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Mechanism of Cryoinjury In Biological Systems

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Several theories have been advanced to explain the mechanism of cryoinjury. Lovelock (24) suggested that damage is caused by the high electrolyte concentration inside and outside the cell which increases when water is removed from the solution during ice formation. The observation (28) that intracellular fluids remain in the liquid state well below −15°C served as a basis for several hypotheses. Since the free energy of undercooled liquids, as manifested by higher vapor pressure, vp, is higher than their solid modification, a dehydration process results. Injury was thought to be caused as a result of reducing the water content in the cell, viz., the severe reduction of the cell volume (34) and the loss of the protecting water layer leading to denaturation of the protoplasmic proteins (19). It was also suggested that intracellular freezing of the undercooled water is responsible for injury to the cells (27). Each of these theories has good experimental support but none of the proposed mechanisms is able to account for all the relevant observations (18, 29, 33, 35).

Perhaps the most important of the unexplained phenomena is the action of cryoprotective agents (30). These chemicals, when added to biological tissues, increase the survival rate from zero to even 100% in a freeze–thaw cycle. Customarily, the additives are divided into two groups according to their ability to migrate through biological membranes.

It appears that most of the observations concerning the freezing phenomena in biological systems can be explained by a single, integrated theory based on the following points.

Adsorbed molecules, including water, in close proximity to inorganic (23, 43) or polymer (16) surfaces remain in a liquid-like state at temperatures below their bulk triple point. Failure of crystallization is attributed not to lack of nuclei but to the effect of the adsorption forces. The interaction between the surface and adsorbate molecules restricts the mobility of the sorbed species to such an extent that they are unable to attain the configuration required by the ice crystal lattice.

No doubt exists that water in the vicinity of surfaces also in biological systems is subjected to strong forces and has properties uncommon to those of bulk water (4, 6, 8, 17, 22, 37, 42, 46). Specifically, extensive investigations have been carried out on red blood cells, the main component of which is the hemoglobin macromolecule (mol wt 64,500). The water is adsorbed on the relatively large specific surface and in the 15-Å diameter channels formed by adjacent hemoglobin molecules. It was found that at least part of the water is "bound," not available as solvent (12, 38) and has higher activation energy for diffusion (48) consonant with significant water surface interaction. Thus it seems reasonable to assume that the water rigidly held in close proximity to cell proteins is also inhibited from crystallization.

At slow cooling rates crystal formation is restricted to extracellular spaces (28, 32). At rapid cooling, when intracellular ice forms, cytolysis invariably ensues (1). This is true for the case of plant tissues (20) and those of animal cells. The results of the microscopic study of frozen insect cells (2) showed that extracellular ice formed in every case of freezing while intracellular ice, if it occurred at all, formed subsequent to extracellular freezing and was associated with destruction of the cell membrane. Survival after intracellular ice formation has recently been reported. Glycerolated frog blood (40) and ascites sarcoma cells of rats (3) when frozen to form a translucent solid after thawing were proved to be viable. Similarly, rabbit corneal cells were found to survive intracellular ice formation (14). Disregarding these exceptions for the time being, it may be concluded that in general intracellular ice formation is lethal. The present hy-

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pothesis is based on the assumption that intracellular ice does not form in intact cells and in cases where it was observed it did not precede and cause cell destruction but rather followed it.

Let us consider the consequences of the coexistence of nonfreezable intracellular and freezable extracellular water.

Pure water and crystalline ice can be in equilibrium only at 0°C. At subzero temperatures, the vp of undercooled water exceeds that of ice and the metastable liquid, if unable to freeze, evaporates and condenses onto the ice. The driving force of this process, the difference in free energy and consequent vp difference (Δp), increases with decreasing temperature.

Mazur (27) showed that in biological systems intracellular water remains undercooled due to lack of nuclei and recognized the resulting Δp. He suggested that equilibrium is restored by water migrating out of the cell leading to increased salt concentration and diminished vp of the remaining cellular fluid. Damage was thought to be caused by the high salt concentration and the eventual formation of intracellular ice.

