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Freezing Characteristics of Cultured *Catharanthus roseus* (L). G. Don Cells Treated with Dimethylsulfoxide and Sorbitol in Relation to Cryopreservation[1]

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ABSTRACT

The freezing behavior of dimethylsulfoxide (DMSO) and sorbitol solutions and periwinkle (*Catharanthus roseus*) cells treated with DMSO and sorbitol alone and in combination was examined by nuclear magnetic resonance and differential thermal analysis. Incorporation of DMSO or sorbitol into the liquid growth medium had a significant effect in the temperature range for initiation to completion of ice crystallization. Compared to the control, less water crystallized at temperatures below −30°C in DMSO-treated cells. Similar results were obtained with sorbitol-treated cells, except sorbitol had less effect on the amount of water crystallized at temperatures below −25°C. There was a close association between the per cent unfrozen water at −40°C and per cent cell survival after freezing for 1 hour in liquid nitrogen. It appears that, in periwinkle suspension cultures, the amount of liquid water at −40°C is critical for a successful cryopreservation. The combination of DMSO and sorbitol was the most effective in preventing water from freezing. The results obtained may explain the cryoprotective properties of DMSO and sorbitol and why DMSO and sorbitol in combination are more effective as cryoprotectants than when used alone.

Cryoprotective compounds are essential for the freeze preservation of plant tissues with little or no natural tolerance of freezing (14). Since the discovery of glycerol as a cryoprotective agent in 1949, many types of cryoprotective compounds which differ in structure and cryoprotective properties have been reported (2, 3, 7, 13). DMSO has been used most extensively in the freeze-preservation of plant materials (3, 4, 18). Recently, sorbitol has been used successfully in preserving plant suspension cultures at liquid nitrogen temperature (16). However, it is not known how these compounds act as cryoprotectants. Results have shown that in certain cases a combination of cryoprotectants is more efficient than a single cryoprotectant (4, 18). Although cryoprotectants have been applied to a wide range of plant material, almost all the published protocols for cryopreservation have been developed from an empirical approach.

The interpretation of how DMSO and other cryoprotectants protect living cells against freezing injury has not been conclusive (3). Lovelock (*cf.* 3) suggested that DMSO and glycerol prevent excessive increases in electrolyte concentration which occur during freezing. Meryman (13) suggested that penetrating solutes such as DMSO and glycerol protect against the detrimental effects of cell shrinkage. Farrant (2) also suggested that the efficacy of DMSO may be due to a reduction in cellular shrinkage.

Cell lines derived from *Catharanthus roseus* have been described as a potential source for the industrial production of alkaloids (10). A procedure for the cryopreservation of periwinkle cell line no. 916, which does not produce detectable amounts of alkaloids has been reported (9). However, this procedure was unsuccessful in cryopreserving the high alkaloid producing cell line no. 200. This research was conducted to develop a suitable protocol for cryopreserving this cell line.

In this report, we used NMR[2] techniques to examine ice crystallization of DMSO and sorbitol solutions and periwinkle cells treated with these cryoprotectants. The results indicate that the DMSO treatment enables cells to retain a larger fraction of unfrozen water during freezing. A high percentage of unfrozen water at temperatures below −30°C appears to be a critical factor in cryopreservation.

MATERIALS AND METHODS

Cell Cultures. An alkaloid producing periwinkle, *Catharanthus roseus* (L). G. Don, cell line designated as PRL no. 200 and a cell line which does not produce detectable levels of alkaloids, designated as PRL no. 916 were used in this study. These cell lines were initiated with anthers of periwinkle and maintained on solid and liquid media as previously reported (10). Here, suspension cultures of cell line no. 200 were grown in B5 liquid medium (5) supplemented with 1 mg/l 2,4-D, 1 g/l casein hydrolyzate, 200 mg/l ribose, 200 mg/l glucose, and 200 mg/l arginine. Cell cultures of no. 916 were grown in liquid B5 medium supplemented with 1 mg/l 2,4-D and 1 g/l casein hydrolyzate. All cultures were subcultured as 50-ml batches in 250 ml Delong Flasks on a gyratory shaker (130 rpm) under a continuous light of about 10 wm^{-2} at 24 ± 1°C. Three- to 5-d-old cultures were used in all studies except for the cultures of cell line no. 200 which were precultured over agar medium supplemented with 1 M sorbitol. For preculturing, 3- to 5-d-old cultures of cell line no. 200 were transferred to 15-ml sterile conical centrifuge tubes and centrifuged at 100 *g* for 3 min. The liquid was removed and the packed cells were placed over 30 ml B5 medium supplemented with 1 mg/l 2,4-D and 1 g/l casein hydrolyzate, 1 M sorbitol, and solidified with 0.5% agar in a sealed jar.

