Patrick. Bovine semen frozen in liquid nitrogen vapor. *J. Dairy Sci.* 47:1403-1406. 1964.
- (174) Sherman, J. K. Preservation of bull and human spermatozoa by freezing in liquid nitrogen vapour. *Nature* 194:1291-1292. 1962.
- (175) Weseli, D. F., T. M. Ludwick, E. A. Hess, and F. Ely. Variations in quality of liquid and frozen semen at various temperatures. *J. Dairy Sci.* 41:1278-1281. 1958.
- (176) Fowler, A. K., B. W. Pickett, D. G. Gosslee, and W. A. Cowan. Effects of  $-196^{\circ}\text{C}$  and  $-76^{\circ}\text{C}$  storage on the resistance of bull sperm to three repeated freeze-thaw treatments. *J. Dairy Sci.* 44:715-720. 1961.
- (177) Pickett, B. W., W. A. MacDonald, D. G. Gosslee, and W. A. Cowan. Correlation between certain laboratory stress tests and fertility of frozen bovine spermatozoa. *J. Dairy Sci.* 44:1134-1140. 1961.
- (178) Kelly, J. W., and V. Hurst. Evaluation of frozen bovine semen packaged in glass and plastic ampules for 3 methods of storage and shipping. *Amer. J. Vet. Res.* 25:1146-1149. 1964.
- (179) Pickett, B. W., A. K. Fowler, and W. A. Cowan. Effects of continuous and alternating storage temperatures of  $-79^{\circ}\text{C}$  and  $-196^{\circ}\text{C}$  on motility of frozen bull semen. *J. Dairy Sci.* 43:281-283. 1960.
- (180) Sullivan, J. J., and J. P. Mixner. Effects of storage temperature and length of storage time upon the post-thawing motility and metabolic activity of frozen bull semen. *J. Dairy Sci.* 46:850-853. 1963.
- (181) Etgen, W. M., T. M. Ludwick, H. E. Rickard, E. A. Hess, and F. Ely. Use of mechanical refrigeration in preservation of bull semen. *J. Dairy Sci.* 40:774-778. 1957.
- (182) Larson, G. L., and E. F. Graham. Effects of low temperatures in storage of bovine semen. *A.I. Digest.* 6(12):6-8. 1958.
- (183) Salisbury, G. W. Fertilizing ability and biological aspects of sperm storage *in vitro*. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. II:1189-1204. 1968.
- (184) Salisbury, G. W., and R. G. Hart. Gamete aging and its consequences. *Biol. Reprod. Supp.* 2:1-13. 1970.
- (185) Stewart, D. L. Observations on the fertility of frozen semen stored at  $-79^{\circ}\text{C}$  and  $-196^{\circ}\text{C}$ . 5th Inter. Congr. Animal Reprod. and Artif. Insem. Trento. IV:617-623. 1964.
- (186) Salisbury, G. W. Aging phenomena in spermatozoa. III. Effect of season and storage at  $-79$  to  $-88^{\circ}\text{C}$  on fertility and prenatal losses. *J. Dairy Sci.* 50:1683-1689. 1967.
- (187) Clegg, E. D., and B. W. Pickett. Effect of storage at  $-196^{\circ}\text{C}$  on fertility. *A.I. Digest.* 14(3):12-13. 1966.

semen frozen in liquid

spermatozoa by freezing in

. Variations in quality of  
*J. Dairy Sci.* 41:1278-

. Cowan. Effects of -196  
to three repeated freeze-

W. A. Cowan. Correlation  
of frozen bovine spermatozoa.

semen packaged in glass  
stripping. *Amer. J. Vet. Res.*

Effects of continuous and  
on motility of frozen bull

temperature and length of  
metabolic activity of frozen

. Hess, and F. Ely. Use of  
men. *J. Dairy Sci.* 40:774-

temperatures in storage of

aspects of sperm storage  
Artif. Insem. Paris. II:1189-

and its consequences. *Biol.*

semen stored at -79°C  
and Artif. Insem. Trento.