It is now suggested that equilibrium is restored by another mechanism, the gradual release of bound water (desorption) and subsequent excretion of the free water from the cell. This process approaches the $\Delta p = 0$ condition by reducing the amount of adsorbed water, a, since a functional relationship exists between a and the relative pressure p/p° (p is the partial and p° the saturation pressure). It has been shown (23) that in order to maintain $\Delta p = 0$ as the temperature decreases, p/p° of the intracellular water has to decrease from unity at 0°C to 0.69 at −40°C and further at lower temperatures.

This mechanism accounts for both the severe dehydration of cells and the continuous and gradual nature of the phenomenon. The desiccation process has been found (49) to proceed even at −60°C, a temperature well below the eutectic temperature of most salt solutions. Accordingly, it is to be expected that denaturation occurs (19) because, as the amount of water decreases, the protein molecules lose their protective layer and also, because of this loss, the cell volume decreases.

On slow cooling the desorbed water leaves the cell in a nondestructive fashion; sufficient time is available for removal of the water before a further amount is desorbed. The chief cause of cellular damage is probably due to dehydration and consequent denaturation. On rapid cooling, however, the water in the cell is released at a rate faster than the permeability of the membrane allows it to flow out, a condition that leads either to total membrane destruction or merely to the creation of numerous small holes in the membrane. The latter has been noted if erythrocytes were cooled at a rate exceeding 5000°C per min (29). It should be emphasized that the value of Δp is small and in itself is not damaging. The resulting release of bound water is, however, sudden, due to the extremely steep rise of the adsorption isotherm at high p/p° values for the protein–water system (5), and this increase of pressure and transfer from the cell can be lethal.

Mazur *et al.* (31) showed that survival rate vs. cooling rate curves exhibited a maximum and noted that this is the effect of two factors: one of which is directly, and the other inversely, proportional to cooling velocity. They were identified as solution effect and intracellular freezing. The model now suggested implies that the two factors are dehydration and membrane destruction.

The freezing behavior of plant tissues is consistent with the outlined mechanism. Figure 1 shows the differential thermogram and the simultaneously determined length changes of a potato sample during a temperature cycle where the rate of temperature change was 4°C/min. The details of experimentation were identical to those already described (23).

The DTA curve on cooling indicates that freezing took place in at least two stages: the first exothermic peak is characteristic of sudden freezing and the second of slow, continuous freezing extending over a wide temperature range. It is proposed that the first peak is due to the nucleation at −7°C of the undercooled extracellular water which triggers the desorption, transfer, and crystallization which occurs extracellularly of the originally intracellular water.

The second process, associated with large expansion, extends to −40°C because desorption is the rate controlling process which in turn is a function of temperature. The process is essentially terminated when on further cooling no significant amount of water becomes unstable.

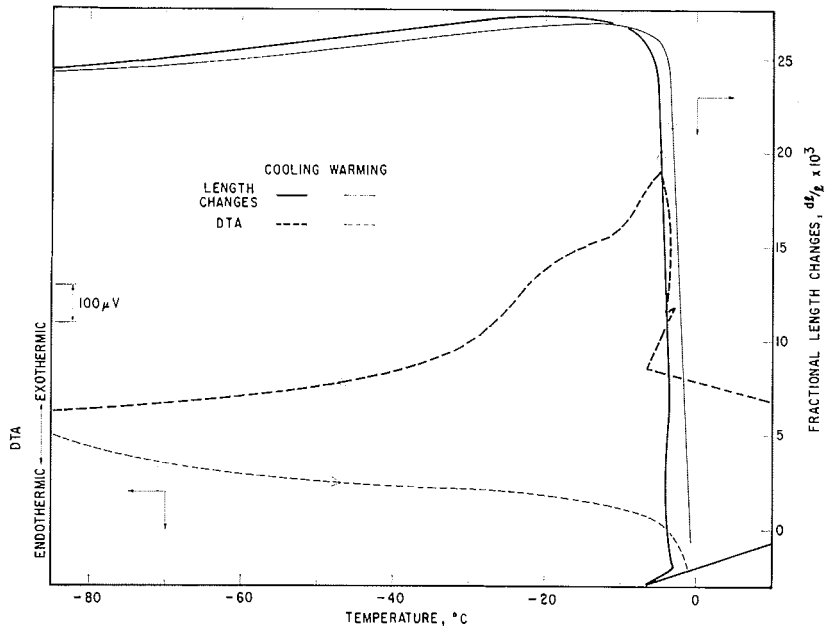

FIG. 1. Fractional length changes and thermogram of potato during a temperature cycle (4°C/min).