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[2] Abbreviations: NMR, nuclear magnetic resonance; DTA, differential thermal analysis; TTC, 2,3,5-triphenyl tetrazolium chloride.

DTA. Differential thermal analysis was used to determine the temperatures at which spontaneous freezing initiated for solutions of DMSO and sorbitol and the time for freezing to reach equilibrium. For each determination 200 μl of solution was placed in a small aluminum foil cylinder (5 mm in diameter and 15 mm in length), and the foil was crimped around a copper-constantan thermocouple to provide good contact between the aluminum foil and the thermocouple. Samples and DTA reference (aluminum foil cylinder without solution) were inserted into wells in an aluminum block, placed into a deep freezer at −60°C and cooled at a rate of 0.85°C/min. Exotherms were recorded at 0.1 mv on a potentiometric recorder. Each sample solution was measured for 10 to 20 times and only the average values are presented.

NMR. The liquid water content of cryoprotective solutions or cell cultures treated with cryoprotectants was determined at subzero temperatures in a Bruker Mini Spec p. 20 spectrometer equipped with a programmable temperature controller as described by Gusta *et al.* (6). Cultures in cryoprotective solutions were collected on a dry paper towel to remove excess liquid.

Cell culture samples (0.2 g fresh weight), and 100 μl of solution sample were carefully placed into the bottom of a NMR tube (5 mm in diamter). The samples were cooled to −3°C and freezing was initiated with ice crystals from a glass rod dipped in liquid nitrogen. After initiation of freezing, samples were slowly cooled to the desired freezing temperatures and held at that temperature until freezing reached equilibrium as determined from the quantity of liquid water. In order to minimize errors due to differences in the water content due to different treatments, the data are expressed as the per cent ratio of liquid water to total water content.

Cryopreservation and Viability Tests. The procedure for cryopreservation of cell suspensions was as reported by Kartha *et al.* (9). One-ml aliquots of cell suspension were dispensed into cryogenic glass ampoules (1.2 ml capacity, Wheaton), flame-sealed, and arranged in the freezing chamber (CRYOMED 972 Freezer) precooled to 0°C. The specimens were then cooled at 1°C/min with a programmable CRYOMED 1000 controller to −40°C followed by immersion in liquid nitrogen. After 1 h storage in liquid nitrogen, the ampoules were rapidly thawed in a 37°C water bath. The viabiity of frozen thawed cells was compared to controls by means of the TTC reduction assay procedure as described by Kartha *et al.* (9).

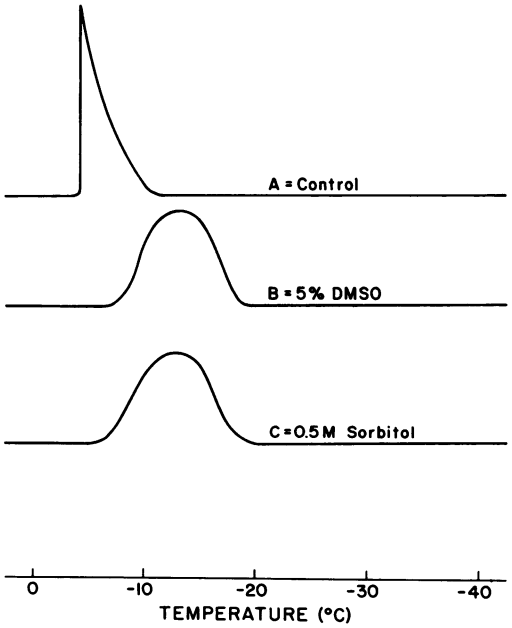

FIG. 1. Typical DTA profiles of 20 μl of B5 medium (A) and 5% DMSO (B) and 0.5 M sorbitol (C) in B5 medium.

