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# COLLIGATIVE AND NON-COLLIGATIVE FREEZING DAMAGE TO THYLAKOID MEMBRANES

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**When spinach thylakoid membranes were frozen in vitro in solutions containing constant molar ratios of cryotoxic to cryoprotective solute, maintenance of functional integrity strongly depended on initial osmolarities. Optimum cryopreservation of cyclic photophosphorylation was observed when the membranes were suspended in solutions of intermediate osmolarities (approx. 50–100 mM NaCl, 75–150 mM sucrose). Both higher and lower initial osmolarities were found to result in decreased cryopreservation. In the absence of added salt, more than 100 mM sucrose were needed for full cryopreservation of the membranes. When thylakoids were frozen in solutions containing low concentrations of NaCl (2 mM), the ratio of sucrose to salt necessary to give full protection was high (up to 50). When the salt concentration was about 60 mM, ratios as low as 1.5 were sufficient for maintaining membrane integrity. This ratio increased again, as the initial NaCl concentration was increased beyond 60 mM. During freezing, proteins dissociated from the membranes, and the amount of the released proteins was correlated linearly with inactivation of photophosphorylation. The gel electrophoretic pattern of proteins released at low initial osmolarities differed from that of proteins released at high initial osmolarities. Cryopreservation was also found to depend on membrane concentration. Concentrated membrane suspensions suffered less inactivation than dilute suspensions. The protective effect of high membrane concentrations was particularly pronounced at high initial solute concentrations. It is proposed that damage at low initial osmolarities is caused predominantly by mechanical stress and by osmotic contraction/expansion. Damage at high initial osmolarities is thought to be caused mainly by solute effects. Under these conditions, both the final volume of the unfrozen solution in coexistence with ice and the membrane concentration affect membrane survival by influencing the extent of the loss of membrane components through dissociation reactions. Membrane protection by sugars is caused by colligative action under these circumstances.**

## Introduction

Damage to cellular membranes is responsible for cell damage by freezing. Many aspects of freezing damage can be studied more profitably with isolated membranes than with complex cellular systems, if membrane integrity and membrane damage can be properly assessed. The degree of thylakoid inactivation by freezing can be easily measured by the loss or alteration of biochemical activities such as light-dependent photophosphorylation and electron transport [1]. Also, loss of membrane proteins can be used to characterize membrane damage [2–4]. It has been shown that a variety of compounds, such as sugars, glycerol, amino acids and organic acids are able to protect membranes from damage, and that a variety of inorganic salts is cryotoxic. The degree of damage can be manipulated experimentally by freezing to

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different temperatures and by varying the composition of the suspending medium.

Protection is thought to be based mainly on a colligative mechanism [5,6]. It has been recognized that the accumulation of some solutes during freezing leads to membrane damage, and that membrane protection may be observed when the presence of membrane-compatible solutes moderates the increase in the concentration of membrane-toxic solutes, which accompanies freezing. The ratio of potentially membrane-toxic solutes to cryoprotectant has been found to determine the extent of damage during freezing (for review see Ref. 7).

In this work we provide evidence that the colligative mechanism of freeze/thaw injury and protection is effective predominantly at high initial solute concentrations. At low initial solute concentrations, a non-colligative mechanism prevails. Under these conditions, membrane damage can occur, even in the presence of substances considered to be cryoprotective.

## Materials and Methods

*Plant material. Spinacia oleracea* cv. Yates was grown in the greenhouse.

*Isolation of thylakoids.* 100 g of washed leaves were blended in the cold in 200 ml 160 mM NaCl/240 mM sucrose/1 mM $MgCl_2$/1 mM $MnCl_2$/2 mM EDTA/1 mM $KH_2PO_4$/50 mM Tris/1.25 mM sodium ascorbate/0.04% cystein (pH 7.8). The homogenate was filtered through 20 $\mu$m nylon mesh and centrifuged for 5 min at $2000 \times g$. The chloroplasts were resuspended in distilled water and washed twice in 10 mM NaCl/15 mM sucrose. Other isolation and washing media are given in the figure legends. The final pellet was resuspended in washing medium and diluted to a chlorophyll concentration of about 1 mg/ml.

