CHAPTER II

REVIEW OF LITERATURE
II.1. Extenders

II.1.a. Sodium citrate

Since the chance discovery of Polge et al. (1949) on the protective action of glycerol on spermatozoa during freezing, freezing of cattle semen had become a fascination for many biologists, mainly due to its economic advantages. Smith and Polge (1950) obtained good survival rates by dilution of bull semen with 3.9% sodium citrate buffer containing 15% glycerol and by slowly cooling the ampoules by transferring them successively in previously cooled alcohol bath with solid carbon dioxide (dry ice). Polge and Rowson (1952) was successful with this method but modified the procedure further. The semen was diluted with equal volume of egg yolk-citrate buffer, cooled to 5°C, then further diluted with equal volume of glycerol-citrate solution to get a final glycerol concentration of 10%. This was stored in the refrigerator for 18 hours (now referred to as equilibration period) and then frozen slowly over a period of 45 minutes in an alcohol bath to -79°C. After storing at -79°C up to 8 hours the first inseminations resulted in 79% pregnancy in a total of 38 cows. This procedure has become a stepping stone for the innumerable reports of research on the topic. This procedure still stands good, with slight modifications.

Eventhough many diluters were tried for the freezing procedures, the sodium citrate solution still remains to be
the choice of many, probably because of its simplicity.
Kinney and VanDemark (1954) observed that a final concentration of 1.55% to 1.95% of sodium citrate in the diluter gave optimum results. These concentrations were obtained by diluting the semen initially in a diluent containing equal volumes of egg yolk and 2.9% sodium citrate solution and subsequently in an equal volume of 2.9% sodium citrate solution containing 14% glycerol.

Cragle and Myres (1954) and Cragle et al (1955) estimated the optimum level of citrate as 2.9%. Mixner and Wiggins (1957) used a 2.175% solution of sodium citrate for freezing bull semen and obtained comparable fertility results with control semen. Emmens and Martin (1957) used a buffered citrate containing 80 parts of 3% sodium citrate solution and 20 parts of 0.1 M sodium phosphate buffer, to give a pH of 7.0.

Blackshaw (1960) had tried 3 levels of sodium citrate (3.63, 2.94 and 2.18%) and observed that 2.94% which was iso-osmotic was more favourable than the other two levels for sperm survival.

II.1.b. Milk

Milk heated at 90 - 93°C for 10 minutes has been found to be an acceptable diluent for preservation of bull semen at refrigeration temperature, but unheated milk was not acceptable for this purpose (Thacker and Almquist, 1951, 1953, and 1959; Dreher and Webb, 1953; Almquist, 1954; Boyd et al 1954; Flipse et al 1954). The harmful component
in unheated milk was named as 'lactoenin' which will be destroyed during heating.

The effect of heat treatment had been studied by Rowland (1937) and found that milk albumin and globulin were completely denatured by heating to 95° C. for 10 - 15 minutes. No change was noticed in the non-protein nitrogen up to a temperature of 100°C, but on continued heating small amounts of proteose were produced by the hydrolysis of whey proteins.

In an attempt to identify the factor in unheated milk which is toxic to the sperm, Thacker et al (1954) studied the effect of milk proteins upon the spermatozoan viability. It was observed that casein and protein free milk serum were devoid of toxic factor and the toxicity was associated with the albumin containing fraction only. Heating these fractions to 92°C eliminated the toxicity. The possibility of release of reactive sulph-hydryl group from albumin and other proteins by heat denaturation has been discussed by these authors. These sulph-hydryl groups may prevent the inhibitory effect of the milk and thus giving an explanation for the beneficial effect of heated milk. But based on the observations obtained it was concluded that it was unlikely that sulph-hydryl groups account for the differences in sperm survival observed in heated and unheated milk.

Bruce (1956) in a pilot field trial found that a diluent containing a final concentration of 10% skim milk powder and 10% glycerol gave a conception rate of 57% compared
with 56.7% in usual yolk-citrate-glycerol diluent. Heated reconstituted milk was compared with heated fresh skim milk for freezing bull semen by Amman and Almquist (1957) and obtained equal survival rates with both diluents.

O’Dell and Almquist (1957) observed that skim milk diluents maintained higher motility after thawing and storage at 5°C than those in egg yolk-citrate. In another comparative study of yolk-citrate, milk, skim milk and yolk-glucose-glycine diluters to find out their efficacy in the freezing of bull semen, Erickson and Graham (1969) observed that the first three are acceptable. Cossmann (1963) got comparable results in survival rates after freezing with skim milk diluent and commercial diluents. He also observed that skim milk without egg yolk is suitable for freezing.

Boyd et al (1956) studied the effect of addition of cysteine (free base) and thioglycolic acid, both sulph-hydryl containing compounds, and observed that thioglycolic acid had beneficial effects on sperm survival and was better than heated skim milk and cysteine added milk. Jones et al (1956) compared the effectiveness of yolk citrate, heated skim milk and thioglycolic acid treated milk in their effectiveness as a diluent and observed that chemically treated milk was superior to others for freezing.

Johnson et al (1955) studied the effect of cysteine hydrochloride on the livability of bull spermatozoa at 4°C. The addition of 1 mg. per ml. of cysteine hydrochloride to
unheated skim milk gave similar survival as that in heated skim milk. Glutathione also had this effect but methionine was ineffective indicating that the beneficial effect was due to SH-groups. This was shown to exert its effect by acting on the nondialysable portion of the unheated skim milk rather than acting directly on the spermatozoa.

O'Dell and Almquist (1957) showed that heated as well as cysteine treated skim milk gave similar survival rates after freezing.

II.1.c. Sugars

Probably the first report of the use of sugar diluent for freezing is that of Luyet and Hodapp (1939) who used 1 - 2 M sucrose for vitrifying frog spermatozoa in liquid air and obtained 20 - 40 % survival.

Sugar diluents are widely being used in case of freezing of semen in the form of pellets. Nagase and Graham (1964) had compared six sugar diluents, composed of egg yolk and glycerol with glucose, lactose, raffinose and stachyose singly and in combinations of lactose-raffinose and glucose-lactose-raffinose. They observed that there was no significant differences in conception rates due to type of diluents. Again, they had compared the effectiveness of egg yolk-glycerol-glucose-lactose-raffinose diluent in pellet form and whole milk-glycerol diluent in alcohol bath for freezing the semen of three low fertility bulls. The difference in conception rate with frozen semen was highly significant in favour of sugar diluent.
Different sugars were again tried for their efficacy as a diluent for freezing bull semen in pellet form by Nagase et al. (1964 b, c, d). They observed that survival increased with the increase of molecular weight of sugar.

II. Tris buffer

Recently, Tris buffer has been tried successfully in deep freezing of semen of different animals. Davis et al. (1963) had studied the survival of bovine spermatozoa in 0.2 M and 0.25 M tris buffered yolk-glycerol at +5\(^{\circ}\), -25\(^{\circ}\) and -35\(^{\circ}\)C and observed that they were generally superior to standard citrate-yolk-glycerol. Steinbach and Foote (1964 a) in a study with tris-fructose-yolk-glycerol found that 6 hour equilibration with glycerol and a freezing rate of 3\(^{\circ}\)C per minute in the critical zone of -5 to -15\(^{\circ}\)C was the most satisfactory procedure. This diluter was better than citrate-fructose-yolk-glycerol.

