FACTORS CONTRIBUTING TO INACTIVATION OF ISOLATED THYLAKOID MEMBRANES DURING FREEZING IN THE PRESENCE OF VARIABLE AMOUNTS OF GLUCOSE AND NaCl

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ABSTRACT During freezing of isolated spinach thylakoids in sugar/salt solutions, the two solutes affected membrane survival in opposite ways: membrane damage due to increased electrolyte concentration can be prevented by sugar. Calculation of the final concentrations of NaCl or glucose reached in the residual unfrozen portion of the system revealed that the effects of the solutes on membrane activity can be explained in part by colligative action. In addition, the fraction of the residual liquid in the frozen system contributes to membrane injury. During severe freezing in the presence of very low initial solute concentrations, membrane damage drastically increased with a decrease in the volume of the unfrozen solution. Freezing injury under these conditions is likely to be due to mechanical damage by the ice crystals that occupy a very high fraction of the frozen system. At higher starting concentrations of sugar plus salt, membrane damage increased with an increase in the amount of the residual unfrozen liquid. Thylakoid inactivation at these higher initial solute concentrations can be largely attributed to dilution of the membrane fraction, as freezing damage at a given sugar/salt ratio decreased with increasing the thylakoid concentration in the sample. Moreover, membrane survival in the absence of freezing decreased with lowering the temperature, indicating that the temperature affected membrane damage not only via alterations related to the ice formation. From the data it was evident that damage of thylakoid membranes was determined by various individual factors, such as the amount of ice formed, the final concentrations of solutes and membranes in the residual unfrozen solution, the final volume of this fraction, the temperature and the freezing time. The relative contribution of these factors depended on the experimental conditions, mainly the sugar/salt ratio, the initial solute concentrations, and the freezing temperature.

INTRODUCTION

According to the theory originally proposed by Lovelock (1953*a*, *b*), inactivation of cells, such as erythrocytes, in the course of slow freezing is considered to be chiefly due to the increase in the concentration of membrane-toxic solutes, such as electrolytes, in the unfrozen part of the system during ice formation (see also Meryman et al., 1977). However, already Lovelock (1953*b*) noticed that at least at temperatures below −35°C factors other than the external salt concentration determine cell damage. Experiments performed by Mazur's group (Souzu and Mazur, 1978; Rall et al., 1978; Mazur, 1981) in which erythrocytes were suspended in glycerol/NaCl solutions revealed that hemolysis at given cooling and warming rates was determined by a number of factors. Mainly, the final salt concentration reached near the cells, the initial concentrations of solutes, and the time of storage in the frozen state contribute to cell survival. Recently, Mazur et al. (1981) concluded that hemolysis of erythrocytes during freezing is far more dependent on the fraction of water that remains unfrozen than on the concentration of salt in this unfrozen portion.

The electrolyte concentration reached during freezing is considered the most important factor contributing to inactivation of chloroplast membranes (Heber and Santarius, 1973, 1976; Heber et al., 1979, 1981; Lineberger and Steponkus, 1980). According to this view, membrane survival in a system containing potentially membrane-toxic salts and neutral compounds, e.g., polyols, is determined at a given freezing temperature predominantly by colligative action of the solutes, i.e., the polyol prevent nonspecifically the increase in the electrolyte concentration in the residual unfrozen liquid (Lineberger and Steponkus, 1980; Santarius and Giersch, 1983*a*, *b*; see also Meryman et al., 1977). However, results obtained with isolated spinach thylakoids have shown that both during freezing at variable temperatures and in the presence of extremely low solute concentrations membrane inactivation is determined by factors other than the salt concentration reached in the unfrozen part of the system (Santarius and Heber, 1970; Santarius, 1982*a*, *b*; Santarius and Giersch, 1983*a*, *b*).

In this study, we attempted to separate the factors contributing to inactivation of isolated biomembranes during freezing. For that purpose spinach thylakoids were used as model systems. The membranes were subjected to freeze-thaw cycles in the presence of various concentrations of glucose and NaCl. Calculation of final solute concentrations in the unfrozen portion of the system at their respective freezing temperatures allowed discrimination between the effect of electrolyte concentration and other factors in freezing damage.

MATERIAL AND METHODS

Thylakoid membranes were isolated from greenhouse-grown spinach leaves (*Spinacia oleracea* L. cv. Monatol) according to a procedure described recently (Santarius, 1982*a*; Santarius and Bauer, 1983). Washed membranes corresponding to 0.4–0.8 mg chlorophyll/ml were suspended in solutions containing NaCl and D-glucose at concentrations indicated. Aliquots (0.5 ml each) were transferred into glass tubes and either kept at temperatures ~0°C or frozen at an initial cooling rate not exceeding 10°C/min. Suspensions were inoculated with small ice crystals at temperatures slightly below the freezing point to prevent extensive supercooling and rapid ice growth. After storage for 3–5 h at the respective temperature, frozen samples were thawed in a water bath at room temperature at an initial warming rate exceeding 100°C/min. The integrity of unfrozen and freeze-thawed chloroplast membranes was estimated by measuring phenazine methosulfate-mediated cyclic photophosphorylation as described earlier (Santarius, 1982*a*). Chlorophyll was determined according to Arnon (1949). For evaluation of the chloride concentrations in the thylakoid suspension before freezing, a microchlorocounter (Labo International B.V., Marius, Krimpen, Holland) was used. The final solute concentration in the unfrozen part of the system, which was in equilibrium with ice at a given freezing temperature, was calculated as recently described (Santarius and Giersch, 1983*a*).

RESULTS

A thermodynamic description of ideal solutions that contain one or more solutes states that only the freezing temperature determines the total solute concentration in the unfrozen part of the system. It is assumed that this concept holds also for nonideal solutions and in the presence of biomembranes. Thus, if two solutes such as glucose and NaCl are present in the membrane suspension, the final concentrations of these predicted compounds near the membranes during freezing are solely dependent on the initial molal ratio of the compounds at a given freezing temperature. At high concentrations electrolytes such as NaCl are believed to be toxic to biomembranes. If the salt concentration reached during freezing would be predominantly responsible for membrane inactivation, one would expect that membrane damage at a given initial glucose/NaCl ratio would increase with decreasing the freezing temperature. This was indeed observed. When isolated thylakoids were frozen at temperatures not lower than about −15°C in the presence of a constant amount of NaCl and variable concentrations of glucose, G_f^{50} values increased with decreasing freezing temperature