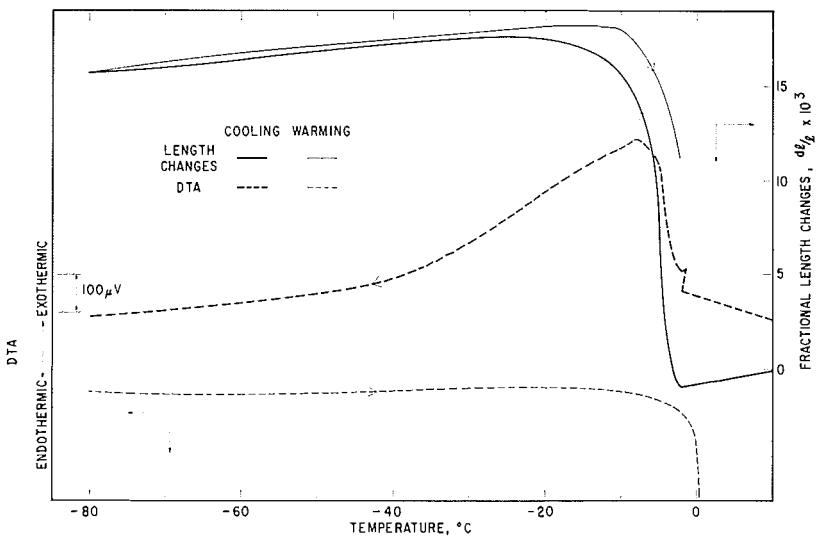

FIG. 2. Fractional length changes and thermogram of potato during temperature cycle (2°C/min).

The main features of these curves remain the same when the cooling rate was half as much, i.e., 2°C/min (Fig. 2) but certain quantitative changes are apparent. Extracellular freezing was initiated at higher temperature, 2.3°C, presumably because the longer time period at subzero temperature increased the probability of nucleation. The amount of heat released during the second phase of freezing appears to be about the same, as expected, because the amount of water frozen is identical, but the process proceeded at a more even rate. Most significantly, the fractional expansion dl/l is only 18×10^{-3} instead of 27×10^{-3}. It should be noted that in both cases

(Figs. 1 and 2) freezing is completed essentially at the same low temperature of approximately −40°C. Melting in both instances was sharp and occurred at 0°C, since the ice was located outside the cell and had bulk properties. It may be mentioned that hardy winter plants show multiple exothermic peaks (49) because the cell membranes are intact and permeable. Conversely, nonhardy plants or dead stems exhibit a single freezing process, presumably due to a fragile or already destroyed membrane structure.

MECHANISM OF CRYOPROTECTION

The proposed mechanism of cryoinjury permits the establishment of conditions under which damage is minimal or totally avoided.

Cooling rate. It follows from the aforesaid that the intermediate velocity renders optimum survival because it is fast enough to minimize dehydration but slow enough to avoid membrane injury. From the point of view of dehydration, high cooling rates are advantageous because, in spite of the high dehydration rate, the absolute amount of redistributed water is small. The finding of Lovelock (24), that damage of red cells occurs between −3 and −40°C and if the time spent in this temperature region is more than 5 sec, supports this view. The lower boundary value of the critical region is probably determined by the drastically decreased mobility of the water molecules.

Although only the cooling process has been discussed thus far, the same considerations also apply to the warming process. There is no reason to assume that thawing is less injurious than freezing. In fact, perhaps the opposite is true. On warming, a long time is required for the complete disappearance of the ice crystals, and during this period the unfrozen cells can warm to well above 0°C. Under these conditions large deleterious vp differences may exist. The situation is aggravated by the relatively high temperature at which water diffusion is rapid.