The optimum level of sodium and potassium in extenders for freezing has been found to be less than the normal intracellular concentration of these ions (Yassen and Foote, 1967). In their study using 0.25 M tris buffer with 20% yolk having freezing point depression of 0.55\(^{\circ}\)C, the osmotic pressure of the extender was increased by adding NaCl, KCl and CaCl\(_2\) singly and in all combinations. As the osmotic pressure increased motility of spermatozoa after freezing declined in a linear manner.

Steinbach and Foote (1967) had tried different levels of glycerol-tris molarity and pH and came to the conclusion
that an extender adjusted to 6.5 pH, 0.2 M-tris and 6.4% glycerol with 1% fructose was most suitable for freezing bull spermatozoa.

II.1.e. Egg yolk

Since the introduction of Phillips and Lardy (1940) of a diluter containing egg yolk, many researchers had turned their attention to find out the actual substance in egg yolk which have the protective effect on the spermatozoa. Mayer and Lasley (1945) isolated an active resistance factor from egg yolk which was shown to be more effective than the original egg yolk buffer mixture. Bogart and Meyer (1950) observed that the resistance factor present in yolk not only protects spermatozoa from drastic temperature changes but in addition protects them from many types of adverse environmental conditions such as changes in pH, osmotic pressure and presence or accumulation of harmful substances.

Kampschmidt et al (1953) extracted the lipid portion of the complex from egg yolk. The lipid portion consisted of the phospholipids, lecithin and cephalin and these obtained from a variety of sources were effective in protecting bull spermatozoa. They had observed that for prolonged storage of the spermatozoa some other substances are also needed and that these lipoproteins should be used as fresh preparations.

Srivastava et al (1963) isolated an active principle from egg yolk which could extend the period of storage of
buffalog semen 2 - 3 times than yolk itself.

Blackshaw (1954) observed that egg yolk could prevent the temperature shock to spermatozoa while cooling suddenly. The activity of egg yolk in this respect was in the alcohol soluble acetone insoluble fraction. The phospholipid lecithin could be isolated from this fraction which had the capacity of preventing cold shock in as low concentration as 0.12%. Dally et al (1957) had described a method of extraction of the lipoprotein from egg yolk, the addition of which at 5% level to the diluent gave significantly higher proportion of survival after freezing where as 2.5% had no effect and 10% did not give a higher recovery rate than did 5%.

The addition of yolk in the non glycerol and the glycerol portion of the diluter had been studied by Hafs and Elliot (1955). The addition of 25% yolk in both the fractions gave higher survival rates (30.5%) than addition of 50% yolk in the second fraction (22.3%) of the diluter only. Platov (1966 c) have shown that egg yolk have definite protective effect on freezing.

Yassen and Foote (1967) in a study with tris buffer observed that the supernatant and the precipitated portion of the egg yolk had equal effect on spermatozoa during freezing. Washed precipitate was poor in protecting the spermatozoa. The dialysed portions of the egg yolk was better than contral indicating that high survival of bull spermatozoa can be achieved with an extender low in all ions except those supplied with tris.
II.1.f. Glycerol

De Groot (1952) reported that 7.5% glycerol gave best motility after freezing. The highest level that the spermatozoa could tolerate without damage during freezing was found to be 10% by motility observations (Folge, 1953). Miller and VanDemark (1953) had observed that 6 and 8% levels of glycerol gave best results compared with other different levels higher and lower than these. Graham and Marion (1953) found 10% glycerol better than either 15 or 20% in a yolk citrate buffer. Cragle et al (1955) had estimated the optimum level of glycerol as 7.6% with sodium citrate diluent.

Amman and Almquist (1957) obtained optimum motility when the percentage of glycerol was equal to the percentage of total solids of skim milk and when both levels were 9 or 11%. In fresh skim milk with 9% of total solids a glycerol level of 11% was better than 9 or 13%. Almquist and Wickerson (1962) studied the fertility and motility of bovine spermatozoa in skim milk with various levels of glycerol and methods of glycerolation. They had observed that the beneficial effect of glycerol was related to level and mode of addition. The optimum was found to be at 5°C and added step-wise and 5% level was better than 10%.

A sugar diluent containing 8% glycerol was found to be superior to 4 or 6% if the activity of the sperm after thawing was the scoring criterion, but there was no difference in the percentage of sperm stained by congo red-nigrosine when frozen in diluents containing these
levels of glycerol (Martin, 1963a). Roussel et al (1964) observed significant differences among 6, 7, 9 and 11% glycerol levels and 7% was optimum for post thawing survival after liquid nitrogen vapour freezing.

Martin (1965b) with citrate-phosphate-fructose diluent had observed that out of 3,10 and 12% glycerol levels 10% gave best motility after freezing and thawing but percentage of stained spermatozoa had increased with increasing level of glycerol. A similar observation was reported by Rathore (1966) using 7 and 14% glycerol levels with egg yolk-citrate diluent.

Steinbach and Foote (1964b) had tried different levels of glycerol in citrate, tris and skim milk diluents and found that optimum levels were 7.2%, 8.8% and 9% respectively.

Nagase et al (1964a,c) had tried 1-7% glycerol in egg yolk-sugar diluents for normal as well pellet freezing and observed that all levels gave good results. Pellet freezing gave better survival at lower glycerol levels. With egg yolk-glucose diluent having 3.5 or 7% glycerol, Nagase et al (1964d) observed that the conception rates were 67.6 and 55.5% respectively and the difference between them were highly significant. They further observed that with egg yolk-lactose diluent, reducing the concentration of glycerol from 3.5 to 1.75% did not significantly affect the conception rate.

II.2. Technique

II.2.a. Addition of glycerol

Although the addition of glycerol is generally done
at 5°C as recommended by Polge and Rowson (1952), studies by Dunn and Hafs (1953) and later Polge and Jakobsen (1959) could find no difference in motility when glycerol was added at room temperature itself. A similar observation was made by Graham et al (1958) in a study on the temperature effect at the time of glycerol addition. They had studied the effect of adding glycerolated yolk-citrate buffer to the diluted semen at 5°C, 10°C, and 20°C as well as direct dilution of fresh semen with glycerolated diluent and failed to observe any significant difference among the four treatments. Similarly Blackshaw (1960) had observed that the temperature at which glycerol was added (5°C to 29°C) had no effect on the survival of bull sperm.

Contrary to this, Miller and VanDemark (1954) in a study on the various aspects of freezing of bull semen with yolk-citrate diluent showed that the addition of glycerol to the diluted semen at temperatures of 4 - 5°C was better than at a higher temperature of 10 - 15°C. Blackshaw (1955), working with a glycerol-arabinose diluent observed that the adverse effect of addition of glycerol portion occurs only at a temperature of 15-20°C.

