Natural Environment Research Council

Institute of Terrestrial Ecology

CRYOPRESERVATION

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An introduction to cryopreservation
in culture collections

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The cover photograph shows the
removal of samples from liquid nitrogen.
Photograph Chris Chalk.

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The Institute of Terrestrial Ecology (*ITE*) was established in 1973, from the former Nature Conservancy's research station and staff, joined later by the Institute of Tree Biology and the Culture Centre of Algae and Protozoa. ITE contributes to and draws upon the collective knowledge of the fourteen sister institutes which make up the *National Environment Research Council*, spanning all environmental sciences.

The Institute studies the factors determining the structure, composition and processes of land and freshwater systems, and of individual plant animal species. It is developing a sounder scientific basis for predicting and modelling environmental trends arising from natural or man-made change. The results of this research are available to those responsible for the protection, management and wise use of our natural resources.

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Contents

THE TEMPERATURE RANGE ENCOUNTERED IN CRYOBIOLOGY

0°C	Freezing point water
−20°C	Domestic deep freeze
−21.8°C	Eutectic temperature NaCl :water
−79°C	Solid carbon dioxide (dry ice)
−88.7°C	Lowest recorded environmental temperature
−139°C	Limit for ice crystal growth
−196°C	Boiling point nitrogen
−213°C	Melting point nitrogen
−273°C	Absolute zero.

INTRODUCTION

Cryobiology, the study of the effects of low temperatures upon biological systems, has a long history. The first well documented experiment was by Henry Power in 1663, when eel-worms in vinegar were frozen for several hours and upon thawing "danced and frisked about as lively as ever". The observation that life could be arrested at low temperatures and subsequently "resurrected" upon thawing aroused much speculation, both of a scientific and theological nature. However, with the exception of a few naturally resistant forms, little practical success was achieved. Cryobiology gained real impetus only in 1949, when it was reported that glycerol protected fowl spermatozoa against freezing injury. Since this fortuitous discovery, storage under liquid nitrogen has become a standard method for the long-term maintenance of a large variety of cell types and tissues (Table 1). At this temperature (−196°C), cellular viability is almost independent of the period of storage and biological systems are genetically stable.

In the medical sciences, methods for the storage of organs before transplantation are being developed. It has even been suggested that bodies could be stored in a state of "suspended animation" in terminal illness or for interstellar travel. However, there is little scientific basis for removing this concept from the

Table 1. Cryopreservation in biology and medicine

Cell-type	*Applications*	*Advantages of Cryopreservation*
Red blood cells, platelets	Transfusion	Blood stored in the conventional manner at 4°C has a limited 'shelf life' after which it is not suitable for transfusion. Cryopreserved blood cells can be successfully transfused after years of storage.
Bone marrow, lymphocytes, skin, cornea	Transplantation	Material can be tissue-typed and stored when it becomes available and used as required.
Spermatozoa	Artificial insemination	Widely used in veterinary science for the long-term improvement of stock.
Mammalian Embryos —(Plate 4)	Implantation into foster mothers	As for spermatozoa, with the additional advantages that frozen embryos are easy to transport and overcome many quarantine restrictions.
Seeds	Conservation of plant genetic resources	Although many types of seed can be stored in the dried state, there is a group of plants in which reduction in water content of the seed below a critical level decreases the period of viability, such seeds are thus difficult to preserve by conventional methods. Examples include tropical crops such as rubber, oil-palm, coffee and cocoa.
Plant shoot-tip and tissue-culture cells	These cultures have the potential to regenerate into plants and thus offer an alternative method of preservation when seed storage is not suitable i.e. for discrete clones.	The selection pressures inherent in any sub-culturing programme are avoided. In addition, many mutants of plant tissue-culture cells have been selected and characterized. These novel genotypes are of potential importance in producing new cultivars of high yielding or disease resistant crops, the economic importance of which may take several years to evaluate. Cryopreservation is a simple method of stabilizing these cultures.
Suspension cultures of yeasts, bacteria, protists, mammalian tissue culture cells etc.	Culture and reference collections. Starter cultures for industrial processes.	The cell suspensions are maintained in a stable state and any genetic drift is avoided. Once under liquid nitrogen there is no possibility of mislabelling or contamination of the cultures and there are also economies of time, space and materials.

realms of fantasy (Plate 1). Freezing is used for the removal of warts and surface tumours (Plate 2) and in the relief of pain.

There has been extensive research into the harmful effects of environmental chilling and freezing on crop plants. An understanding of the biochemistry of this injury may allow development of practical methods of increasing crop hardiness. This would be of major importance for world food production as, for example a 2°C increment in the frost hardiness of wheat could extend cultivation into areas which are now marginal because of cold stress, resulting in a potential increase in world wheat production of between 25 and 40%. A complementary area of research lies in the ecology of polar and alpine regions. Some algae have evolved to grow on the surface of snow (Plate 3), where they are exposed to a daily temperature range of −35 to +5°C. The mechanisms by which these and other plants survive such extremes of temperature may provide insights into the nature of injury during freezing and thawing.

The technique of freeze-drying (lyophilization) is widely employed for the storage of prokaryotic organisms. In this method the culture is frozen and water is then removed directly by a reduction in pressure. The dried sample is sealed under vacuum in which state it can be stored at room temperature. The cultures are reconstituted simply by the addition of fresh medium. Unfortunately, this technique cannot as yet be applied to eukaryotic cells because survival rates obtained with these more highly organized cell-types are very low, invariably <0.1%, survival rates decline with increasing time of storage, and the process of lyophilization has been said to be mutagenic.

This account concentrates on the long-term preservation of cellular viability at low temperatures (cryopreservation). Other related topics of cryobiology will be discussed only when directly relevant.

STRATEGIES FOR CRYOPRESERVATION

The outstanding success of cryopreservation has been in the medical and veterinary sciences. However, the direct application of the methods empirically evolved for mammalian cell-types are often unsatisfactory for the storage and recovery of other organisms. This appears to be especially true for plant material. At the ITE Culture Collection of Algae and Protozoa (CCAP), research into the cryopreservation of algae and protozoa is in progress. Whilst it is often possible, using "recipe" methods, to cryopreserve these organisms such methods are not satisfactory because of very low recovery rates. For the long-term storage of material it is essential to minimize the loss of viability. The reasons for this need are:—

(1) Practical difficulties may arise in re-establishing growth if the viable cell density falls below a minimum inoculation level.
(2) The processes of freezing and thawing are not mutagenic. However, at low levels of recovery, pre-existing, freeze-resistant mutants may be selected.
(3) Cryopreservation can select sub-populations of a cell-type. This selectivity may have practical applications e.g. in eliminating organized structures from cell suspensions or resting stages from vegetative cells, but must be avoided for genetic conservation.

It is not intended to describe the many 'recipe' methods that have been used for the storage of cells. Further progress can best be achieved by an understanding of cryobiology, the basic principles of which are outlined in five sections:—

1. Physical stresses to which biological materials are exposed during freezing and thawing.

2. Effects of low temperatures on biological membranes.

3. The effect of these stresses upon cellular viability and the empirical methods by which cell survival is increased.

4. Current theories of the physico-chemical mechanisms of cell damage.

5. The site of cellular injury. Specific methods of cryopreservation can best be evolved if the biochemistry of freezing injury is understood.

The specialized equipment and methods used in cryobiology are described in Appendix 1. Details of a procedure developed at CCAP for the storage of Chlorococcales at −196°C are presented in Appendix 2. Key references to the literature are given in Appendix 3.

FREEZING AND THAWING

The freezing process

If water is cooled below its freezing point without the formation of ice, it is said to be super- or sub-cooled. Ice formation from the super cooled state is a random event initiated by the nucleation of a single crystal of ice. This may be the result of an ordering of water molecules, i.e. homogeneous nucleation, or induced by other substances, i.e. heterogeneous nucleation. As ice forms latent heat is liberated and until this heat has been dissipated no further cooling occurs (Figure 1).

When pure water freezes there is a discrete phase transition and all the liquid water turns to ice. In aqueous solutions, e.g. biological systems, only a proportion of the water is converted to ice and this removal of liquid water increases the concentration of

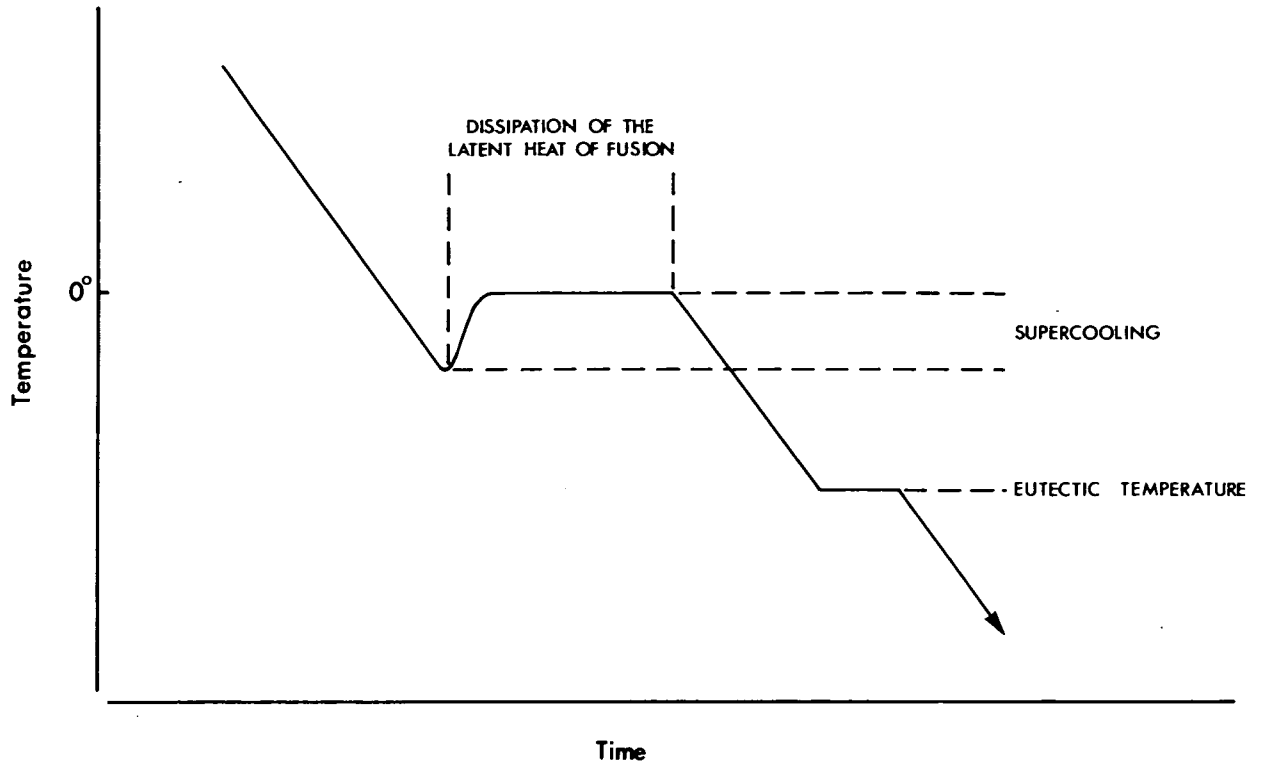

Figure 1—The freezing processes in a dilute salt solution.

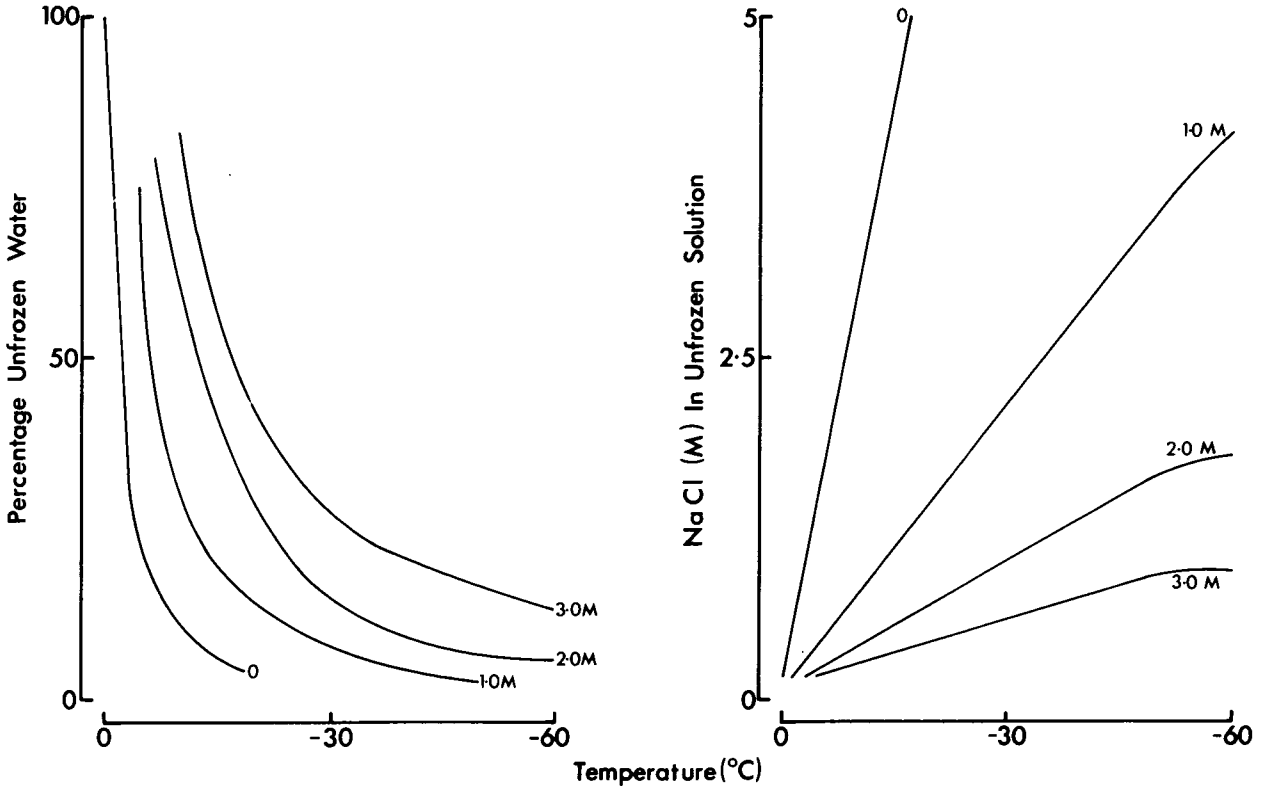

Figure 2—Percentage of unfrozen water at various subzero temperatures (left). Molarity of sodium chloride in the unfrozen portions of solutions at different subzero temperatures (right). The values on the curves refer to the concentration of glycerol before freezing. The initial molarity of NaCl was 0.15M.

solutes in the remaining aqueous phase. As the temperature is further reduced more ice forms and the residual solution becomes increasingly concentrated. Figure 2 illustrates these events in a dilute solution of sodium chloride. Ice co-exists with concentrated salt solutions until the eutectic temperature is reached, at which point the solutes crystallize. With sodium chloride this occurs at −21.8°C. At the eutectic transition the temperature remains constant until solidification is complete (Figure 1). The incorporation of a non-ionic component, e.g. glycerol, reduces the amount of ice at any temperature during cooling, thus reducing the increase in ionic concentration.

What happens to cells in such a system? Most cell-types can be supercooled without any deleterious effect and injury occurs only with the formation of ice. At high sub-zero temperatures biological membranes are an efficient barrier to ice crystal growth and extracellular ice does not initiate nucleation within the cell. There is no evidence of mechanical injury to cells by extracellular ice and the damaging effects of freezing and thawing are associated with the formation of concentrated solutions. Following extracellular freezing the interior of the cell is at a much lower ionic concentration than the surrounding solution and water must be lost from the cell to maintain equilibrium. There are two ways in which this loss can take place.

(a) Water can move osmotically from the cell to the hypertonic solution around it.

(b) Liquid water can be removed by the formation of intracellular ice.

Which of these processes predominates depends largely on the rate of cooling.

Cooling rates

In the natural environment, with slow rates of cooling, ice formation is invariably extracellular. In the laboratory, a wide range of cooling techniques can be used. The effects of three rates of cooling on cells are represented diagrammatically in Figure 3.

At slow rates of cooling, there is sufficient time for osmotic equilibrium to be maintained by cell shrinkage. Dehydration occurs until a minimum cell volume is attained, beyond which further shrinkage is physically limited. In most plant cells the response is complicated by the presence of a relatively rigid cell wall which restricts volume changes. Following exposure to hypertonic solutions, shrinkage of the protoplast away from the cell wall occurs (plasmolysis). Upon rehydration there is the reverse process of deplasmolysis.

During cooling, two types of cellular response can occur. The most commonly observed is that of true plasmolysis, often accompanied by a collapse of the cell wall so that total cellular dehydration occurs (Figure 3a). Upon thawing, the cell wall, which is naturally elastic, returns to its original shape. In damaged cells, the protoplast does not swell and remains contracted, producing so-called "frost plasmolysis". Alternatively, ice may form between the cell wall and protoplast (Figure 3b). Because of differences in chemical potential between supercooled water within the protoplast and the ice outside, the protoplast shrinks away from the cell wall (pseudo-plasmolysis).

As the rate of cooling is increased the cells are exposed to the potentially damaging effects of hypertonic solutions for shorter periods. However, there is less time for osmotic dehydration to occur so the cell interior becomes increasingly supercooled and the probability of intracellular ice nucleation increases.

It is now possible to directly observe cells during freezing and thawing using a specialised microscope system (cryomicroscope), in which the temperature of the microscope stage and the rate of change of temperature are accurately controlled. In Plate 5 the response of two filaments of the green alga *Spirogyra* are illustrated. Nucleation of extracellular ice occurred at −2°C, cells are then surrounded by concentrated growth medium. As the temperature is reduced one filament loses water osmotically and plasmolyses. During thawing the protoplast partially rehydrates but is extensively damaged. In the other filament only a small amount of shrinkage occurs and consequently the cell becomes supercooled. At −7·5°C ice forms within the cells, as visualized by a sudden darkening of the cellular contents. This is due to many small crystals of ice which interfere with the passage of light through the cell. As this ice melts gas bubbles appear in the cells, these bubbles persist after thawing (Page 19).

The actual rate of cooling at which intracellular ice formation occurs is dependent on the water permeability of a cell and its surface area to volume ratio. A human red blood cell has a very high permeability coefficient to water and osmotic equilibrium is maintained at rapid rates of cooling. Intracellular ice forms only at rates greater than 3,000°C min^{-1}. In contrast a white blood cell has a lower water permeability and becomes supercooled at slower cooling rates and nucleation is observed within the cell at rates in excess of 10°C min^{-1}. With ultrarapid rates of cooling, there is insufficient time for ice nucleation and subsequent growth of crystals, so water reaches low temperatures in an amorphous glass state.