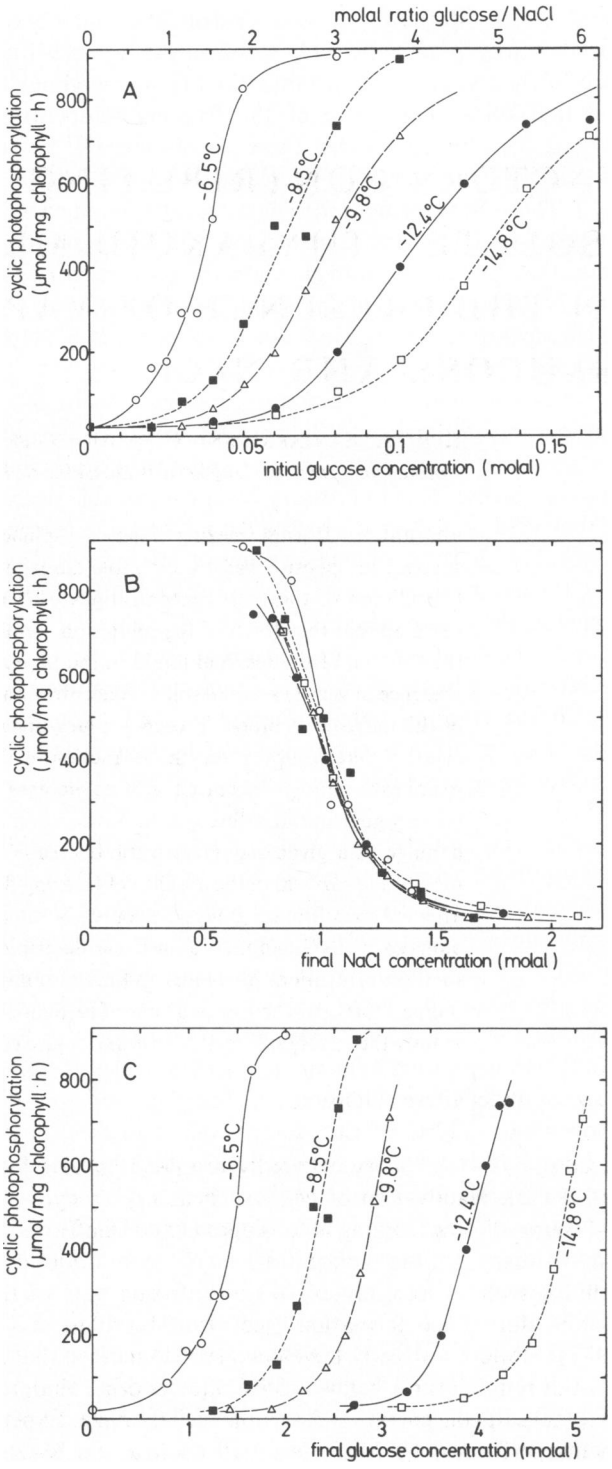

FIGURE 1 The effect of freezing temperature on isolated thylakoid membranes suspended in solutions containing initially 26.7 mmol/kg NaCl and various concentrations of glucose. Membranes were stored at their respective freezing temperatures for 3–5 h. Rates of cyclic photophosphorylation were plotted vs. (*A*) the glucose concentration before freezing (*lower* abscissa) and the molal ratio of glucose/NaCl (*upper* abscissa), (*B*) the final molality of NaCl, and (*C*) the final glucose concentration. Final concentrations are those predicted in the surroundings of the thylakoids during freezing.

(Fig. 1 *A*).[1] Moreover, it was evident that membrane survival was practically only determined by the final electrolyte concentration attained in the surroundings of the thylakoids irrespective of the freezing temperature (Fig. 1 *B*), although under these conditions, G_f^{50} values drastically increased with decreasing temperature (Fig. 1 *C*). This is in agreement with data recently obtained with sucrose (Santarius and Giersch, 1983*b*). This result suggests that thylakoid survival is chiefly a function of the final NaCl concentration and not influenced essentially by the temperature or the final sugar concentration in the unfrozen fraction.

However, under more severe freezing conditions, a considerably different result was obtained. At freezing temperatures below −20°C it was evident that the molal ratio of glucose/NaCl necessary for a comparable degree of thylakoid protection decreased with decreasing temperature (Fig. 2 *A*). A plot of membrane survival vs. final NaCl concentration shows that thylakoid inactivation occurs at extremely different electrolyte levels (Fig. 2 *B*); however, below about −20°C, membrane damage seems to be determined by the final glucose concentration irrespective of the freezing temperature (Fig. 2 *C*). Note that eutectic crystallization in glucose/NaCl solutions does not occur down to temperatures of about −30°C (Santarius, 1973).

The total initial solute concentrations in the experiments of Figs. 1 and 2 did not exceed ~0.2 mol/kg.[2] When thylakoids were suspended in solutions containing considerably higher starting solute concentrations, freezing at variable temperatures showed a partially different result (Fig. 3). Here, the initial sugar concentration was kept constant and that of NaCl was varied. As shown in Fig. 3 *B*, S_f^{50} values varied with the temperature even for mild freezing under these conditions. At freezing temperatures below about −20°C a result comparable with that shown in Figs. 2 *B* and *C* was obtained. S_f^{50} values drastically increased with decreasing temperature (Fig. 3 *B*), and membrane damage appeared to be determined by the final concentration of glucose (Fig. 3 *C*). In the presence of low solute concentrations the $(G/S)^{50}$ values at −10° and −20°C are ~3 and 4.5, respectively (Figs. 1 *A* and 2 *A*), and ~3.8 and 2.2 under high solute conditions (Fig. 3 *A*), i.e., at low initial solute concentrations the $(G/S)^{50}$ value at

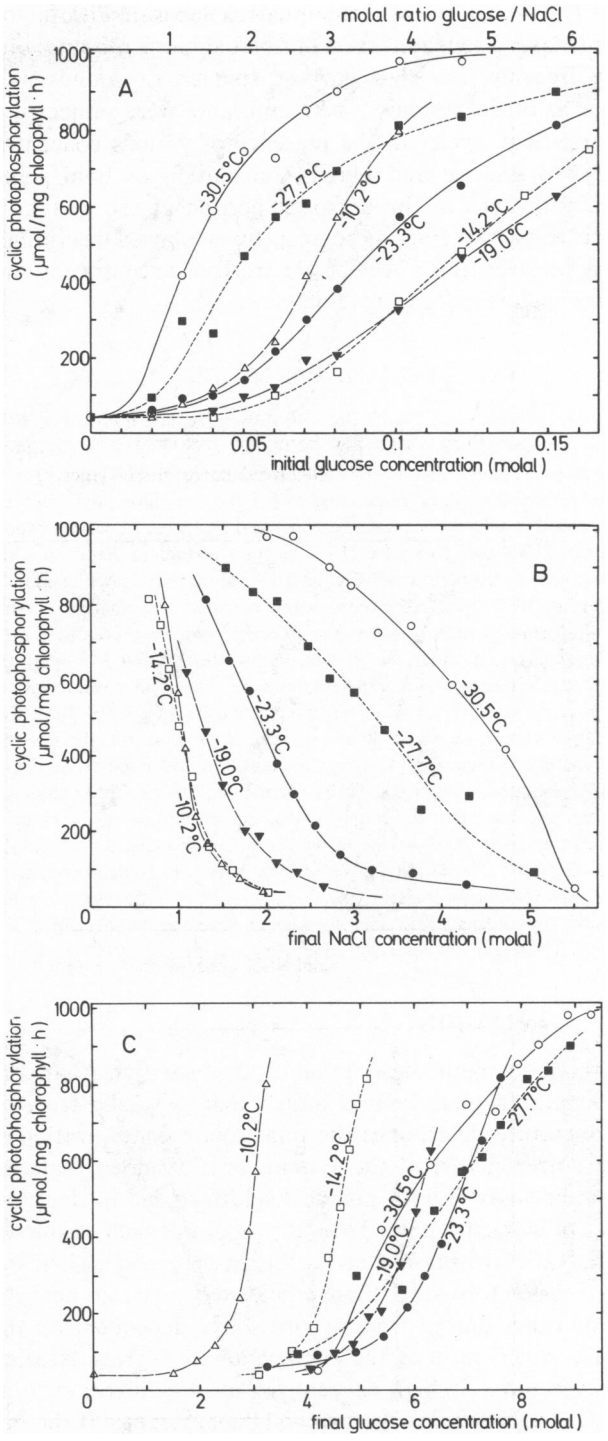

FIGURE 2 The effect of freezing temperature on isolated thylakoid membranes suspended in solutions containing initially 26.3 mmol/kg NaCl and various concentrations of glucose. See Fig. 1 for presentation of the data.