In a study using milk-glycerol diluent for freezing of bull semen Almquist (1969) has shown that with this diluent glycerol had an adverse effect unless it is added at 5°C. It has also been shown that the addition of glycerol portion in three steps at 10 minutes intervals gave better results. This observation was again confirmed by Almquist and
Ickerson (1962), Cossmann (1963) also reported beneficial effect of adding glycerol portion after cooling the diluted semen in a skim milk diluent.

Settergren (1963) had tried the glycerolation at room temperature after 10 minutes of primary dilution and got comparable survival rates with traditional methods, after freezing and storage.

Eiblik (1964) experimented a new procedure with the intention of reducing the total period of time between collection and freezing. The semen was cooled to 20°C with in 25 minutes of collection and diluted with yolk-citrate diluent to half the final volume followed by dilution with equal quantity of diluent containing fructose and glycerol. The diluted semen was ampouled and then cooled to 5°C with in 2½ hours. After equilibration for 15 - 16 hours it was frozen in stages. The new procedure gave better conception rates compared to traditional method but oxygen uptake in both the cases were similar.

Clegg et al (1965) in a study on the glycerol entry into the bovine spermatozoa in yolk-citrate diluent obtained best pre- and post-thawing motility and survival with semen cooled to 5°C over a 4 hours period and glycerolated at 5°C (than at 32°C) followed by equilibration for 4 hours (than 15 hours). The glycerol was added over a period of 45 minutes. Martin (1965 a) had stored the diluted semen for different periods and added the glycerol containing portion at the beginning or at the end of this period or half way through it. He got best survival rates in samples stored for 8 hours
and when the glycerol portion was added after half the storage period had elapsed.

El-Kafrawi (1966) observed that dilution and glycerolation done at 20°C was better than at 30°C at different glycerol levels. There was no significant difference in sperm survival between semen to which glycerol was added at dilution at 20°C and that in which glycerol was added 4 hours later at 5°C.

II.2.b. Equilibration period

The necessity of adding the glycerol portion to the diluted semen at low temperature and subsequent storing overnight at that temperature before freezing was pointed out by Polge and Rowson (1952). This storage period later came to be known as equilibration period. Miller and VanDemark (1954) observed that an equilibration of 6 hours was adequate. But Saroff and Mixner (1954) studied the effect of 3 different levels of yolk, 3 levels of glycerol and 4 levels of equilibration periods and observed that there was a progressive increase in sperm survival in all diluters when the equilibration period was increased from 2 to 8 hours.

Using a milk diluent O'Dell and Almquist (1954) could not find any difference in the frozen thawed motility for the equilibration periods of 30 minutes, 4 hours and 18 hours. Again, O'Dell and Hurst (1955) using yolk citrate as well as skim milk diluters compared 13 hours of equilibration with no equilibration and got better results in samples without equilibration.
Cragle et al. (1950) observed that an equilibration time of 14.9 hours gave optimum results. Based on non-return rates, Graham et al. (1957) observed that out of 4, 8 and 12 hours of equilibration periods, longer periods were superior.

Using a yolk-citrate-phosphate-arabinose medium, Emmens and Martin (1957) studied the effect of equilibration and observed little difference between one hour and 18 hours equilibration periods. But, Martin and Emmens (1961) observed that longer equilibration periods gave higher revival rates and the use of fructose in the diluent significantly improved the revival of spermatozoa after freezing. The improvement in fertility after longer equilibration was highly significant when 1.25% fructose was present in the diluent.

Using a skim milk diluent, Cossmann (1963) observed that an equilibration period of 12 - 18 hours was preferable to 4 - 6 hours.

Martin (1963c) using a citrate-phosphate-fructose-glycerol diluent with lecithin or egg yolk observed that 4 hour equilibration was better than 0 or 1 hour periods. Martin (1963a) studying the equilibration phenomena again showed that an improvement in survival after deep freezing occurred if diluted semen was stored up to 12 hours after dilution but this was not dependent on the presence of glycerol in the diluent during aging process. Martin (1963b) had again observed that there was no significant difference between mean effect of addition of glycerol either immediately after the diluted semen had been cooled to 5°C.
(equilibration) or just prior to freezing after a period of storage at 5°C equivalent to the equilibration period (ageing).

Roussel et al (1964) in a 4 x 4 factorial experiment with 4 levels of glycerol and 4 equilibration periods observed that 12 hours equilibration period was optimum compared to 6, 8 and 18 hours.

Steinbach and Foote (1964a) could not find any difference in survival rates between the equilibration times of 6 and 18 hours in citrate-fructose-yolk-glycerol and tris-fructose-yolk-glycerol diluters. Martig and Alquist (1966) got slightly better results which was significant at 1% level for samples equilibrated for 18 hours than 2 hours in a milk-fructose-glycerol medium. Nikulenkov (1967) had observed that an equilibration period of 4 - 12 hours was better than a shorter or longer one.

Nagase et al (1964b) introducing the new technique of pellet freezing observed that for such freezing the optimum equilibration period was 5 - 10 hours.

A multitude of different factors like difference in bulls and ejaculates, diluters, handling procedures, etc., come into play and probably this explains the many contradictory observations reported.

II.2.c. Container for semen

Small glass ampoules of about 2 ml. capacity which are heat sealed or closed with tight fitting rubber corks are commonly used as containers for diluted semen during freezing. Burgmann and Schmidt (1959) had frozen semen in plastic and glass ampoules and observed that both were satisfactory except
that the plastic vials will float on the alcohol bath. A comparative study of the efficacy of glass ampoules and plastic vials was conducted by Graham and Erickson (1959) for freezing bovine spermatozoa and on the basis of nonreturn rates observed that glass ampoules were superior to plastic vials.

Gaul (1966) obtained almost similar motility for semen frozen in ampoules and in PVC straws. Aamdal (1966) had frozen semen in yolk-glycerol-raffinose diluent in plastic straws over solid carbon dioxide and stored them in liquid nitrogen. This was compared with semen frozen in skim milk-glycerol diluent by conventional ampoules. Both the treatments gave similar results on the basis of nonreturn rates. Nikulenkov (1967) found that glass ampoules, polythene ampoules and polyester test tubes were equally satisfactory for the storage of semen.

Adler et al. (1967) have reported significantly better results from inseminations for semen frozen in straws than in pellet form, based on 60-90 day nonreturns. Meding (1967) observed that semen frozen in straws over liquid nitrogen gave poorer conception rate than fresh semen.

II.2.d. Freezing rate

Graham and Marion (1953) got best results with a freezing rate of 3 - 5°C per minute in yolk-citrate diluent and 2 - 3°C per minute in whole milk. Miller and VanDemark (1954) while studying the various aspects of freezing bull spermatozoa observed that the rate of cooling from 1°C to 4°C
per minute in the range of +5 to -20°C gave best results.

The interaction between glycerol levels and rates of freezing was studied by VanDemark and Kinney (1954). A cooling rate of 2°C per minute from +5 to -19°C and 4°C per minute from -19 to -79°C resulted in higher sperm survival than cooling at a rate of one fourth as fast or four times as fast with different glycerol levels. The fastest freezing rate was least injurious with high citrate-glycerol level combinations.