*Freezing of thylakoids.* Salt or sucrose were added to the thylakoids from concentrated stock solutions, diluting the original thylakoid suspension by a factor of 2. The samples were frozen to $-20$°C. In some experiments, cooling was recorded by a thermocouple connected to a potentiometric recorder. The cooling rate close to 0°C was approx. 0.5 K/min. After 3 h (if not noted otherwise), the samples were thawed rapidly in a water-bath at room temperature. For the analysis of protein release, the samples were centrifuged for 25 min at $10\,000 \times g$. The colorless supernatant was used for protein determination and gel electrophoresis.

*Cyclic photophosphorylation.* Thylakoids were illuminated with white light (approx. 200 $W \cdot m^{-2}$) in a reaction medium comprising 50 mM KCl/5 mM $MgCl_2$/1 mM $KH_2PO_4$/1 mM Tris/1 mM ADP/20 $\mu$M phenazine methosulfate (pH 7.7) at 20°C. The irreversible, light-dependent alkalization of the medium, a consequence of the reaction $ADP^{3-} + HPO_4^{2-} \rightarrow ATP^{4-} + OH^-$, was recorded by a pH electrode [8]. Small aliquots of 0.01 M HCl were added to calibrate observed pH changes.

*Gel electrophoresis.* Gel electrophoresis was performed as described previously [4]. Samples were mixed with concentrated sample buffer and used without further concentration. Staining was done using the sensitive silver nitrate procedure of Ansorge [9].

All other techniques have been described previously [4].

## Results

At equilibrium, the final osmolality of a solute or a mixture of solutes coexisting in solution with ice is solely determined by the freezing temperature. In its most simplified form, the colligative theory of freezing damage and protection [5,6] demands that the ratio of cryoprotective to cryotoxic solutes determines the extent of freezing damage at a given temperature regardless of the initial concentration of the solutes before freezing. Damage is assumed to be caused by the increase in the concentration of cryotoxic solutes affected by the removal of water as ice, and it is moderated in the presence of membrane-compatible solutes. If initial concentrations of NaCl and sucrose are varied so that molar ratios are kept constant, it should therefore be expected that thylakoid membranes suspended in the solutions are damaged by freezing in a comparable manner. In our experiments, we have chosen a molar ratio of sucrose to NaCl of 1.5. On freezing thylakoids in media containing sucrose and NaCl in this proportion, the capacity for photophosphorylation was par-

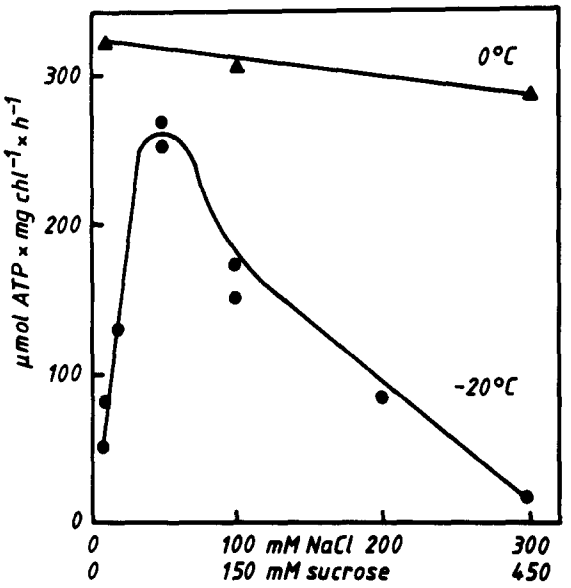


Fig. 1. Rate of cyclic photophosphorylation after freezing to −20°C. Thylakoids were suspended in solutions containing NaCl and sucrose at a constant molar ratio of 1:1.5.

tially but not uniformly retained. Fig. 1 shows that there was a marked effect of the initial osmolarity on the cryopreservation of the membranes as revealed by photophosphorylation. Optimum cryopreservation was obtained when the initial concentration of NaCl was about 50–100 mM and that of sucrose was 75–150 mM. When the initial