Permeability. It is expected that increased membrane permeability is beneficial by virtue of permitting the application of higher cooling rates without membrane injury, or alternatively, by reducing the extent of the damage at a given cooling rate.

In agreement with this view is the observation that during the increase of winter hardiness the cell permeability of plants increases. Only plants whose frost resistance actually increased on exposure to "hardening" temperatures showed this change (21, 49). It has also been reported (45) that peptone protects T_4 bacteriophage of Escherichia coli, presumably by increasing the permeability (35).

Williams and Meryman (50) demonstrated that winter-hardened or artifically protected spinach grana avoid injury if a reversible influx of solute is possible. Subsequently, Meryman suggested (36) the increased permeability induced by cryoprotective additives as the efficacy of nonpenetrating agents.

Viscosity. In terms of the proposal presented in this paper, cells are damaged during slow cooling mainly by dehydration. Accordingly a certain degree of protection may be achieved by increasing the viscosity of the intracellular fluid which retards the flow of water. It seems significant that the aqueous solutions of the penetrating cryoprotective agents (glycerine, DMSO, glycol, ammonium acetate, trimethylamine acetate) are noted for having high viscosity, particularly at low temperatures.

Minimization of Δp. Elimination of Δp, the primary cause of cryoinjury, is possible theoretically in at least two ways: by decreasing the vp of the intracellular fluid and by increasing the vp of the extracellular solid.

The former can be achieved by the addition of one of the known penetrating agents. A property common to these additives is the low vp of their aqueous solutions. The values of p and $p/p°$ of three solutions, of a concentration customarily applied for complete protection of biological systems, are given in Table 1. Most agents in the undiluted state are deliquescent because the vp of their saturated solution is less than the partial pressure of water in air.

Increase of the vp of the extracellular solid, relative to crystalline ice, can be achieved by glass formation. As glasses do not represent

TABLE 1

VAPOR PRESSURE OF AQUEOUS SOLUTIONS OF CRYOPROTECTIVE AGENTS AT 0°C

Solution	Concentration %	Pressure, Torr	Relative pressure
Ammonium acetate	36	3.27	0.72
DMSO	39	3.48	0.76
Glycerine	40	3.85	0.84

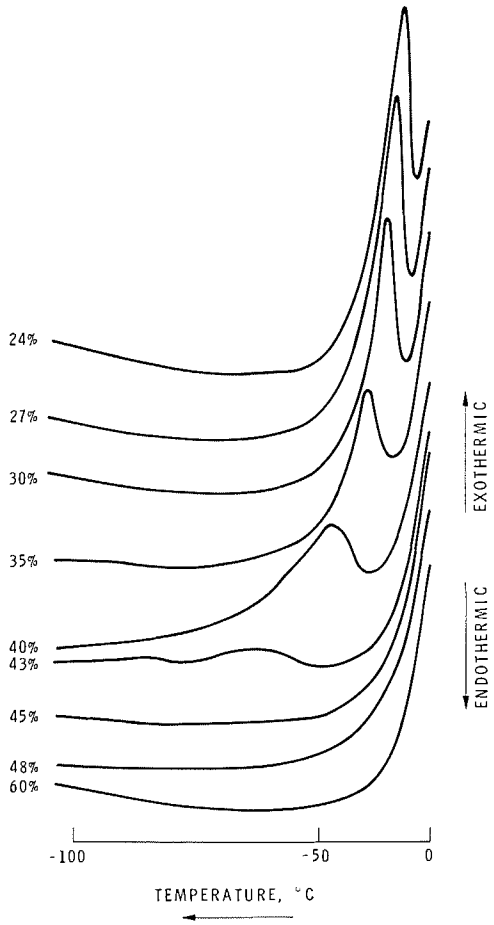

FIG. 3. Differential thermograms of aqueous glycerine solutions. Concentrations are expressed in v/v% and indicated on the right-hand side of the curves. Cooling rate approximately 130°C/min.

states of thermodynamic equilibrium with respect to configurational changes of their molecular structures their free energy and vp are greater than in the equilibrium crystalline form (47). Glass formation occurs with certain substances under unique conditions, e.g., amorphous ice is formed below −120°C when water vapor is cooled very rapidly, but other substances undergo glass formation under almost all conditions. Liquids belonging in the latter group are characterized by high viscosity which is a recognized criterion for glass formation.