−10°C is lower than at −20°C, but at elevated initial solute concentrations the $(G/S)^{50}$ value is lower at the lower temperature. This illustrates that, depending on the initial solute concentration, membrane damage at a given

[1]Molal concentrations of glucose and NaCl at which 50% membrane activity (measured as the rate of cyclic photophosphorylation) is left are denoted by G^{50} and S^{50}, respectively. Where necessary, these concentrations are further specified as initial and final concentrations by indices i and f, respectively. Thus, S_f^{50} denotes the final NaCl concentration present in the liquid portion of the frozen system at which 50% of the membrane integrity of the control is observed. $(G/S)^{50}$ is the molal ratio of glucose to NaCl at 50% membrane damage. Indices are omitted in this case as this ratio is not altered by freezing.

[2]In the figures the concentration unit molal is used instead of mol/kg.

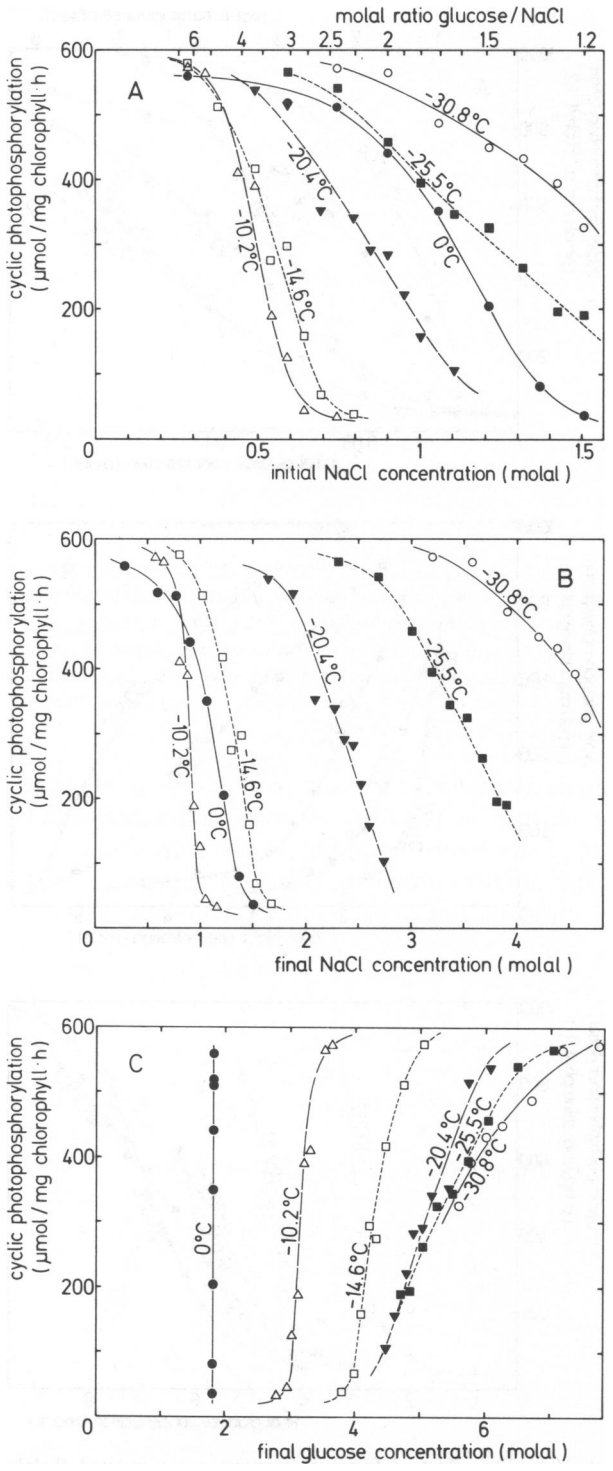

FIGURE 3 The effect of freezing temperature or storage at 0°C on isolated thylakoid membranes suspended in solutions containing initially 1.81 mol/kg glucose and various concentrations of NaCl. Samples were stored at 0°C or at their respective freezing temperatures for 3.5–5 h. Photophosphorylation was plotted (*A*) as a function of the NaCl concentration before freezing (*lower* abscissa) and the molal ratio of glucose/NaCl (*upper* abscissa), vs. (*B*) the final molality of NaCl, vs. (*C*) the final glucose concentration. Note that the *upper* abscissa in *A* runs in opposite direction from Figs. 1 *A* and 2 *A* because the salt concentration was varied in the presence of a fixed glucose concentration.

freezing temperature not only occurs at different molal ratios of glucose to salt; moreover, deviations from the behavior expected from the colligative concept occur in opposite ways at different solute concentrations.

The concentration of salt achieved in the residual unfrozen portion during freezing at a given temperature depends only on the molal ratio of glucose to NaCl in the initial solution, i.e., variations in the starting concentrations at a constant molal ratio of sugar to salt do not affect the final concentrations of glucose and NaCl. However, the total initial solute concentration determines the amount of liquid unfrozen at a given temperature. Therefore, it is likely that the differences between the experiments shown in Figs. 1 and 2 and those shown in Fig. 3 are due to differences in the initial solute concentrations that were increased by a factor of 10 to 40. If so, thylakoid inactivation appears to be influenced by the volume of the residual unfrozen portion of the system.

In the experiment demonstrated in Fig. 3 membrane inactivation during freezing was compared additionally with that occurring at 0°C in the presence of glucose and high NaCl concentrations. Surprisingly, the $(G/S)^{50}$ value of thylakoids stored at 0°C was close to that observed for samples frozen at −20 to −25°C (Fig. 3 *A*). The S_f^{50} value at 0°C was between those obtained for −10° and −15°C (Fig. 3 *B*), although under these conditions the glucose concentration in the unfrozen sample at 0°C was lower than the G_f^{50} values for all other samples (Fig. 3 *C*). This indicates that the temperature itself also affects the activity of the thylakoid membranes.