Jones et al (1956) found that a cooling rate of 2.2 to 3.3°C per minute was better than 1.1°C per minute for freezing bovine spermatozoa in various extenders. Luyet and Keane (1955) tried two freezing rates: (a) semen in ampoules frozen suddenly (20°C per minute) from 0°C to -27°C and transferred after 5 minutes to liquid air (cooled at 15°C per minute), (b) first stage cooling was done only up to -20°C. In the first case there was a recovery of 75% while there was none in the second. This suggested that rapid cooling above -27°C is harmful to sperms and that slow cooling at the critical range (-15 to -25°C) rendered the sperms relatively immune to rapid cooling below that temperature.

O'Dell et al (1958) in a study on the effect of freezing rate on bovine spermatozoa has found that the recovery rate was best with fresh skim milk and homogenised whole milk by cooling at the rate of 1°C per minute from +5 to -15°C, at the rate of 5°C per minute from -15 to -50°C and then at 20°C per minute to -79°C. With yolk-citrate
diluter they got best results with a cooling rate of 3° per minute from +5 to -15°C and 10° per minute from -15 to -50°C.

Lot of time and labour can be saved if the freezing bath is kept at a low temperature at the start of the procedure. Such an experiment was designed by Kennelly et al (1960) in which the ampoules at +50°C were transferred directly to previously cooled baths whose temperatures varied from +5 to -40°C. The best results were obtained from baths having initial temperature varying from -20 to -40°C.

Bruemmer et al (1963) observed that a cooling rate between 4°C and 20°C over a temperature range of -10 to -30°C were superior to other cooling rates in an egg-yolk-citrate-glycerol diluter. Steinbach and Foote (1964a) could not find any significant difference between the freezing rates of 0.8, 3.0 and 8°C per minute in the critical range of -5 to -15°C using citrate-fructose-yolk-glycerol and fructose-yolk-glycerol diluents.

Martin (1963b) had observed that the initial cooling of diluted semen from +35 to +5°C when done in 30 minutes instead of 2 hours did not affect the activity of spermatozoa but the proportion of the stained spermatozoa were increased. Effects of different rates of cooling had again been examined by Goffaux (1966) and observed that very slow rate of cooling initially as well as after the onset of crystallisation was deleterious to sperm survival. Nikulenko (1967) tried two rates of freezing, one taking
30-40 minutes and the other 10-15 minutes to reach the temperature of \(-100\,^\circ C\) and found that the latter rate gave better results.

II.2.e. Freezing in alcohol bath

The freezing of semen in ampoules are usually done in an alcohol or acetone bath, the temperature of which is brought down slowly by the addition of solid carbon dioxide. A thermometer which can read temperatures below \(-70\,^\circ C\) is kept in the bath. The temperature fall is adjusted by the amount of solid carbon dioxide put in the bath, checking the time from a stop watch kept side by side.

Many mechanical freezing contrivances are available on the market which can handle large quantity of diluted semen with good manoeuvrability (Henderson, 1956; Ehlers and Rice, 1957; Anderson, 1963).

II.2.f. Freezing as pellets

A new technique in deep freezing was introduced by Nagase and his associates by freezing the diluted semen by placing them directly over solid carbon dioxide. Nagase et al (1964b) in a study on the factors affecting freezing of bull spermatozoa observed that the bull semen diluted in an egg yolk-glucose-glycerol medium and frozen as pellets by dropping over solid carbon dioxide gave similar survival to that of normal method. This diluter was found better than yolk-citrate-glycerol. The optimum equilibration period was 5-10 hours. The pellets were stored in containers in solid carbon dioxide bath.
The various aspects of pallet freezing as extender composition, glycerol level, thawing solution and their effect on fertility were studied by Nagase and Graham (1964) and Nagase et al (1964 a).

Nagase et al (1964c) studied the protective action of various sugars on the pellet freezing of bovine spermatozona. Thirteen different sugars and sugar alcohols were added singly to egg yolk diluter with egg yolk-glucose-glycerol as control. Survival rates were best in glucose followed by lactose, raffinose and sucrose diluents. Different instantaneous methods of freezing as dropping diluted semen into liquid nitrogen, plunging into alcohol-solid carbon dioxide bath were compared with normal methods and using egg yolk-sugar diluents containing 0-7% glycerol. Normal and pellet freezing gave best results at all glycerol levels. Pellet freezing gave better survival of these two methods at lower glycerol levels.

The fertility rate of pelleted semen was compared with conventionally frozen semen by Nagase et al (1964 d). Conception rates were better in the case of pellets than conventional methods. In egg yolk-glucose diluent the conception rate of pelleted semen with 3.5% of glycerol was better than with 7% glycerol. With egg yolk-lactose diluent, reducing the concentration of glycerol from 3.5 to 1.75% did not significantly affect the conception rate. Egg yolk-glucose diluent and egg yolk-lactose diluent with 5% glycerol gave similar results.
Nagase et al. (1964b) observed that a pellet size of 0.013 - 0.2 ml. significantly influenced sperm survival rate in yolk-citrate-glycerol but not in yolk-glucose-glycerol. Leipnitz (1965) observed that pellets of 0.1 ml. gave 16% more motility than those containing 0.2 ml.

Koslowska and Maik (1967) had compared the pellet freezing method of Nagase and the conventional method. After storing for different periods the motility ratings were higher in the case of conventional method, but the difference was not statistically significant.

II.2.g. Freezing in liquid nitrogen vapour

Equilibrated semen contained in ampoules or straws were successfully frozen by keeping them directly over the liquid gases. The cooling was much faster than alcohol bath freezing but the rate of cooling could be controlled by adjusting the distance between the semen containers and the liquid gases below, (Forgason and Berry, 1961; Martig and Dickey, 1963; Roussel et al., 1964; Juscenko et al., 1968).

Rajamannan (1966) had evolved a suitable container for such type of freezing after many trials. Uwland et al. (1967) had frozen semen diluted in a lactose-glycerol-yolk medium by dropping on to a steel grid kept at -50°C over liquid nitrogen. These semen pellets were stored in plastic straws in liquid nitrogen containers. By this method survival rates of 67.2% were obtained.

II.2.h. Freezing in powdered dry ice

In this rapid and simple method, equilibrated semen
contained in ampoules were kept immersed in powdered dry ice in a wide mouthed flask. Freezing was quite rapid but slower than that in case of pellet, as the quantity of semen is more in this case and it is not directly exposed to the dry ice.

Bruce (1953) tried this type of rapid freezing with cattle semen equilibrated overnight and obtained reasonable recovery rates. A comparison of semen subjected to this type of freezing was again done by Bruce (1956) and based on 112 day nonreturn rates a conception rate of 56.7% was obtained as against 63.0% for semen frozen in alcohol bath. Polge and Jakobsen (1959) had compared this method with the usual and observed better survival rates. Bolt (1960) also had described this method of freezing.