III. Effect of season and  
losses. *J. Dairy Sci.* 50:1683-

at -196°C on fertility. *A.I.*

- (188) Ström, B. Influence upon fertility of bull semen of storage time in liquid nitrogen. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. II:1171-1174. 1968.
- (189) Cassou, R. Evolution des pourcentages de fécondation en fonction de l'âge de la semence. 7th Inter. Congr. Animal Reprod. and Artif. Insem. Munich. II:1421-1425. 1972.
- (190) Graham, E. F. The usefulness of useless data, field tests and responsibilities. Proc. 2nd NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 88-90. 1968.
- (191) Foote, R. H. Aging of spermatozoa during storage in liquid nitrogen. Proc. 4th NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 28-30. 1972.
- (192) Hayden, T. E., Jr., J. W. Kelly, and V. Hurst. The effect of exposure of frozen bull semen to high temperature in the neck of a liquid nitrogen refrigerator (LNR-35 Linde) during semen transfer. South Carolina Agr. Exp. Sta. Dairy Sci. Res. Series No. 41. 1965.
- (193) Mazur, P. Causes of injury in frozen and thawed cells. *Fed. Proc.* 24:175-182. Supp. 15. 1965.
- (194) Pickett, B. W., R. C. Hall, Jr., J. J. Lucas, and E. W. Gibson. Investigations on thawing frozen bovine spermatozoa. *Fert. Ster.* 16:642-651. 1965.
- (195) Pickett, B. W. Factors affecting the utilization of frozen bovine semen for maximum reproductive efficiency. *A.I. Digest.* 19(2):8-23. 1971.
- (196) Brugman, H. H., M. E. Poore, and R. P. Schmidt. Investigation of motility of sperm thawed at different temperatures and time intervals. *J. Dairy Sci.* 41:737-738. 1958.
- (197) Rowson, L. E. A. The storage of bull semen at low temperatures. *Vet. Rec.* 65:559-560. 1953.
- (198) Hafs, H. D., and F. I. Elliott. Effect of thawing temperature and extender composition on the fertility of frozen bull semen. *J. Animal Sci.* 13:958. 1954.
- (199) Dunn, H. O., H. D. Hafs, and G. F. Young. Laboratory and field studies with frozen semen. *J. Animal Sci.* 12:893. 1953.
- (200) Boyd, L. J., and H. D. Hafs. Motility and fertility after rapid and slow thawing of semen in ice water. *A.I. Digest.* 16(12):8. 1968.
- (201) Kelly, J. W., and V. Hurst. Fertility results with frozen bull semen thawed for four and six hours. *J. Amer. Vet. Med. Assoc.* 141:1171-1172. 1962.
- (202) Robbins, R. K., L. E. Gerber, and R. G. Saacke. Influence of thaw rate on maintenance of the acrosomal cap. *J. Animal Sci.* 35:253. 1972.
- (203) Buch, N. C., V. R. Smith, and W. J. Tyler. Bull and line differences in the survival of spermatozoa after freezing and thawing. *J. Dairy Sci.* 39:1712-1716. 1956.
- (204) Shannon, P., and S. R. Searle. Heritability and repeatability of conception rate of bulls in artificial breeding. *J. Dairy Sci.* 45:86-90. 1962.