Table I. *Effect of DMSO on the Temperature for Spontaneous Ice Crystallization and Time for Freezing to Reach Equilibrium*

DMSO Concn.	Freezing Initiated	Freezing Completed	Difference	Time[a]
%		°C		min
0	−3.9	−10.2	6.3	7.8
1	−5.3	−13.6	8.3	9.8
2	−5.7	−16.7	18.8	12.7
5	−6.9	−17.8	10.9	12.8
10	−8.2	−19.8	11.6	13.6
15	−8.5	−22.6	14.1	16.6

[a] The time from freezing initiation to completion was calculated by the temperature difference divided by cooling rate (0.85°C/min).

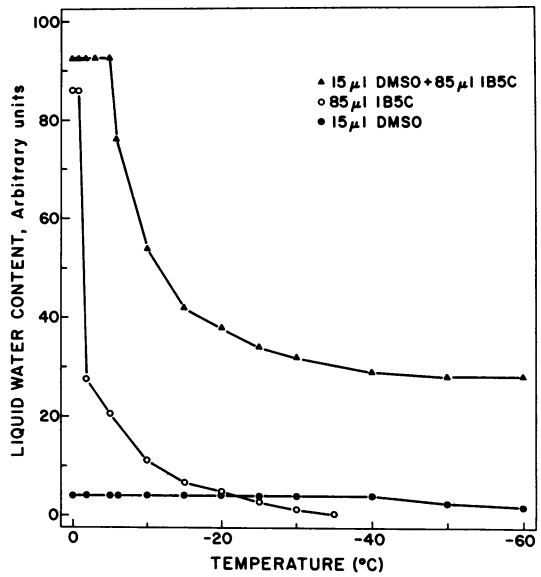

FIG. 2. NMR freezing curves of DMSO (15 μl), B5 medium (85 μl), and 15% DMSO (85 μl B5 medium plus 15 μl DMSO).

RESULTS

DMSO and Sorbitol Solutions. Typical DTA profiles of B5 liquid medium, alone and in combination with DMSO (5% v/v or 0.64 Osmolar) and sorbitol (0.5 M) are shown in Figure 1. Freezing initiated spontaneously at temperatures ranging from −3°C to −11°C in B5 liquid medium. In general, once freezing was initiated complete freezing occurred over a very narrow temperature range, resulting in a very sharp DTA profile (Fig. 1A). In contrast, the DMSO solution initiated freezing at a lower temperature and required a much longer period before freezing was complete (Fig. 1B; Table I). For example, 5% DMSO in liquid culture medium, the cryoprotectant prescribed for cryopreservation of periwinkle cell line no. 916 (9); the time from initiation to completion of freezing was 12.8 min, whereas it was 7.8 min for the liquid medium without DMSO. For sorbitol solutions, the results were similar to the DMSO solution (Fig. 1C). Since the samples were of the same size, heat removal due to freezing was not the main factor in determing time for freezing to reach equilibrium.

The freezing of water in DMSO and sorbitol solutions was studied using pulsed proton nuclear magnetic resonance spectroscopy (NMR). Because of the presence of H in DMSO molecules, the proportion of NMR signal due to DMSO and water had to be clarified. Figure 2 shows the freezing curves of liquid

medium (85 μl), DMSO (15 μl), and the mixture of both (15% DMSO and 85 μl liquid medium, v/v). The reasong 15% DMSO was chosen was because this was the highest concentration tried for cryopreservation of periwinkle cell cultures. The results revealed that a DMSO concentration up to 15% did not contribute significantly to the NMR signal.