II.2.1. Storage temperature

Storage of frozen semen for shorter duration is done usually in alcohol bath cooled by adding solid carbon dioxide pieces. This will keep the temperature to nearly \(-79^\circ C\). This method is quite cumbersome and the cost involved will be high when storage for longer periods are necessary. Storage for longer duration are usually done in liquid nitrogen (\(-196^\circ C\)) or in liquid oxygen (\(-183^\circ C\)) or liquid air (\(-190^\circ C\)) in special containers.

O'Dell et al. (1968) had studied the effect of storage at \(-70^\circ C\) and observed that the greatest decline in motility of bovine spermatozoa occurred in the first four weeks of storage regardless of the diluent used. The effectiveness
of liquid nitrogen and solid carbon dioxide for storage of frozen bovine semen was compared by Pickett et al. (1959) and observed that the former to be superior. The effect of continuous and alternate storage of frozen semen in solid carbon dioxide and liquid nitrogen in different combinations were tried by Pickett et al. (1960a) and observed that continuous storage in liquid nitrogen was superior to any other combinations.

Pickett et al. (1960b) studied the fertility of frozen semen stored at -79°C and at -196°C and obtained slightly higher fertility with liquid nitrogen storage but the difference was not statistically significant.

Sulliwan and Mixner (1963) in a detailed study on the effect of temperature and length of storage time upon post thawing motility and metabolic activity observed that the mean motility, sugar utilisation and lactic acid production were significantly greater for semen samples stored at -196°C than at -79°C.

II.2.1. Thawing procedure

Miller and Vandemark (1954) had observed that thawing of frozen semen at 5°C was better than at 38°C. On the other hand no significant difference on the motility of frozen semen was observed after thawing semen at 5°C, 40°C and intermediate by Dunn et al. (1953) and Elliot (1954). Zakrazevska and Genowefa (1962) also could not observe any difference due to thawing of the semen at 18 or 40°C.
Blackshaw (1955) working with a glycerol-arabinose diluent observed that thawing the frozen semen at 40°C gave 45% revival whereas thawing at 5°C gave only 24%. Kelly and Hurst (1962) thawed frozen semen in warm water and tried to store it in ice water for 4-6 hours. On the basis of fertility results from 1342 first inseminations they concluded that the thawed semen can be stored in ice water for 4 hours without loss of fertilising capacity.

Bruemmer et al. (1962) observed that rapid warming of frozen semen was better than slow warming. This observation was confirmed by Martin (1963) who observed that 40°C was superior to 0°C as a thawing bath temperature. Pickett et al. (1965) had observed that thawing at 1°C or 40°C was better than any other temperature in between.

Different thawing diluents were tried for pelleted semen by Nagase et al. (1964a). Out of the different diluents as buffered yolk-milk, minnesota 0.0., 3% sodium citrate, milk, 0.9% sodium chloride, 3% buffered glycine, 10.5% lactose and tris buffer, significant increase in conception rate was observed only in the case of buffered glucose-milk.

Many failures in recovery following freezing and thawing of glycerolated cells may be attributed to their direct transfer to a glycerol-free medium following thawing. Evans et al. (1962) reported improved recovery of cell cultures frozen with glycerol when they were suspended by stepwise dilution in the glycerolated medium rather than being transferred directly to a glycerol-free culture medium.
Farrent (1966) has observed that thawing of frozen materials stored in the presence of non-electrolytes is best carried out rapidly as the composition of the medium will return almost immediately to that of the solution before freezing where as slow thawing will prolong exposure to salt concentrations. Aamdal and Anderson (1968) have shown that semen frozen in straws and thawed at 75°C for 12 seconds gave better motility and fertility than those thawed at 35°C for 30 seconds. In this case the semen was diluted in lactose-yolk-glycerol as well as in skim milk-fructose-glycerol and frozen in polyvinyl chloride straws.

II.3. Additives

Various substances had been tried to be incorporated into the diluent for increasing the viability of the spermatozoa. Graham and Marion (1963) observed that cysteine and catalase had increased the motility after freezing but not to a great degree. Steinbach and Foote (1964a) could not observe any favourable influence of catalase on the freezability.

Lipoproteins separated from egg yolk when added at 5% gave significantly higher motility than the control after freezing, (Bialy et al, 1957). Addition of 1% lecithin was compared with 25% egg yolk (Martin, 1963) and found that lecithin was superior to egg yolk. 1% lecithin gave better motility than 0.5 or 2% and proportion of unstained sperm were higher in 2% than in 0.5%.

Zakrazewski and Genowefa (1962) observed that semen
samples which are best in surviving at room temperature in glycine containing diluents are likely to survive best in deep freezing.

Choong (1963) had observed that the non dialysable milk solids added to synthetic diluents improved revival of deep frozen spermatozoa. Similar effect was found with the casein also among dialysable portion. Lactose was found to have protective properties in synthetic diluents but it was inferior to fructose. Addition of potassium, magnesium and calcium ions or sodium citrate had no beneficial effect with the synthetic diluent studied.

Various types of sugars were found to give some amount of protection to the spermatozoa during freezing. Hafs and Elliot (1956) had added fructose, glucose and xylose at 1% level in nonglycerol portion, glycerol portion and in both and obtained survival rates of 21, 28 and 28½ respectively. Out the three sugars fructose gave better average results. Blackshaw (1957) described a suitable diluent for freezing bull semen incorporating fructose with egg yolk-citrate-glycerol diluent.

Martin (1963) observed that 62 mM fructose mixed with buffered sodium citrate gave better result than the control. On replacement of part of the citrate buffer by 62, 123 and 185 mM fructose solution there was no significant change in the activity of thawed semen but percentage of unstained sperm was significantly lower in diluents containing 185 mM fructose than in those with 62 mM. Best results on freezing
were obtained in 12 hour equilibration in a mixture of 62 mM fructose, 60 mM sodium citrate, 20 mM phosphate buffer and 2.0 M glycerol.

Amman and Almquist (1957) and O'Dell and Almquist (1957) observed that the addition of 1.25% fructose to reconstituted skim milk resulted in highly significant increase in post thawing motility and reduced the variation among ejaculates in sperm survival. O'Dell and Almquist (1957) claimed that any beneficial effect of addition of sugar to milk diluents was not necessarily associated with a shortening of equilibration period.

Choong (1963) had observed that addition of fructose after chilling improved revial of bull spermatozoa after freezing even when 144 mM fructose was already present in the diluent. Nagase and Graham (1964) tried different sugars like glucose, lactose, raffinose and stachyose singly and in combination for freezing in pellet form and observed no differences in conception rates between the diluters.

Steinbach and Foote (1964b) also observed beneficial effect of fructose by adding at 1% level to skim milk-glycerol and citrate-yolk-glycerol diluents but not with tris-yolk-glycerol.

Lanz et al (1965) studied the effect of different lipid additives on pre- and post-freeze survival of bovine spermatozoa using NJ2 and ETC extenders with different levels of cholesterol, diolein, triolein, milk fat glycerides and lecithin. Out of these only cholesterol and lecithin
protected the cells during freezing and thawing. Combining 2% cholesterol with 2, 4 and 8% lecithin in different extenders reduced motility of frozen semen compared to that with out cholesterol. Two percent lecithin was superior to 4 and 8% levels.