Three conclusions emerge from the results demonstrated in Figs. 1 to 3. (*a*) The final salt concentration reached near the thylakoids during freezing is not the only factor determining the extent of membrane damage. (*b*) Variation of absolute initial concentrations at a given molal ratio of glucose/NaCl and, thus, variation of the amount of residual liquid influence thylakoid survival during freezing. (*c*) Also the temperature to which thylakoids were exposed seems to affect the activity of membrane-bound photochemical reactions. These observations are considered in detail in the following experiments.

The suspicion that the temperature has an influence on membrane survival can easily be confirmed by varying the temperature in the absence of freezing. Fig. 4 shows that membrane integrity in the presence of a constant glucose level and variable concentrations of NaCl was strictly dependent on the temperature to which thylakoids were exposed; with decreasing temperature the membranes became extremely more sensitive to salt. It is also likely that in the presence of the higher electrolyte concentrations, which exist near the membranes during freezing, the temperature itself drastically affects the condition of the thylakoids. The decrease in the maximum activity of cyclic photophosphorylation with an increase in storage temperature (Fig. 4) is due to the well-known progressive thermal

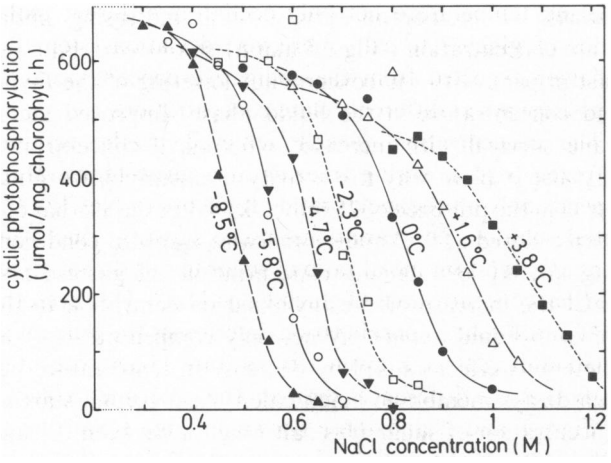

FIGURE 4 Survival of isolated thylakoid membranes after exposure for 4.5–6 h to various temperatures in solutions containing 1.81 mol/kg glucose and various concentrations of NaCl as a function of the electrolyte concentration. At the lower temperatures, the solutions were kept in the supercooled state.

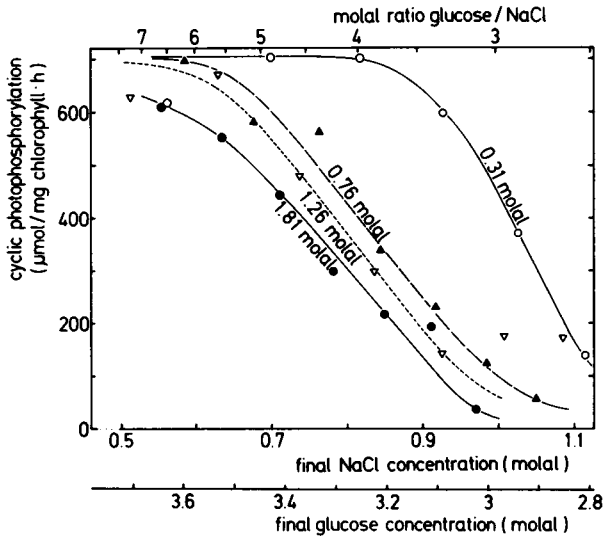

FIGURE 5 Freezing of isolated thylakoid membranes that were suspended in solutions containing initially different constant amounts of glucose and variable concentrations of NaCl. After storage at −10.1°C for 3–4.5 h, membrane integrity was measured and plotted as a function of the final molalities of NaCl and glucose (*lower* abscissae) and of the molal ratio of glucose/NaCl (*upper* abscissa). Initial glucose concentrations as indicated in the curves.

inactivation of biochemically active membranes and enzymes, which increases with raising temperatures. Thus, the activity of biomembranes at a constant high salt level is affected differently by the two effects.

As outlined above, membrane survival during freezing also depends on the total initial solute concentration in the thylakoid suspension before freezing. This fact is clearly documented when isolated thylakoids are frozen at a given temperature in the presence of stepwise additions of increasing amounts of glucose and varying concentrations of NaCl (Fig. 5). The G_f^{50} values increased (and the S_f^{50} values correspondingly decreased) with an increase in the initial glucose concentration from 0.31 to 1.81 mol/kg. Membrane survival at a given glucose/NaCl ratio dropped when the initial concentrations of glucose plus NaCl were increased.

As expected, a comparable result was obtained when thylakoids were frozen in the presence of increasing amounts of NaCl and varying concentrations of glucose (Fig. 6). Again, at a constant freezing temperature, the degree of membrane survival decreased with increasing starting concentrations of glucose plus NaCl at a fixed ratio of sugar to salt. Obviously, the volume of the unfrozen part of the system and/or the amount of ice formed may also play a role in membrane survival.

In the experiment as shown in Fig. 6 membranes were also exposed at 0°C to the calculated concentrations of glucose and NaCl reached during freezing at −6°C for given molal glucose/NaCl ratios. In this case, membrane survival without freezing was considerably reduced in comparison with freezing to −6°C in the presence of 25 mmol/kg NaCl. This result does not agree with the observation that membrane damage decreases with lowering the temperature at a given glucose/NaCl ratio (Fig. 4).

However, note that the results of Fig. 4 were obtained in the absence of freezing, whereas frozen and unfrozen samples are compared in Fig. 6. As freezing reduces the volume of the unfrozen liquid and, consequently, increases the thylakoid concentration in the remaining liquid por-

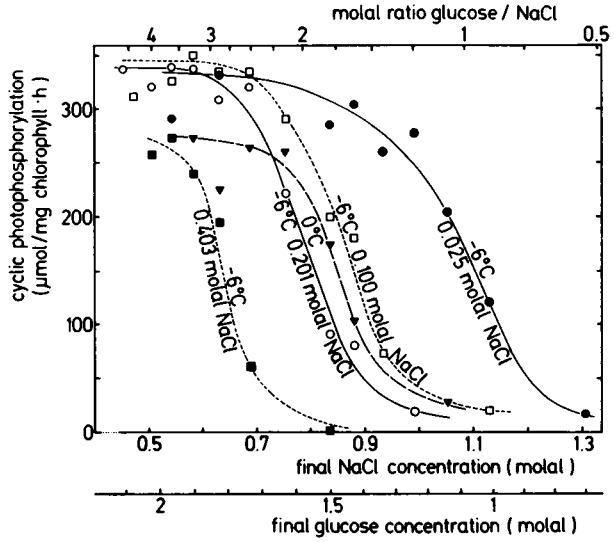

FIGURE 6 Isolated thylakoid membranes were exposed at 0°C and −6°C in the presence of given ratios of glucose/NaCl. Freezing took place in the presence of variable starting concentrations of glucose plus NaCl. For exposure at 0°C membranes were suspended in solutions containing those concentrations of glucose and NaCl that were calculated to exist in the unfrozen portion of the frozen samples. Exposure time was 3–4.5 h. Presentation of the data is the same as for Fig. 5. Starting concentrations of NaCl before freezing to −6°C is as indicated in the curves.

tion, comparison of Figs. 4 and 6 again suggests that the volume of the unfrozen part affects membrane survival.

