CHAPTER I

INTRODUCTION
INTRODUCTION

I.1. Historical

It has been a constant source of stimulus and a challenge to the biologists for centuries in entertaining the idea that by holding the reaction rate in tissues at minimum they can be kept in suspended animation to be revived again at a later stage. This can be achieved only by reducing the biochemical activities of tissues to a very low temperature as that of solid carbon dioxide (-79°C) or of liquid nitrogen (-196°C). But there occur in the cells and tissues many changes in the process of cooling which can cause damage to the integrity of cell structures, thus spoiling the whole aim itself. It is a story of strenuous and hard work of many decades which has been rewarded by success in overcoming many of the hurdles. At present a stage has reached in which many tissues are able to be kept in a deep frozen state to be revived again without much deterioration of its vital processes.

The concept of anabiosis or suspended animation in nature has originated in the observations of Leuwenhock that 'animalcules' found in gutters can be stored in dry state which regain normal life again when water is added to it. The origin of this concept and its fascinating development has been told by Keilin (1959) in his lecture to the Royal Society on "The problem of anabiosis or latent life:"
History and current concept. He had defined anabiosis as the state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable or comes reversibly to a standstill.

The studies of frozen storage of biological material have grown into a separate branch of science, "Cryobiology." This has already given innumerable benefits to medical, agricultural and many other sciences. This branch has proved useful to the technique of tissue transplantation, where a ready supply of grafting material can be made available; in the field of microbiology to store organisms avoiding the disadvantages of continuous reculture; in the field of blood transfusion; as well as in the field of artificial insemination of livestock.

Ageing is an unavoidable consequence of living. The continuous interplay of physical and biochemical processes that undergo in living cells, which is necessary for life itself, necessarily leads to ageing and finally death of the cells. By lowering the temperature of activity the rate of physical and biochemical processes are also lowered. Ionic exchanges between the cells and their environments are reduced to a great extent at ice cold temperature but to reduce the physical process of diffusion a much lower temperature of about 

-40°C is necessary. The problem of cooling to such an extent becomes complicated by the changes
that occur in the cell structure during the process of freezing.

1.2. Cellular alterations on freezing

Were it not for the crystallisation of water, it is probable that cells and tissues could be reduced in temperature sufficiently to induce complete suspension of animation permitting indefinite preservation. However, water does crystallise to ice at few tenths of a degree below 0°C with a multitude of consequences. Other than the physical development of ice crystals and the histological distortion they produce, the only other known direct result of freezing is dehydration. As water is removed as ice, there is a gradual concentration of cell solutes.

An understanding of the role of water in living systems is fundamental to a study of biological freezing. Although this is superficially self-evident, the ways in which the water is directly related to freezing injury are not so distinct.

When an aqueous system is frozen, two main changes that occur are the formation of ice crystals with great increase in viscosity and the concentration of dissolved salts. This increase in the concentration goes on till their eutectic temperature is reached when the remaining water in the system also separates as ice releasing the salts as crystals. Both these changes can cause damage to the cellular system involved in the process of freezing as well as when these process are reversed during thawing.
It cannot be assumed that the relation between temperature and rate of injury during freezing will be the same as that during thawing. More water remains frozen during warming than would have frozen at the same temperature during cooling. Thus -3°C or -2°C would be more injurious after warming from a lower temperature than if the tissue had been frozen only to that temperature. Exceptions to this also have been observed (Meryman, 1966).

The damages caused to the cells may be due to the physical rupture of cell structures by the ice crystals formed inside and outside the cells or by the concentration of substances to toxic levels or as a result of alteration of pH caused by the concentration of buffering salts. It seems that more proof is accruing in evidence of the theory that it is the exposure of the cellular membranes to the high salt concentrations that causes major damage during freezing (Ashwood-Smith, 1966).

Other changes that occur in such an aqueous system are the release of dissolved gases and liberation of heat at the rate of 80 calories per gramme.