Cell survival following freezing and thawing can therefore be interpreted as a result of two damaging processes, namely the effects of concentrated solutions at slow rates of cooling and the formation of intracellular ice at faster rates (Figure 4). The effects of a range of cooling rates on the recovery of a wide variety of cell-types (Figure 5) indicate that:—

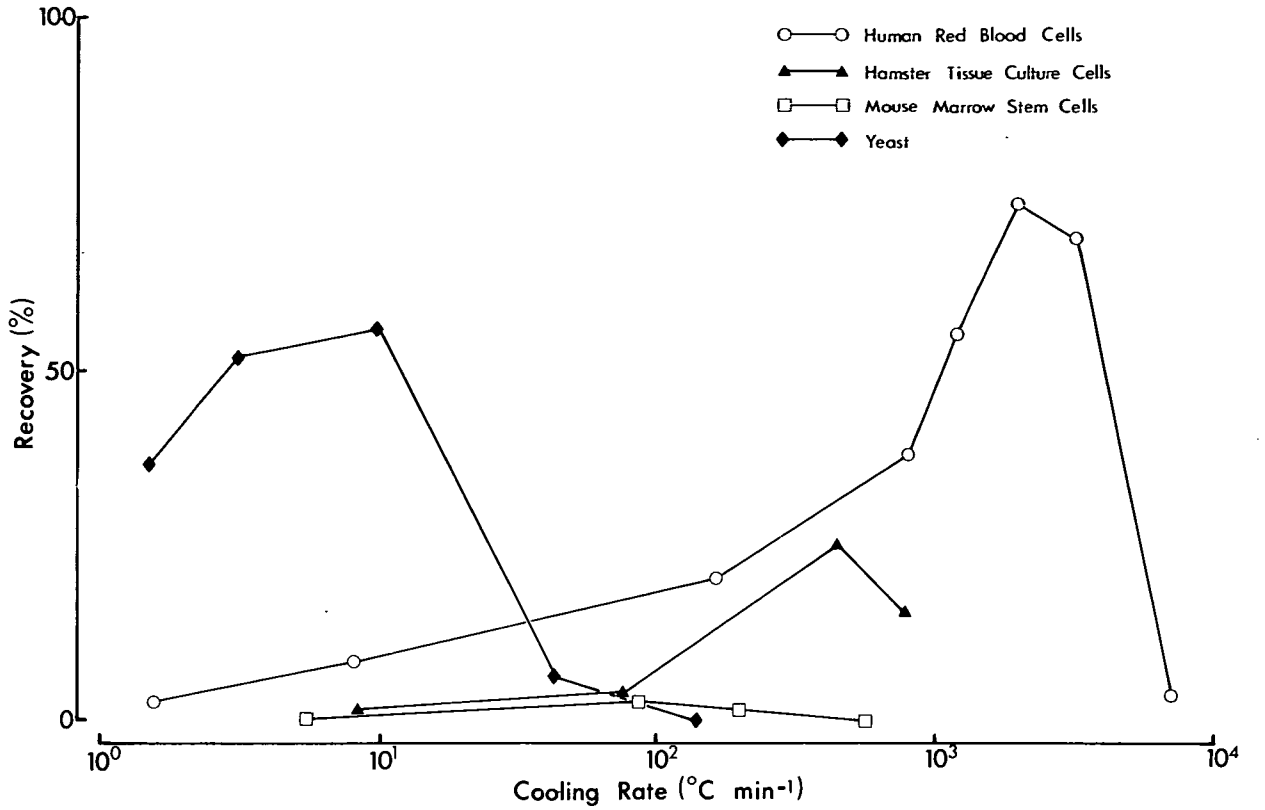

Figure 5—Recovery of various cell-types following cooling at different rates to -196°C.

(a) There is often a rate of cooling which gives optimal survival so that at both faster and slower rates, survival decreases. Slow and fast rates can be defined for a cell-type, but have no absolute values. To use an extreme example, a rate of less than 3,000°C min^{-1} is sub-optimal for the maximum survival of red blood cells but is too rapid for the survival of most other cells.

(b) Maximum survival varies for different cell-types and is determined by the extent to which the two damaging processes overlap.

The differences between cells account for some of the difficulties experienced in preserving organs, as each of the cell-types composing the organ has a specific cooling rate requirement for optimum survival.

Warming rates

The response of cells to different warming rates after freezing is determined by the method of cooling (Figure 6). With slow rates of cooling, cells reach low temperatures in a shrunken state and rehydration of the cell occurs upon warming. This process is independent of the rate of warming. At rapid rates of cooling the probability of intracellular ice formation increases. This ice is thermodynamically unstable and recrystallization occurs at slow rates of warming so that large ice crystals are formed within the cell. As the rate of warming is increased, there is less time for recrystallization to occur before thawing.

EFFECTS OF LOW TEMPERATURES UPON BIOLOGICAL MEMBRANES

All cells are bounded by a plasma membrane which encapsulates the cytoplasm. In eukaryotic cells the organelles are also surrounded by membranes thus forming internal compartments. Any alterations in the surrounding micro-environment first impinge on the plasma membrane and it is generally assumed that the cellular response to freezing and thawing is largely determined by the properties of this membrane. Analysis of isolated membranes show them to be composed of lipids and protein. The current conception of how these molecules are organized in biological membranes is shown in Figure 7.

Phospholipid molecules possess polar head groups and have two fatty acids esterified to a glycerol group. These fatty acids can be of different chain length and degree of unsaturation (number of double bonds per fatty acid molecule). Fatty acids are hydrophobic and the most stable configuration of phospholipids in aqueous solutions is in the form of bilayers, in which the polar head groups face the aqueous solution, whilst the fatty acid chains occupy the non-aqueous centre of the bilayer. The lipid composition of the membranes is heterogeneous. Phospholipids have different polar head groups and fatty acids and other lipids e.g. sterols and neutral lipids, also occur within the bilayer. The lipid composition varies within the plane of any membrane and between different membranes in a cell. Membrane proteins are either localized on the surfaces of a bilayer or pass entirely through the hydrophobic

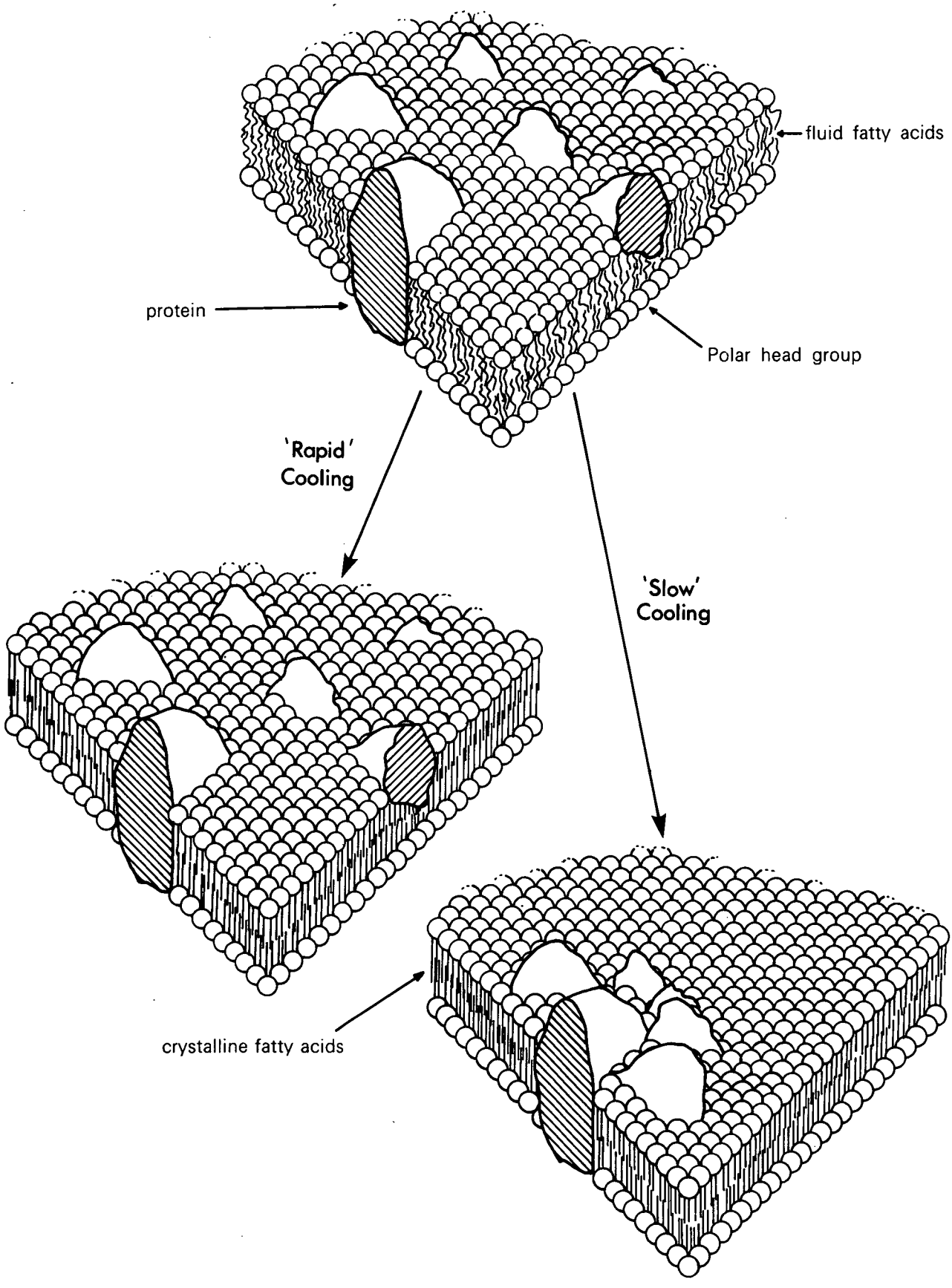

Figure 7—The fluid mosaic model of the structure of cell membranes and the effects of slow and rapid cooling on membrane protein distribution. Reproduced in part from Singer & Nicholson, 1972, Science, *175 : 720. Copyright 1972 by American Association for the Advancement of Science.*

core. The whole membrane structure is dynamic with both lipid and protein components capable of lateral and rotational diffusion.

The lipid bilayer can pass through a thermal phase transition. At a temperature below the transition, the fatty acids of the phospholipids are in a relatively rigid, crystalline state; at a temperature above the transition these side chains assume a more random fluid structure. The temperature at which the phase transition occurs is largely determined by the fatty acid composition, especially the degree of unsaturation. The more double bonds a fatty acid molecule contains, the lower the temperature of phase change. Because of the heterogeneity of biological membranes the temperature range of the phase transition is very broad (c. 10°C). Upon cooling, the membrane lipids become solid and therefore the membrane proteins are no longer free to diffuse. The topography of these proteins at low temperatures is dependent on the rate of temperature reduction. At rapid rates of cooling there is insufficient time for migration of proteins and they are "set" in a configuration similar to that found at normal temperature. During slow cooling a gradual phase separation occurs and proteins are pushed into areas of still-fluid membrane so, at low temperatures, the membrane proteins will be aggregated and large areas of protein-free lipid occur.

The processes of membrane synthesis, transmembrane transport and the activity of membrane-bound enzymes can occur only when the membrane is sufficiently fluid, Therefore living organisms maintain a lipid composition that renders the membrane semi-fluid at the growth temperature by regulating the degree of unsaturation of the membrane lipids.

CELLULAR VIABILITY FOLLOWING FREEZING AND THAWING

The many variables which determine the response of cells to the stresses of freezing and thawing can be separated into two classes; the intrinsic or cellular factors and the extrinsic or physical factors. Although many of these factors are interrelated, and all contribute to the viability of cells upon thawing, they will be considered separately.

The response of unicellular algae will be used to illustrate these factors as the viability of such cells can be accurately determined by serial dilution and colony formation in agar. Indirect methods of assaying viability are available e.g. dye exclusion, uptake of vital stains and motility, but, although these methods are simpler to use, they usually over-estimate the recovery potential. Regrowth and division are definitive indicators of viability which should be used whenever practical.

Intrinsic factors

Cellular viability following freezing is partly determined by the choice of cell material and the culture conditions before and after freezing. A natural resting stage, with a low water content, is more resistant to injury than is hydrated vegetative material. Therefore a dehydrated form such as seed, zygote or cyst should be selected for preservation. However, in many cases this selection is not possible, and it is therefore important to determine the growth conditions which produce vegetative cells in a state most resistant to freezing injury. The culture conditions which have been most frequently examined include the following.

Growth temperature

The frost resistance of many plants increases with a decrease in ambient temperature. This cold hardening process occurs naturally in autumn, allowing plants to survive the extremes of winter. It is often assumed that this phenomenon is restricted to plant tissue, but similar results have been described for bacteria, ciliated protozoa and insects.

A typical pattern of cold acclimatization is observed with the unicellular alga *Chlorella emersonii* (Figure 8). The biochemical adaptations which allow metabolism to continue at 4°C increase the resistance to freezing injury compared with cells cultured at 20°C.

Cold acclimatization has been found useful in increasing the recovery after freezing to −196°C of a variety of cell-types, but the response is variable. Some species are killed at the hardening temperature (page 13), but, of those which adapt to the reduced temperatures, the freezing tolerance is either unaffected or significantly increased.

Extensive attempts to determine the nature of the cold-hardening process have been made from comparisons of the frost-hardy and non-hardy states. Many biochemical and ultrastructural alterations have been described and emphasis has been placed on the increase in the degree of unsaturation of the membrane phospholipid fatty acids (Table 2). However, in all these studies, it is impossible to differentiate between the adaptations necessary for cells to metabolize at the lower temperature, those which are a consequence of reduced growth rate, and the specific modifications (if any) conferring freezing resistance.

Age of culture

When suspension cultures are grown by batch methods they pass through definable phases of culture. For algae, cells from the actively dividing exponential phase are more sensitive to freezing injury than are cells from the older stationary phase. In the stationary phase of culture, cell growth is limited by many physical and nutritional factors and under these conditions cells accumulate lipid, become less vacuolated and modify the membrane fatty acids (Table 2).

Table 2. Phospholipid fatty acid composition (percent) of *Chlorella emersonii* following different treatments.

Fatty Acid	a	b	c	d
12 : 0	—	0.28	—	—
13 : 0	—	0.06	—	—
14 : 0	0.28	0.89	0.12	0.13
15 : 0	0.20	0.54	0.33	0.16
16 : 0	15.24	15.20	11.79	15.53
16 : 1	2.19	3.26	1.71	0.85
16 : 2	3.70	3.69	2.50	0.57
16 : 3	11.79	8.81	5.66	6.21
16 : 4	13.86	10.93	17.51	20.64
17 : 0	0.56	0.65	1.04	0.66
18 : 0	0.29	1.23	0.13	0.55
18 : 1	10.38	11.86	8.52	9.45
18 : 2	11.63	10.23	13.16	6.16
18 : 3	19.11	13.93	23.63	31.11
18 : 4	6.86	6.96	9.78	5.60
19 : 0	1.27	2.19	1.52	0.68
20 : 0	1.73	4.05	1.95	1.07
20 : 1	0.27	1.88	0.33	—
20 : 2	0.33	0.45	—	—
20 : 4	—	1.09	—	—
Unknowns	0.31	1.82	0.52	0.63
Mean mol. wt.	265.50	267.68	267.32	265.60
db/mol	2.22	1.95	2.42	2.44

Cells were from cultures maintained at 20°C for (a) 14 days, (b) 14 days then frozen to and thawed from −196°C, (c) 35 days, (d) at 4°C for 14 days; Fatty acids are noted by two numbers, the first of which gives the number of carbon atoms and the second the number of double bonds. db/mol = mean number of double bonds per molecule of fatty acid; — = not detected.

Table 3. Response of sixty strains of *Chlorella* to freezing to, and thawing from—196°C

Species	Recovery following freezing and thawing		Presence of a large vacuole
	<10%	>60%	
C. emersonii	8	0	+
C. luteoviridis	9	0	+
C. kessleri	2	0	+
C. saccharophila	7	0	+
C. vulgaris f. tertia	3	1	+
C. vulgaris var. vulgaris	5	10	±
C. protothecoides	0	15	—

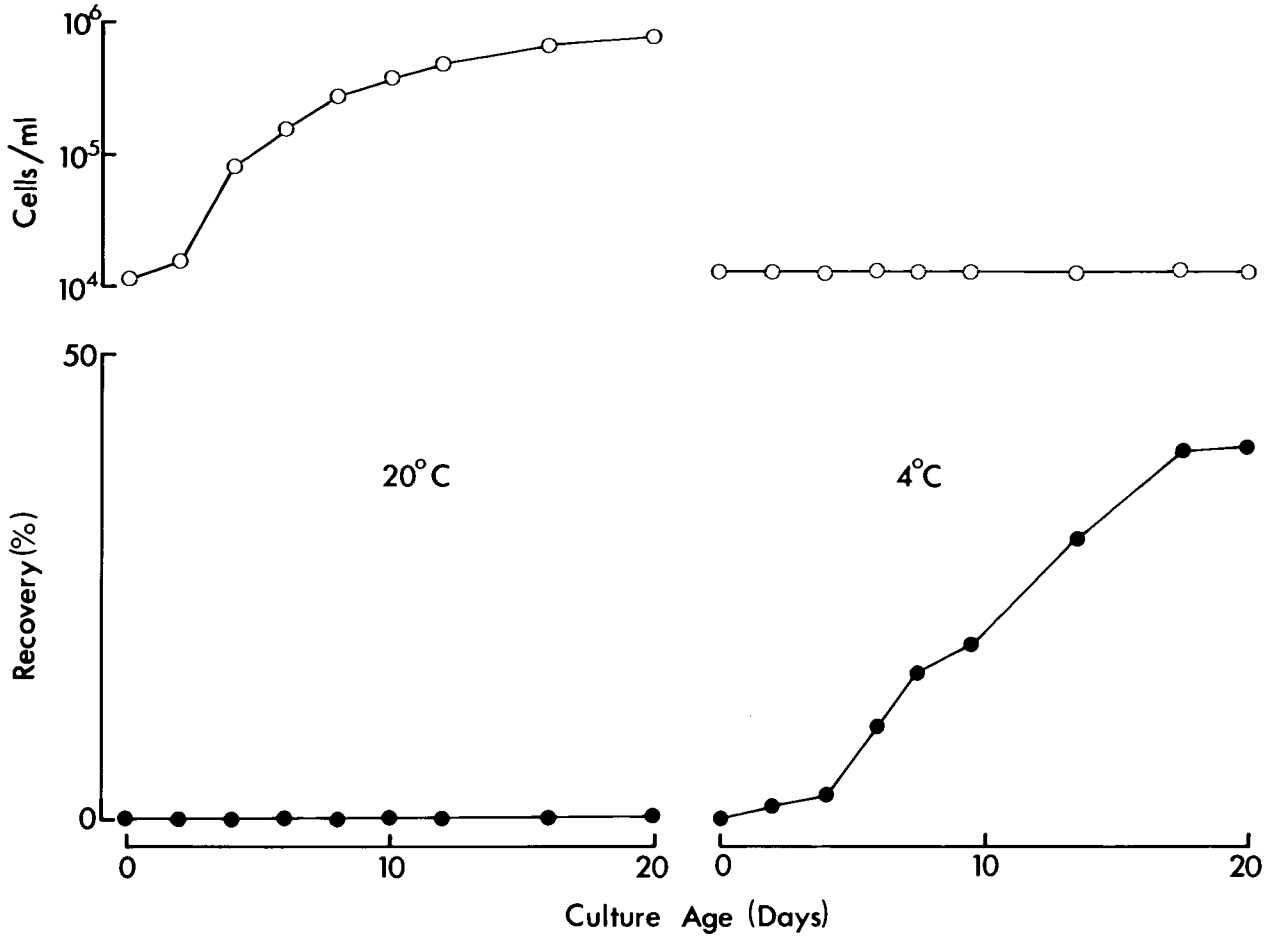

Figure 8—Recovery (%) of Chlorella emersonii *following freezing to and thawing from -196°C on successive days of culture at 20 and 4°C.*

As a general principle, the susceptibility of plant cells to freezing injury is associated with the presence of a large vacuole. This is clearly demonstrated by the response to freezing and thawing of 60 strains of *Chlorella* (Table 3). For the four species that contain a large vacuole the recovery from −196°C was less than 10% and in most cases below 0.1%. The presence of a vacuole in *C. vulgaris* is strain specific and some strains (11/19) without vacuoles survived freezing and thawing with a recovery greater than 60%. *C. protothecoides* does not have a prominent vacuole and all strains examined were resistant to freezing injury. A similar relationship between the extent of vacuolation and sensitivity to freezing injury exists for many plant tissue-culture cells.