Salt buffering. Farrant (9–11) demonstrated that the harmful threshold concentration of NaCl occurs at a lower temperature when penetrating cryoprotective agents or PVP are present than when they are absent and that the reduced salt concentration contributes to protection during freezing.

ACTION OF CRYOPROTECTIVE AGENTS

Additives possessing cryoprotective qualities greatly affect the pattern of ice formation in solutions as shown by Luyet and co-workers (26, 41).

Most of the additives that possess cryoprotective qualities are known to form glasses on solidification. Glycerine, DMSO, glycol, ammonium acetate, PVP (16), trimethylamine acetate (36), and sucrose (13) are good examples of such compounds. It is obvious that extracellular glass formation is one of the means by which protection is provided. The concentration of the aqueous solutions of three additives at which the transition is completely glassy has been established in this laboratory. The differential thermograms, obtained at a cooling rate of 130°C/min (Figs. 3–5), indicate that no crystalline ice forms in glycerine above 45% concentrations, in DMSO above 40, and in ammonium acetate above 37% concentrations. It is most significant that for complete protection of blood the concentration is required to exceed 45% in the case of glycerine (1), 34% for DMSO (15) and 31% for ammonium acetate (33). PVP provided good but not complete protection when the concentration was 15% in freezing of chinese hamster and marrow stem cells (31). This is to be expected in view of the very high concentrations [60% (16)] required to avoid ice formation of PVP solutions.

In a classical experiment, Lovelock (25) demonstrated that glycerine must penetrate the red cells to attain good protection. He found that the recovery decreased from 97 to 63% when permeation was blocked by added copper ions and thus proved his point but, at the same time, the results had also a puzzling aspect, in that survival was 63% instead of 2% which would be expected in the absence of glycerine. It is suggested that survival increased from 2 to 63% by virtue of glass formation but could not achieve 97% due to the absence of penetration, i.e., vp lowering of the intracellular fluid.

Figures 6 and 7 show the fractional length changes and thermal effects obtained on cycling of potatoes containing glycerine and DMSO ad-