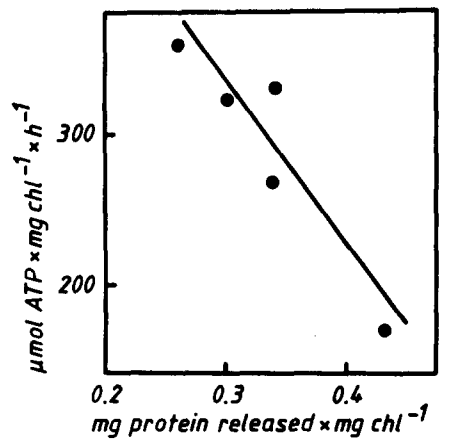


Fig. 2. Correlation of the rate of cyclic photophosphorylation with protein release after freezing. The line has been fitted to the data using linear regression ($r = 0.93$).

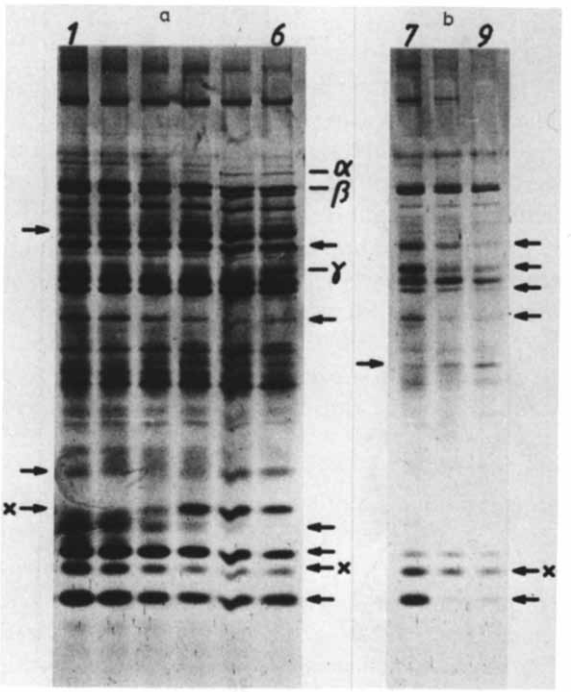


Fig. 3. Gel electrophoretic analysis of the proteins released during freezing. (a) Variation of the initial concentration of the medium at a constant molar ratio of NaCl to sucrose.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| mM NaCl | 10 | 20 | 50 | 100 | 200 | 250 |
| mM sucrose | 20 | 40 | 100 | 200 | 400 | 500 |

(b) The thylakoids were washed in 10 mM sucrose and frozen in the concentrations of sucrose indicated. Lane 7: 10 mM; lane 8: 100 mM; lane 9: 500 mM. The arrows point to the direction in which individual bands increase. Bands which are possible markers for damage at high or low initial osmolarities, respectively, are marked (×). Three subunits of the coupling factor are indicated ($\alpha$–$\gamma$).

concentrations of both solutes were increased or decreased at constant molar ratios, freezing damage increased.

The amount of protein released from the membranes was correlated linearly with freezing damage (Fig. 2). Certain proteins were released predominantly at low initial concentrations, others at high initial concentrations (Fig. 3a).

The existence of an optimum in the cryopreservation of thylakoids at a constant ratio of cryoprotective to cryotoxic solute indicates that two different mechanisms caused freezing damage.