It is evident that, before freezing, cells should be cultured under conditions which reduce the degree of vacuolation. In the algae so far investigated this condition is correlated with an accumulation of storage lipid at low growth rates. Freezing tolerance is increased following limitation of nutrients, reduction in temperature or light intensity, addition of metabolic inhibitors and the use of hypertonic growth media.

Culture conditions after thawing

The growth requirements of frozen and thawed cells are often different from those of unfrozen material. The recovery of algae upon thawing is partly determined by the plating medium used to estimate viability. Survival is maximal when a complex medium is employed. It is thus probable that different levels of injury occur in cells, some of which may be reversible, depending upon the culture conditions.

Extrinsic factors

If the maximum intrinsic tolerance to freezing injury has been achieved by modifications in the growth conditions, cellular survival is determined by the complex interactions between the physical determinants of the freezing and thawing process and the addition, if any, of protective compounds.

Chilling injury

Some cells are damaged by a reduction in temperature. The first step in any cryopreservation method is to cool the sample to a temperature at which ice nucleation is initiated and therefore a chilling stress is applied. Longer times of exposure to low temperatures occur in cold-hardening (page 11). Chilling injury is of two types:—

(1) Direct chilling injury or cold shock, which is

expressed immediately upon a reduction in temperature. This injury is dependent on the rate of cooling; more damage occurs at rapid rates than at slow rates. Cold shock occurs in rapidly dividing cells of blue-green and green algae and in many species of tropical plants and suspension cultures derived from them.

(2) Indirect chilling injury. For cellular damage to result, a long period, often days at the reduced temperature is required. Injury is independent of the rate of cooling.

Protein denaturation has been suggested as the primary cause of chilling injury. The stability of many proteins is maintained by hydrophobic interactions. These forces become weaker at low temperatures, resulting in irreversible denaturation. However, it is now generally accepted that both types of chilling injury are the result of cell membrane phase transitions. The membrane lipid phase change from a liquid-crystalline to a solid-gel state, which has been demonstrated in many cell-types, is apparently damaging to chilling sensitive cells.

Cold shock injury is due to the direct loss of selective permeability of the membrane with a leakage of intracellular constituents occurring at temperatures below that of the phase change. With cell-types that are sensitive to this stress, injury can be avoided by using stationary phase cultures because only rapidly dividing cells are sensitive. In addition, injury is a function of growth temperature, suspensions of a blue-green algae being extremely susceptible to chilling when cultured at 35°C but resistant when grown at 25°C.

Indirect chilling injury is a consequence of the effect of membrane phase changes upon cellular metabolism. The effects of phase separations on the membrane-bound enzymes of the mitochondria have been considered especially important. Cell death is then due to an accumulation of toxic substances or the depletion of intermediate metabolites. Cultures which show a significant loss of viability due to the effects of indirect chilling injury are not suitable for cryopreservation because the selection of atypical, chilling resistant cells has been shown to occur.

Chilling injury may occur at temperatures below 0°C, even in cells not previously damaged. Exposure to the concentrated solutions formed during freezing (page 8) increases the sensitivity to a subsequent reduction in temperature.

Rate of cooling

Some algal cells are intrinsically resistant to injury following freezing to, and thawing from, −196°C. The survival, as determined by the ability to form colonies in agar, is dependent on the rate of cooling (Figure 9). For *Scenedesmus quadricauda* and *Chlamydomonas nivalis* the optimal rates of cooling were 2 and 12°C min $^{-1}$, with survival decreasing at both faster and slower rates. It is thought that cells are damaged at suboptimal rates of cooling by exposure to concentrated solutions, whilst the formation of intracellular ice is lethal at rates faster than the optimum. The observed optimum rate of cooling is then a result of the cellular response to these two stresses (page 8). *S. quadricauda* exists as a colony of four cells and its

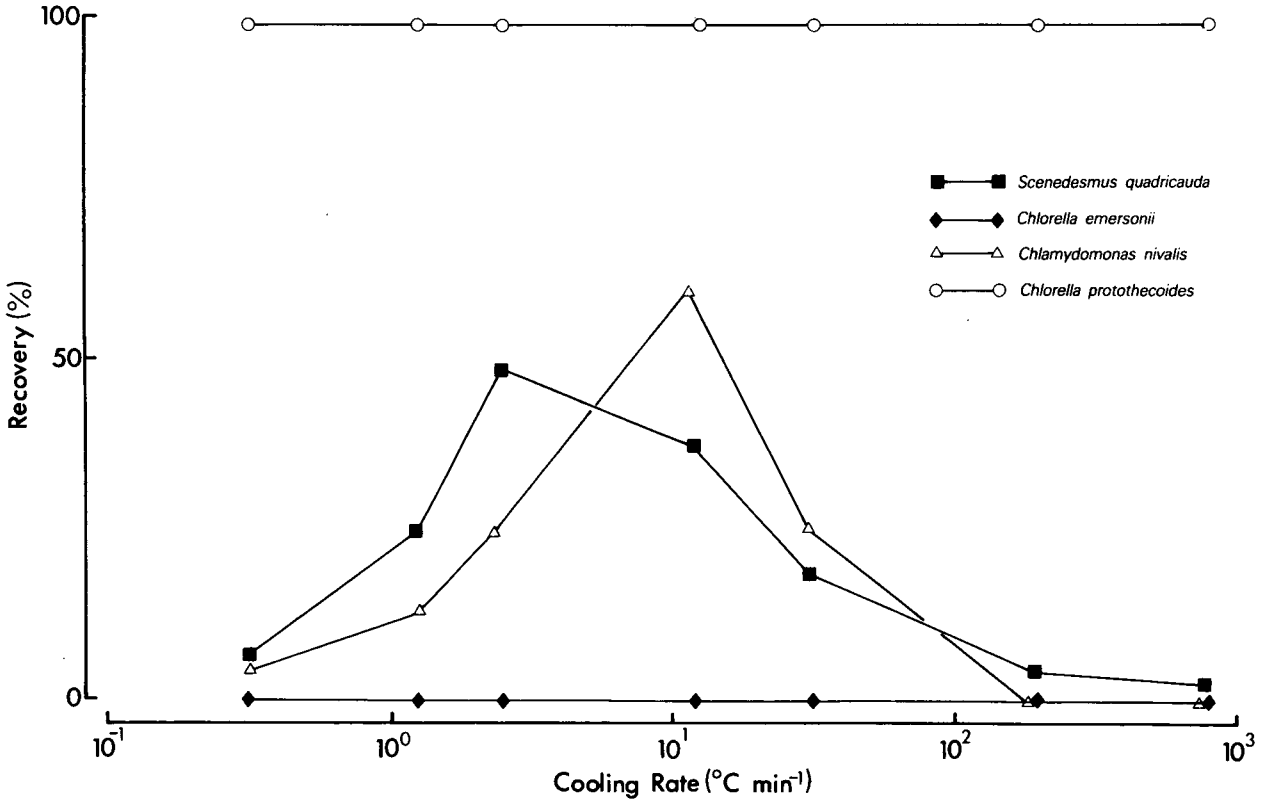

Figure 9—Recovery (%) of algal cells after cooling at different rates to -196°C.

Plate 1—Still from the film 'Dark Star', a crew member communicates electronically with the cryogenically preserved captain (Printed with permission of Opidan Films Ltd.).

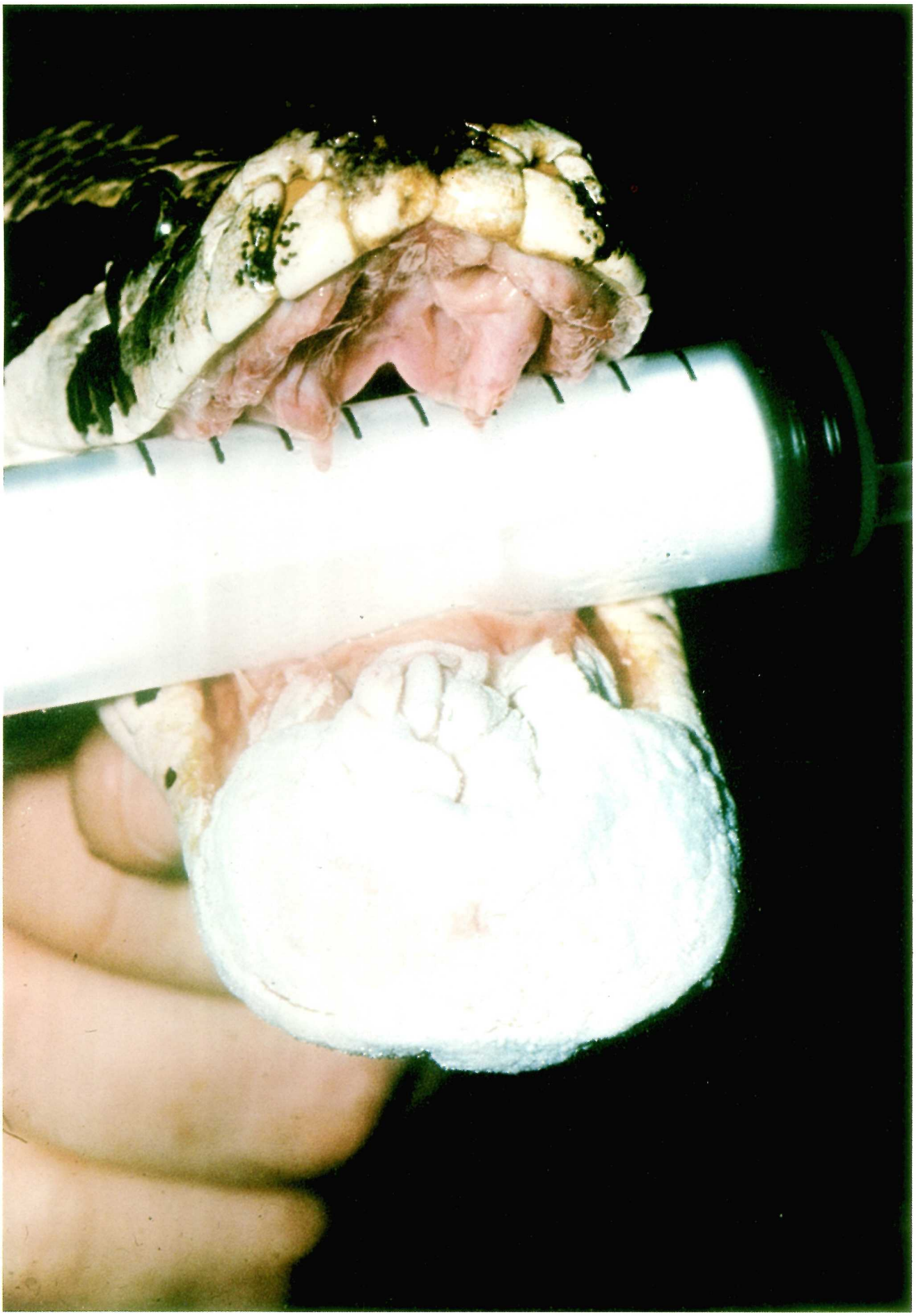

Plate 2—The destruction, by freezing with a spray of liquid nitrogen, of a surface tumour on the lower jaw of a snake. Photograph Dr C. Green.

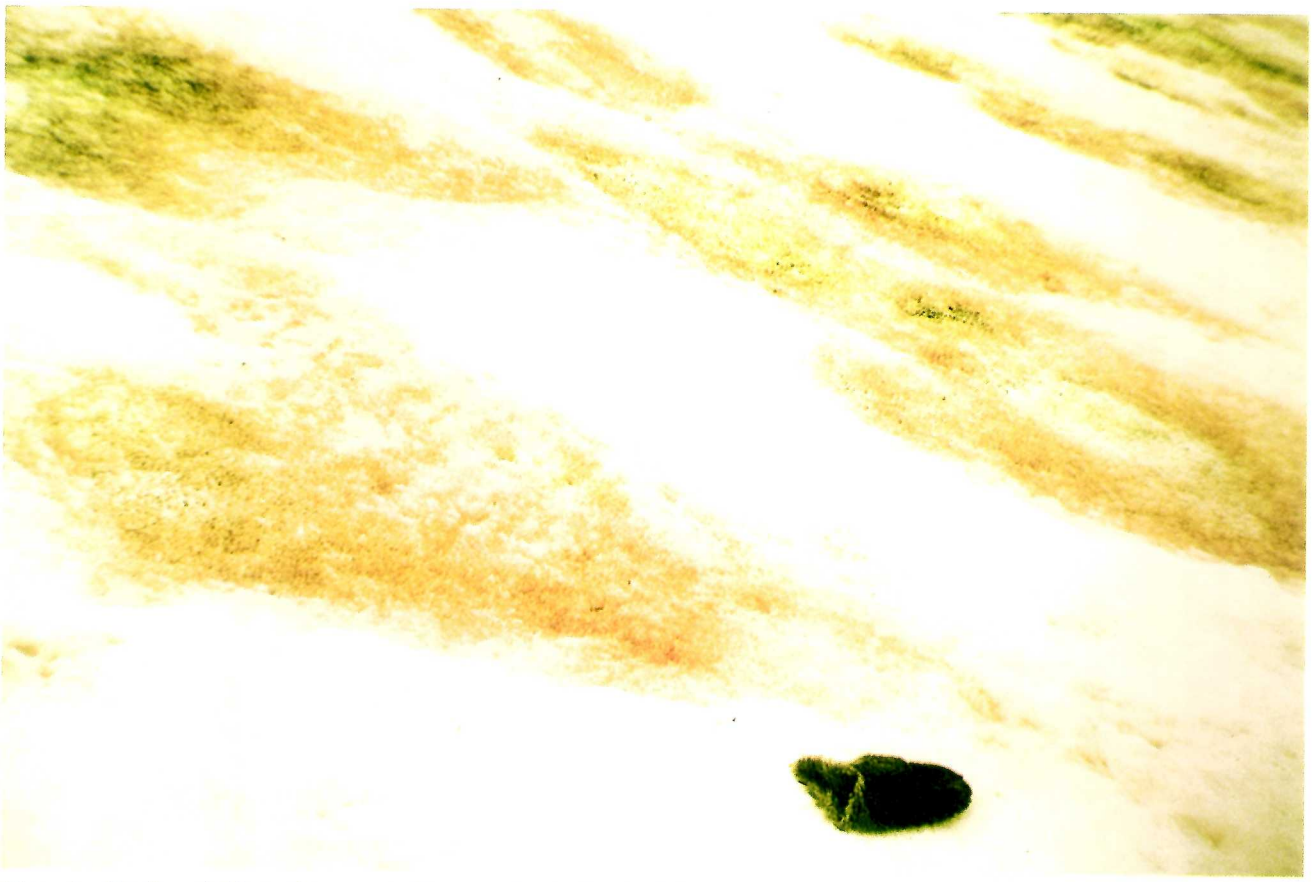

Plate 3—'Red' and 'Green' snow at Signey Island, South Atlantic. Photograph Dr. J. Light.

Plate 4—The young twin lambs (left) resulted from the transfer of two sheep morulas collected 5 days after ovulation and stored for two months at -196°C. The older lamb (centre foreground) is the progeny of the ewe and the ram on the right, both of which developed from embryos which had been stored at -196°C. Photograph Dr S. Willadsen.

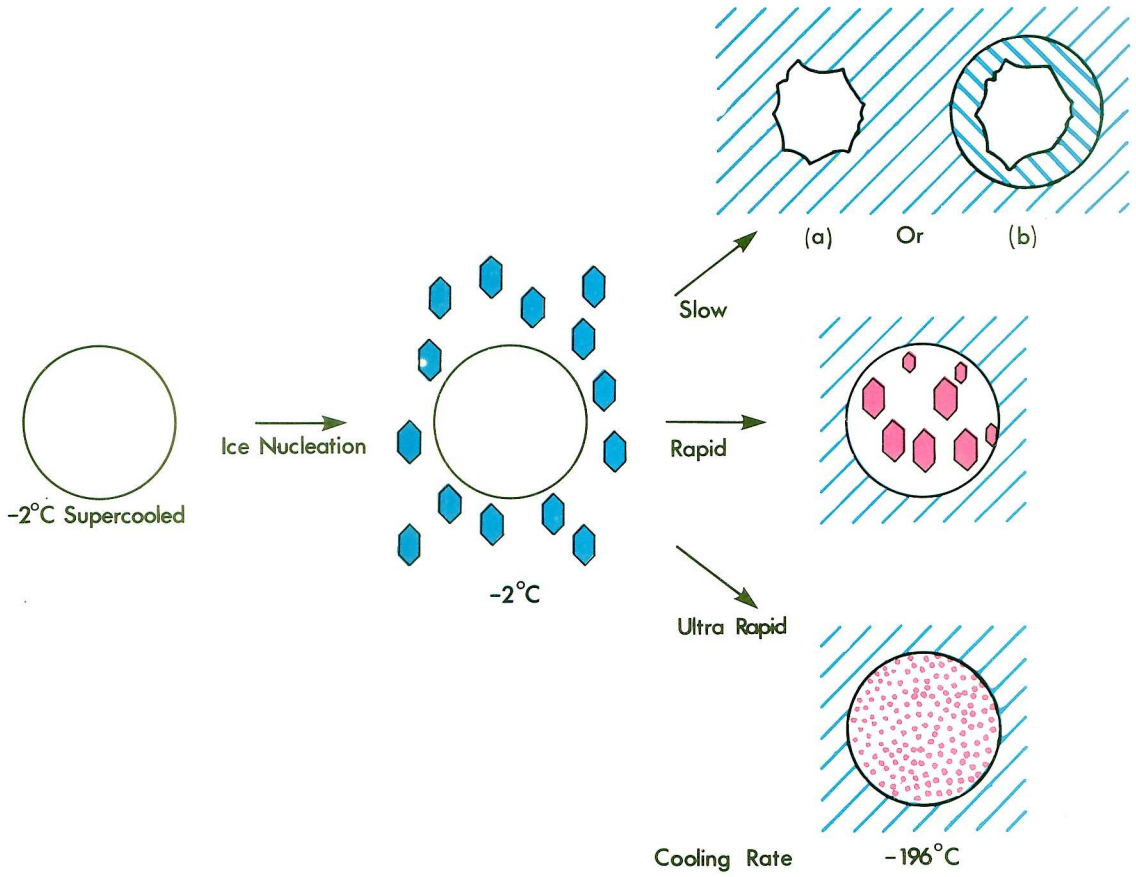

Figure 3—Diagrammatic representation of the effects of different rates of cooling on cells.