Several mechanisms by which freezing injures biological materials have been proposed (Meryman, 1966). The more tenable of these are:

1) mechanical rupture of structural elements by the ice crystal growth,

2) denaturation due to electrolyte concentration,
iii) pH changes,
iv) dehydration sufficient to precipitate proteins
    from solution, or
v) to bring them into contact and permit abnormal
cross linking, or
vi) the direct effect of removal of the structurally
important water.

One or more of these may provide an explanation for the freezing
injury.

During the early days itself Becquerel (1936, 1938) had
suggested that the main source of danger to the cellular
structures at low temperatures is at the freezing zone, caused
by the separation of water and electrolytes from the
colloidal particles. He had pointed out that this damage
can be substantially reduced if freezing is carried out in
the presence of certain organic substances such as sucrose,
glucose, fructose, glycerol, ethylene glycol, gelatin,
albumin and various gums.

Later, it has been shown that inclusion of certain
nonelectrolytes like glycerol and dimethyl sulphoxide in
freezing system reduces the changes from the normal and
affords protection of biological material during freezing.
They appear to act in a generalised way on the colligative
properties of the solutions and more specifically on the
kinetics of ice formation and the structure of ice and water.
Glycerol reduces the ice formation and at a particular
temperature the amount of ice formed is inversely proportional
to the amount of glycerol present in the system. It thus directly reduces the electrolyte concentration resulting from ice formation in the cells. Thus the addition of nonelectrolytes lowers the percentage of ice formed and the consequent concentration of electrolytes inside the cells at a particular temperature during freezing and allows the salts to remain in solution at a temperature far below its eutectic points. This directly helps in avoiding damages to the cell system in a physical way which otherwise would have occurred.

A polymer additive, polyvinyl pyrrolidone has been found to give protection to erythrocytes in a mechanical way by giving a protective coating to the cells during freezing and thawing (Meryman et al, 1962; Farrant, 1966).

It is evident that these protective agents should be nontoxic to the cells at the concentration needed for protection and it should be able to penetrate inside the cells also, for which sufficient time has to be allowed. Thus with different biological systems the optimal conditions of concentration and period allowed for penetration of the protective agent as well as the rate of cooling may vary. Each biological material may also vary in the maximum concentration of salts thus separated out which it can stand without damage. There are other factors such as permeability of cells both to water and to protective nonelectrolytes as these are related to the amount of osmotic stress produced.

The formation of ice crystals during freezing processes
ceases at -130°C and so the storage of frozen material is best done below this temperature. **Liquified nitrogen**, oxygen and air are found to be most convenient for this purpose. A well insulated container for these liquids will maintain a temperature of below -160°C up to the top of the container due to the vapour from the liquid below.

**1.3. Freezing of spermatozoa**

Attempts to induce a temporary quiscence in spermatozoa and store it for later resuscitation form a part of the wider problem anabiosis. Eventhough many types of tissues and cells have been successfully frozen, the largest volume of work turned out in this aspect is on the freezing of semen of different species of animals. Due to economic reasons the freezing of cattle semen gained much importance and wider acceptance. Probably no other technique in the field of animal husbandry has gained as much popularity as that of deep freezing of bull semen. At present a stage has reached where the farmer can get the offspring of a good bull even after years of its death. The semen of good bulls are being transported across continents in frozen state to be stored and used for long time.

Experiments in cooling the semen to subzero temperatures were started as early as the middle of 19th century. Prevost (1840), de Quatrefages (1853), Mantegazza (1866) and Schenk (1870) had exposed semen to temperatures ranging from 0°C to -17°C. Davenport in 1897 subjected human spermatozoa
to a temperature of $-17^\circ C$. Later, it was shown that frog spermatozoa frozen in liquid air ($-192^\circ C$) failed to survive but in the presence of 1 to 2 M sucrose solution at least 20-40% of them resumed motility on warming (Luyet and Hodapp, 1938). Jahnel in 1938 and Shettles in 1940 tried to freeze human spermatozoa to $-79^\circ C$, $-196^\circ C$ and $-269^\circ C$ and met with partial success. Shaffner et al (1941) had tried to freeze rooster semen after partial dehydration and addition of levulose. The first report of fertilisation from frozen semen came from Shaffner (1942) eventhough it ended in embryonic mortality.