The NMR freezing curves of DMSO solutions from 0 to 15% are shown in Figure 3. With increasing concentrations of DMSO, less water froze at a given temperature. The solutions deviated from ideal freezing behavior because less ice was found. For example, at −40°C, the per cent liquid water was 0, 3, 15, 30, and 40% for 0, 2, 5, 10, and 15% DMSO solution, respectively. Figure 4 shows the freezing curves of sorbitol solutions (0.5 and 1 M) and sorbitol plus 5% DMSO. Although 0.5 or 1 M sorbitol was effective in preventing water from freezing at temperatures from −5°C to −25°C, at temperatures below −30°C, the effect of sorbitol was much less than DMSO. The combination of DMSO and sorbitol showed the additive effect in preventing water from freezing. Such additive effects are clearly illustrated when comparing the freezing curves shown in Figures 3 and 4.

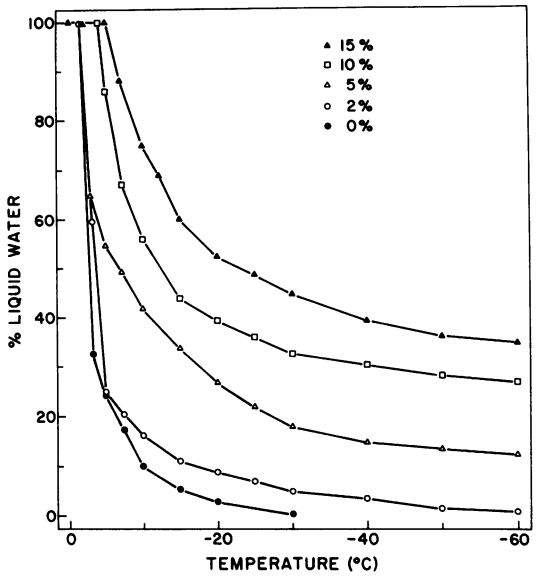

FIG. 3. NMR freezing curves of 0 to 15% DMSO solutions in B5 medium.

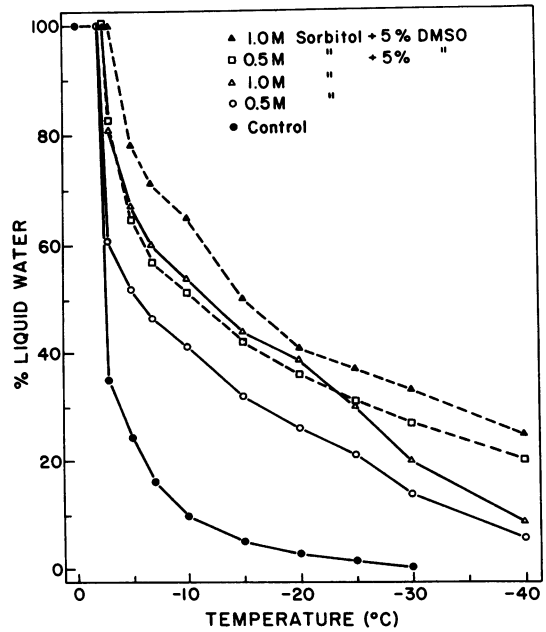

FIG. 4. NMR freezing curves of sorbitol (0.5 and 1.0 M) and sorbitol plus 5% DMSO solutions in B5 medium.

Cells Treated with Cryoprotectants. A 24-h preculturing of no. 916 periwinkle cells in culture medium containing 5% DMSO prior to freezing was found to be essential for cryopreservation (9). Recent results have shown that a 1-h preculturing treatment was equal or better than a 24-h preculturing treatment (Chen and Kartha, unpublished). However, attempts to preserve the alkaloid-producing periwinkle cell line no. 200 in liquid nitrogen by the same procedure were not successful. As shown in Figure 5, cells of cell line no. 916 and no. 200 differ in cell size and vacuolation when viewed under Nomarski optics. Cells of no. 916 were characterized by smaller cell size, dense cytoplasm, and many small vacuoles in a single cell. Cells of cell line no. 200 were much bigger in cell size, less dense in cytoplasmic components, and most cells are characterized by a large central vacuole.