In Fig. 7 it is demonstrated that this dependence of membrane activity on the volume of the remaining liquid portion in a frozen system can be ascribed, at least in part, to changes of the thylakoid concentration: Membrane survival at a given glucose/NaCl ratio increases with increasing chlorophyll concentration. This occurs both in the course of freezing (Fig. 7 *A*) and during exposure at 0°C (Fig. 7 *B*). As the volume of the unfrozen part of the system increases with the total initial osmolality, the decrease in membrane survival with increasing initial solute concentration (Figs. 5 and 6) can be correlated, at least in part, with the concomitant lowering of the thylakoid concentration in the liquid phase. Improved membrane survival with increased chlorophyll concentration may also explain why the activity in a sample, frozen at −6°C in the presence of 25 mmol/kg salt, exceeds that of a sample stored at 0°C under otherwise identical conditions (Fig. 6). At low starting concentrations of glucose plus NaCl the increase of the thylakoid concentration in the unfrozen liquid apparently not only compensates for the deleterious effect caused by decrease in temperature but even delays membrane inactivation. At a certain starting concentration of sugar plus salt (Fig. 6, between 0.1 and 0.2 mol/kg NaCl and corresponding amounts of glucose) the damage caused by decreasing the temperature was just compensated for by the increase in the concentration of the thylakoids in the liquid phase of the membrane suspension. Only when the initial solute concentration before freezing was relatively high (e.g., at −6°C > 0.2 mol/kg NaCl and appropriate concentrations of glucose) did the injurious effect of the temperature overcome the protective effect caused by concentrating the thylakoids; as a result the S^{50} value at 0°C was higher than the S_f^{50} value at −6°C, although the final glucose concentration in the frozen sample was even higher. Apparently, the differences in the NaCl concentration at which 50% of membrane inactivation was observed at 0° and −10.2°C (as shown in Fig. 3 *B*) can also be explained in the same way (see also Fig. 3 *C*).

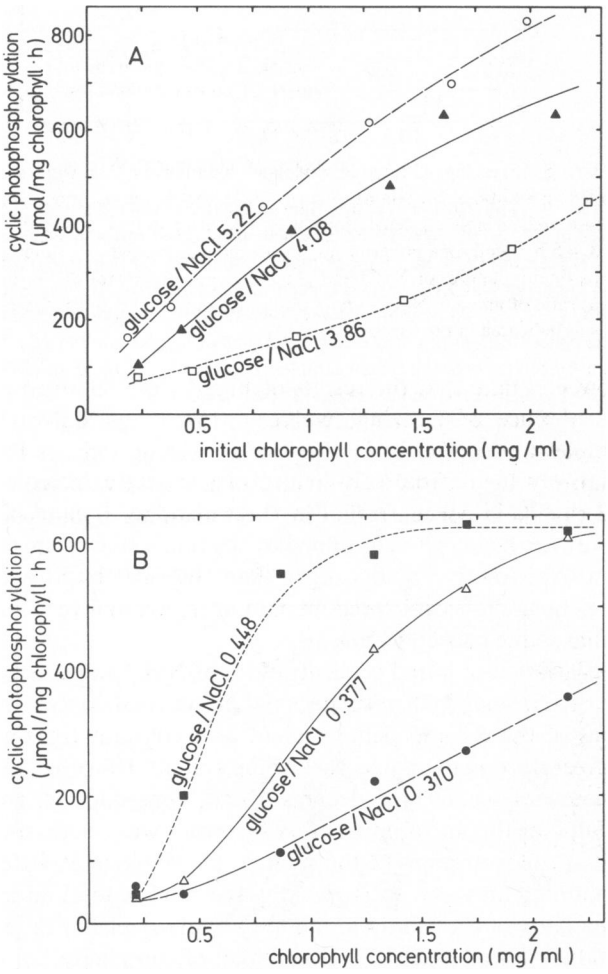

FIGURE 7 Freezing of isolated thylakoid membranes (*A*) at −9.8°C and (*B*) exposure at 0°C in the presence of given ratios of glucose/NaCl and variable amounts of membranes. In these experiments the isolation procedure was modified. After rupture of chloroplasts in distilled water, aliquots of the released thylakoids were washed by centrifugation for 4 min at 20,000 *g* and resuspended in the glucose/NaCl solutions used subsequently during (*A*) freezing or (*B*) in 0.22 mol/kg glucose/0.1 mol/kg NaCl. Exposure time at the respective temperatures was 3–4 h. The chlorophyll concentration indicated on the abscissae corresponds to the amount of thylakoids present in the membrane suspension. (*A*) The molal ratios of glucose/NaCl of 5.22, 4.08, and 3.86 were obtained by adding appropriate amounts of salt in the presence of initial glucose concentrations of 0.42, 0.205, and 0.311 mol/kg, respectively. (*B*) The molal glucose/NaCl ratios given in the figure were obtained by varying the NaCl concentration in the presence of 0.197 mol/kg glucose.

In earlier investigations it was shown that, even in the absence of freezing, inactivation of thylakoids by high concentrations of electrolytes can be prevented by addition of polyols, such as sugars and sugar derivatives, to the membrane suspension before salt exposure (Santarius, 1971, 1982*a*, *b*; Santarius and Bauer, 1983); in the absence of freezing, addition of carbohydrates does not alter the concentration of membrane-toxic electrolytes near the membranes. Thylakoids were exposed in those experiments to increasing concentrations of NaCl, which led to progressive membrane inactivation; addition of polyols, i.e., increase in the molal ratio of polyol to salt, resulted in increasing membrane survival. However, as shown here, differences in the starting solute concentrations at a fixed molal ratio of glucose/NaCl also affect membrane integrity even at 0°C (Fig. 8). The $(G/S)^{50}$ values increased with increasing the total initial solute concentrations. This clearly shows that both salt and sugar specifically act on biomembranes. The drop in the maximum activity of cyclic photophosphorylation, which occurs in the absence of freezing at elevated glucose/NaCl ratios, is mainly due to the high concentration of NaCl that damages the thylakoids even in the presence of high sugar levels; the high glucose concentration plays a minor role in lowering the membrane integrity, e.g., exposure of thylakoids for 3.5 h at 0°C in the presence of 2 mol/kg glucose and 0.025 to 0.1 mol/kg NaCl led to a drop in the activity of cyclic

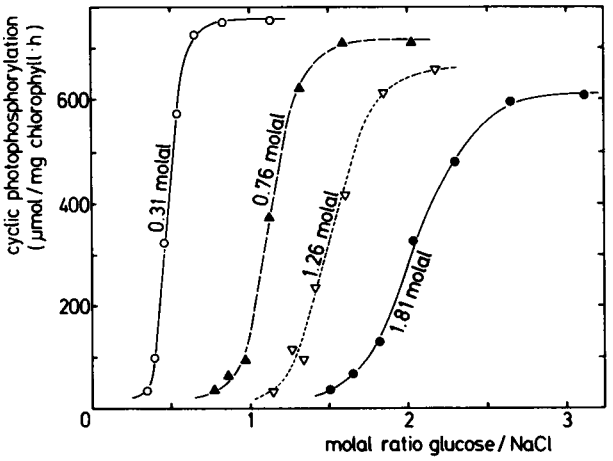

FIGURE 8 Exposure of isolated thylakoid membranes for 3–4.5 h at 0°C in solutions containing different constant amounts of glucose and variable concentrations of NaCl. Membrane survival was plotted as a function of the molal ratio of glucose/NaCl. Glucose concentrations as indicated in the curves.