The earlier workers in the field of deep freezing of spermatozoa were handicapped in as much as that they were unaware of the various alterations in the integrity of the cells during freezing procedures and that the protective agents which can prevent these changes were not known at that time. One such agent is glycerol which is now being widely used and which seems to be the best in the category. It has an efficient protective action on the spermatozoa during the process of freezing and alters the crystallisation pattern favourably with reduction in the total amount of ice formed at a particular temperature, thus reducing the mechanical stress on the cells during freezing (Lovelock, 1953). It also exerts an influence on the electrolyte balance in the system during the process of freezing (Lovelock and Polge, 1954).

The remarkable discovery that glycerol can protect
the spermatozoa from the effect of freezing was made by Polge, Smith and Parkes (1949) working on fowl spermatozoa. This observation was later supplemented by the same workers (Smith and Polge, 1950; Polge, 1951; Polge and Rowson, 1952; Parkes, 1956).

Although Costand (1946) had shown that motility of frog spermatozoa could be preserved in glycerol at subzero temperatures, this discovery remained in the dark till its rediscovery by Polge and coworkers independently. This had aroused widespread interest in freezing of bull semen for artificial insemination purposes and Stewart (1951) and Smirnov (1951) came out with reports of getting calves out of insemination with frozen semen in cattle.

1.4. Buffaloes over the world

The contribution of buffaloes to the food supply of the world is considerable. The majority of the world's buffaloes are located in countries of South Asia. The various breeds of buffaloes (Bubalus bubalis) are separated into two groups: river buffaloes, which are found mostly in India and the swamp buffaloes which are plentiful in east and south of Burma (Cockrill, 1967). River buffaloes are better suited to milk production than swamp buffaloes.

In India the river buffalo is known to have been domesticated since 2500 B.C. and the swamp buffalo in China about 1000 years later. From these two countries they have spread out to different parts of the world. The estimation of total population of buffaloes over the world was about
-10-

119 million out of which India's contribution was over 55 million for the year 1964-65. This was about 46% of the total population (F.A.O., 1966).

Cockrill (1967) had presented the distribution of buffaloes over the world in four categories depending on the number of buffaloes per 1000 of human population. In the first category of having over 100 buffaloes per 1000 population are the countries like India, West Pakistan, Thailand, Cambodia, Laos and Philippines. In the second category of having 50 to 99 buffaloes per 1000 population are the countries like Ceylon, Burma and Egypt. In the third category of 10 to 49 animals per 1000 population are China, Tunisia, New Guinea, Malasian islands, Iran, Iraq, Turkey, Bulgaria and Rumania. In the last category of less than 10 animal per 1000 population are Brazil, Italy, Greece, Yugoslavia, Hungary, Syria and Afghanistan.

The region-wise buffalo population for 1959-60 were as under (Singh and Parnerkar, 1966): (numbers in thousands) Europe 500; Near East 3,700; Far East 68,400; Africa 400; and world total 94,000.

In most of the countries buffaloes are used as a work animal and milk is only a secondary one, if at all they are used for that purpose. But only in India buffaloes are important for milk production as well as draught purposes. Its qualities as a meat animal is equal to that of cattle and its meat is almost indistinguishable in taste and quality from that of cattle.
The milk of buffaloes is richer in nutrients than that of cows'. Buffalo gives milk of almost double the amount of butterfat and its nonfat solids are also higher than that of cows' milk.

Had the modern practices of selective breeding and husbandry been adopted in the buffaloes, the potentialities of them with regard to milk and meat qualities would have improved, alleviating in part the shortage of food supply over the world.