- (205) Foote, R. H. Inheritance of fertility-facts, opinions, and speculations. *J. Dairy Sci.* 53:936-944. 1970.
- (206) Collins, W. E., E. K. Inskeep, W. H. Dreher, W. J. Tyler, and L. E. Casida. Effect of age on fertility of bulls in artificial insemination. *J. Dairy Sci.* 45:1015-1018. 1962.
- (207) Hahn, J., R. H. Foote, and G. E. Seidel, Jr. Quality and freezability of semen from growing and aged dairy bulls. *J. Dairy Sci.* 52:1843-1848. 1969.
- (208) Bean, B. H. Eastern Artificial Insemination Cooperative, Inc., Ithaca, N.Y. Personal communication. 1972.
- (209) Kinney, W. C., Jr., and N. L. VanDemark. The effect of yolk and citrate levels and stage of maturity on the survival of bull sperm at sub-zero temperatures. *J. Dairy Sci.* 37:650. 1954.
- (210) O'Dell, W. T., J. O. Almquist, and R. P. Amann. Freezing bovine semen. V. Practicability of collecting and freezing large numbers of successive ejaculates. *J. Dairy Sci.* 42:1209-1215. 1959.
- (211) Martig, R. C., J. O. Almquist, and J. Foster. Reproductive capacity of beef bulls. V. Fertility and freezability of successive ejaculates collected by different methods. *J. Animal Sci.* 30:60-62. 1970.
- (212) Branton, C., G. D'Arensbourg, and J. E. Johnston. Semen production, fructose content of semen and fertility of dairy bulls as related to sexual excitement. *J. Dairy Sci.* 35:801-807. 1952.
- (213) Lindahl, P. E., and J. E. Kihlström. Alterations in specific gravity during the ripening of bull spermatozoa. *J. Dairy Sci.* 35:393-402. 1952.
- (214) Masaki, J., and T. Tomizuka. Change in phospholipid content of spermatozoa during maturation and aging. I. Effect of frequency of collection on plasmalogen content of bull and goat spermatozoa. *Bull. of National Institute of Anim. Ind.* No. 9. pp. 53-57. 1965.
- (215) Pickett, B. W., and R. J. Komarek. Lipid and dry weight of bovine seminal plasma and spermatozoa from first and second ejaculates. *J. Dairy Sci.* 50:742-746. 1967.
- (216) White, I. G., and R. G. Wales. The susceptibility of spermatozoa to cold shock. *Inter. J. Fert.* 5:195-201. 1960.
- (217) MacMillan, K. L., C. Desjardins, K. T. Kirton, and H. D. Hafs. Relationship of glycerylphosphorylcholine to other constituents of bull semen. *J. Dairy Sci.* 50:1310-1313. 1967.
- (218) Reid, J. T., G. M. Ward, and R. L. Salsbury. Acid and alkaline phosphatase levels in consecutive semen ejaculates from bulls. *Am. J. Physiol.* 153:235-241. 1948.



- d speculations. *J. Dairy Sci.*
- ler, and L. E. Casida. Effect  
m. *J. Dairy Sci.* 45:1015-
- and freezability of semen  
:1843-1848. 1969.
- erative, Inc., Ithaca, N.Y.
- t of yolk and citrate levels  
at sub-zero temperatures.
- Freezing bovine semen. V.  
rs of successive ejaculates.
- ctive capacity of beef bulls.  
ollected by different meth-
- lemen production, fructose  
ated to sexual excitement.
- specific gravity during the  
102. 1952.
- id content of spermatozoa  
ollection on plasmalogen  
nal Institute of Anim. Ind.
- weight of bovine seminal  
lates. *J. Dairy Sci.* 50:742-
- spermatozoa to cold shock.
- I. D. Hafs. Relationship of  
bull semen. *J. Dairy Sci.*
- and alkaline phosphatase  
*Am. J. Physiol.* 153:235-
- (219) Elliott, F. I. Comparative history of dairy and beef semen production. Proc. 20th Ann. Conv. NAAB pp. 109-115. 1967.
- (220) Sullivan, J. J., and F. I. Elliott. Season and fertility in artificial insemination. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. 1:329-332. 1968.
- (221) Meryman, H. T., and E. Kafig. Survival of spermatozoa following drying. *Nature* 184:470-471. 1959.
- (222) ———. Freeze-drying of bovine spermatozoa. *J. Reprod. Fert.* 5:87-94. 1963.
- (223) Albright, J. L., R. E. Erb, and M. H. Elhers. Freeze-drying bovine spermatozoa. *J. Dairy Sci.* 41:206-207. 1958.
- (224) Bialy, G., and V. R. Smith. Freeze-drying of bovine spermatozoa. *J. Dairy Sci.* 40:739-745. 1957.
- (225) Leidl, W. Experiments in freeze-drying of bull semen. 3rd Inter. Congr. Animal Reprod. and Artif. Insem. Cambridge. III:39-41. 1956.
- (226) Saacke, R. G., and J. O. Almquist. Freeze-drying of bovine spermatozoa. *Nature* 192:995-996. 1961.
- (227) Smith, A. U., and C. Polge. Storage of bull spermatozoa at low temperatures. *Vet. Rec.* 62:115-116. 1950.
- (228) Roussel, J. D., M. A. Pinero, T. E. Patrick, and H. C. Kellgren. Preliminary results on the freezability and fertility of bovine semen with either amylase, dialyzed egg yolk or sodium glutamate. *J. Dairy Sci.* 54:454. 1971.
- (229) O'Hagan, C. O., E. Linford, F. Lawton, D. L. Stewart, and D. R. Westgarth. The influence of beta-amylase and beta-glucuronidase on the fertility of frozen bull semen. *Brit. Vet. J.* 130:292-298. 1974.
- (230) Dimitropoulos, E. The fertility of bull spermatozoa in media with additives of alpha-amylase, thiamine prophyl disulfide or ascorbic acid. Abstr. in *Animal Breeding Abstr.* 39:493. 1971.
- (231) Almquist, J. O. Effect of sperm numbers on fertility of frozen bull spermatozoa in skim milk diluent. *J. Dairy Sci.* 58:420-422. 1975.
- (232) Stewart, D. L. Ministry of Agriculture, Fisheries and Food, Agricultural Development and Advisory Service, Reading Cattle Breeding Centre. Annual Report. 1968.
- (233) ———. Ministry of Agriculture, Fisheries and Food, Agricultural Development and Advisory Service, Reading Cattle Breeding Centre. Annual Report. 1969.
- (234) ———. Ministry of Agriculture, Fisheries and Food, Agricultural Development and Advisory Service, Reading Cattle Breeding Centre. Annual Report. 1973.
- (235) Milk Marketing Board. Report of the Breeding and Production Organization. No. 17. 1966-67.