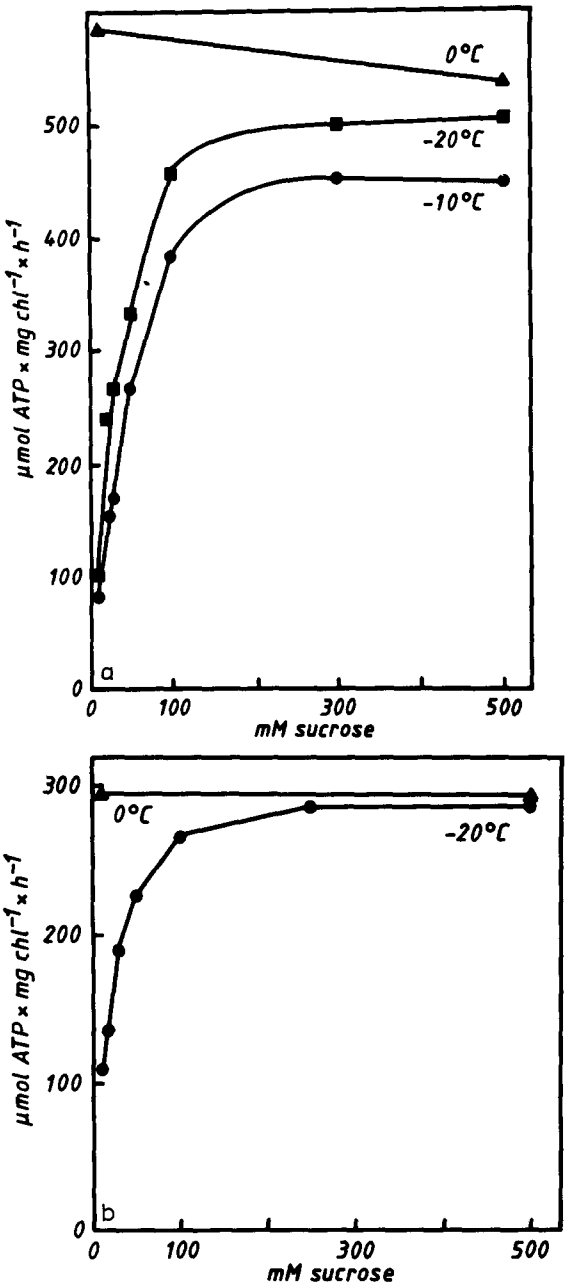


Fig. 4. Protection of thylakoids suspended in 2 mM NaCl/10 mM sucrose (a) or 10 mM sucrose (b) against inactivation during freezing as shown by the rate of cyclic photophosphorylation. The thylakoids used in (b) were isolated in a medium in which NaCl was replaced by sucrose and washed in 10 mM sucrose.

It was of interest to examine the influence of both types of solute during freezing at high or low initial osmolarities. Thylakoids which are suspended in solutions containing high concentrations of sodium chloride (100 mM) can be completely protected against freeze/thaw inactivation by adding sucrose in 2- to 3-fold excess [4]. When the NaCl concentration was increased, correspondingly higher sucrose concentrations were required for full protection [1]. However, the ratio of sucrose to salt required for full protection varied within wide limits. If thylakoids were suspended in a dilute solution of NaCl (2 mM), and titrated with sucrose, protection against damage during freezing was not observed before a sucrose concentration of more than 100 mM had been reached (Fig. 4a). Freezing to −10°C resulted in a slightly higher damage than freezing to −20°C (Fig. 4a, see Discussion). A similar curve was obtained, when the thylakoids were washed and frozen in sucrose solutions in the absence of added salt (Fig. 4b). Smaller amounts of protein were released when sucrose predominated as a solute, but the gel electrophoretic pattern of proteins released from the membranes during freezing in the presence of sucrose and of sucrose/NaCl mixtures was similar in principle (Fig. 3b).

It has been mentioned above that the final osmolarity of solute during freezing is determined by the freezing temperature. It follows that different initial solute concentrations determine different final volumes of the liquid phase in equilibrium with ice above the eutectic point. The dissociation of peripheral proteins from the membrane has been recognized to be a corollary, if not the cause, of freezing damage (Refs. 2, 4 and 10, and Figs. 2 and 3). It should be expected that high initial solute concentrations should favor protein dissociation by providing a larger volume of liquid during freezing. Likewise, increased membrane concentrations may decrease injury [3] by shifting the association/dissociation equilibrium towards association. We have examined the protective effect of higher membrane concentrations as a function of the initial solute concentration. Fig. 5a shows that at all initial solute concentrations used, higher thylakoid concentrations had a protective effect on the rate of cyclic photophosphorylation. The slope of the activity curves at different initial solute concentrations was found to be variable. This slope can be taken as an indicator of the sensitivity of the membranes to freeze/thaw in-