The freezing curves of line no. 916 and no. 200 cells before and after a 1-h DMSO preculture treatment are shown in Figure 6. The control cells froze as an ideal solution with all of the freezable water frozen at −40°C for cells of both lines. More than 20% of the total water remained unfrozen at −40°C in no. 916 culture pretreated for 1-h in either 5 or 10% DMSO. The same treatment when applied to no. 200 cells resulted in only 10% of the water being unfrozen at −40°C. The reduction in ice formation by DMSO cannot be explained fully by osmotic effects. Cell

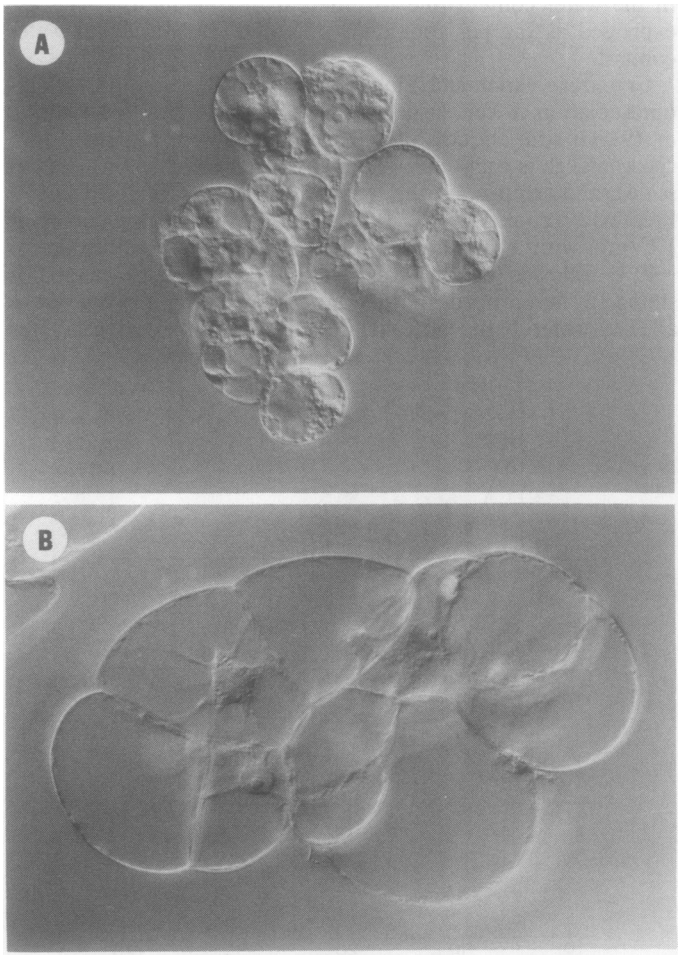

FIG. 5. Cells of periwinkle line no. 916 (A) and no. 200 (B). (Nomarski optics, × 400.) Note for the differences in cell size and vacuolation between these two cell lines.

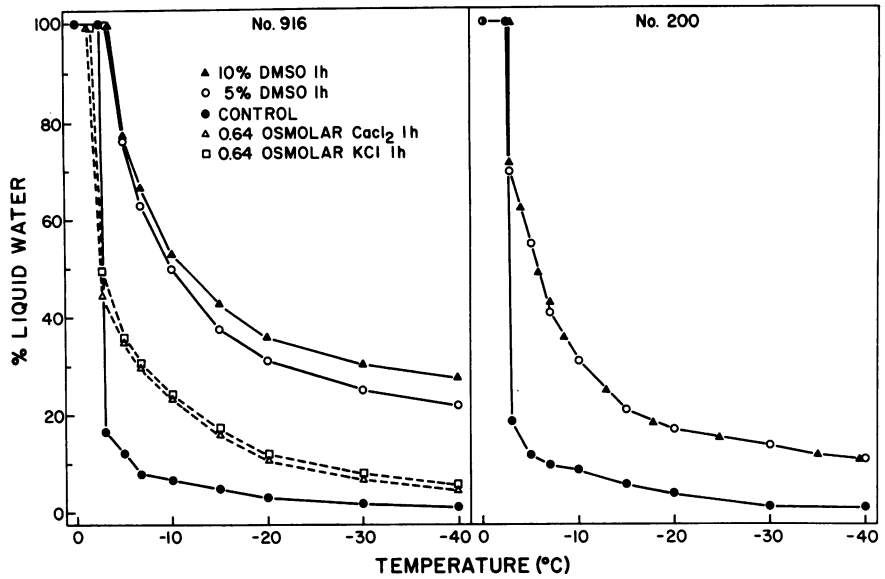

FIG. 6. NMR freezing curves of periwinkle cells (no. 916 and no. 200) precultured for 1 h with DMSO, $CaCl_2$, and KCl in B5 medium.