The addition of alpha and beta amylase at two different levels were tried for freezing bull semen by Kirton et al (1968). Average fertility with amylase was significantly greater than controls. Higher level of amylase in the case of alpha type had better effect than the other.

An oxytocin preparation from posterior pituitary was found to have some favourable action on the spermatozoa during freezing (Habibullin and Bereznev, 1965). Five units of the preparation was added to glucose-yolk-citrate extender and after freezing the motility was 5-10% higher than the control semen.

II.4. Dilution rate of semen

Dunn et al (1953) had frozen bull semen at the rate of 5, 12 and 30 million spermatozoa per ml. diluter and obtained a post thaw motility of 34, 38 and 47% respectively, the differences between them being highly significant.

Bruce (1956) observed no significant difference in conception rate between the two dilution rates of 1:20 and 1:50. Pickett et al (1964) had diluted bull semen to contain about 20 and 30 million live spermatozoa per ml. and after freezing observed that there was no significant difference
between them in conception rate. Goffaux (1965) had concluded from his study that there should be 10-15 million live sperm per ml. in the thawed sample as the minimum level.

Freund and Weidermann (1966) working with human semen observed a true dilution effect and recommended a dilution rate of 1:5.

II.5. Variation between bulls

Variation on freezability between semen of different bulls were reported by Rowson (1953) and Swanney (1953) based on motility assessment of frozen thawed semen. This was confirmed by extensive studies by Dunn et al (1953).

Emmens and Martin (1957) in a study of the fertility of frozen semen observed that there was highly significant variation between ejaculates, the fertility varying from 42 to 80%. Nearly all the variation was between bulls and not within bulls. The bulls most frequently used was the lowest in fertility.

Steinbach and Foote (1964b) observed highly significant difference between bulls on freezability. Adler (1967) studying the fertilizing efficiency of frozen semen observed that there were significant differences between bulls.

II.6. Variation within bulls

The effect of maturity of spermatozoa on the freezability had been studied by many workers. Kinney and VanDemark (1954) had compared the freezability of 20 consecutive ejaculates (in 4 hours) and found that freezability increased up to fourth and decreased there after. Second ejaculate was found to be better than the first in freezability by Willet and
Ohmes (1953). They further observed that the superiority is due to the cells and not to the plasma. First and second ejaculates were compared again in freezability and the second was found to be superior by Burgman and Schmidt (1958).

Contrary to the above observations O'Dell et al. (1959) observed no significant difference between first five ejaculates while studying the suitability of successive ejaculates for freezing. A similar observation was reported by Salamon and Lightfoot (1967) who found no significant difference in successive ejaculates by pellet freezing technique in yolk-glucose-citrate medium.

II.7. Interaction of various factors

As a large number of factors come into play during the various procedures of freezing, thawing and finally getting the cow conceived, an elucidation of the factors having interaction among them is necessary. Saroff and Mixner (1954) in two different sets of trials observed a significant interaction between the levels of yolk and glycerol in a sodium citrate diluter. As yolk levels were increased glycerol levels had to be increased for optimum sperm survival after freezing. A diluter having 20% yolk and 7% glycerol gave best sperm survival after freezing.

An interaction between glycerol and citrate levels were observed by Cragle et al. (1953). Using a final egg yolk level of 24%, the optimum final levels of sodium citrate and glycerol were found to be 2.9 and 7.6% respectively.

Steinbach and Foote (1964a) observed that there is
large interaction of bulls with different other variables studied, such as addition of catalase, sealing under partial pressure with nitrogen or argon, equilibration period, rate of freezing, etc. This indicated that single freezing procedure was not optimum for all semen samples. It was again reported that the freezability differed with bulls (Steinbach and Foote, 1964b). Interaction between bulls and extender, rate of freezing and glycerol level, equilibration time and extender as well as equilibration time and fructose level were significant indicating that a procedure optimum for one step may no longer be optimum when another step is changed.

The findings of Lovelock and Polge (1954), Polge (1957) and Howson (1956) indicated the possibility of an interaction between equilibration time and rate of cooling.

Blackshaw (1955) showed that egg yolk plus lecithin save best spermatozoal survival rates when compared with egg yolk alone or milk plus lipoprotein.

**II.8. Other protectants**

Various other hydrophylic non-electrolytes were tried as protective substances in addition to glycerol. Lovelock and Bishop (1959) had tried dimethyl sulphoxide (DMSO) at 15% level to a sodium chloride-glucose diluent at room temperature and observed that the motility persisted for several hours showing that it is not toxic to bovine spermatozoa. Working with suspensions of mitochondria Grieff and Myers (1961) and Dickinson et al. (1967) had shown
that DMSO was equal or superior to glycerol for protecting the oxidative and phosphorylative capacities following freezing.

The toxicity and pharmacological actions of DMSO on smooth muscle of guinea pig during freezing was explored by Farrent (1964) and observed that it has got only a low toxicity.

The protective action of DMSO on human spermatozoa during freezing was compared with glycerol by Sherman (1964a). It was found that DMSO had equal protective action as glycerol but DMSO was more toxic during storage at -22°C, both for freezing and after thawing. DMSO at 10% level was most suitable. Working with human spermatozoa Zimmerman et al (1964) observed that based on motility assessment glycerol was superior to DMSO but based on actual survival DMSO was better.

Jones (1965) had reported unfavourable effects with DMSO in a study of freezing ram spermatozoa in skim milk diluents. The revival rate was less than 10% only.

Shaffner (1964) observed with freezing chicken semen that ethylene glycol, propylene glycol, xylitol, DMSO and sugars were inferior to glycerol as protectants of spermatozoa. Different levels of glycerol and ethylene glycol were tried in lactose-yolk diluter for freezing ram semen in pellet form (Platov, 1965). The motility of the thawed spermatozoa were maximum at 3.5% glycerol level and 1.75% ethylene glycol. Various percentages of ethylene
glycol (1.75 to 16%) were again tried (Platov, 1966a) for freezing of ram semen in alcohol bath with glucose-yolk-citrate diluent. The levels of 1.75 and 3.5% were found to be optimum and were better than 15% glycerol.

Zverev (1966) had compared different levels of ethylene glycol and glycerol for the freezing of bull semen and observed that the levels of 3.5% glycerol and 1.75% ethylene glycol gave optimum results after freezing.

Platov (1966b) had tried diethylene glycol at different levels as a protectant for bull and ram semen during freezing. It was found that best motility after thawing at 38°C was in ram semen with glycerol concentration of 0.88 - 3.5% and in bull semen with those of 0.88 - 1.75%. The protective action of glycerol was not considered to be inferior to that of glycerol.