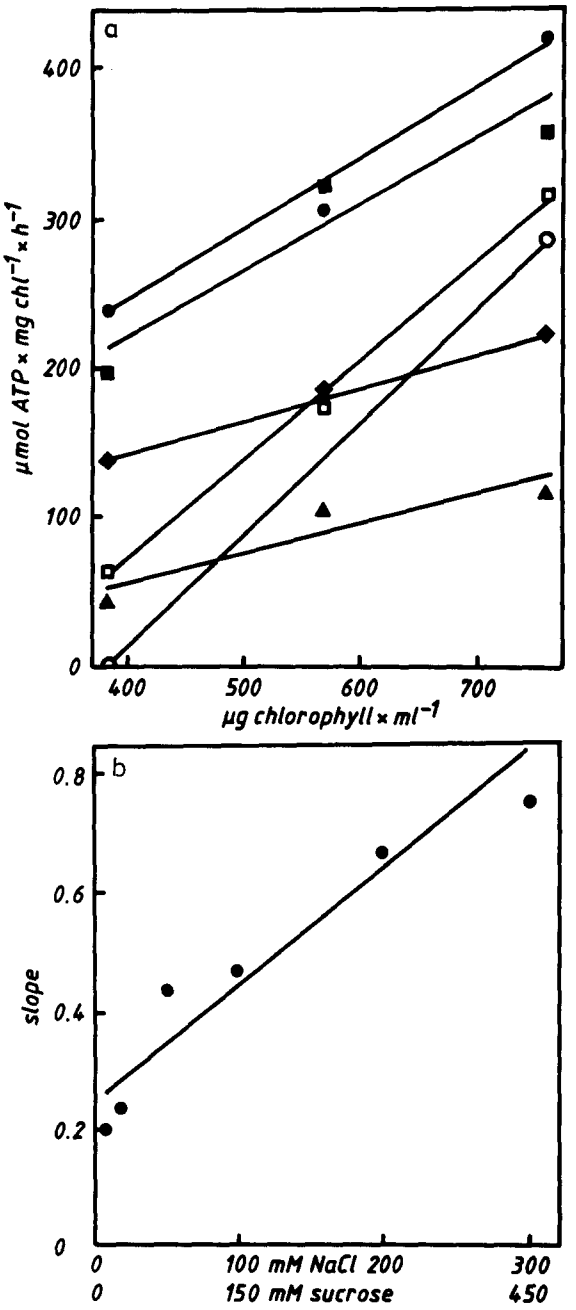


Fig. 5. The rate of cyclic photophosphorylation as a function of membrane concentration. The thylakoids were suspended in solutions of constant molar ratios of NaCl and sucrose at different initial osmolarities. (a) Rate of ATP synthesis at different chlorophyll concentrations. The lines were fitted by linear regression analysis ($r = 0.92$–$0.99$). The initial solute concentrations were: ▲, 10 mM NaCl/15 mM sucrose; ◆, 20 mM NaCl/30 mM sucrose; ■, 50 mM NaCl/75 mM sucrose; ●, 100 mM NaCl/150 mM sucrose; □, 200 mM NaCl/300 mM sucrose, ○, 300 mM NaCl/450 mM sucrose; (b) The slope of the lines in (a), indicating sensitivity to membrane dilution, is plotted against initial solute concentration ($r = 0.97$).

activation as a function of membrane concentration. If it is plotted against the initial solute concentrations (Fig. 5b) it can be seen that the sensitivity of the membranes is correlated linearly with the initial solute concentration, the membranes being more sensitive to dilution if they are frozen in solutions of high initial osmolarities.