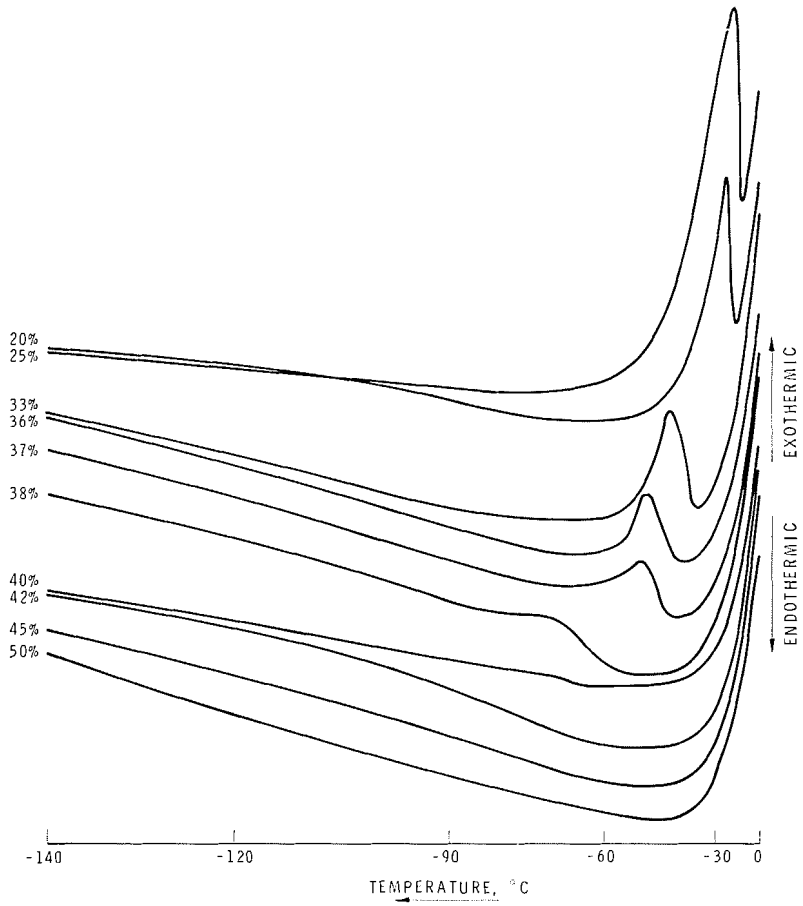

FIG. 4. Differential thermograms of aqueous DMSO solutions. Concentrations are expressed in v/v% and are indicated on the right-hand side of the curves.

ditives. The cryoprotective agents have dramatically reduced the exothermic heat effect, i.e., solidification was essentially glass formation and the concomitant changes in dimension have also diminished.

It is most probable that the aforementioned survival of ascites tumor (3) and glycerolated frog blood cells (40) of intracellular crystallization was due to the vitreous character of the formed ice. This is supported by the observation that viability was preserved only if the solid was translucent. In fact Rapatz and Luyet (40) observe that "The similarity of the results (of the glycerolated blood) with those obtained with non-glycerolated blood cells frozen at −150° and recrystallized at −10° is striking." It is a well-known fact that water cooled rapidly to below −120°C forms vitreous ice.

According to the proposed mechanism, penetrating agents protect by the elimination of Δp utilizing both the lowering vp of the fluid and increasing the vp of the solid, while the hitherto unsatisfactorily explained action of the nonpenetrating additives consist mainly of external glass formation. Penetrating agents also increase the viscosity of the intracellular fluid. Is is of great importance that although "bound" water excludes salt it does dissolve nonionic species such as glucose (44) and, presumably, the penetrating additives.

It should be emphasized that the proposed hypothesis does not deny the operation or the importance of the solution, minimum cell volume, and sulfhydryl–disulfide mechanisms but rather suggests the primary causes leading to the creation of conditions under which they be-

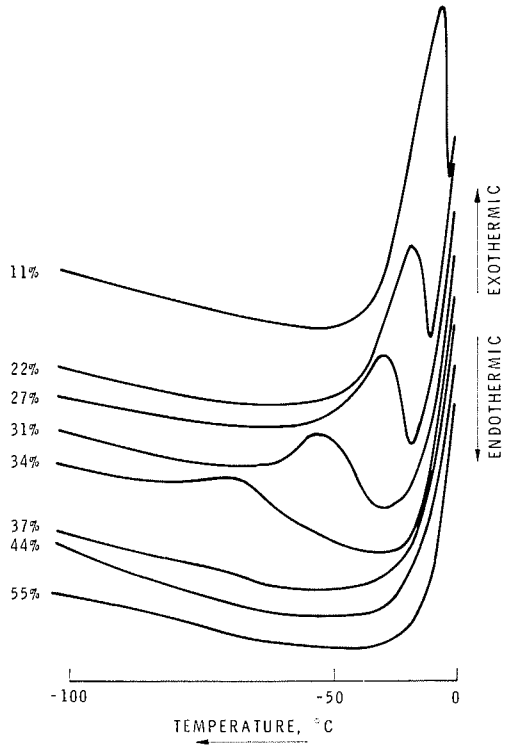

FIG. 5. Differential thermograms of ammonium acetate solutions. Concentrations are expressed in w/v% and are indicated on the right-hand side of the curves.

come injurious and demonstrates their compatibility.