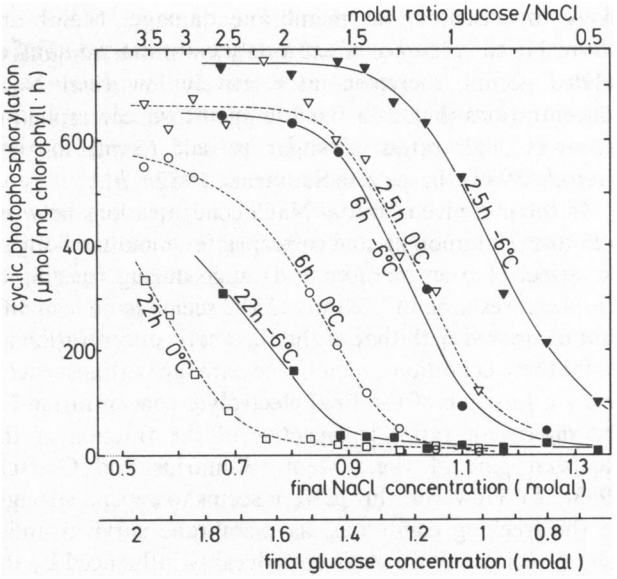

FIGURE 9 The effect of exposure time on isolated thylakoid membranes stored at 0°C or at −6°C in the presence of indicated ratios of glucose/NaCl. Freezing (▼, ●, ■, —) took place in solutions containing initially 31.4 mmol/kg NaCl and various concentrations of glucose. For exposure at 0°C (▽, ○, □, ---) membranes were suspended in solutions containing those concentrations of glucose and NaCl that were calculated to exist in the unfrozen parts of the frozen samples. Presentation of the data as for Fig. 5. Exposure time (in hours): ▼,▽ 2.5; ●,○ 6; ■,□ 22.

photophosphorylation of only ~5% (see also Santarius and Ernst, 1967). In the presence of extremely high concentrations of glucose, e.g., ~6 mol/kg, roughly 25% of the phosphorylating capacity was lost after 3.5 h exposure at 0°C (not shown). Therefore, it is not too surprising that the combined action of high salt and sugar causes the reduction of maximum activity shown in the figure.

Both in the absence and in the presence of freezing, membrane inactivation caused by electrolytes and other factors is a strictly time-dependent process. If membranes were exposed to conditions as already outlined (either frozen at −6°C in the presence of varying ratios of glucose/NaCl or exposed at 0°C to calculated concentrations of sugar and salt reached in the membrane suspensions during freezing at −6°C; see Fig. 6), it was evident that the extent of membrane survival drastically dropped with increasing exposure time (Fig. 9). However, the differences in membrane survival occurring at a given molal ratio of glucose/NaCl between −6 and 0°C are in agreement with the data shown in Fig. 6. These differences are maintained at least over 24 h.

DISCUSSION

Effect of Variation of Salt and Sugar Concentrations at Fixed Temperatures

The preceding results confirm earlier data that show that only under certain conditions is thylakoid survival determined by the final electrolyte concentration reached in the unfrozen part of the system. Evidence that membrane survival at a given freezing temperature is mainly a function of the final salt concentration was given recently (Santarius and Giersch, 1983*a*). A behavior different from this is observed at very low (e.g., 1–4 mmol/kg NaCl at around −20°C; Santarius and Giersch, 1983*a*, *b*) and at relatively high initial salt concentrations (e.g., exceeding 0.2 mol/kg NaCl at −6°C, Fig. 6; see also Fig. 5); here, S_f^{50} values are no longer determined by the final salt concentration. Apparently, factors other than the final electrolyte concentration become predominant at higher and very low initial solute concentrations.

Variation of initial concentrations of NaCl and glucose at a fixed sugar/salt ratio does not affect final concentrations of these components present at a constant freezing temperature in the liquid part of the system. However, the amount of ice formed decreases and, consequently, the volume of the unfrozen fraction increases with increasing initial concentrations of the solutes. The fraction of water remaining unfrozen, q, is given by the ratio of total initial osmolality of the solutes to the total final osmolality in the residual liquid part. For the sake of simplicity, q is approximately given by $q = 1.86\ (osm_i/-t)$, where osm_i is the initial osmolality of salt plus sugar, and t the freezing temperature in degrees Celcius. Thus, freezing in the presence of very low initial solute concentrations drastically reduces the volume of the liquid phase in the frozen system; e.g., q is ~0.002 (or 0.2%) if a solution containing 3 mM NaCl and 15 mM glucose is frozen at −20°C. It is likely that during severe freezing in the presence of very low solute concentrations, the thylakoids become highly compressed and thereby mechanically damaged in the drastically decreased volume of the unfrozen part of the system. Apparently, in addition to the high electrolyte concentration, the extreme reduction of the liquid phase is

likely to contribute to membrane damage. Membrane survival in the presence of extremely low initial amounts of solutes occurs, therefore, at relatively low final NaCl concentrations that, at a fixed temperature, correspond to relatively high ratios of sugar to salt (Santarius and Giersch, 1983*a*, *b*; see also Santarius, 1982*a*, *b*).

In the presence of initial NaCl concentrations between ~25 and 75 mmol/kg and appropriate amounts of sugar, the effect of q on membrane damage during freezing at temperatures around -20 to -25°C seems to be insignificant compared with that of the final salt concentration as, under these conditions, membrane damage is almost exclusively a function of the final electrolyte concentration (or the sugar/salt ratio) irrespective of the fraction of the unfrozen part of the system (Santarius and Giersch, 1983*a*, *b*). However, this pattern seems to depend strongly on the freezing conditions, as membrane survival under mild freezing conditions is considerably influenced by the volume of the residual liquid when the initial salt concentration is increased from 25 to 100 mmol/kg (Fig. 6). In this case S_f^{50} values already decrease with increasing initial NaCl concentrations above 25 mmol/kg NaCl and corresponding amounts of glucose, a pattern generally observed at higher initial concentrations of sugar plus salt (Figs. 5 and 6). Clearly, the fraction q of the unfrozen part of the system is relatively high under these conditions; thus, q is ~0.3 (or 30%) when a solution containing 1 mol/kg glucose and 0.3 mol/kg NaCl is frozen to -10°C. The higher the amount of unfrozen liquid, the more pronounced is the inactivation of the thylakoids at a given molal ratio of sugar to salt. Obviously, the effect of relative dilution of the membranes itself contributes to increased salt sensitivity (Fig. 7; see also Meryman et al., 1977).