Table II. *Effects of Pretreatments on the Per Cent of Unfrozen Water at −40°C and Survival of Periwinkle Cells after 1-Hour Storage in Liquid Nitrogen*

Cultures	Treatment	Cryoprotectant	Unfrozen Water at −40°C	Survival[a]
			%	%
PRL no. 916	None	None	1	0
	5% DMSO. 1 h	5% DMSO	22	43
	10% DMSO, 1 h	10% DMSO	28	15
PRL no. 200	None	None	1	0
	5% DMSO, 1 h	5% DMSO	11	0
	0.5 M sorbitol, 20 h	0.5 M sorbitol	4	0
	1 M sorbitol, 20 h	1.0 M sorbitol	3	3
	0.5 M sorbitol, 20 h	5% DMSO	18	10
PRL no. 200 cultured over B5 agar + 1 M sorbitol for 4 d	None	None	5	0
	5% DMSO, 1 h	5% DMSO	14	5
	1.0 M sorbitol, 20 h	1.0 M sorbitol	9	8
	1.0 M sorbitol + 5% DMSO, 1 h	1.0 M sorbitol + 5% DMSO	22	33

[a] Survival based on TTC reduction assay and represents average of at least three experiments.

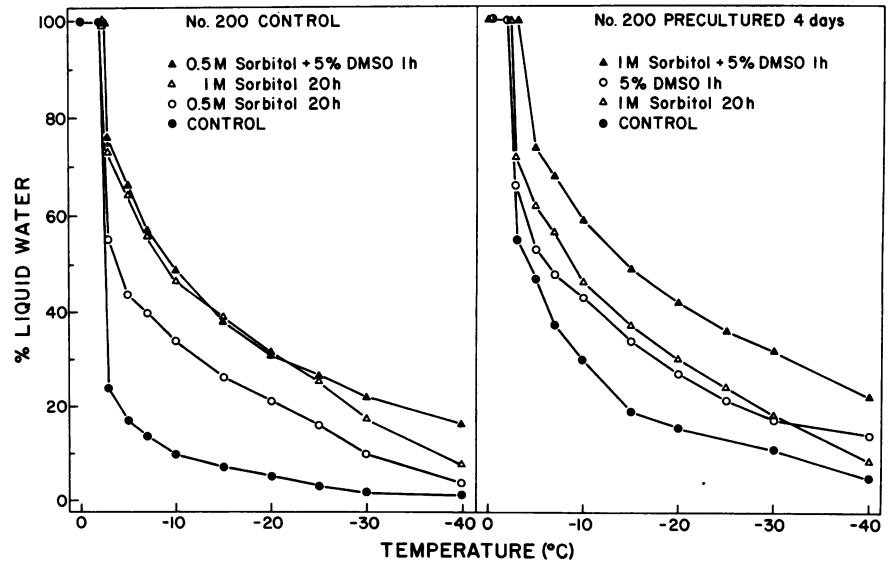

FIG. 7. Freezing curves of periwinkle cell line no. 200 cells with or without preculturing over 1 M sorbitol agar medium for 4 d and treated with various cryoprotectants.

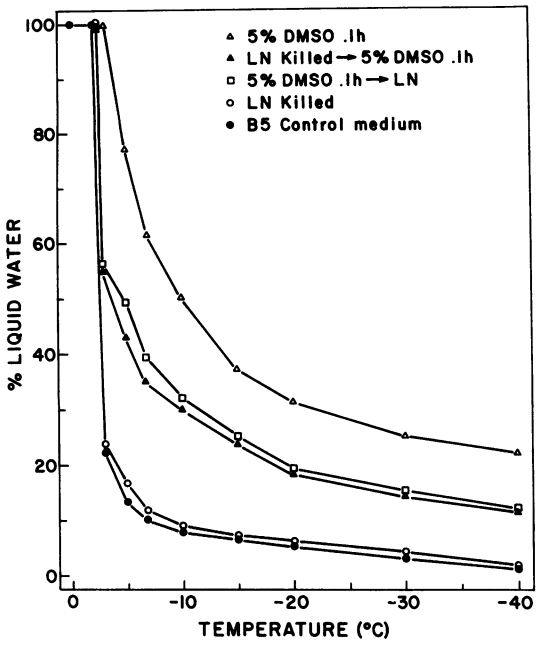

FIG. 8. Freezing curves of periwinkle cells (no. 916) before and after immersion in liquid nitrogen to rupture the cellular membrane.