SUMMARY

A new hypothesis, based on the assumption that intracellular water remains liquidlike below 0°C and therefore its vapor pressure (vp) is greater than that of ice, is proposed to explain the mechanism of cryoinjury. On cooling, extracellular ice forms and a vp difference, Δp, is created which increases with decreasing temperatures. A spontaneous process of water desorption and subsequent redistribution is prompted by the nonequilibrium state. Injury is the result of dehydration at slow cooling rates and membrane rupture at rapid rates. Penetrating cryoprotective agents improve survival rate by diminishing the migration rate and Δp. The latter is achieved by lowering the vp of the intracellular fluid colligatively and increasing the vp of the extracellular solid. Differential thermal analysis of the aqueous solutions of glycerine, DMSO, and ammonium acetate provides experimental evidence that at concentrations at which these cryoprotective agents render full protection for red blood cells the frozen solid is amorphous glass instead of crystalline. Nonpenetrating agents protect only extracellular glass formation. Certain additives increase membrane permeability which at slow cooling rates is bene-

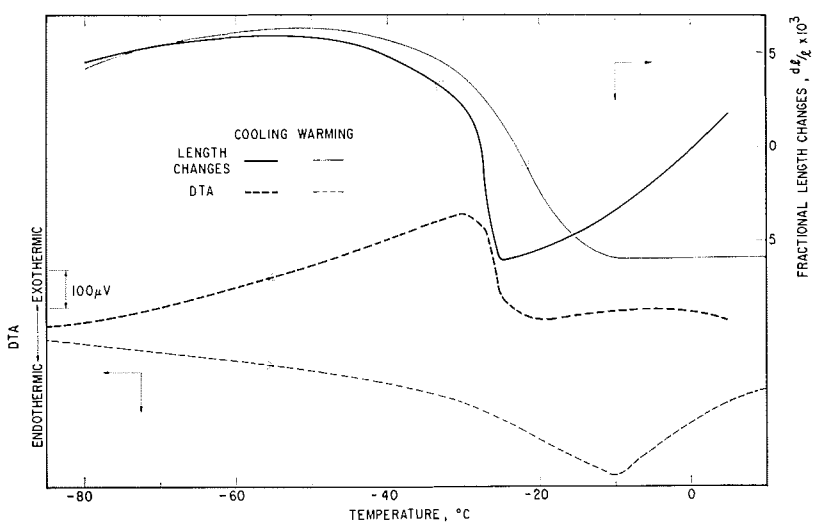

FIG. 6. Fractional length changes and thermogram of potato presoaked in 50% glycerine–water solution. Rate of temperature change 4°C/min.

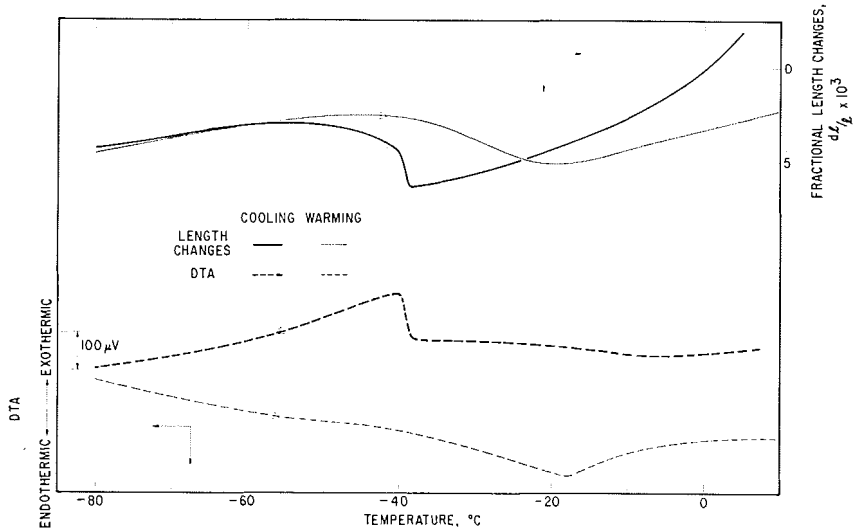

FIG. 7. Fractional length changes and thermogram of potato presoaked in 50% DMSO-water solution. Rate of temperature change 4°C/min.

ficial. The dimensional changes and thermogram of potato samples during temperature cycles are explicable by the proposed model. It is shown that the hypothesis is compatible with the most important modes of freezing injury already proposed, suggesting the primary cause leading to the creation under which they became operational.

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