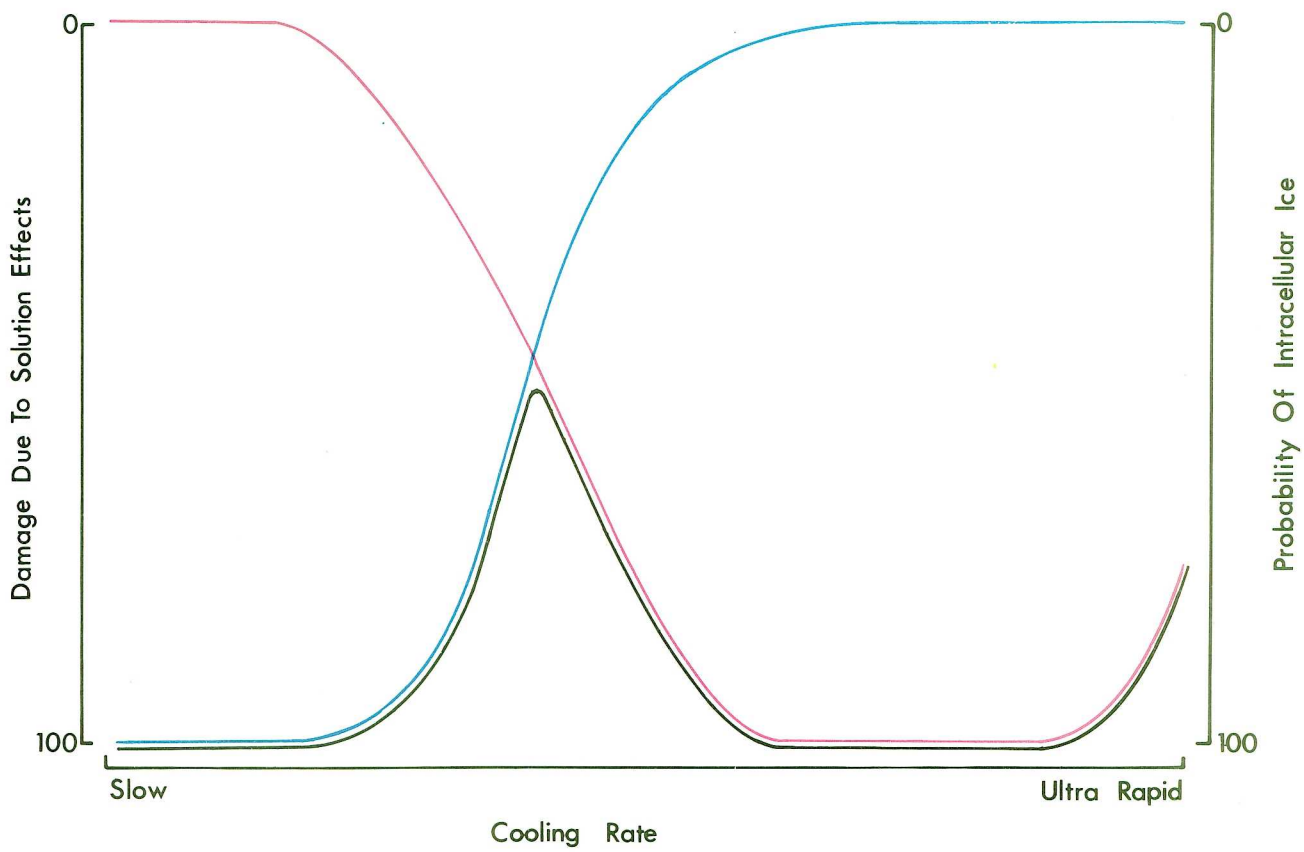

Figure 4—The damaging stresses of freezing. At slow cooling rates 'solution effects' (Blue lines) are important. At rapid rates the formation of intracellular ice (Red lines) is damaging. The interaction of these two factors result in the observed optimum cooling rate.

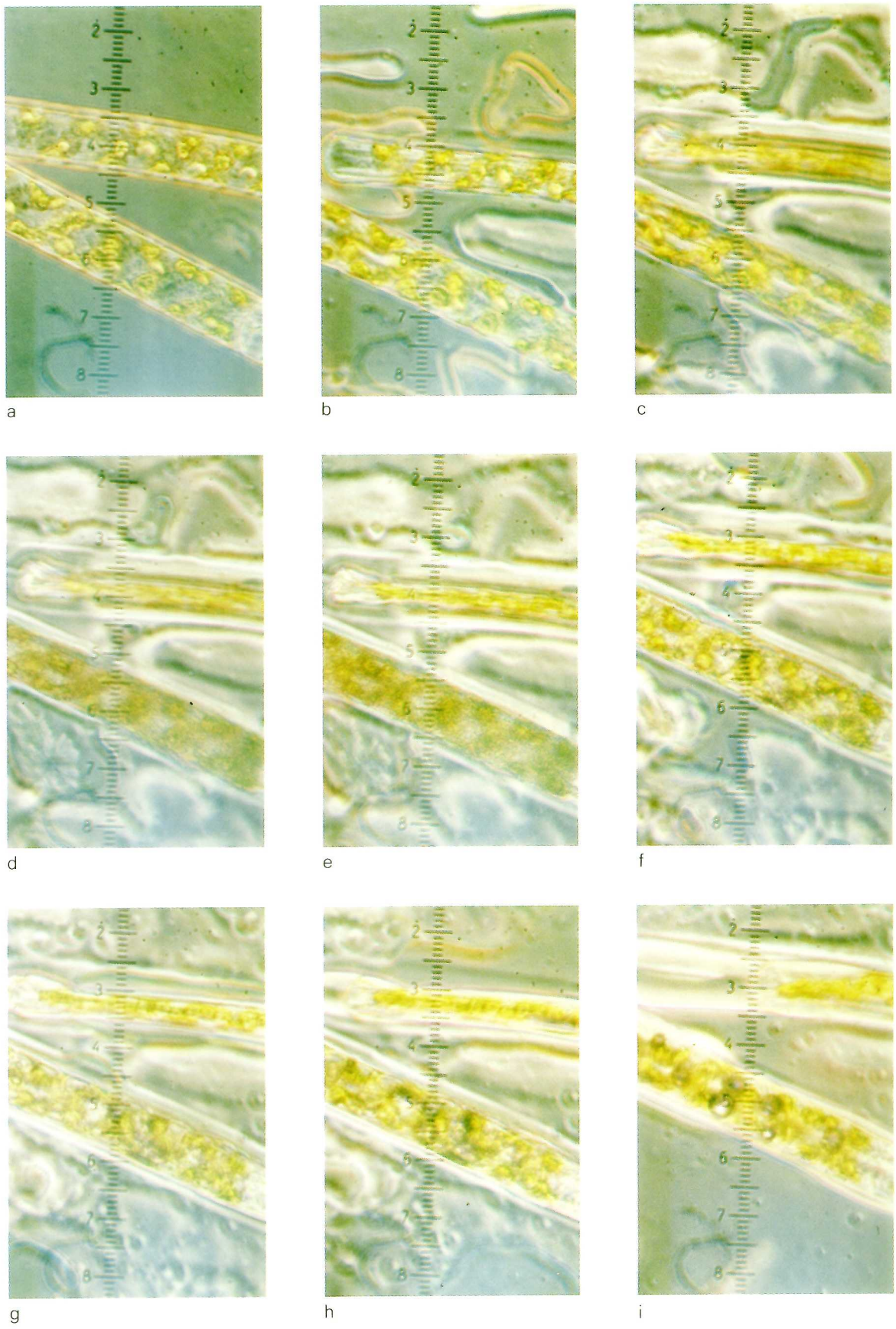

Plate 5—Response of two filaments of Spirogyra *to freezing and thawing, the rates of cooling and warming were 10°C^{-1}, (Magnification x 625). (a) Control, (b) -2.5, (c) -5, (d) -7.5, (e) -10°C, (f) during warming -7.5, (g) -5, (h) -2.5, (i) +5°C. Photograph J. J. McGrath, J. Morris.*

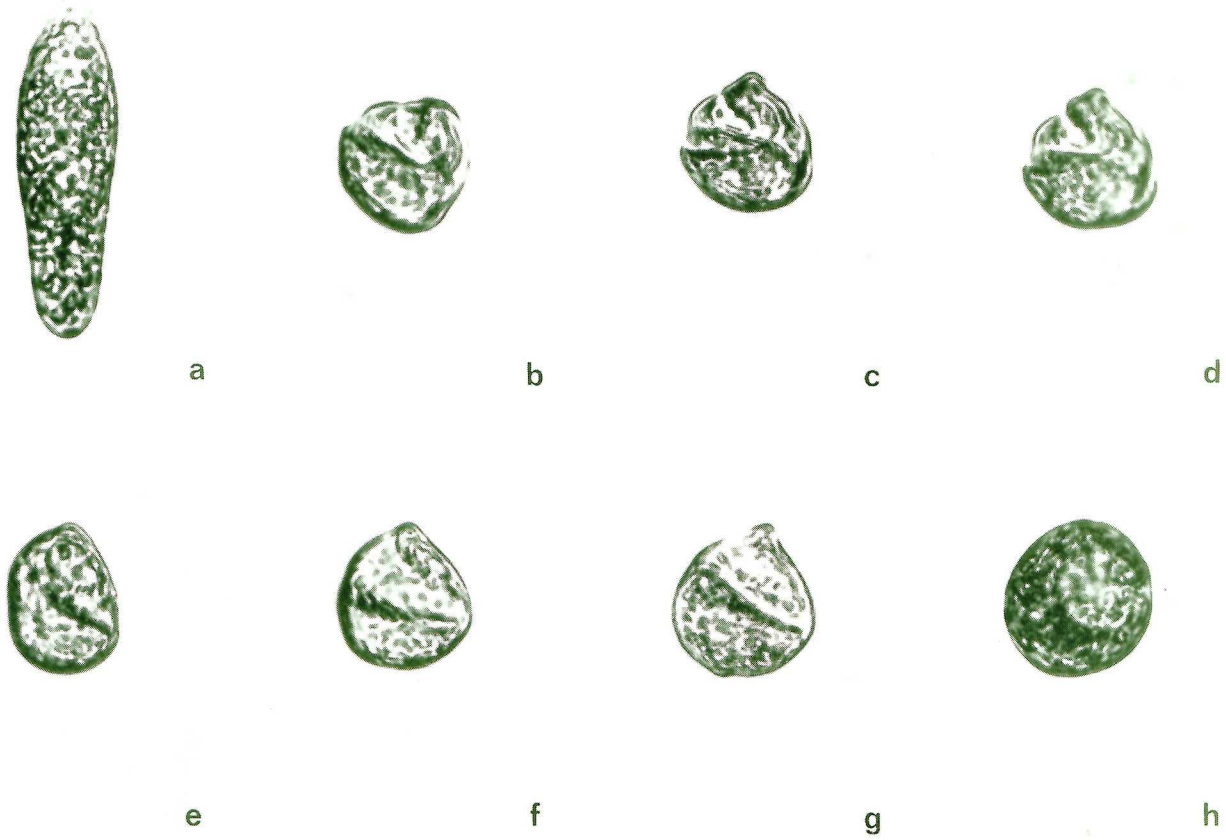

Plate 6—The morphology of Euglena gracilis *after exposure to either glycerol for 5(b), 15(c) and 60(d) minutes, dimethylsulphoxide for 5(e), 15(f) and 60(g) minutes, or methanol for 10(h) minutes. All additives were at a final concentration of 1.5M. Untreated control cell (a).*
Photograph Glyn Coulson.

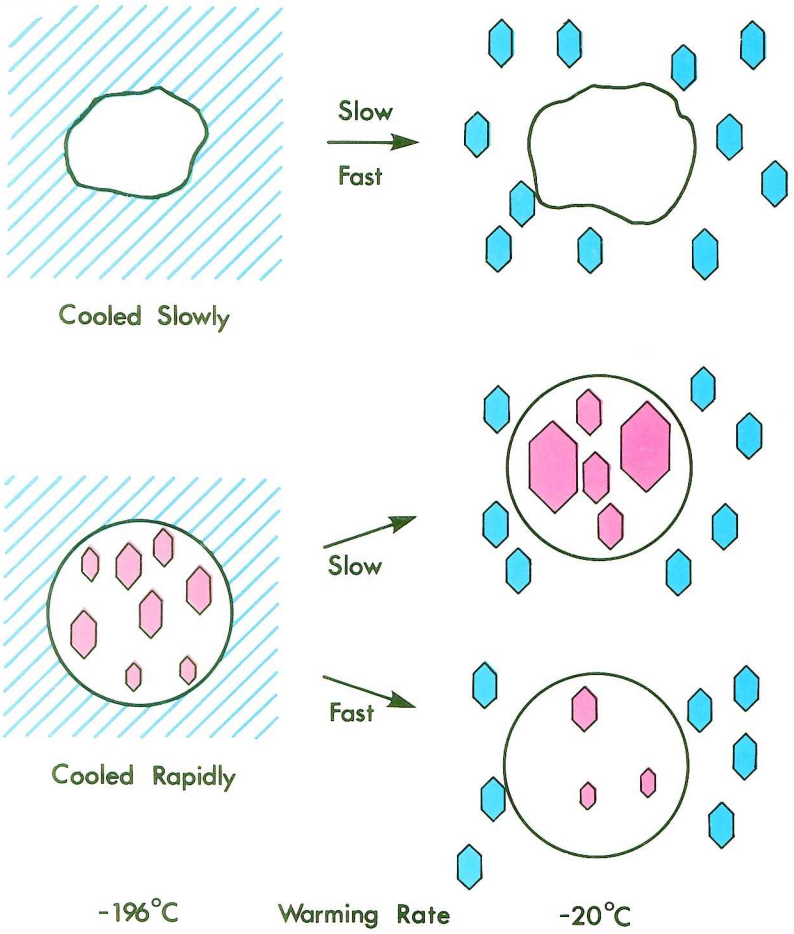

Figure 6—Diagrammatic representation of the effects of different rates of warming on cells.

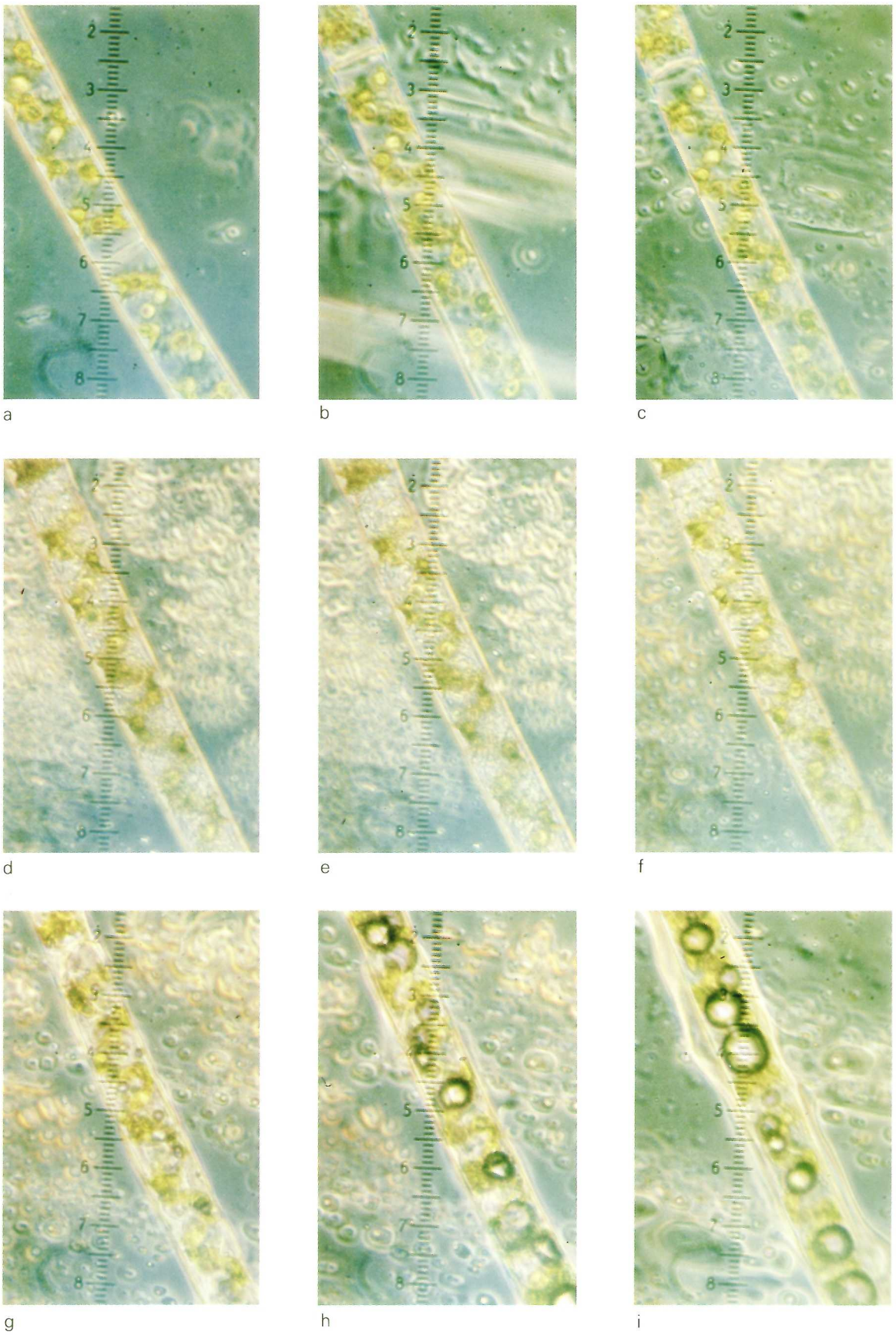

Plate 7—Gas bubble formation in a filament of Spirogyra *during freezing and thawing (Magnification × 625). (a) Control, (b) during cooling at 10°C min^{-1} -2.5, (c) -5, (d) 7.5, (e) -10°C, (f) warming at a rate of 1°C min^{-1} -5, (g) -3, (h) -2, (i) -1°C. Photograph J. J. McGrath, J. Morris.*

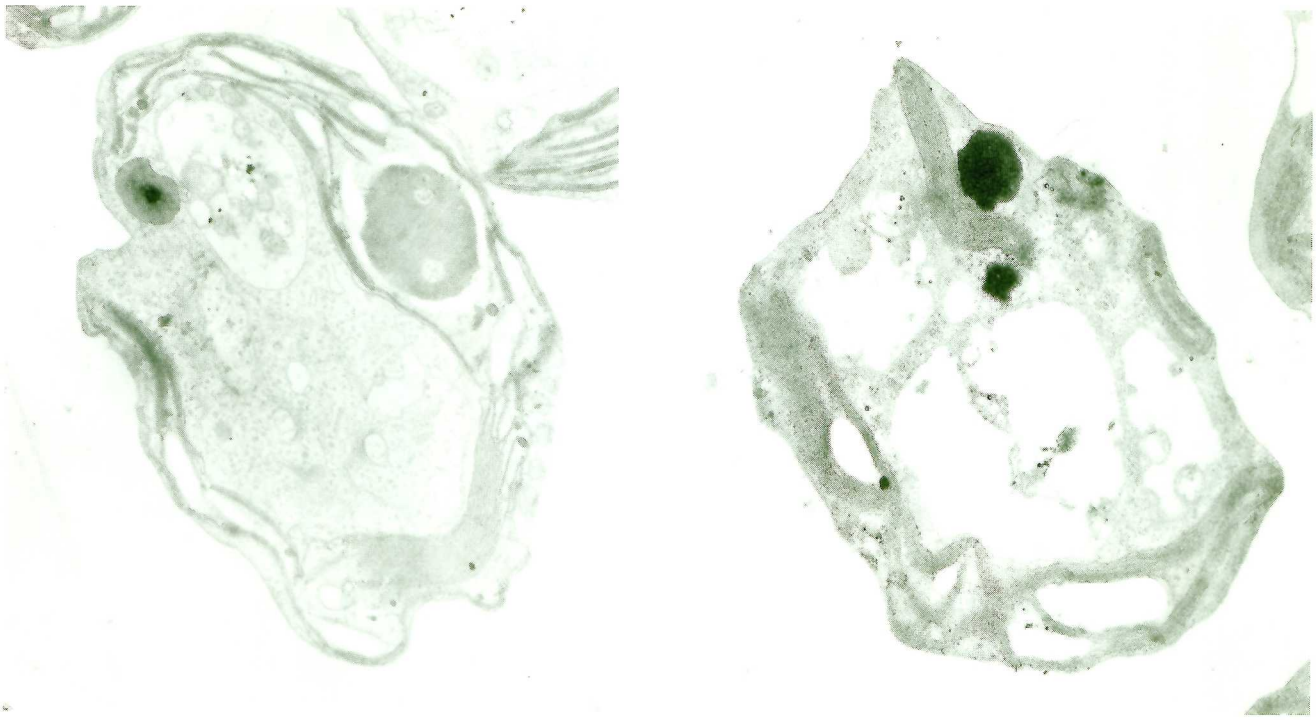

Plate 8—Thin section electron micrograph of Chlamydomonas reinhardii *(left), control cell (right), following freezing and thawing from -5°C.*
Photograph Glyn Coulson.