cultures frozen in solutions of $CaCl_2$ or KCl of the same osmolarity as DMSO had only 5% of the total water as liquid at −40°C. No attempt was made to determine the freezing curve of no. 200 cell after incubation in solution containing DMSO higher than 10% (v/v) due to its lethal effects.

Preculturing no. 200 cells over agar medium containing 1 M sorbitol for 4 d followed by slow freezing (1°C/min) in 1 M sorbitol + 5% DMSO to −40°C prior to immersion in liquid nitrogen resulted in more than 30% cell survival after thawing (Table II). As shown in Figure 7, only those cells precultured for 4 d in 1 M sorbitol and 5% DMSO had more than 20% of their water unfrozen at −40°C. There was over a 30% survival rate after freezing in liquid nitrogen in such pretreated cells (Table II).

There was a linear relationship between the per cent unfrozen water at −40°C (y) and per cent cell survival (x). The linear regression equation obtained was $y = 0.47x + 7.33$. The correlation coefficient was $r = 0.75$ which is significant at the 1% level. The analysis indicated that the amount of unfrozen water at −40°C, and the temperature prior to immersion into liquid nitrogen, was closely associated with the success of cryopreservation of periwinkle suspension cultures.

When comparing the freezing curve for 5% DMSO solution (Fig. 3) and the freezing curve for cells after incubation in 5% DMSO (Fig. 6), it was found that only the no. 916 cells and not the no. 200 cells, had more unfrozen water at −40°C than the 5% DMSO solution. The reason why no. 916 cell cultures had more unfrozen water than the no. 200 cell cultures is not known. Possible reasons for this difference may be due to the stability of the plasma membrane, density of the cytoplasm, or rigidity of the cell wall. To test the hypothesis that the stability of the plasma membrane is required for maintaining water in a liquid state at −40°C, cells of line no. 916 were plunged in liquid N_2 to disrupt the plasma membrane (Fig. 8). The freezing curves for the control cells and cells which had been dipped in liquid nitrogen to rupture the membranes were essentially the same. At −10°C, more than 90% cellular water was frozen. At −40°C, there was only a small portion of cellular water remaining in the liquid state for both the control and liquid nitrogen killed cells. There was more than 20% water unfrozen at −40°C for no. 916 cells precultured in 5% DMSO for 1 h. The freezing curves for no. 916 cells immersed in liquid nitrogen to rupture the membranes and then incubated 1 h in 5% DMSO solution, or no. 916 cells incubated in 5% DMSO solution first and then immersed in liquid nitrogen, were very similar to each other, and also similar to the freezing curve for the 5% DMSO solution (Fig. 3). The results imply that the integrity of cellular membranes is essential for the effect of DMSO to increase the amount of unfrozen water at −40°C. The results suggest that the cells may concentrate DMSO. However, our data were not sufficient to pinpoint the exact differences between cells of lines no. 916 and no. 200.

DISCUSSION

DMSO, glycerol, sugars, and sugar alcohols are the most extensively used cryoprotectants (4, 18). Cryoprotective compounds vary dramatically in structure and also possibly in their mode of protection. Theoretically, a cryoprotectant may protect living cells against freezing damage in at least three locations; extracellularly, the membrane itself, and intracellularly (4). A cryoprotectant could act to stabilize the intra- and intermolecular arrangements of structural components and water associated with the membrane (4). Some cryoprotectants penetrate the plant cell quickly (*e.g.* DMSO), some slower or not at all (*e.g.* sorbitol). An obvious difference between DMSO and sorbitol in respect to cryopreservation may be in the ability to penetrate the plasma membrane.