The suggestion that membrane damage is increased in the presence of both extremely low and relatively high values of q is, at least in part, in agreement with results recently found by Mazur et al. (1981). These authors show that during slow freezing of human erythrocytes in glycerol/NaCl solutions cell survival is more dependent on the fraction of water remaining unfrozen than on the concentration of NaCl in the unfrozen solution; more strictly, electrolyte concentration affects survival of erythrocytes significantly only when q is between 10 and 25%, i.e., when 75–90% of the extracellular water is frozen to ice. However, direct comparison of the results obtained by Mazur et al. (1981) with those results concerning thylakoids are not possible as erythrocytes did not survive the freezing of more than 90% of the extracellular water. In contrast to the relatively large cells, small membrane vesicles such as thylakoids are extremely insensitive to excessive osmotic dehydration. This insensitivity was apparent both after transferring the membranes in solutions of high concentrations of neutral solutes (as mentioned above; see also Santarius and Ernst, 1967) and after drying them over $CaCl_2$ in the presence of sugars, which caused extremely high sugar concentrations near the membranes (Santarius and Heber, 1967). Williams and Meryman (1970; see also Meryman et al., 1977) suggest that thylakoids, which are impermeable to various solutes including glucose, become permeable to these compounds under severe osmotic stress thereby avoiding excessive shrinkage. The data shown here do not signify whether those changes are also involved in membrane damage and protection.

The molecular mechanism by which changes in the volume of the unfrozen liquid and, thus, alterations in the relative concentration of the membranes affect thylakoids is not yet known. Mazur et al. (1981) suggest that in aqueous solutions containing glycerol and NaCl the geometry of liquid-filled channels present between the precipitated pure ice may influence erythrocyte survival. It was also shown with erythrocytes that cell crowding increases the chance of cell-to-cell interactions (Nei, 1981; Pegg, 1981). Therefore, it appears possible that similar effects are involved in both inactivation and survival of thylakoid membranes at 0°C and during freezing.

Direct Effects of Temperature on Membrane Survival

That membrane survival in the absence of freezing depends on the temperature is clearly demonstrated in Fig. 4. Here, S^{50} values decrease with decreasing temperature. Comparison between membrane exposure at 0°C and membrane exposure to freezing at -10.2 (Fig. 3) or -6°C (Fig. 6, initial NaCl concentration >0.2 mol/kg) revealed that the differences in membrane damage between unfrozen and frozen samples at a given molal ratio of glucose/NaCl can be explained, at least in part, by the result documented in Fig. 4, i.e., by a direct effect of the temperature on the membranes. Nevertheless, freezing in these cases concentrated the membranes in the residual unfrozen liquid by a factor of ~2 to 4 compared with the solutions kept at 0°C, which partially reduces the injurious effect of the lower temperature. Therefore, it is evident (see Fig. 6) that the amount of the liquid fraction also modifies membrane survival as discussed above.

The direct effect of the temperature on thylakoid membranes is probably due to the reported cold lability of the coupling factor (CF_1), which is especially conspicuous in the presence of high electrolyte concentrations (McCarty and Racker, 1966; Lien et al., 1972; K. A. Santarius, unpublished results). This may explain differences in results obtained with different membrane systems, such as erythrocytes and thylakoids. If the erythrocyte membrane does not contain cold labile proteins, the conclusions drawn by Mazur et al. (1981), which suggest that the temperature has no observable effect on cell survival other than its role in affecting the fraction of the solution that remains unfrozen and the final molality of NaCl, are intelligible. A recent spin-label study on erythrocytes suggests that irreversible protein conformational changes mainly due to increase in the ionic strength of the medium

during freezing are responsible for membrane damage after a freeze-thaw cycle (Nunes, 1981).

Effect of Freezing at Varying Temperatures

From the data discussed above it can be concluded that thylakoid survival under otherwise constant conditions is affected by at least four factors: (*a*) the concentration of membrane-toxic salts in the residual unfrozen fraction, (*b*) the amount of ice formed and, thus, the corresponding volume of the residual liquid, (*c*) the temperature itself, and (*d*) a specific noncolligative action of neutral compounds such as sugars on biomembranes (Fig. 8; see also Santarius, 1982*a*, *b*; Santarius and Bauer, 1983; Santarius and Giersch, 1983*a*, *b*). Moreover, under certain conditions the manner in which crystallization occurs also directly affects thylakoid survival; e.g., mechanical damage is always observed after eutectic freezing (Asahina, 1967; Santarius and Heber, 1970; Santarius, 1973; see also Santarius and Giersch, 1983*a*). Finally, note that thylakoid survival is also affected by freezing and thawing rates and the time intervals of exposure to the respective temperatures (e.g., Fig. 9; see also Santarius and Heber, 1970; Heber and Santarius, 1973, 1976; Santarius and Giersch, 1983*b*). Separation of factors involved in thylakoid damage during freezing permits, at least in part, interpretation of the behavior of the membranes during freezing at variable temperatures (Figs. 1–3).

Comparison of membranes frozen at different temperatures in the presence of relatively low initial solute concentrations revealed that under mild freezing conditions (down to temperatures of about −15°C) membrane survival was mainly determined by the final NaCl concentration reached near the thylakoids (Figs. 1 *B* and 2 *B*). This is surprising in view of a direct effect of the temperature on membrane damage, which was clearly demonstrated in the absence of freezing (Fig. 4) and that was supposed to exist also at lower temperatures under freezing conditions (see above). The apparent insensitivity of membrane survival to the temperature could mean that the deleterious effect of decreasing temperatures is just compensated by the noncolligative protective effect caused by the simultaneously rising amount of sugar molecules in the unfrozen part of the system (Figs. 1 *C*, 2 *C* and 8). In this case, changes in the volume of the unfrozen fraction can be neglected (for the data of Fig. 1 the q values for 50% membrane damage are ~0.025 for all temperatures between −6.5 and −15°C). However, as already discussed, at higher initial solute concentrations a decrease in q diminishes membrane damage during freezing (Figs. 5 and 6). Therefore, the extent of membrane survival at temperatures down to −15°C cannot be expected to be determined mainly by the final electrolyte concentration if simultaneously q was changed. Indeed, at higher initial amounts of glucose plus NaCl, membrane inactivation was not determined solely by the final NaCl concentration even at mild freezing temperatures (Fig. 3 *B*). Here, the lower volume of the unfrozen liquid at −14.6°C (q is ~0.32) compared with that at −10.2°C (q is ~0.44) may contribute to increase in the S_f^{50} value at the lower freezing temperature.