- (236) Alder, H. C. Storage and transport of semen in cellophane straws, single dosage glass vials, and larger glass vials. Abstr. in *Animal Breeding Abstr.* 30:191. 1962.
- (237) Aamdal, J. A new insemination appliance. Abstr. in *Animal Breeding Abstr.* 35:246. 1967.
- (238) Aamdal, J., O. Lyngset, and O. Filseth. Insemination experiment with new type of insemination equipment for straws. Abstr. in *Animal Breeding Abstr.* 37:56. 1969.
- (239) Morales, J. R. and O. Duverger. Three different techniques for the freezing of cattle semen and their conception results. Abstr. in *Animal Breeding Abstr.* 38:422. 1970.
- (240) Aamdal, J., and K. Andersen. Fast thawing of semen frozen in straws. *Zuchthygiene* 3:22-24. 1968.
- (241) Filseth, O. Conception rates using the French straw. Abstr. in *Animal Breeding Abstr.* 38:594. 1970.
- (242) Linares, I. A. A comparison of the methods of freezing semen in straws and in ampoules for artificial insemination. Abstr. in *Animal Breeding Abstr.* 38:84. 1970.
- (243) Rajamannan, A. H. J. A method of packaging semen—the straw. Proc. 3rd NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 49-51. 1970.
- (244) Mortimer, R. G. Frozen Bovine Spermatozoa: U.S. straws vs ampules. M. S. Thesis. Colorado State University, Fort Collins. 1975.
- (245) Almquist, J. O. Fertility results with straws favorable. *A.I. Digest.* 23(7):7. 1975.
- (246) American Breeders Service (ABS). DeForest, Wisconsin. Personal communication. 1975.
- (247) Pickett, B. W., and W. E. Berndtson. Preservation of bovine spermatozoa by freezing in straws. A review. *J. Dairy Sci.* 57:1287-1301. 1974.
- (248) Pickett, B. W., W. E. Berndtson, and J. J. Sullivan. Influence of seminal additives and packaging systems on fertility of frozen bovine spermatozoa. *J. Animal Sci.* Supp. (in press). 1978.
- (249) Saacke, R. G., R. K. Robbins, and P. T. Chandler. Effect of freezing rate, thaw rate and glycerol level on bovine sperm frozen in straws. Proc. 5th NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 41-46. 1974.
- (250) Graham, E. F., E. V. Larson, and B. G. Crabo. Freezing and freeze-drying bovine spermatozoa. Proc. 5th NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 14-20. 1974.
- (251) Hammond, J., and S. A. Asdell. The vitality of the spermatozoa in the male and female reproductive tracts. *J. Expt. Biol.* 4:155-185. 1926.
- (252) Lee, A. J., G. W. Salisbury, L. J. Boyd, and W. Ingales. *In vitro* aging of frozen bull semen. *J. Dairy Sci.* 60:89-95, 1977.