At present, the hypotheses to explain the mechanisms of freezing damage of living cells can be grouped into three categories (3), namely, the salt concentration theory, structural water hypothesis, and minimum cell volume hypothesis. Although each of these hypotheses explains some part of freezing injury, none of them explains the freezing damage fully. There is a common denominator to all of these theories, however; freeze-induced cell dehydration results in reduced cell volume, increased salt concentration, and the loss of water which protects cellular components. The response of cells to an increase in osmotic environment may be the same as a result of freezing, addition of extracellular solutes, or dehydration. All three types of stress result in a loss in cellular water. Thus, cryoprotection could be achieved by postponing or reducing critical cellular water during freezing.

It is generally thought that as cells suspended in aqueous solution freeze, the electrolytes in the external solution progressively concentrate. If the cooling is slow the cell undergoes progressive dehydration resulting in either excessive salt concentration or excessive cell shrinkage. However, results of recent experiments do not support this concept. For example, Mazur *et al.* (12) found in human red cells that the fraction of solution remaining unfrozen has a major effect on cell survival over the excessive salt concentration or cell shrinkage. They found that when the fraction of unfrozen water exceeded 35%, the majority of the cells survived even when the salt concentration in the unfrozen portion exceeded 2 molal. Our results also suggested a strong assocation between unfrozen water and cell survival during slow freezing to liquid nitrogen temperature. Karrow and Webb (8) proposed that death due to freezing occurs primarily as a result of extraction of bound water from vital cellular structure. A similar vital water hypothesis has been suggested for the freeze killing of plant cells (17). Thus, it seems our results confirm that a minimal amount of liquid water is essential for a cell to survive freezing.

It is generally thought that a certain amount of liquid water is essential for maintaining the integrity of the membrane (15). Progressive removal of water from the membranes to lower than a critical level could result in destabilization. Chen and Gusta (1) concluded that slow freezing injury of plant cells may result

from the freeze-induced dehydration stress. The observed increase in salt concentration (3) and reduced cell volume (13) may not be the direct cause of freezing injury. If this is true, compounds which prevent loss of cellular water during freezing may give cryoprotection. This is supported by our NMR data from the freezing of periwinkle cells treated with DMSO. The major function of DMSO as a cryoprotectant may be to prevent excessive loss of water at sub-zero temperatures to prevent disorganization of cellular structures.

Hellergren and Li (7) suggested that the cryoprotective properties of proline and sucrose are due to the removal of excessive intracellular water by osmotic effects. However, it has been demonstrated that no cryoprotection was obtained by dehydration alone in spite of how dehydration was achieved (11). Sakai and Sugawara (14) demonstrated that prefreezing and the use of cryoprotectants enhance innocuous intracellular ice crystals and reduces the rate of intracellular ice formation. We also observed that both DMSO and sorbitol reduced the rate of ice crystallization in solutions (Fig. 1; Table I). A slower freezing rate of extracellular solution would reduce the stress on the plasma membrane during the initial stages of freezing. Thus, osmotica such as proline and sucrose (7) and sorbitol act to protect cells from freezing injury by reducing cellular water content (7, 16) and at the same time reduces the rate of both intracellular and extracellular ice formation. Our NMR data indicate that sorbitol is effective in preventing water from freezing at temperatures above −25°C. Interestingly, the cryoprotective effect of sorbitol is not effective at freezing temperature below −25°C (4).

The use of a mixture of cryoprotectants is in general better than using a single cryoprotectant alone (18). Most of the cryoprotectant combinations involve DMSO plus sugars or sugar alcohols (4, 18). The combination of DMSO with other cryoprotectants may enhance cryoprotection for the following reasons. (a) Nonpenetrating cryoprotectants, such as sorbitol, may reduce cellular water and reduce the rate of initial ice crystallization. (b) The penetrating cryoprotectant DMSO enters the cells to reduce freeze-induced cellular dehydration. (c) The combination of DMSO and sorbitol maximizes the effects due to the initial freezing stresses and subsequent dehydration. The additive effects of DMSO and sorbitol in the reduction of ice crystallization (Fig. 7) and the increase of periwinkle cell survival during cryopreservation (Table II) supports this view.

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