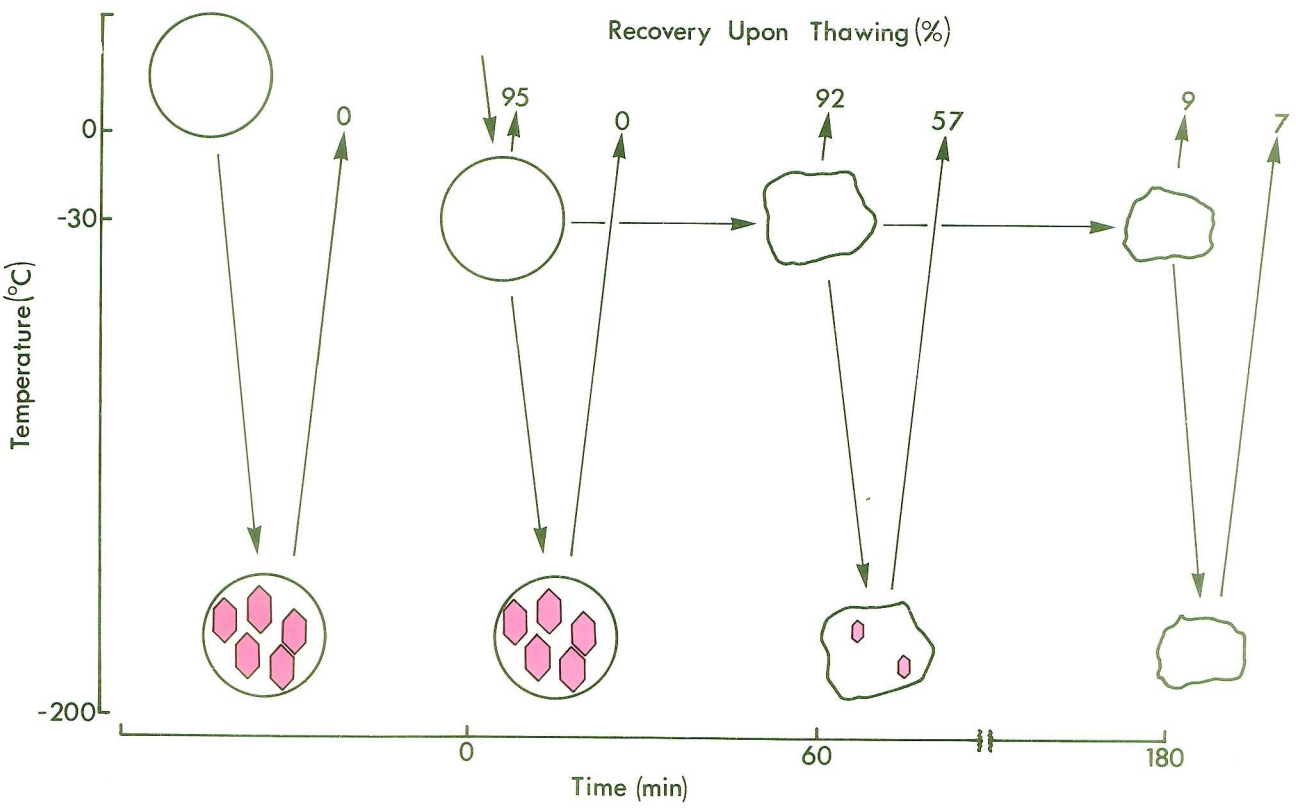

Figure 14—Depiction of the effect of different times at -30°C during a two-step cooling method upon cell volume and the subsequent probability of intracellular ice formation during the secondary stage of cooling.

surface area to volume ratio is thus much lower than that of the unicellular *C. nivalis*. Assuming an approximately equal coefficient of water permeability, loss of intracellular water would be predicted to be slower for *S. quadricauda*, and intracellular ice formation would thus occur at lower rates of cooling than in *C. nivalis*.

C. nivalis is one of the causal organisms of red and green snow (Plate 3) and is therefore exposed to diurnal freezing and thawing. To survive such extremes a high degree of intrinsic freezing resistance has been evolved. It is surprising that the optimum rate of cooling to −196°C is at a rate much faster than the cells would be exposed to in the environment.

The recovery of *Chlorella protothecoides* upon thawing from −196°C is greater than 99% at all rates of cooling studied. This response to a spectrum of cooling rates is unusual, but not unique. *C. protothecoides* is found as an epiphyte on tree bark, where it is exposed to periodic freezing and to drying. The biochemical adaptations which allow it to survive these environmental stresses also confer protection during freezing to, and thawing from, −196°C.

With the majority of plant cells, survival upon thawing from −196°C is very low. It is significantly increased only by the addition of protective compounds or following treatments such as cold-hardening. For *Chlorella emersonii*, cold-acclimatization (Figure 8) increased the recovery at rapid rates of cooling but this increase was little related to cold hardening under natural conditions, where the rates of cooling are extremely low. In contrast, the recovery of the colourless alga *Prototheca* following a similar period of cold acclimatization was at a maximum at the slowest rates of cooling examined. In any comparison of growth treatments upon cellular freezing tolerance, a range of cooling rates must be examined because protective effects may not be observed at a single rate.

Ultrarapid cooling of *Chlorella*, achieved by spraying a cell suspension into liquid nitrogen, results in cells in which no intracellular ice can be detected by freeze-fracture microscopy and, if thawing is also rapid, there is complete recovery of viability. The advantage of ultrarapid cooling as a preservation method is that cryoprotectants are not essential. However, many practical problems exist in recovering adequate amounts of axenic cultures after spray freezing. The technique is also applicable only to unicellular organisms. In multicellular organisms heat transfer is slower and intracellular ice nucleation and crystal growth are inevitable.

Cryoprotective additives

For most cell-types it is necessary to include compounds, so called cryoprotective additives, to reduce injury upon thawing. The effects of a large number of possible cryoprotective additives on the recovery of frozen and thawed mammalian cells, especially erythrocytes and spermatozoa, have been examined empirically. These

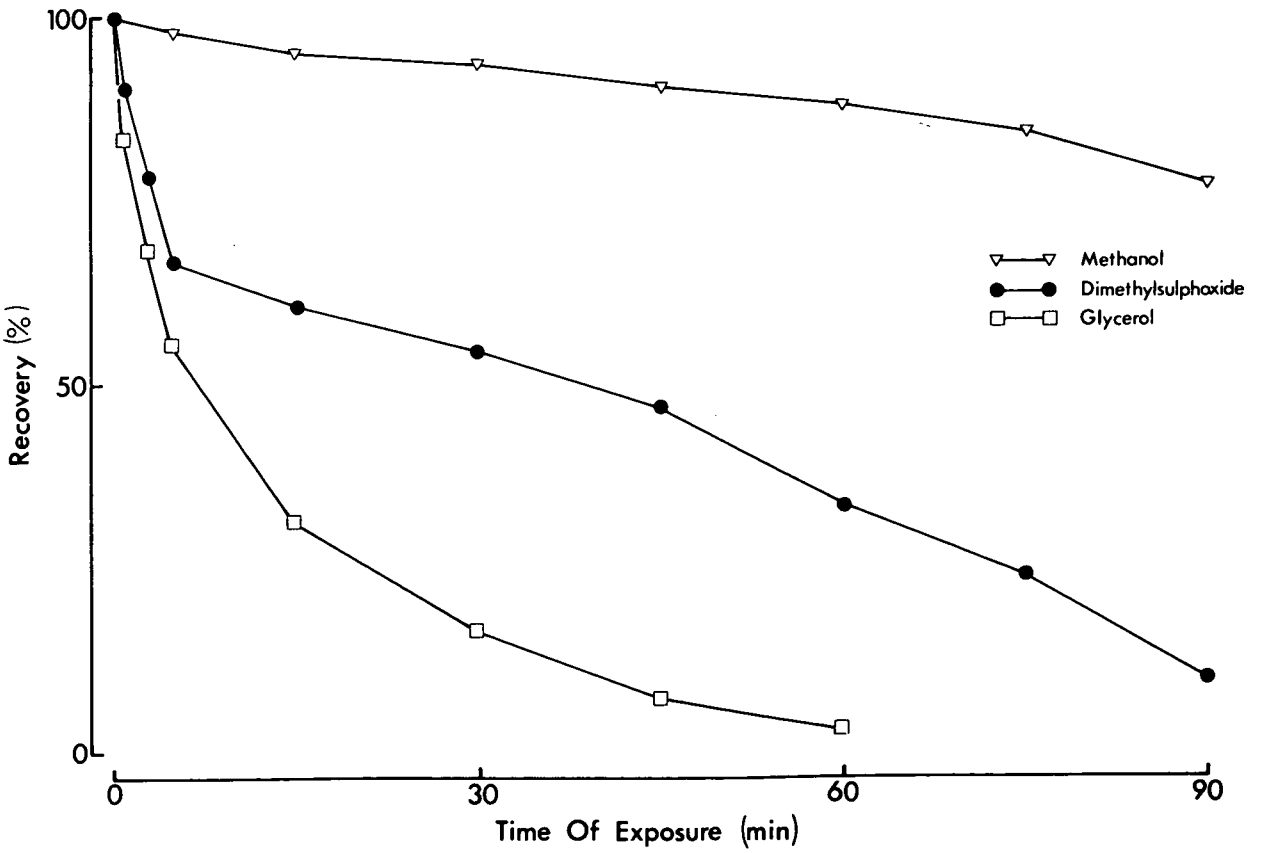

Figure 10—Recovery (%) of Euglena gracilis *after exposure to either dimethylsulphoxide, glycerol, or methanol for different times at 20°C. The final concentration of additive was 1.5M*

studies have enabled practical methods of cryopreservation to be developed. No such basic studies have been reported for other cell-types and it has been assumed that the additives effective for mammalian cells, such as glycerol and dimethylsulphoxide (Me_2SO) would also be effective for plant cells. However, it is now apparent that other compounds may be more suitable as cryoprotectants for plant cells.

Toxic effects may occur at the high concentrations at which additives are used. These are of two types, osmotic and biochemical. The osmotic stresses include dehydration following exposure and during uptake, if any, of the additives, and rehydration upon subsequent dilution. The extent of shrinkage will be determined by the cellular permeability to the additive; one with a high permeability will enter the cell rapidly and normal volume will be retained, but an additive with lower permeability will dehydrate the cell for longer periods. However, bulk uptake of the additive, whilst reducing osmotic stress, is itself potentially damaging. The biochemical effects of high intracellular concentrations of cryoprotectants are poorly understood and will depend on the type of additive, temperature and duration of exposure. The observed toxicity is then a result of the osmotic and biochemical effects. The morphology of *Euglena gracilis* during exposure to the three additives, glycerol, methanol and Me_2SO at 20°C, is illustrated in Plate 6. The recovery of cells following rapid dilution from similar treatments are presented in Figure 10.

Glycerol. For *Euglena*, this additive is apparently non-penetrating, the cells remain shrunken for 1 hr at 20°C and there is a rapid loss of viability.

Dimethylsulphoxide (Me_2SO). *Euglena* has a higher permeability coefficent for Me_2SO than for glycerol. Following initial shrinkage there is a slow uptake of the additive and the cells increase in volume, but are non-motile and morphologically altered. These effects are reversible; upon removal of Me_2SO motility, typical euglenoid morphology and cell viability are regained. Me_2SO is less cytotoxic than glycerol, but prolonged exposure to the intracellular effects of Me_2SO are damaging.

Methanol. This additive penetrates cells very rapidly; *Euglena* returns to approximately normal volume within 1 min of exposure and, as with Me_2SO, there are reversible changes in morphology. Compared with other additives, methanol is surprisingly non-toxic, even after long periods of exposure. Other alcohols are also cryoprotective for *Euglena*, but the protection obtained decreases with increasing chain length of the molecule.

The protection afforded by cryoprotective additives during freezing and thawing is dependent on the rate of cooling and the concentration of the additive. This effect is demonstrated with Me_2SO (Figure 11). The recovery of *Chlorella emersonii* was at a maximum in 0.75 M Me_2SO; both higher (1.5, 2.25 M) and lower (0.38 M) concentrations were less effective. As the initial concentration of the additive increased, the rate of cooling giving optimal survival became lower.

When these three additives are compared at one

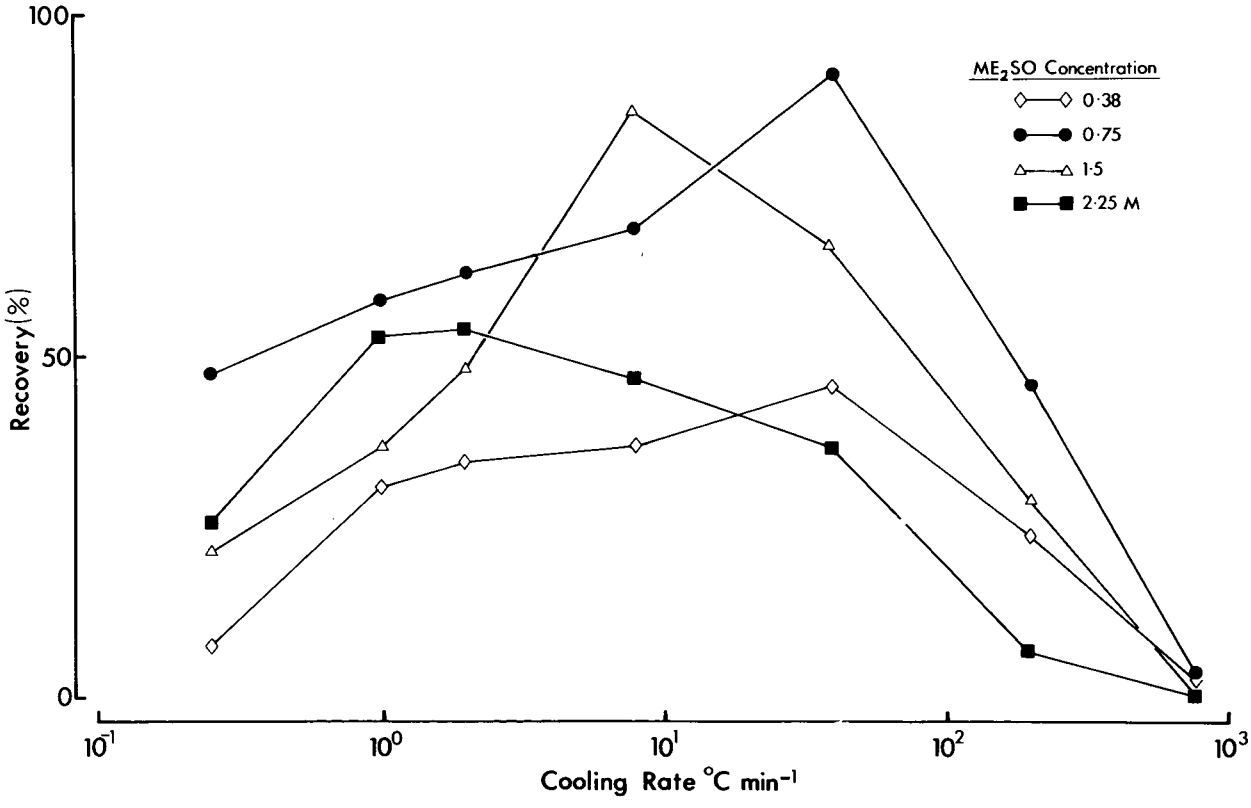

Figure 11—Recovery (%) of Chlorella emersonii *after cooling at different rates to −196°C in 4 levels of dimethylsulphoxide.*

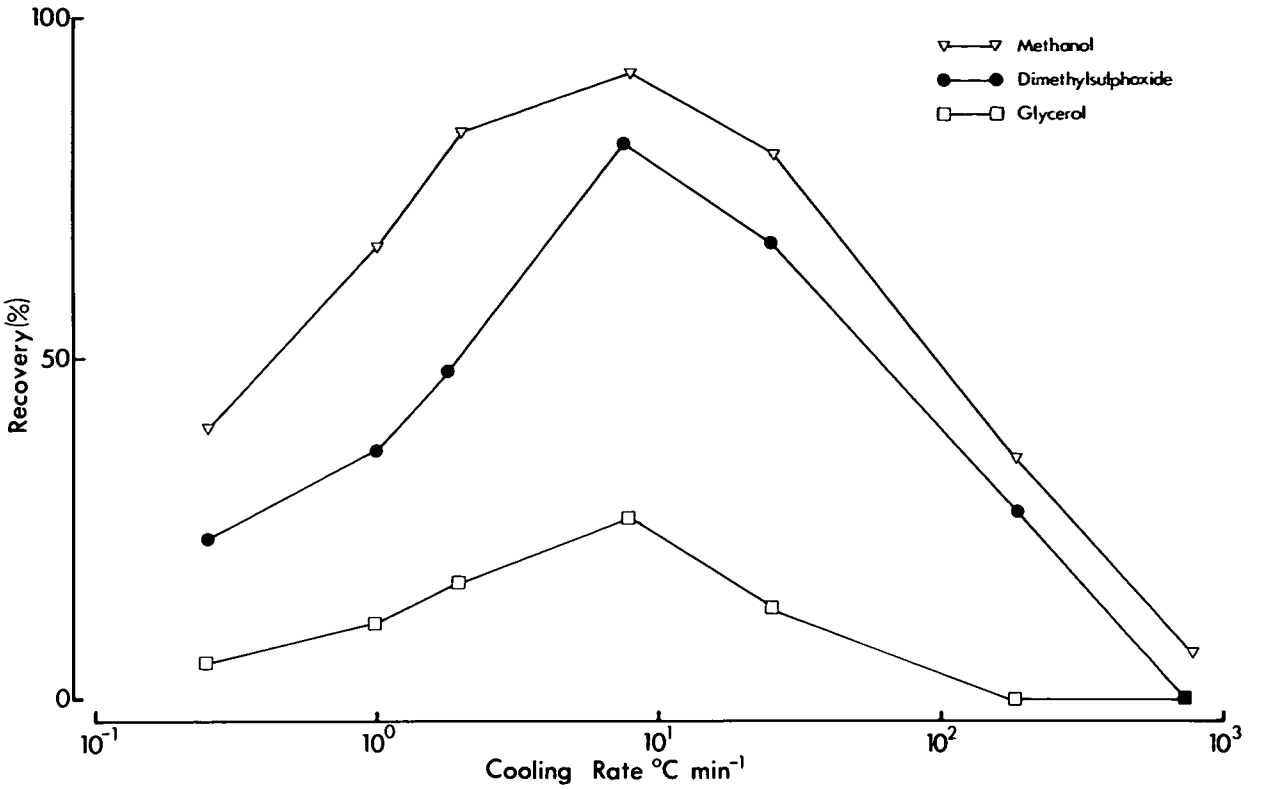

Figure 12—Recovery (%) of Chlorella emersonii *after cooling at different rates to -196°C in the following additives, methanol dimethylsulphoxide and glycerol. The final concentration of additive was 1.5M.*

concentration (1.5 M), differences are observed in the maximum recovery obtained and in the general shape of the survival curves (Figure 12).