At more severe freezing, i.e., at temperatures below −20°C, S_f^{50} values drastically increased with decreasing temperature (Figs. 2 *B* and 3 *B*; see also Santarius and Heber, 1970; Santarius and Giersch, 1983 *b*). The lower the freezing temperature, the lower the molal ratio of glucose/NaCl at which comparable degrees of membrane survival were observed (Figs. 2 *A* and 3 *A*), whereas G_f^{50} values showed relatively small differences at these temperatures (Figs. 2 *C* and 3 *C*). Glucose concentrations are extremely high at these low freezing temperatures, and the limit of the solubility of the sugar is likely to be reached. It is suggested that membrane inactivation under these conditions is mainly determined by the final sugar concentration reached near the thylakoids. Presumably, under severe dehydration almost no free and very little bound water is left; the sugar molecules are densely packed close to the membrane sites and may replace bound water by means of specific bindings of the OH groups of the sugars to surface proteins (see also Steponkus, 1971; Parker, 1972; Heber and Santarius, 1973; Santarius, 1982*a*, *b*; Santarius and Bauer, 1983). This may stabilize membranes against the injurious effect of electrolytes. Therefore, it is likely that relatively more NaCl is necessary for membrane inactivation, in the presence of an extremely low water potential, than it is at higher vapor pressure. This would explain the finding that the $(G/S)^{50}$ values decrease with decreasing temperature at severe freezing (Figs. 2 *A* and 3 *A*) and agrees well with earlier results, which show that during severe dehydration survival of thylakoids was observed at relatively low sugar/NaCl ratios (Santarius and Heber, 1967). Under conditions at which extreme sugar concentrations were reached, the volume of the residual liquid fraction seems to be less important for the extent of membrane inactivation: q values for 50% membrane damage changed either by a factor of ~3 (Fig. 2) or were practically not affected (Fig. 3) when the temperature dropped from −20° to −30°C, whereas the corresponding final sugar concentrations were ~5 mol/kg in both cases (Figs. 2 *C* and 3 *C*). As mentioned earlier, even when the volume of the residual unfrozen liquid becomes exceedingly small, mechanical damage by ice crystals might contribute to membrane inactivation, which then occurs at relatively low final NaCl concentration, although sugar concentration in the unfrozen fraction reaches very high values (Santarius and Giersch, 1983*a*, *b*).

CONCLUSIONS

It is evident that survival of thylakoid membranes during freezing is determined by various factors: the initial and final salt and sugar concentrations, the molal ratio of

glucose/NaCl, the amount of thylakoids present in the membrane suspension, the temperature, and the time of exposure. From these data a more general conclusion can be drawn concerning the effect of freezing on biomembrane systems.

The dependence on initial solute concentrations of membrane survival, measured as S_f^{50}, G_f^{50}, and $(G/S)^{50}$ values at fixed freezing temperatures and exposure times, is shown schematically in Fig. 10 *A*. An increase in the initial solute concentration corresponds to an increase in the volume of the residual liquid and, at fixed initial amounts of membranes in the suspensions, to a decrease in the thylakoid concentration in the unfrozen fraction. At very low initial solute concentrations, S_f^{50} values drastically increase and G_f^{50} values decrease with an increase in the initial solute concentration. At these very low initial concentrations, the portion of the residual unfrozen liquid q is extremely small, and the presence of ice crystals is, therefore, likely to contribute considerably to membrane damage. At low and medium initial solute concentrations similar degrees of membrane inactivation are observed at relatively high NaCl and low glucose concentrations. In this region membrane survival is nearly independent of q but is mainly determined by the final electrolyte concentration. S_f^{50} values decrease again at still higher initial solute concentrations, as the decrease in chlorophyll concentration, i.e., the dilution of the thylakoid membranes due to an increase of q contributes to membrane damage in addition to the final salt concentration. The relative magnitude of the ratio $(G/S)^{50}$ can be estimated from the slopes of the G_f^{50} and S_f^{50} curves; the dependence of this ratio on the initial solute concentration resembles that of the G_f^{50} curve.

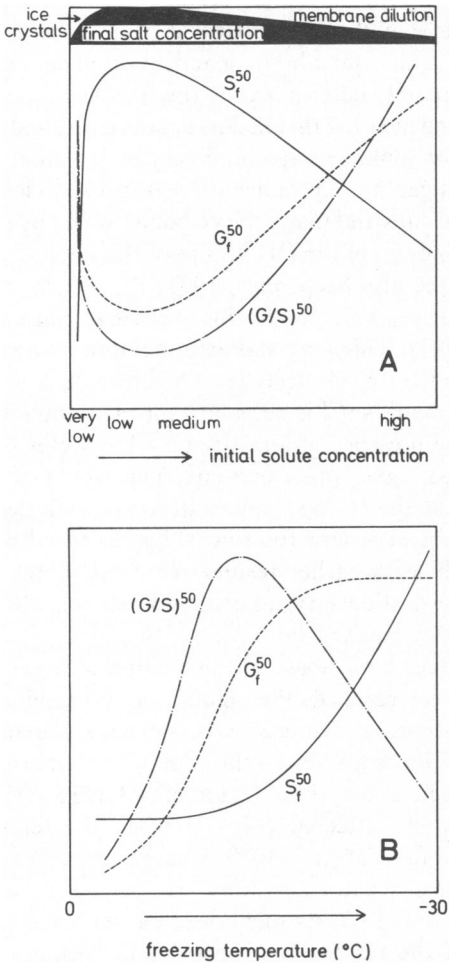

FIGURE 10 Simplified scheme of survival of isolated thylakoid membranes during freezing in the presence of variable amounts of glucose and NaCl. The plots show relative values of final concentrations of glucose and NaCl or of the glucose/NaCl ratio at which membrane activity is decreased to 50% after subjection to a freeze-thaw cycle. Note that each of the three curves in the figures are plotted at individual arbitrary scales of the ordinate. (*A*) Dependence of membrane survival on initial solute concentration under otherwise constant conditions. The relative contributions of ice crystal formation, final salt concentration, and thylakoid concentration to membrane damage are shown at the top of the figure. (*B*) Dependence of membrane survival on the freezing temperature under otherwise constant conditions. See text for further explanations. The definitions of G_f^{50}, S_f^{50}, and $(G/S)^{50}$ are given in footnote one.

The variation of these values with the freezing temperature is shown diagramatically in Fig. 10 *B*. This scheme applies to low, medium, and high initial solute concentrations but does not consider the extremes on both sides of the concentration scale; nevertheless, separation of factors contributing to membrane damage is less obvious here. In the presence of relatively low initial solute concentrations and relatively mild freezing conditions, S_f^{50} values do not vary significantly with the temperature, indicating that freezing damage is governed mainly by the final electrolyte concentration. S_f^{50} values increase at lower temperatures, whereas the temperature dependence of the G_f^{50} values is less pronounced in this range of temperature. The dependence of $(G/S)^{50}$ values on the temperature shows an optimum curve: values first increase with decreasing temperature and, after reaching a maximum at about -15°C, gradually decrease under more severe freezing conditions. At higher initial solute concentrations ice formation occurs only at relatively low temperatures; under these conditions the rising branch of the $(G/S)^{50}$ curve may be less pronounced or even nonexistent, i.e., only the declining part of the curve is observed. Factors that predominantly contribute to membrane damage under mild freezing conditions are changes in the sugar/salt ratio and the final concentration of NaCl and glucose, and changes in the volume of the residual liquid. At severe freezing conditions it is likely that the extreme solute concentrations reached in the surroundings of the thylakoids also affect membranes by other factors, e.g., severe dehydration.

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