Richardson and Sadlier (1967) had investigated the toxicity of various non electrolytes to human spermatozoa and their effects during freezing. At concentrations varying from 2.5 to 10% glycerol, DMSO, ethylene glycol, methyl formamide, and methyl acetamide had approximately equal toxicity effects on human spermatozoa, whereas dimethyl formamide and dimethyl acetamide were considerably more toxic at the same concentrations. Each of glycerol and ethylene glycol could retain approximately 50% of original motility after thawing. Methyl formamide and methyl acetamide, dimethyl formamide and dimethyl acetamide gave negligible protection. With DMSO only 34% of the original motility could be retained.
11.9. Laboratory tests

The ability of certain dyes as methylene blue and resazurin to act as final hydrogen acceptor in the respiratory process of the spermatozoa has been utilised as an indirect laboratory test in evaluating the vitality of semen samples (Beck and Salisbury, 1943; VanDermark et al, 1944; Erb and Ehlers, 1950).

Buckner et al (1954) observed that the only correlation with a magnitude of 0.9 or higher were those among bulls between nonreturn rates and (a) motility of sperm after incubation at 38°C in yolk citrate and antibiotics, (b) the combination of methylene blue reduction test, drop in progressive motility after 120 minutes in 3% aniline blue solution at 4°C and initial motility in yolk citrate. Bishop et al (1954) observed a relationship of fertility with methylene blue reduction time and fructolysis.

The relative usefulness of laboratory tests in predicting the fertility of bovine semen had been investigated by Braton et al (1956). They had observed significant linear correlation between fertility and concentration of sperm, percent motile sperm, methylene blue reduction time, pH, percent unstained sperm, livability at 5°C and oxygen uptake at 37.5°C.

Semakov (1963) observed a positive correlation between the dehydrogenase and cytochrome oxidase activity of bull semen with motility, concentration and sperm survival at 0°C and -79°C. The activity of enzymes with fertilizing ability also was demonstrated by 110 inseminations.
using 15 ejaculates from 10 bulls Upenskii (1967) showed that fertility was directly related to dehydrogenase activity of semen.

Dimitropoulos (1967) had evaluated the significance of the more rigorous test in evaluating fertility of frozen bull semen. The percentage of progressively motile spermatozoa after 5 hour incubation in water bath at 38°C was highly significantly correlated with fertility.

Salomon and Lightfoot (1967) had observed that there was no firm relationship between cold shock values and subsequent viability during liquid storage or recovery after pellet freezing.

Exhaustive reviews on the different aspects of preservation of bull semen has been published: Bhatia (1960), Maule (1962), Sharma (1962), Sherman (1964c) and Sadlier (1966).

II.10. Freezing damage on cells

II.10.a. Biochemical alterations

Earlier conceptions on the damaging effect on the cells due to freezing were centered round the idea of ice crystal formation in and out of the cells and its prevention or alteration by glycerol.

It was first suggested by Lovelock (1953a) working with human red blood cells that the principal destructive process occurring during freezing and thawing was due to the effect of the concentration of electrolytes occurring inside and outside the cells. He further observed (Lovelock, 1953b) that
in a suspending medium of sodium chloride, when the concentration of electrolytes reached a mole fraction of about 0.014, haemolysis of red cells developed regardless of the temperature or concentration of glycerol. He assumed that the mechanism of injury was some form of denaturation in the membrane.

Hansen and Nossel (1955) studied the structure and metabolism of yeast cells during freezing. The cells became permeable to added organic acids and lost amino acids, protein, carbohydrates, coenzymes and inorganic salts when washed with water or buffer. The response to cold shock varied with different strains. The action of liquid nitrogen on in vitro fibroblasts were studied by Roy (1957) and the results were discussed with regard to different theories related to cold-injury.

Lovelock (1957) suggested the possibility of denaturation of lipoprotein complex of cell structures also as a damaging factor during freezing. He observed that these complexes are "inherently unstable and probably maintained in living cells by continuous synthesis". These may dissociate following small changes in the environment such as increase in electrolyte concentration and resulting increase in the ionic strength of the suspending medium, changes in pH, the concentration of substances such as urea and dissolved gases to toxic levels or sufficient removal of water to bring the cells and their structures into actual physical contact.
The above observation was later supported by Pickett and Komarek (1964) who had presented evidence of loss of lipid from bovine spermatozoa due to freezing. The amount of lipid extracted from spermatozoa before and after freezing were significantly different. This probably reflected the cellular destruction during freezing and thawing.

Luyet (1957) had described the growth of ice phase in aqueous colloids and its significance on deep freezing of semen. The oxidative phosphorylation after freezing and thawing was studied by Privitera et al (1958). They observed that slow freezing preserved the oxidative phosphorylation at higher levels than rapid freezing.

Boljkevic et al (1959) have postulated that calcium is released from the external lipoprotein layer of spermatozoa on cold shocking and passes into the cell causing disorganization of protoplasm and inhibition of enzyme systems. They have suggested that the protective action of various substances such as lecithin, casein, etc., against cold shock is due to their ability to bind the released calcium and prevent it passing into spermatozoa.

The loss of enzymes had been reported from rat liver due to freezing (Porter et al, 1953). Waravdekar et al (1963, 1964) studied the activities of various enzymes in rapidly frozen and thawed mouse liver. It was observed that some of the enzymes were not affected but some others were affected at different levels with different methods of freezing.
Working with rat liver mitochondria, Lusena (1965) observed the release of enzymes during freezing. The effect of freezing and thawing was similar to the effect of exposure to higher sucrose concentration and redilution. The maximum damage occurred at about -15°C while below -40°C no damage was detectable. Heber and Santarius (1964) by tracer experiments with isolated cells and organelles showed that ATP synthesis is primarily damaged by freezing. The effect of freezing on the synthesis of ATP in ram spermatozoa was again studied by Platov and Nikolaev (1966).

The structural changes associated with release of enzymes from rat liver mitochondria by freezing were studied by Lusena and Dass (1966). They observed that when dehydrogenases were not released by a freezing treatment, no structural changes were detected. Release of these enzymes were associated with mitochondrial disruption and drastic rearrangement of membranes. Partial release of enzymes was not associated with partial structural modifications of all mitochondria but with drastic structural changes in only some of them.

The effect of the size of the intracellular ice on consumption of oxygen in mouse kidney cells was studied by Sherman (1964). It was observed that survival of cells was greater after formation of large rather than small intracellular artifact. The intracellular artifact was 5-17 times larger in slow than rapid freezing.

Mazur (1964) postulated that survival of cells will be higher when they are cooled slowly, as slow cooling decreases
the probability of intracellular freezing by permitting water to leave the cell rapidly enough to keep the protoplasm at its freezing point. He had derived a quantitative relation between amount of water in a cell and temperature, enabling to calculate the water content which permit predictions as to the likelihood of intracellular ice formation.

Farrent (1965) in a study on the mechanism of cell damage during freezing and thawing and its prevention, developed two new methods which prevent any rise in the electrolyte concentration during cooling to low temperature. This had greatly improved the functional recovery of organised cells. The results of this study supports the theory that the protective action of hydrophobic nonelectrolytes like glycerol and DMSO is due to a reduction in the percentage of electrolytes at any temperature during freezing.

The effect of cold shock and deep freezing on the concentration of major cations in spermatozoa was studied by Quinn and White (1966) and observed in bull and ram spermatozoa an influx of sodium to and an efflux of potassium and magnesium from the spermatozoa. Calcium was actively accumulated in the spermatozoa after cold shocking.