Compounds are cryoprotective because of their colligative effect on other solutes, especially salts, at any temperature during freezing (Figure 2). The removal of liquid water as ice will also increase the relative concentration of the additive in the residual aqueous solution. The actual extent of shrinkage at any rate of cooling will be determined by the concentration of impermeable solutes and the cellular permeability to the additive. At any sub-zero temperature all additives, when compared on a molar basis, will reduce the concentration of other solutes by an equal amount. Therefore an additive with a high permeability would be expected to induce less shrinkage and be a more effective cryoprotectant than a slowly penetrating compound. This may explain why methanol, with a permeability coefficient for algae similar to that of tritiated water, is a superior protectant compared with the conventional additives, which are either slowly more penetrating (Me_2SO) or do not penetrate (glycerol).

Two-step cooling

In this technique, which is best considered as interrupted rapid cooling, freezing occurs during an initial period of rapid cooling to a constant holding temperature. After maintenance at this temperature the sample is then cooled rapidly into liquid nitrogen. The practical advantages of this method are (1) it is simple to carry out, (2) it requires no specialized controlled cooling rate equipment and (3) low concentrations of additive are effective.

The recovery following two-step cooling of *Chlamydomonas reinhardii* cells is presented in Figure 13. The cells were suspended in methanol and frozen rapidly to −30°C before either thawing directly or plunging into liquid nitrogen at −196°C and then thawing. The survival of cells thawed directly from −30°C decreased steadily with increasing time of exposure. The recovery from −196°C initially increased with time at −30°C to a maximum value, then slowly decreased in a manner parallel to the loss of viability at the holding temperature. Survival is not due to an appropriate rate of cooling produced by immersing the sample in a constant temperature bath because protection is acquired only after a certain time at the holding temperature. It is assumed that, because of the rapid rate of cooling to the holding temperature, cells are unaffected by the potentially damaging hypertonic solutions during cooling. Shrinkage then occurs in response to the concentration of solutions at the holding temperature and it is this dehydration that reduces the probability of intracellular ice formation on further rapid cooling. These events are depicted in Figure 14.

Sub-optimal times of exposure at −30°C will induce insufficient shrinkage and intracellular ice nucleation will then occur during the second stage of cooling. As the holding time at −30°C is increased more dehydra-

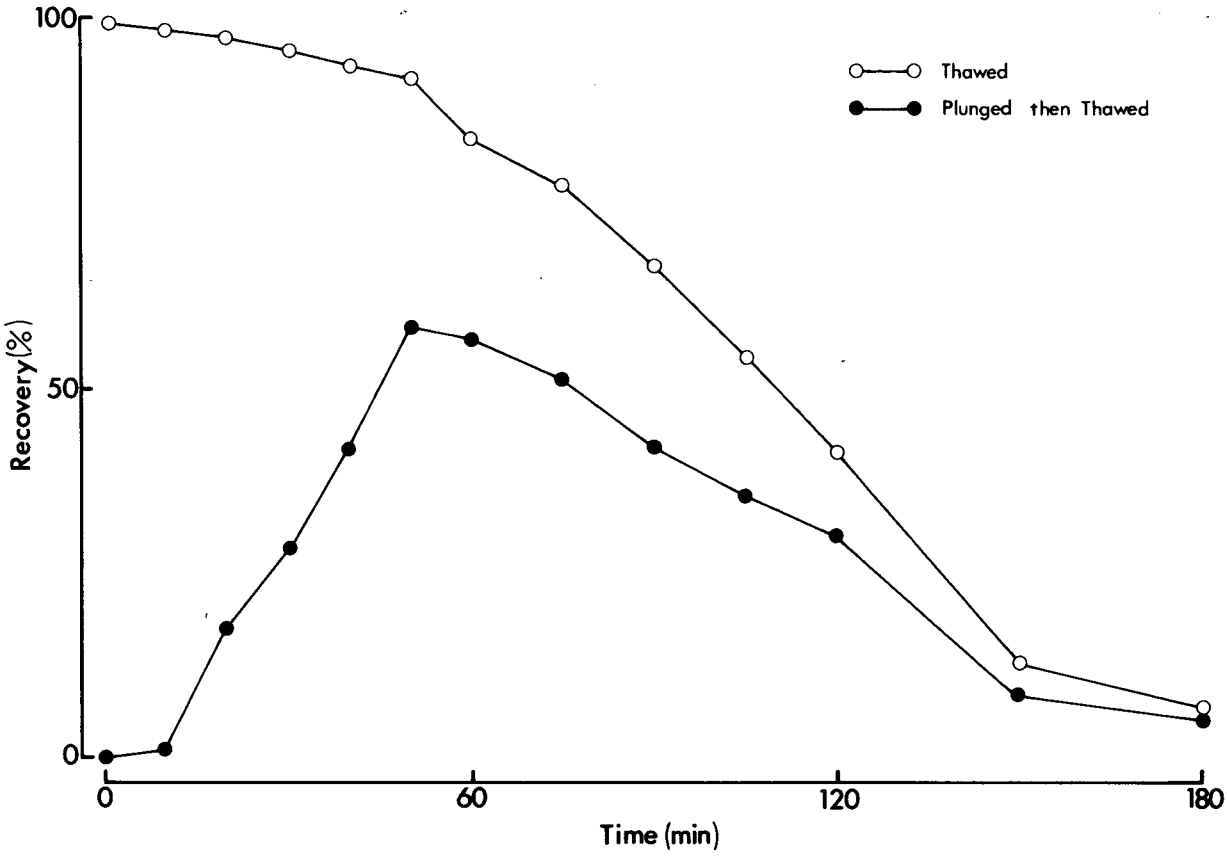

Figure 13—Recovery (%) of Chlamydomonas reinhardii *frozen in methanol (2.5M) at -30°C for different times before either thawing or plunging to -196°C before thawing.*

tion occurs. However, cellular damage induced by exposure to the concentrated solutions becomes more important. The observed optimum time at the holding temperature can then be interpreted as the resultant of these two processes.

Warming rates and post-thaw manipulations

In all studies, where the effects of the rate of warming from −196°C on the survival of algal cells have been examined, rapid rates of warming gave maximal survival. Slower rates have been found in isolated cases, usually following slow cooling, to be as effective as, but never superior to, rapid thawing.

Upon thawing, cryoprotective additives are potentially cytotoxic. Glycerol and methanol can easily be diluted out and cell growth is not inhibited by low levels of these additives. However, dimethylsulphoxide inhibits cellular metabolism, even at very low concentrations and therefore must be removed by washing and centrifuging.

Storage temperature

Cells have been stored at temperatures between −20°C and −79°C, but in is now generally accepted that, for long-term maintenance, temperatures below −139°C are essential. The use of liquid nitrogen (−196°C) is a convenient way of achieving these temperatures. At the higher storage temperatures biochemical and biophysical processes still take place so that there is a progressive reduction of cell recovery with increasing time of storage. At temperatures below −139°C, there is no growth of ice crystals and the rates of other biophysical processes are too slow to affect cell survival. It has been calculated that, if a simple chemical reaction could occur at −196°C, it would be some eight million times slower than at 0°C.

Long-term viability studies with bull semen indicate that no loss of fertilizing capacity occurs within 30 years at −196°C. There are no substantiated reports of a reduction in cellular viability with increasing time of storage at −196°C.

At −196°C the genetic material of cells is still accessible to irradiation. Since no repair mechanism operates at this temperature, any mutations that are induced will accumulate and be expressed upon thawing. It has been calculated that if background irradiation remains at the current level, the median lethal dose or mammalian tissue-culture cells will accumulate within 10,000 years. Therefore, cells should be thawed and sub-cultured periodically or shielded from irradiation.

MECHANISMS OF FREEZING INJURY

From the response of many cell-types to a range of cooling rates (Figures 3, 5, 9, 11, 12), in which an optimal rate of cooling has been observed, it has been assumed that two distinct stresses occur. At rates of

cooling slower than that giving optimal recovery, cell injury is associated with the removal of liquid water as ice and the subsequent alterations in the properties of solutions—'solution effects'. At cooling rates faster than the optimal, the formation of intracellular ice is assumed to be damaging.

'Solution' effects

There are several theories to explain the mechanism by which the increased concentrations of solutes cause harm.

(1) *Shrinkage and rehydration.* Exposure to hypertonic solutions during cooling results in cell shrinkage. It has been suggested that there is a minimum cell volume to which a cell can physically shrink beyond which critical level injury occurs. However, this theory does not explain freezing injury because many non-penetrating compounds, e.g. glycerol (Figure 12), have some protective effects at low rates of cooling. These additives will not affect the extent of cellular shrinkage and therefore some other property of the concentrated solutions must be damaging.

(2) *'Salt effects'* Direct biochemical damage to the structure of the membrane is induced by the concentrated salt solutions. This injury is a function of the ionic strength rather than molarity. Additives are assumed to be protective because they reduce the level of these potentially damaging salt solutions and not simply because they minimise the extent of cell shrinkage.

(3) *Alterations in pH.* The increase in solute levels and the resulting precipitation of salts alters the pH in the residual aqueous solution. In some tissues these changes in pH have been demonstrated to be damaging and the addition of certain buffers improves cell recovery.

(4) *Chilling injury.* There is a range of ionic concentration which does not directly induce cellular injury but which sensitizes cells to a subsequent stress such as a reduction in temperature. During slow cooling, cells are exposed to hypertonic solutions and a simultaneous reduction in temperature, conditions which may result in cellular chilling damage.

Whilst it is apparent that cell injury at low rates of cooling is associated with the relatively long period of exposure to hypertonic solutions, the specific damaging stress is difficult to determine experimentally. It is possible that damage is due to a combination of stresses which vary in importance with the cell-type and at different rates of cooling.

Intracellular ice

At rapid rates of cooling there is insufficient time for cellular water to be lost osmotically and ice nucleates within the cell to maintain thermodynamic equilibrium. It has been suggested that intracellular ice is lethal, but there are examples of cells remaining viable following the formation of intracellular ice (*Chlorella protothecoides,* Figure 9).

The mechanism by which intracellular ice causes cell death is not apparent. The disruption of intracellular membranes by ice crystals has been implicated. However, it is difficult to accept this reason when direct mechanical injury to the outer surface of the plasmalemma by extracellular ice is not considered important at slower rates of cooling. In addition, the mechanism of injury is not due to the effects of concentrated solutes within the cell; the extent of cellular dehydration at any temperature will be the same if intracellular ice is formed or if the cell is osmotically shrunken.

Recent studies using a cryomicroscope have suggested an alternative mechanism of injury (Plate 7). At a rate of cooling of 10°C min^{-1} there is insufficient time for cells of *Spirogyra* to dehydrate osmotically and intracellular ice forms. The nucleation of ice within cells is initiated from the extracellular compartment. When ice forms within one cell of a filament it is transmitted to adjacent cells via cellular connections. In the extracellular ice many large gas bubbles are formed during freezing. During thawing small bubbles appear within the filament at −5°C, these increase in size during further warming.

The role of dissolved gases as a component of freezing injury has been ignored. In a photosynthetic system solutions will be saturated with gases, as the temperature is lowered their solubility will increase. When some liquid water is removed by the formation of ice the solubility of these dissolved gases will be exceeded and they will come out of solution and a three phase system of ice, residual aqueous solution and gas will form. When ice forms within cells many small gas bubbles are entrapped between crystals of intracellular ice. As this ice melts during warming the gas bubbles coalesce and eventually return to solution. These bubbles are potentially damaging either by mechanical action or due to their high oxygen content which may directly oxidise membranes. In cells which dehydrate during cooling no gas bubbles appear during thawing.

THE BIOCHEMISTRY OF FREEZING INJURY

The problem that is central to all cryobiological research is to determine the biochemical mechanism by which cells are injured during freezing and thawing. There are several physico-chemical theories of the mechanism of damage, but in none of these is an actual site of freezing injury specified. Unfortunately, many technical difficulties exist in any experiment designed to investigate the precise nature of this injury. Some methods by which this problem has been examined, and their limitations will be discussed, and future strategies of research will be outlined.

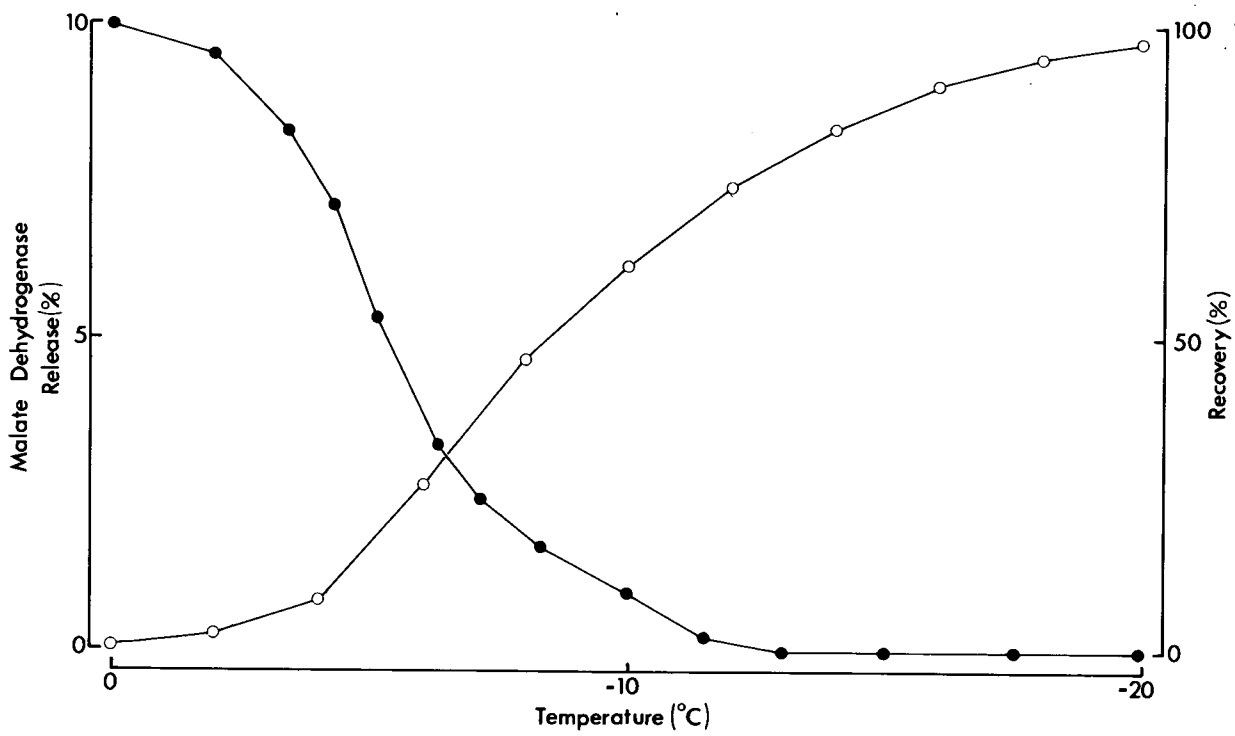

Figure 15—Recovery (%) of Euglena gracilis *following cooling at a rate of 0.25°C min^{-1} to different final temperatures then thawing (●). Release (%) of the cytoplasmic enzyme malate dehydrogenase from* E. gracilis *under equivalent conditions (○).*

A common experimental approach to the problem of freezing injury has been to compare the biochemical composition or ultrastructure with the observed response to freezing injury. By this means, it is hoped to correlate the presence of an intracellular compound or of some cellular structure with susceptibility to damage. However, the number of independent variables which exists renders this type of comparison invalid. For example, many workers have suggested that an accumulation of sugars within plant cells is a factor directly conferring freezing tolerance, but sugar cane is extremely sensitive to freezing injury.

For many cell-types, the reduction in cellular viability upon thawing is directly proportional to the loss of membrane integrity. The reduction in viability of *Euglena gracilis* (Figure 15) is directly related to the loss of the enzyme malate dehydrogenase from the cells. It is now generally accepted that injury to cells is at the level of functional integrity of the membranes, although the biochemistry of this damage has not been elucidated.

Modifications to the composition of the membranes which alter their biophysical characteristics would be expected to modify the response of cells to freezing and thawing. However, there is no direct relationship between membrane lipid composition and freezing tolerance. Recent research has demonstrated that the bulk composition of the various membrane systems within cells is significantly different and that the molecular organization of individual membranes is not homogeneous. Therefore, it is probable that the composition of the membrane at critical microenvironmental conditions, or of a specific "target" organelle, is important in determining the cellular response. In addition, as the membranes have been implicated as a potential area of damage, it is possible that some additives are protective by a direct interaction with the membrane.