## Discussion

The colligative theory of freezing injury and protection [5,6] has provided the framework to interpret the action of damaging solutes to biological membranes during freezing. It is thought that potentially toxic solutes cause membrane damage when accumulated in the unfrozen phase during the crystallization of ice. The establishment of toxic solute levels can be prevented when membrane-compatible solutes are also present, because the freezing temperature determines the total concentration of solutes in the unfrozen solution in coexistence with ice, whereas individual solute levels are a function of the molar fraction of the individual solutes. A considerable body of evidence has accumulated in freeze-thaw studies on thylakoid membranes, confirming the validity of the colligative theory (for review see Ref. 7). In this paper we describe the results of a series of experiments, which are not readily explained assuming only a colligative mechanism of cryoinjury/cryoprotection. The existence of an optimum of cryopreservation which depends on the initial osmolarity of the solution used to suspend the membranes (Fig. 1) suggests at least two different kinds of injury. The demonstration of differential release of proteins from the membranes during freezing at high and low initial osmolarities supports this view (Fig. 3). The polypeptide bands, which seem to constitute markers for injury at high and low initial solute concentrations, respectively, are marked in Fig. 3. Since proteins are also released in the absence of salt at high sucrose concentrations when the membranes are fully protected, it must be concluded that the loss of only a few proteins may be critical for membrane inactivation. For the understanding of Fig. 3, it is

necessary to note that the staining intensity with silver nitrate is not linearly dependent on protein concentration. Furthermore, some proteins (e.g., the alpha subunit of the coupling factor CF1) are only weakly stained under our conditions.

The final solute concentrations at a given temperature are identical during freezing. However, the final volume of the solution coexisting with ice and the membrane concentration in this solution depend on the initial solute concentration. It is suggested that the damage at high initial osmolalities is caused by the concentration of NaCl during freezing, i.e., that it is basically an effect of the excessive concentration of a chaotropic salt. When sucrose is also present, it acts to decrease the salt concentration [7]. Protection by sucrose is based on a colligative mechanism. In addition, however, the final volume of the unfrozen solution has to be taken into account. The greater loss of membrane function at higher initial solute concentrations in the suspending medium (more than 100 mM NaCl, 150 mM sucrose) is thought to be a direct consequence of this larger final volume. It favors the dissociation of membrane proteins. This is in accordance with the finding that the sensitivity of the membranes to cryoinjury caused by membrane dilution is greater at high initial solute concentrations (Fig. 5). In contrast, erythrocytes have been found to be more sensitive to freeze-thaw induced hemolysis when frozen at very high membrane concentrations. This is thought to be a mechanical effect [11], but other mechanisms may also be involved [12].

If the action of cryotoxic solutes is prevented by cryoprotective sugars, full protection can be reached at high initial solute concentrations (see Ref. 7 for review). At very low initial concentrations, sucrose is hardly protective, even if the sugar-to-salt ratio has been maximized by washing the thylakoids in a salt-free medium before freezing (Fig. 4b). Similar observations have been made by Steponkus et al. [3], who examined light-dependent proton uptake and $Ca^{2+}$-dependent ATPase activity.

There are two mechanisms which are possibly damaging in a freeze-thaw cycle using low initial solute concentrations. Firstly, the thylakoids could become mechanically compressed by the ice crystals. This mechanism has been proposed by Santarius and Giersch [13] for thylakoids. Similarly Nei [11] and Mazur et al. [14] described mechanical damage to erythrocytes after crystallization of the major part of the water.

Alternatively, damage could be brought about by the concentration changes during freezing-thawing and the concomitant volumetric contraction/expansion [15,20]. These changes are more drastic at low initial solute concentrations. We favor the second explanation for the following reason: if thylakoids are frozen to a final temperature of $-10°C$ as compared to $-20°C$, they are injured slightly more, although at the higher temperature the available liquid volume at the lowest freezing temperature is approximately doubled (Fig. 4). The slightly higher cryoinjury of the higher temperature could be interpreted to reflect the relative dilution of the membranes at the final temperature as compared to $-20°C$. In addition, the rate of damaging chemical reactions should be increased at higher temperatures. This effect has been discussed in more detail elsewhere [4,21].

Freeze-thaw experiments with isolated thylakoids have been conducted suspending the membranes in solutions of osmolalities below [3,13,16–19] and at or above the optimum initial solute concentration determined in Fig. 1 [4,13,19]. Different views held by different authors as to the mechanism of injury can in part be explained by this fact. It is hoped that the present study will enable the design of experiments to discriminate better the numerous stresses [20] involved in the cryoinjury of plants.

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