1.5. Buffaloes in Indian economy

Buffaloes play a crucial role in the national agricultural economy which is evident from the following facts (Singh and Parmerkar, 1966). During the period of 1959-60, nearly 48% of the national income was derived from agricultural activities out of which the contribution of animal husbandry was 7.2%. The major part of the income from animal husbandry was derived from milk and milk products.

According to 1961 census the total number of female cattle above three years was 176 million, whereas the corresponding figure for buffaloes was only 51 million which was slightly less than one third of the former. But as far as the production of milk is concerned the buffaloes had contributed much more than the larger number of cattle. The tentative estimate of milk production for 1961 for cattle was 8.6 million tons and that for buffaloes was 11.9 million tons.

The superior milk potentiality of buffaloes over
cattle can further be emphasised by the distribution of animals according to the milk yielding capacity. Among cattle 94.3\% yielded less than a Kg. milk per day on an average whereas only 19.2 \% of buffaloes fell in this category. 5.7\% cattle yielded 1 - 2 Kg. per day whereas a larger number, 62.2 \% of buffaloes were in this category. Again, cattle yielding above 2 kg. of milk per day was only 0.4 \% whereas there were 18.8 \% of buffaloes under this category.

Buffaloes in general had a better breeding performance and during 1966, 54.5\% of breedable buffaloes were in milk, whereas only 42.5\% of breedable cattle were in milk. It is gratifying to note that buffalo population shows a better trend of growth than cattle. Buffaloes had increased to 176\% in 1961 over 1920 whereas cattle had recorded an increase of 150 \% during that period.

Till today the attention given to the development of buffaloes were negligible when compared to that accorded for cattle. Considering the better performance as well as the superior nutritive quality of the milk of buffaloes, it is evident that the buffaloes deserve a better deal from our hands.

I.6. Statement of the problem

Due to geographical reasons the deep freezing of semen was mainly concentrated on that of cattle. The semen of buffaloes behaved differently to the usual freezing procedures adopted for cattle. This fact conjoined with
the fact that the buffaloes are mainly distributed in the developing Asian countries contributed to a situation necessitating to explore the possibility of deep freezing buffalo semen aiming at successful freezing and storage of semen without loss of its fertilising capacity and to study different other aspects connected with them.

1.7. Importance of the study

Improving the performance of buffaloe breeds had been hampered by many factors. The first and foremost is the lack of sufficient number of good bulls. Buffaloes have the added disadvantage of high calf mortality. The artificial breeders of buffaloes have been experiencing the additional difficulty in preserving the diluted semen at refrigeration temperature as the keeping quality of buffalo semen is much inferior to that of cattle semen with the conventional diluter. Experiments regarding the suitability of various diluters for buffalo semen and their comparative efficacy had been undertaken by many workers. But none of these diluters has been accepted universally.

This being the position of artificial breeding of buffaloes with chilled semen, the advantages of deep freezing of buffaloe semen for artificial insemination purposes assume great importance. It is true that the initial cost of changing over from liquid semen to frozen semen inseminations may be high, but in the long run it will be definitely advantageous to the breeder taking into consideration the limited availability of good germplasm.
Excepting the problem of initial high cost and availability of cooling medium like liquid nitrogen, the running cost of the artificial insemination programme with frozen semen is as economical, if not more, as chilled semen insemination programme. It must be emphasised that in the frozen semen insemination programme cent per cent utilisation of the semen is possible, whereas with chilled semen the utilisation may be anything between 10-50% only. The poor keeping quality of chilled semen and the nearly seasonal breeding habit of buffalo cows contribute to this poor utilisation of diluted semen.

India is in a fortunate position of having the world's best breeds of buffaloes for milk production. Special mention has to be made to Murrah and Nili (Ravi) breeds of buffaloes whose breed averages are about 2200 Kg. per lactation. This gives us an opportunity to help other buffalo breeding countries by shipping frozen semen for their breeding programmes as well.

Thus the approach of deep freezing of buffalo semen offers tremendous possibilities in improving the economy of the country besides opening new areas of international co-operation.