Many studies on the composition of the membrane lipids before and after freezing have been carried out (Table 2) to determine whether the alterations in membrane function induced by freezing and thawing are simply a reorganization of the membrane components or an actual change in their chemical structure. Upon thawing there is a release of free fatty acids and changes in the phospholipid fatty acid composition. These findings are consistent with the activation of an intracellular enzyme phospholipase D, which is normally contained within cellular vacuoles. However, it is now apparent that the release of this and other lytic enzymes and their subsequent effects on cells are secondary, pathological effects of freezing injury. Damage to the limiting membrane of the vacuole must occur at an early stage of freezing injury. Unfortunately studies of this type have not provided any insight into the primary mechanism by which the organization of the membrane is perturbed.

Many investigations on the ultrastructure of frozen and thawed material have been reported, with the objective of determining a specific lesion of freezing injury. Upon thawing, a large number of ultrastructural alterations are observed in non-viable cells (Plate 8). These changes are so extensive that it is not possible to distinguish between the causes of freezing injury and secondary pathological effects.

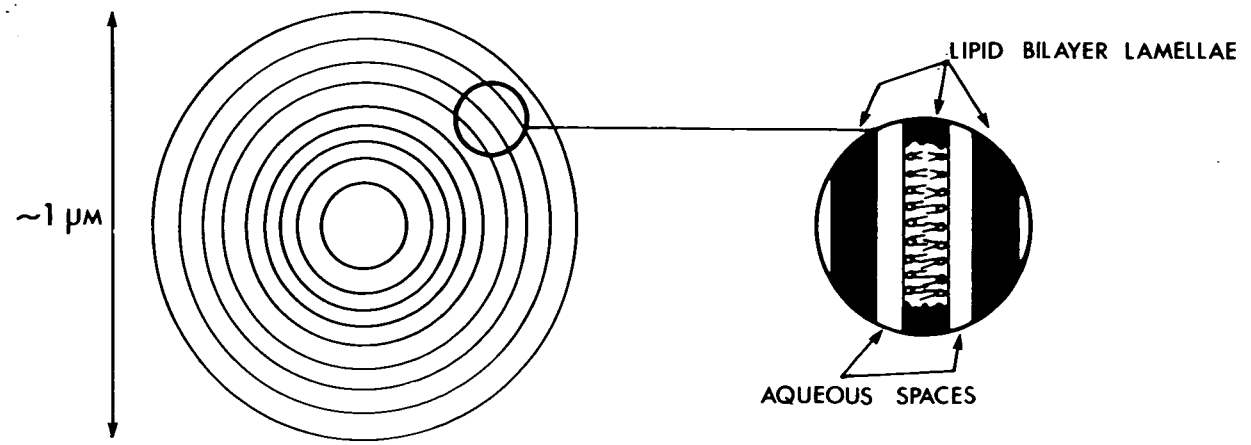

Figure 16—Schematic representation of liposome structure.

From these observations, it is evident that the organization of intact cells is too complex for investigation of the primary mechanism of freezing injury and that a simpler model system is required. Liposomes (lipid bilayer vesicles) have been used extensively to examine the structure and function of biological membranes (Figure 16). Liposomes have many practical advantages in fundamental cryobiological research; their composition can be easily and reproducibly altered and the effects of these modifications on the biophysics of the bilayer are well understood. Liposomes entrap solutions and the integrity of the bilayer can then be assessed by measuring the leakage of these entrapped solutes—a principle which is often used to determine the viability of plant material upon thawing.

Preliminary studies indicate that, during the freezing and thawing of liposomes, much of the morphological behaviour of intact cells was simulated. Following cooling at a range of rates to −196°C, an optimal rate of cooling was observed (Figure 17). In addition, the effects of cryoprotective additives are similar to those seen with many cell-types. These morphological and functional responses suggest that liposomes are a valid model system for investigating freezing injury.

The composition of the liposome bilayer is critical in determining the response to freezing. Liposomes composed of one phopholipid species (dipalmitoyl phosphatidylcholine) are comparatively resistant to freezing injury. Following the incorporation of

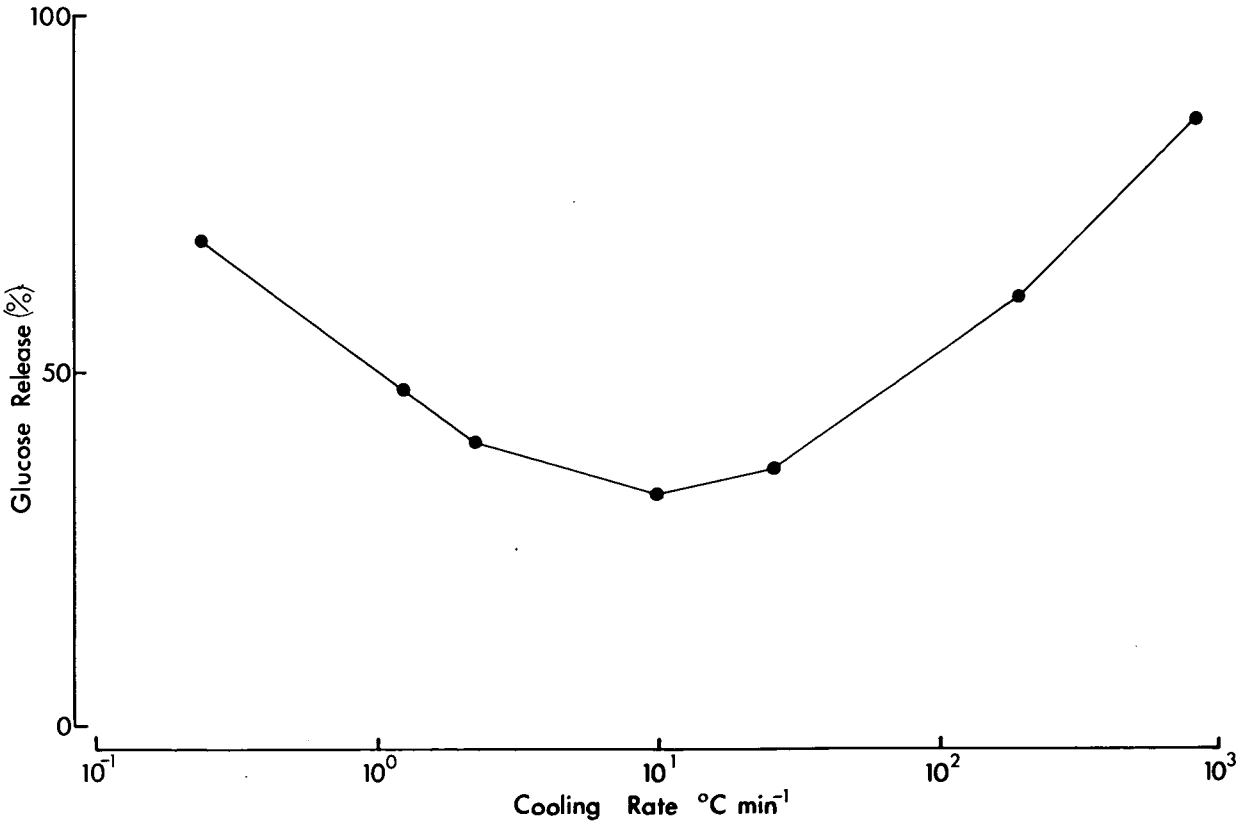

Figure 17—Glucose release (%) from liposomes of dipalmitoyl phosphatidylcholine: cholesterol : dicetyl phosphate (mole ratio 7:2:1) following cooling at different rates to -196°C in the presence of dimethylsulphoxide (0.5M).

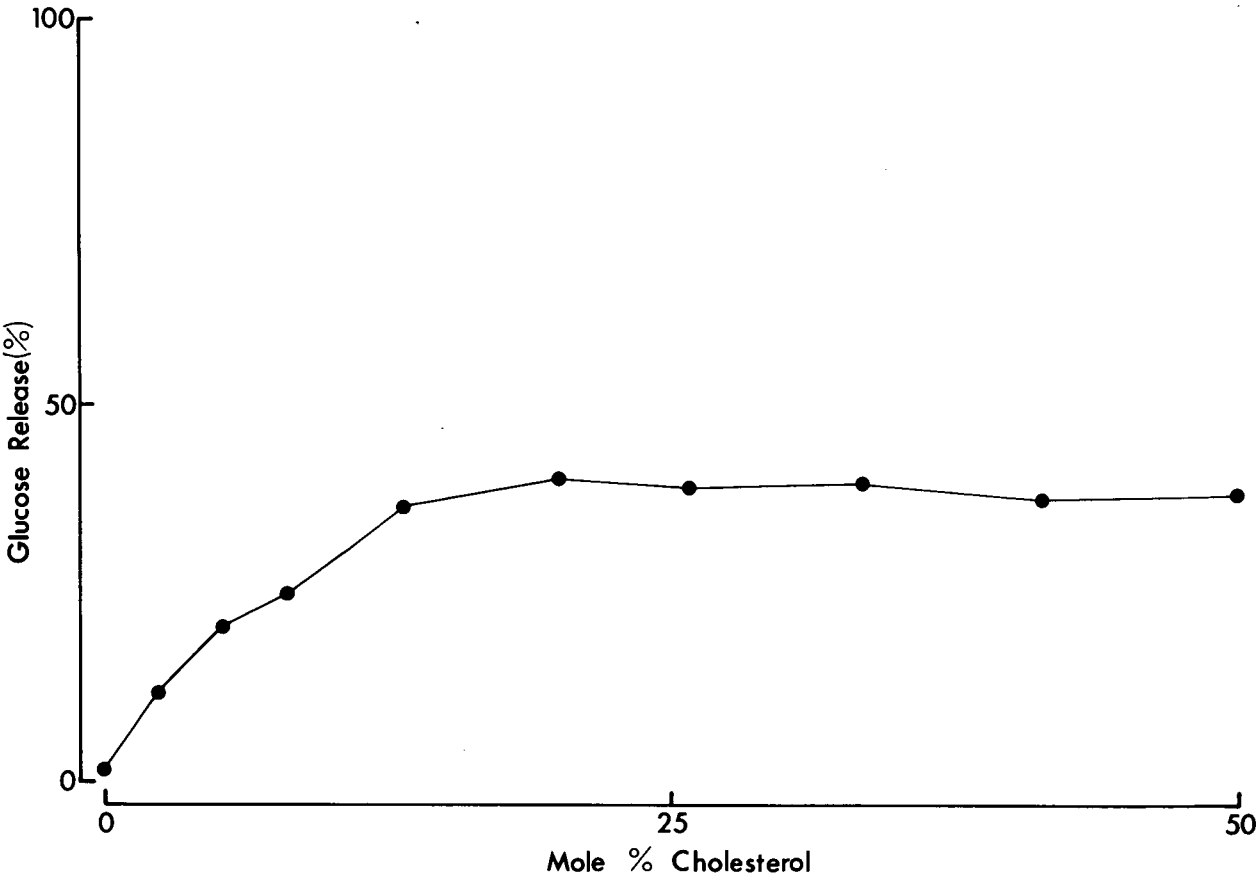

Figure 18—Glucose release (%) from liposomes of dipalmitoyl phosphatidylcholine: cholesterol : dicetyl phosphate, of different mole % cholesterol, following freezing to and thawing from -10°C.

cholesterol the liposomes become more sensitive to freezing injury. At low rates of cooling the response is directly correlated with their cholesterol content (Figure 18). The loss of glucose upon thawing increases as the concentration of cholesterol reaches 20 mole% but in the range 20-50 mole%, there is no further effect. This is the first demonstration that the composition of a lipid bilayer directly determines freeze-thaw injury.

Further studies on modifying the composition of the liposome, and the use of other simple model systems, will elucidate the primary site of injury and thus enable specific methods of avoiding the membrane lesion to be developed. This experimental approach should then be applicable to the protection of sensitive cell-types but, until then, cryobiological experimentation will continue to be empirical in nature.

APPENDIX 1. GENERAL METHODS USED IN CRYOPRESERVATION

Ampoules. Glass containers can shatter when warmed from sub-zero temperatures, so polypropylene screwcap ampoules are now generally used. Polypropylene ampoules designed for low temperature work are commercially available (Gibco, Bio-cult, Sterilin). For rapid rates of cooling, cell suspensions are placed in stainless steel or silver hypodermic tubing or glass capillaries.

Cryoprotective additives. The choice and concentrations of a cryoprotectant is determined by its cytotoxicity and efficiency to the cell-type under investigation. For most studies glycerol, dimethylsulphoxide (Me_2SO) or methanol are used and these should be of Analar or spectroscopic grade. Once prepared at the required concentration, both glycerol and Me_2SO can be autoclaved prior to use or they can be sterilised by filtration. Methanol cannot be autoclaved and, if required sterile, it must be filtered, but methanol dissolves some filters, e.g. Millipore, and therefore a membrane resistant to methanol, e.g. Nuclepore, should be used.

Polymeric compounds e.g. polyvinylpyrrolidone (PVP), hydroxyethyl starch (HES) and dextran have potential cryoprotective properties. Dextrans of different average molecular weight and of high purity are obtainable from Pharmacia. In contrast, PVP is often of variable purity so it should be dialyzed against distilled water or a dilute buffer, then freeze dried. Solutions of PVP are acidic in nature, and they should be buffered to avoid direct cytotoxic effects. Usually the polymer of an average weight of 40,000 daltons is used for cryopreservation.

There are two acceptable methods of adding cryoprotectives to cell suspensions. First, following centrifugation, a cell pellet can be resuspended in medium containing the additive. Second, equal

volumes of a cell suspension can be mixed with a cryoprotectant to give the final required additive concentration. Undiluted additives should not be added directly to cell suspensions, since there is an exothermic heat of mixing which may damage cells.

Temperature measurement. Conventional thermometers have too high a thermal mass to be useful for anything except crude monitoring of the slow cooling of large samples. For accurate work thermocouples are used. For low rates of cooling, a 28 standard wire gauge (SWG) copper-constantan thermocouple is connected to a potentiometric recorder, and a 1 mV deflection is equivalent to approximately 25°C. At faster rates of cooling (>100°C min $^{-1}$) accuracy is limited by the response time of a pen recorder and by the thermal mass of the thermocouple so at rapid cooling rates a 45 SWG thermocouple is attached to a display oscilloscope. Thermocouples are callibrated periodically using a platinum resistance thermometer. Rates of temperature change are usually expressed as °C min $^{-1}$; but this unit is useful only if the temperature range over which it is measured is also defined.

Rates of cooling. Many devices have been described which regulate the rate of cooling during freezing. Commercially available equipment is designed for routine, batch cryopreservation procedures and is therefore not ideally suited to experimental work because the range of cooling rates available is limited, only one cooling rate or temperature may be studied at a time, and there is no facility for nucleating samples. At CCAP, three methods of altering the rate of cooling and of attaining different final sub-zero temperatures are used.

With all methods the ampoules are first placed in a pre-cooled alcohol bath maintained at 1 °C below the melting point of the experimental solution. After allowing the temperature to equilibrate for 5 minutes, the samples are nucleated with the tip of a Pasteur pipette containing a frozen solution identical to that in the ampoule. With axenic cultures or pathogenic organisms ice nucleation may be initiated mechanically by tapping the pre-cooled ampoule. After the dissipation of the latent heat of fusion the ampoule is transferred to the cooling apparatus which has been adjusted so that it is at the same temperature as the sample. If solutions are not nucleated then they freeze spontaneously at different temperatures during cooling resulting in samples with different thermal histories; this difference may affect cell survival. (Figure 19).

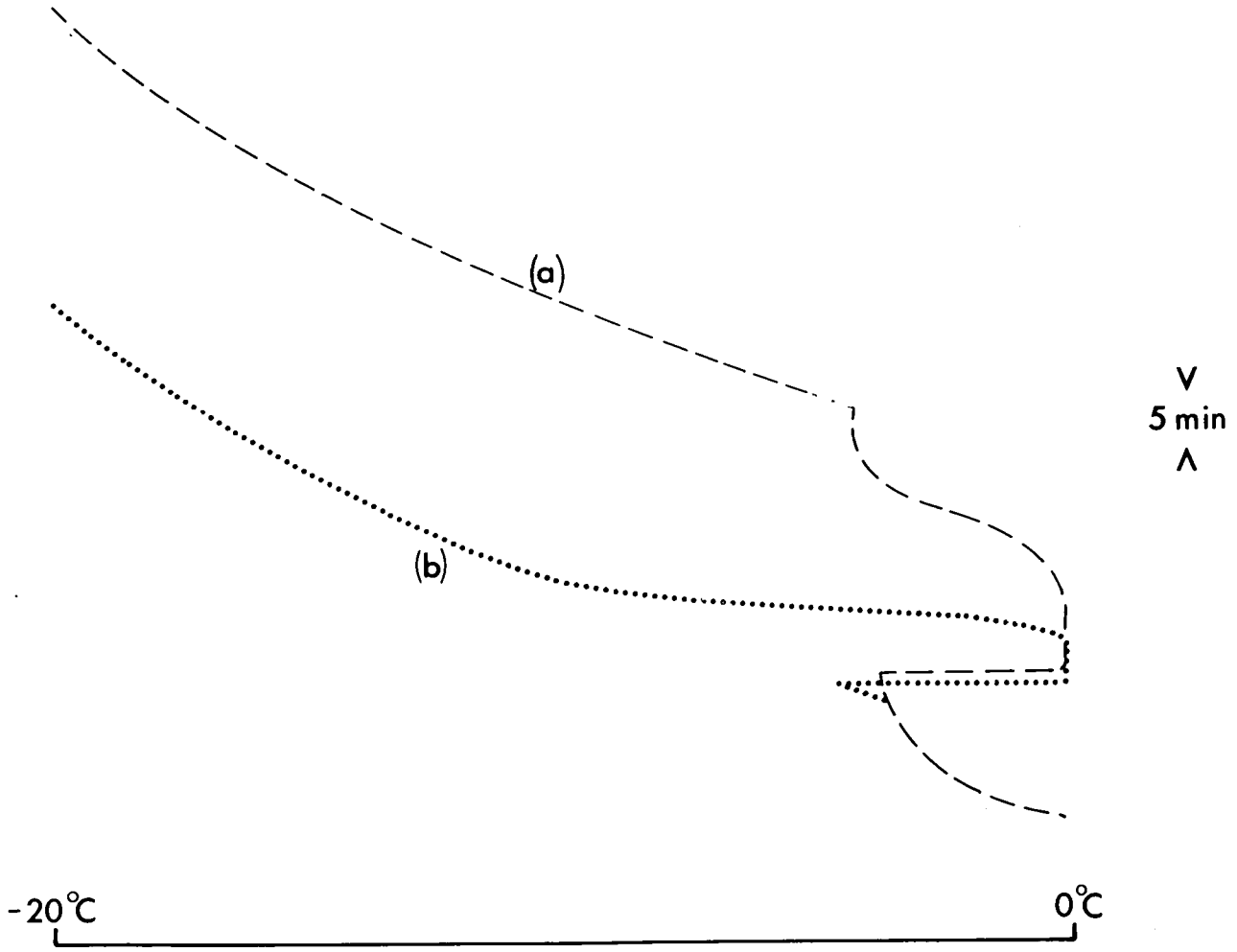

Figure 19—Thermal histories of ampoules containing one ml of distilled water. Sample (a) was nucleated at -3°C and 15 minutes were then allowed for the dissipation of the latent heat of fusion. Sample (b) was cooled without seeding, nucleation occurred spontaneously at -5°C. The rates of cooling from -5 to -10° are (a) 0.5°C min^{-1} and (b) 2.5°C min^{-1}.