The sodium and potassium content of bull spermatozoa during the process prior to freezing was studied by Yassen and Foote (1967). They observed that extending semen with tris buffered egg yolk at room temperature, caused the cells to outflux 85% of sodium and 75% potassium with in 15 minutes of extension. This resulted in an equilibrium between
intra and extracellular sodium but intracellular potassium remained three times as high as potassium in extracellular fluid. Sodium and potassium levels tended to rise during cooling and storage except when glycerol was added. Intracellular concentration of potassium was maintained at a higher level than intracellular sodium during four hour period but neither ion concentration was as high as intracellular levels in fresh semen.

Meryman (1968) pointed out that the salt concentration theory does not explain properly the mechanism of cellular damage to erythrocytes during freezing. Based on experimental findings he proposed a new explanation that the haemolysis of erythrocytes from slow freezing is caused by an osmotic pressure gradient across the cell membrane greater than that which can be compensated by cell volume change. He observed that the function of the protective compounds is simply to prevent concentration of extracellular solute on a purely colligative basis.

The various aspects of mechanisms of freezing, storage, survival and limitations of living cells and spermatozoa were elucidated in the following discussions: Meryman (1956), Meryman (1957), Polge (1957), Parkes (Ed.) (1957) and Meryman (Ed.) (1966).

II.10.b. Mechanism of cryoprotection

Luyet and Gahlenio (1952) observed that glycerol besides dehydrating the tissues and lowering their freezing point also delays the initiation of crystallisation and reduces
the amount of ice formed at a given temperature. The primary factor in protective action of glycerol is not the prevention or delay of crystallisation nor the lowering of the freezing point but the ability of the glycerol to reduce the amount of ice formed. They observed that this effect may be attributed to some water binding properties of glycerol.

Lovelock (1959a,b) working with red blood cells studied the protective action of glycerol during freezing and thawing. He observed that the glycerol reduces the mechanical stress on cells caused by ice crystal formation. He had refuted the suggestion that ice formation is only damaging when it occurs inside the cell and that glycerol protect the cells by dehydrating them, as the red cells are only transiently dehydrated by suspension in glycerol and are rapidly restored to their original degree of hydration when the glycerol permeates the cells. He had put forward the hypothesis that the damage on freezing is a consequence of the increase in concentration of electrolytes within and with out the cells. In the presence of glycerol the electrolyte concentration at temperatures below freezing point is greatly diminished by an amount sufficient to explain the protection afforded by glycerol.

Lovelock (1954) had again studied the protective action of 15 neutral solutes including mono-, di- and poly-hydric alcohols, amides and sugars against haemolysis of human red blood cells by freezing and thawing. He observed that the protection only occurs when the solute is able to permeate
the cells and that the weight concentration of the solute
required for protection is proportional to its molecular weight.
The mechanism of protective action is explained in terms of
colligative properties of solutions.

Lovelock and Polge (1954) had frozen spermatozoa with
different levels of glycerol. They suggested that the damage
suffered by spermatozoa during freezing and thawing was
caused by their exposure to excessive concentration of salt
when water is removed as ice and the protective action of
glycerol is due to its ability to prevent the salt
concentrations.

Grieff et al (1961) studied the effect of glycerol on
freezing and storage of suspensions of mitochondria using
oxidative phosphorylation as a measure of biochemical
integrity. Oxidative phosphorylation was not altered in
suspensions prepared in isotonic sucrose (0.25 M) containing
10% glycerol and frozen slowly to -76°C. The storage at
-65°C for 15 days did not alter phosphorylative capacities of
the suspensions. Other concentrations of 5 and 20%
glycerol were sub optimal or partially toxic.

The protective action of polyvinyl pyrolidone (PVP)
during freezing and thawing was explained by Meryman et al
(1962). Erythrocytes rapidly frozen in solution having or
not having the polymer additive, PVP, showed higher recovery
when thawed by immersion of frozen material in to an
isotonic saline solution. It was suggested that the
interface seeking molecule of PVP, coated the cells
immediately following thawing, giving them mechanical
protection against haemolysis due to osmotic stresses. This was an additional evidence for osmotic effects as a factor in cellular injury during freeze-thaw procedures.

In a study on the chemical constitution of compounds that protects erythrocytes, Nash (1962) had tested different neutral water miscible compounds and observed that the affinity of the compounds for water correlates well with protective ability. It appeared that this character is most important.

Roy and Djerassi (1964) working with DMSO and dextran had observed that extracellular protective agents should act synergistically with intracellular agents.

Karow and Webb (1965) have theorised the conditions of injury and survival of tissue after freezing. He had observed that slow freezing of tissues are beneficial as by the time the temperature had dropped to the point of intracellular ice, adequate water would have been bound to protect protein and other cellular structures. They had explained the protective action of additives, the "solute moderators", as by strengthening the hydrogen bonded water structures by different methods. When glycerol enters the cells, it structures the molecules of water and thus stimulates the formation of progressively growing lattices. This water is available for solution of electrolytes. A similar mechanism of action by induction of lattice and clathrate formation has been ascribed to the protective non-polar agents such as sugars, dextran, and polyvenyl
pyrolidone. Some sugars can penetrate the cell and act as intracellular protectors while dextran and polyvinyl pyrolidone could form an external shield around cells and cause significant proportion of the intracellular water to organise as lattices.

Ling (1965) and Doebbler and Rinfret (1965) also had presented the conclusion that the glycerol protects the cells by stabilising the water coat around proteins.

Quinn and White (1966) observed that lecithin prevented calcium accumulation in bull and ram spermatozoa on cold shocking and glycerol also had this effect on bull spermatozoa. An egg yolk-citrate-glycerol-fructose diluent protected bull and ram spermatozoa to a considerable extent from the influx of sodium and efflux of potassium that occurs after deep freezing to -79°C.

II.11. Freezing of buffalo semen

Published reports on the deep freezing of buffalo semen are few. This might have been caused by the limited distribution of the species combined with the difficulty of freezing its semen with the conventional methods. It is possible that many of the unsuccessful attempts remain unpublished.

Bhattacharya and Srivastava (1965) observed no significant difference between various levels of glycerol in the survival of buffalo spermatozoa after freezing. More rapid cooling in the critical range (-14 to -25°C) resulted in higher percentage of survival. Roy et al (1956) used a
glycine-egg yolk medium for dilution of buffalo semen. After equilibration for 72 hours when frozen and thawed 80% of the spermatozoa were motile. Rathore (1965) tried instantaneous freezing with buffalo semen at two levels of glycerol. Eventhough the survival rate was very low, 7% glycerol was found to be better than 14%.

Settergren (1966) had conducted some trials on the deep freezing of buffalo semen and inseminated 5 buffaloes with frozen semen and got two pregnancies out of it.

Pavithran et al. (1968) had presented some preliminary observations on the deep freezing of buffalo semen. After trials with different diluters it was observed that the addition of lactose to the diluent had beneficial effect and that freezing semen in ampoules in powdered ice gave better results than other methods.