(a) Low temperature baths. Baths which are designed to maintain stable temperatures in the range 0 to −45°C are commercially available (Fryka, Grant). These baths are normally used for nucleating samples, but can be used for cooling if, following the dissipation of the latent heat of fusion, the temperature control is set at minimum and the bath allowed to cool. The rate of cooling obtained is exponential but, providing that the bath has the same volume of coolant, is reproducible. However, only limited cooling rates are obtainable from the Fryka bath (Model KB300). These are in the region of 0.2°C min $^{-1}$ (between −5 and −25 °C).

(b) Temperature gradient bar. A brass rod, 35 mm in diameter and 1m long, has one end in liquid nitrogen and the other in an ice or low temperature bath. Holes are drilled 4 cm apart along the length of the bar to a sufficient depth to take polypropylene ampoules (12.5 mm in diameter). Good thermal contact between the rod and ampoules is achieved by placing 0.01 ml of methanol in each hole. Heat gain to the bar is reduced by a covering of insulation material (Armaflex). When the bar is in equilibrium after about 2-3 hours, the temperatures attained are illustrated in Figure 20. This apparatus is used to study the effects of different temperatures on the survival of cells or for stepwise cooling.

(c) Lagged cooling devices. In the simplest form, samples are placed in different sized polypropylene or polystyrene containers and cooled in a deep freeze or placed directly into liquid nitrogen.

For a more controlled and reproducible method, the ampoules are held equidistant from the centre of a circular aluminium holder to minimize variability between cooling rates of individual tubes. The holder containing the ampoules is then placed in the interior of a freezing vessel whose exterior is cooled in liquid nitrogen. Industrial methylated spirit is used as the coolant within the vessel and is continuously stirred (Figure 21). Typical vessels used and the cooling rates achieved are given in Table 4. Very low rates of cooling (<0.1 °C min $^{-1}$) are obtained using silvered or strip-silvered evacuated Dewar flasks. The advantages of this method are that several cooling rates may be examined simultaneously and that the rates of cooling achieved are reproducible.

Rapid rates of cooling are achieved by plunging ampoules directly into liquid nitrogen. However, as liquid nitrogen is at its boiling point, the immersion of a warm sample produces a layer of gaseous nitrogen which limits heat transfer. The insulating effect of the layer of gaseous nitrogen (Leidenfrost's effect) is reduced if a slurry of solid nitrogen (−210°C) or primary cooling fluids e.g. freon, isopentane, or liquid propane, are used. Heat exchange is more rapid as the specimen remains in direct contact with the coolant. Ultrarapid rates of cooling can be obtained by capillary or spray freezing small volumes of sample.

Two-step cooling. This method is usually carried out

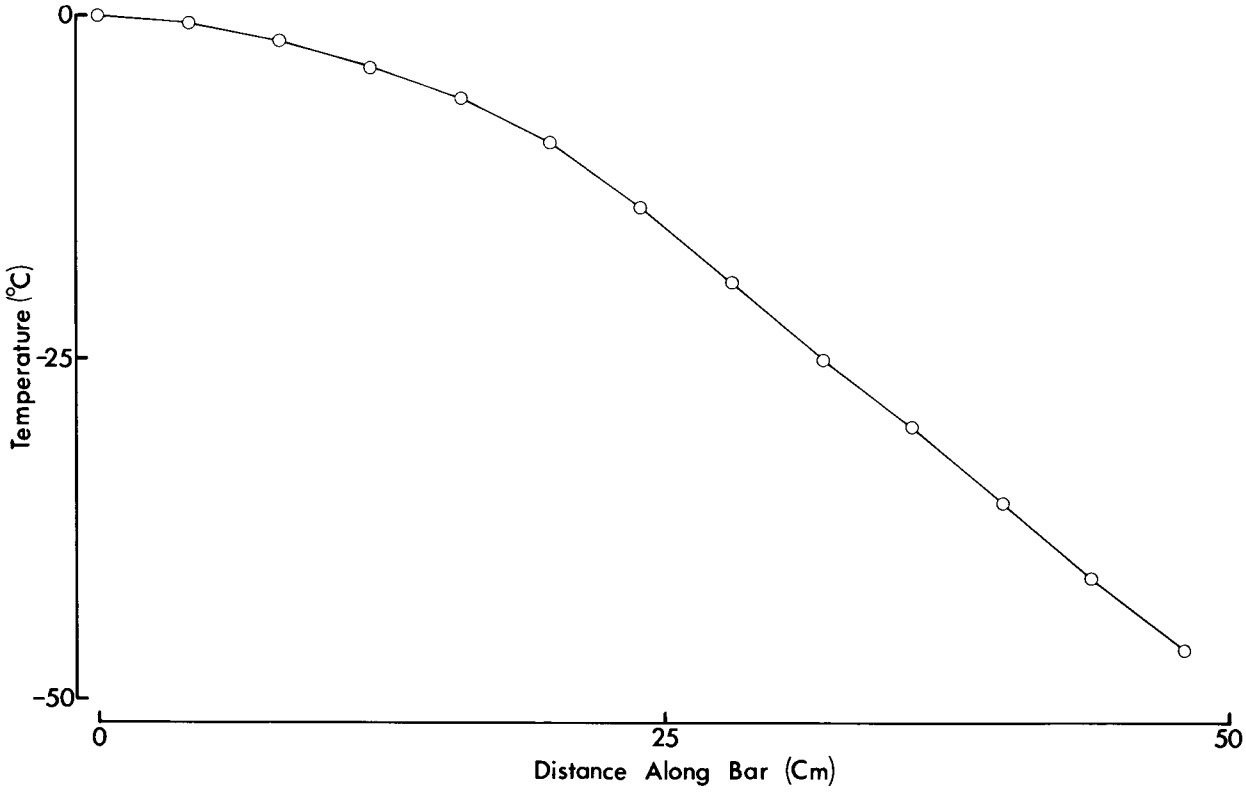

Figure 20—Temperatures attained within a temperature gradient bar, one end of which was maintained at 0°C and the other end in liquid nitrogen.

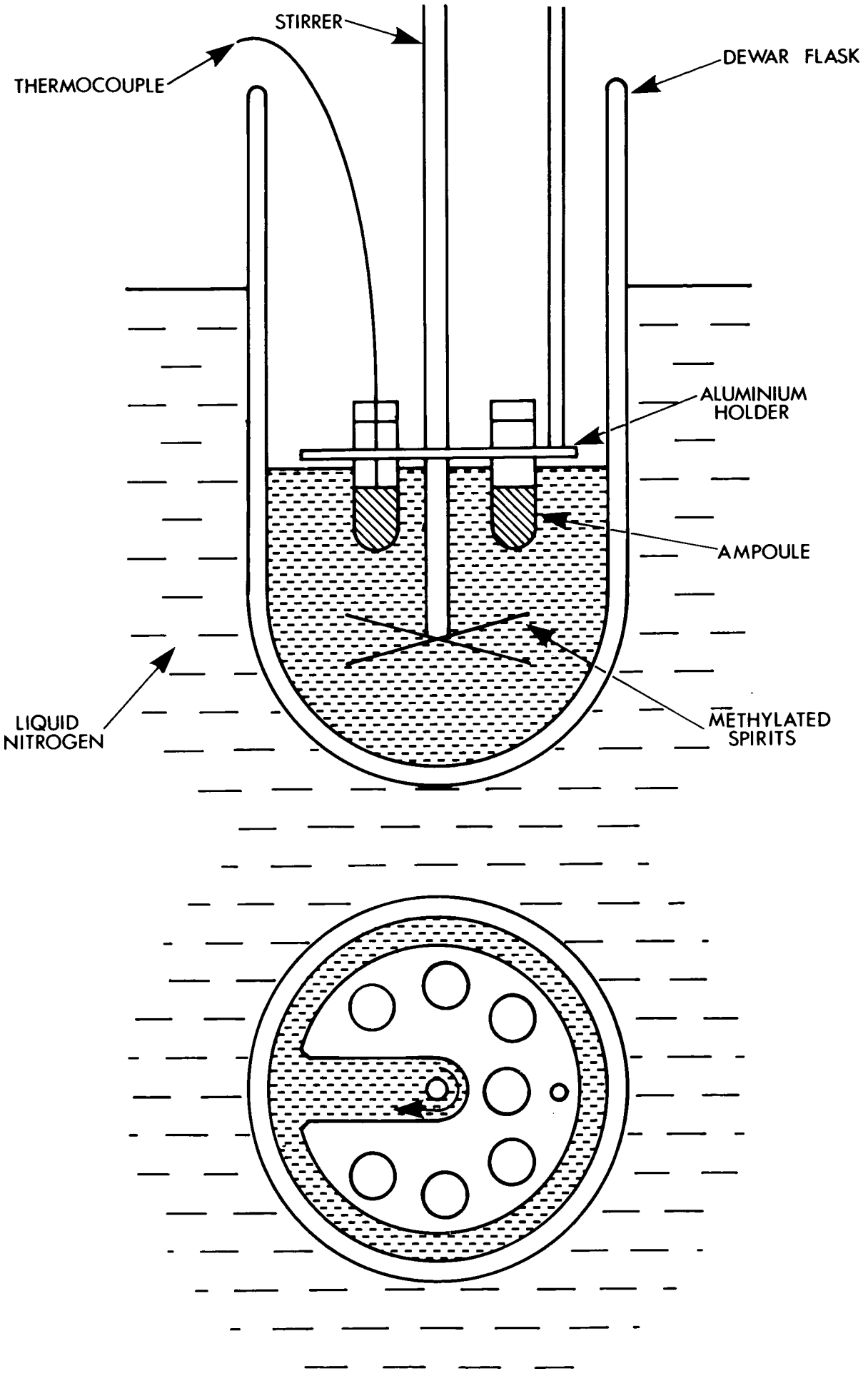

Figure 21—A Dewar vessel set up to obtain a controlled rate of cooling.

using a low temperature bath set at the required holding temperature. If this bath is not available, coolant can be placed in a deep freeze, provided that the volume of coolant is large enough for any temperature fluctuations to be minimized. In two-step cooling, the sample size should be small enough to ensure that cooling is rapid during the initial freezing to the holding temperature.

Rates of warming. For most studies, cells are warmed by rapid agitation of the ampoule in a water bath at 37°C. Slower rates of warming are achieved by using water baths at lower temperatures, allowing the ampoule to warm in the air or by using any of the cooling rate methods in reverse.

APPENDIX 2. CRYOPRESERVATION AT CCAP

The details of a method developed for the cryopreservation of certain algal cultures at CCAP are outlined in Figure 22. Several experimental approaches are integrated into a practical method which is both simple to carry out and yields consistently high recoveries upon thawing.

Cells from an agar slope are transferred to liquid medium and cultured into the late stationary phase (35 days at 20°C), at which stage of culture the cells are non-vacuolated and are most intrinsically resistant to freezing injury. Alternatively, the cellular freezing tolerance can be increased following cold acclimatization or by the limitation of nutrients; however, following these treatments, the cultures have a low cell density and it is thus more difficult to re-establish growth upon thawing than with senescent cultures of higher cell density.

Cells from liquid culture are used without further preparation. 0.5 ml of the cell suspension is added to 0.5 ml methanol (3.0 M, prepared in growth medium) in a 12 × 35 mm sterile polypropylene tube (Nunc). Solutions of methanol were sterilised by filtration. The cells were exposed to the 1.5 M methanol solution for 5 minutes at 20°C to ensure complete uptake of the additive. The ampoules were then frozen by a two-step method, first being placed in an alcohol bath maintained at −30°C and left for 15 minutes, and then transferred directly into liquid nitrogen. Thawing was by rapid agitation of the ampoule in a water bath at 35°C until the last visible crystal of ice had disappeared. Viability was then determined by serial dilution and colony formation in agar.

Long-term storage of ampoules was under liquid nitrogen in a Union Carbide LR 40 refrigerator, 12 ampoules of each strain being routinely preserved. The LR 40 refrigerator has an inventory system allowing easy location and removal of ampoules (Cover photo) and 216 different strains can be stored in each unit. At CCAP, a recovery of 60% is the minimum accepted for long-term maintenance. Using the method outlined, over 300 strains of algae are now successfully stored under liquid nitrogen.

It must be emphasised that the technique, although satisfactory with many strains of Chlorococcales and Euglenophyceae, is less successful with other groups of algae. Improvement with other cell-types can best be achieved by an understanding of the basic principles of cryobiology rather than by following recipes.

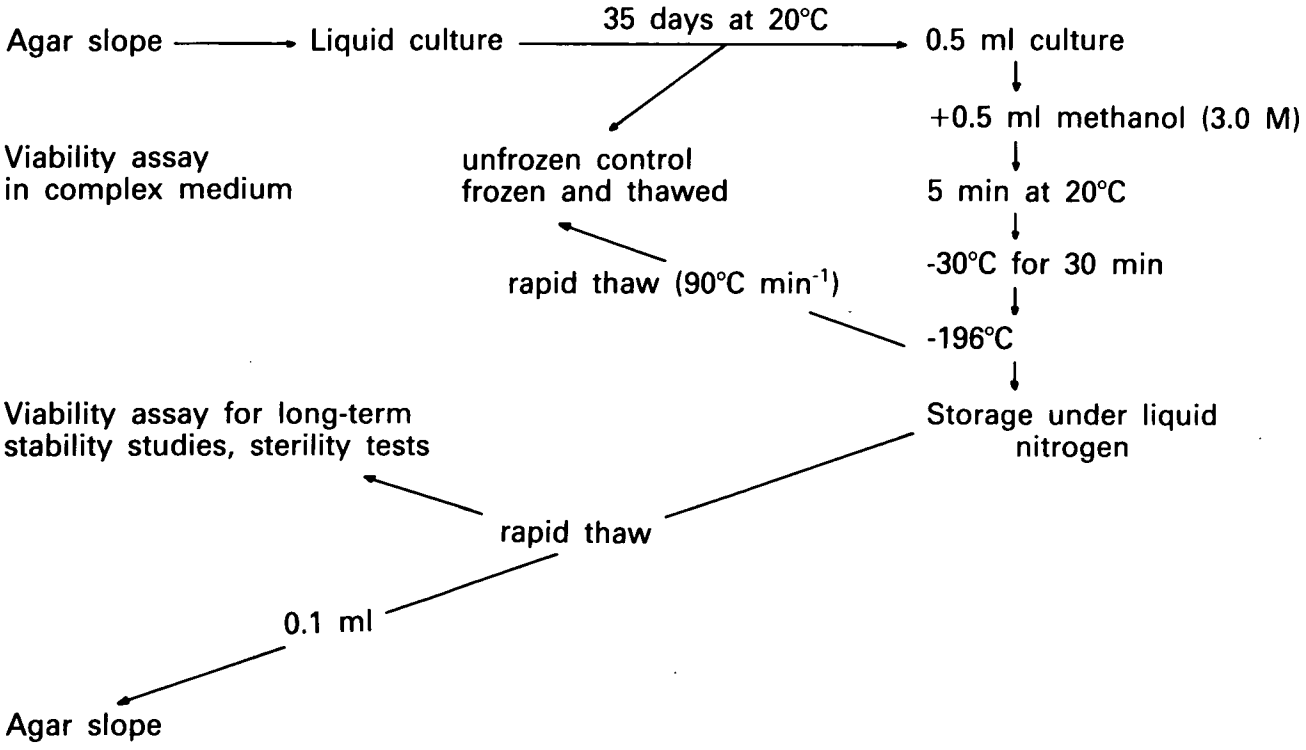

Figure 22—Flow diagram of cryopreservation at CCAP.

APPENDIX 3

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GLOSSARY

Axenic. A pure culture of an organism, having no other kind of organism present.

Buffer. A compound which has the property of reducing the change in pH upon the addition of acid or alkali.

Cold acclimatization. The adaptations, both biochemical and ultrastructural, which allow cellular metabolism to continue at reduced temperatures.

Cold hardening. A special case of cold acclimatization in which the cells become more resistant to a subsequent stress of freezing.

Cold shock. Injury which occurs immediately upon a reduction in temperature.

Colligative effect. A physical property of a system which depends on the number of molecules and not on their nature.

Chilling injury. Damage to cells induced by a reduction in temperature; this is of two distinct types—direct chilling injury or cold shock, which is expressed immediately upon a reduction in temperature and indirect chilling injury, in which a long period, often days, at the reduced temperature is required for cellular damage to occur.

Cryobiology. The study of the effects of low temperatures upon biological systems.

Cryopreservation. The long-term preservation of cellular viability at low temperatures.

Cryoprotective additive. A compound which reduces the extent of cellular injury during freezing and thawing. Common examples are glycerol, dimethylsulphoxide, methanol and polyvinylpyrrolidone.

Eukaryotic. Having the nucleus separated from the cytoplasm by a nuclear membrane and the genetic material is borne on chromosomes. In addition the cytoplasm contains membrane-bound organelles.

Eutectic point. The lowest temperature at which the existence of a liquid phase for a given system is possible.

Hydrophobic bonding. The van der Waals attractive forces which form between nonpolar groups in the presence of water. This results in the nonpolar groups in molecules being arranged so that they are not in contact with water.

Hypertonic. A solution which has a concentration such that it gains water from cells by osmosis.

Ionic strength. A measure of the intensity of the electric field due to the electrolyte ions in a solution.

Lyophilization (Freeze-drying). The process by which water is sublimated, by a reduction in pressure, from a frozen solution.

Median lethal dose. The dose of irradiation at which 50% of a population is killed.

Molarity. The number of moles of a solute dissolved in 1000 grams of solvent.

Nucleation. As applied to aqueous solutions, the ordering of liquid water molecules within a supercooled solution to form ice crystals.

Plasmolysis. Shrinkage of the cell protoplast away from its cell wall when placed in a hypertonic solution, due to the osmotic removal of water. Deplasmolysis is the reverse process, in which the protoplast gains water when placed in an isotonic solution.

Prokaryotic. Cells in which the genetic material is in the form of simple filaments of DNA and not separated from the cytoplasm by a nuclear membrane. Mitochondria, chloroplasts and other membrane bound organelles are also lacking. Examples of prokaryotic cells include bacteria and blue-green algae.

Pseudoplasmolysis. This phenomenon occurs occasionally during freezing when ice forms between the cell wall and protoplast. Due to the differences in chemical potential between supercooled water within the protoplast and ice outside, shrinkage of the protoplast away from the cell wall occurs.

Supercooling. The reduction in temperature of a solution below its freezing point without nucleation occurring.

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