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Cryoprotection by Glucose, Sucrose, and Raffinose to Chloroplast Thylakoids[1]

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R. Daniel Lineberger[2]
Department of Floriculture and Ornamental Horticulture, Cornell University, Ithaca, New York 14853
Peter L. Steponkus
Department of Agronomy, Cornell University

ABSTRACT

Differential cryoprotection is afforded to chloroplast thylakoids against freeze-induced uncoupling of cyclic photophosphorylation by equimolar concentrations of glucose, sucrose, and raffinose. This differential protective effect appears to be due to nonideal activity-concentration profiles exhibited by the sugars during freezing. When cryoprotection is analyzed as a function of the mole fraction of NaCl to which the membranes are exposed during freezing, the pattern of protection to cyclic photophosphorylation and its component reactions is not dependent upon the chemical identity of the protective solute. Cryoprotective efficiency of glucose, sucrose, and raffinose can be accounted for by proposing an activity dependent alteration in the freezing environment rather than specific solute-membrane interactions.

Hypotheses advanced to account for cryoprotection of membrane systems by neutral solutes can be classified into one of two approaches. The neutral solute prevents the attainment of a critical concentration of an injurious solute in a colligative fashion by reducing the mole fraction of the injurious solute at a given level of dehydration to which the system is exposed (8, 10). Protection may be afforded by direct interaction of the protective solute with the membrane in a fashion which precludes the exposure of specific membrane sites to the deleterious effects of freeze concentration (11, 16), or the protective solute may elicit a membrane configuration which is inherently more resistant to freezing stress (19). Experimental evidence supportive of the two alternative mechanisms has been gathered using chloroplast thylakoid vesicles as a model system. Heber and co-workers (4, 5, 16) and Santarius (11, 12) have demonstrated that sugars, amino acids, organic acids, and a low mol wt protein fraction all have cryoprotective activity in preventing freeze-induced uncoupling of photophosphorylation. They have proposed that protection by organic and inorganic solutes may be attributed to a colligative mechanism, while a more complicated membrane interaction mechanism must be proposed to account for the low concentrations of protective protein fraction necessary to maintain thylakoid integrity. Since increasing concentrations of sucrose were required to protect thylakoids frozen to successively lower temperatures, Garber and Steponkus (2) proposed that the lesion to thylakoid membrane semipermeability was protected colligatively. Previous results concerning the differential cryoprotection by glucose, sucrose, and raffinose afforded to thylakoids during freezing and thawing (13) or heat stress (12) were interpreted as evidence supportive of a noncolligative-type mechanism. Here, we present data concerning the anomalous behavior of these sugars during freezing which have necessitated a reinterpretation of this earlier hypothesis concerning the mechanism of cryoprotection by neutral sugars.

[1] This study represents a portion of the Ph.D. thesis of R. Daniel Lineberger.

[2] Current address: Department of Horticulture, The Ohio State University, Columbus, Ohio 43210.

MATERIALS AND METHODS

Chloroplast Isolation. Washed, deveined spinach leaves (*Spinacia oleracea* L. "Winter Bloomsdale") were ground in a Waring Blendor in a medium containing 20 mM Tricine, 250 mM NaCl, 20 mM sodium ascorbate, and 0.1% BSA (pH 7.8). Leaves were sprayed with Foamkill antifoam spray (Nutritional Biochemicals) prior to grinding. Following filtration through two layers of Miracloth, the homogenate was centrifuged for 1 min at 3,000*g*. The pellet was resuspended in 10 ml of 10 mM NaCl and homogenized by sucessive passages through a 10-ml volumetric pipet. The suspension was diluted to 40 ml with 10 mM NaCl and centrifuged for 7 min at 20,000*g*. Thylakoids were washed a second time in 10 mM NaCl and diluted to a Chl concentration of 1.0 mg/ml with 10 mM NaCl. Chl was determined by a modification of the Arnon method (18). Initial isolation was carried out at 4 C, and all washing operations were performed at 0 C.

Freezing and Thawing of Thylakoid Suspensions. Thylakoid suspensions containing 500 μg Chl/ml, 5 mM NaCl, and 0–100 mM glucose, sucrose, or raffinose were frozen to −18 C in a stirred methanol bath freezing unit programmed to cool at 2.8 C/h. Samples were held at −18 C for 3 h and thawed in the same bath at 10 C/h. Biochemical assays were performed immediately after thawing.

Cyclic Photophosphorylation. The reaction mixture (0.5 ml) for cyclic photophosphorylation consisted of the following compounds at the stated final concentrations: 5 μg Chl equivalents; 3 mM KH_2PO_4, 1 mM sodium ascorbate, 50 μM phenazine methosulfate, 3 mM ADP (pH 8.2). Carrier-free ^{32}Pi was added at a dilution of 1 μCi/ml. Illumination was provided by two 300-w flood lamps at a distance of 4 cm from the reaction vessels, and the temperature was maintained at 16 C. The reaction was stopped by adding 0.5 ml of a solution containing 1.5 g ammonium molybdate, 2.75 ml concentrated HCl, 0.5 ml triethylamine, and 0.5 ml saturated bromine water/50 ml of solution (14). The suspensions were allowed to settle 10 min at room temperature and then were centrifuged at 12,000*g* for 10 min. A 0.5-ml aliquot of the supernatant was transferred to a planchet, dried, and counted on a Nuclear-Chicago gas flow counter operated at 1,300 v with a quenching gas flow of 50 ml/min.

Solution Freezing Point Determination. Concentration series of NaCl, glucose, sucrose, and raffinose were prepared by dilution

from concentrated stock solutions. A volume of solution (usually 1.0 ml) providing sufficient heat of fusion to raise the temperature of the solution to the freezing point was used. Samples were cooled in a refrigerator freezer at −20 C, and the temperature was monitored by a 28-gauge AWG copper-constantan thermocouple pair (reference at 0 C) connected to a mv recorder. Recorder output was converted to degrees C using the value −0.038 mv/degree C (17).

Light-induced Proton Uptake. A thylakoid suspension equivalent to 250 μg Chl was suspended in 5 ml of an unbuffered solution containing 15 mM NaCl, 0.2 mM $MgCl_2$, and 40 μM phenazine methosulfate. The reaction mixture was illuminated by two 300-w flood lamps at a distance of 6 cm from the vessel. Temperature was maintained at 16 C and the reaction was monitored by a combination electrode and graphically displayed on a recorder calibrated to 1 pH unit full scale response. The initial pH of the reaction (pH 6.1) was maintained by the addition of 0.001 N HCl and the magnitude of the light-induced proton uptake was expressed as μequivalents HCl added/mg Chl to maintain the initial pH.

Estimation of Thylakoid Osmotic Responsiveness. Thylakoids containing 1.0 mg/ml Chl were diluted with concentrated NaCl stock solutions to give final concentrations of 750 μg/ml Chl with NaCl concentrations of 50, 100, 200, and 250 mM. Suspensions were centrifuged in nonheparinized glass capillaries in an IEC model MB hematocrit centrifuge at 13,000*g* for 15 min. Volume measurements were corrected for any further sedimentation using factors derived from volume determinations of suspensions which had been centrifuged until no further decrease in particle packed volume could be measured. The values reported are the slopes of least-squares regression lines fit to the data (per cent particle packed volume as a function of reciprocal of osmolarity).

Ca^{2+}-dependent ATPase Activity. Activation of membrane associated Ca^{2+} ATPase activity and the ATPase reaction were performed by the procedure described by Vambutas and Racker (15). Determination of Pi was as described by Wharton and McCarty (18).

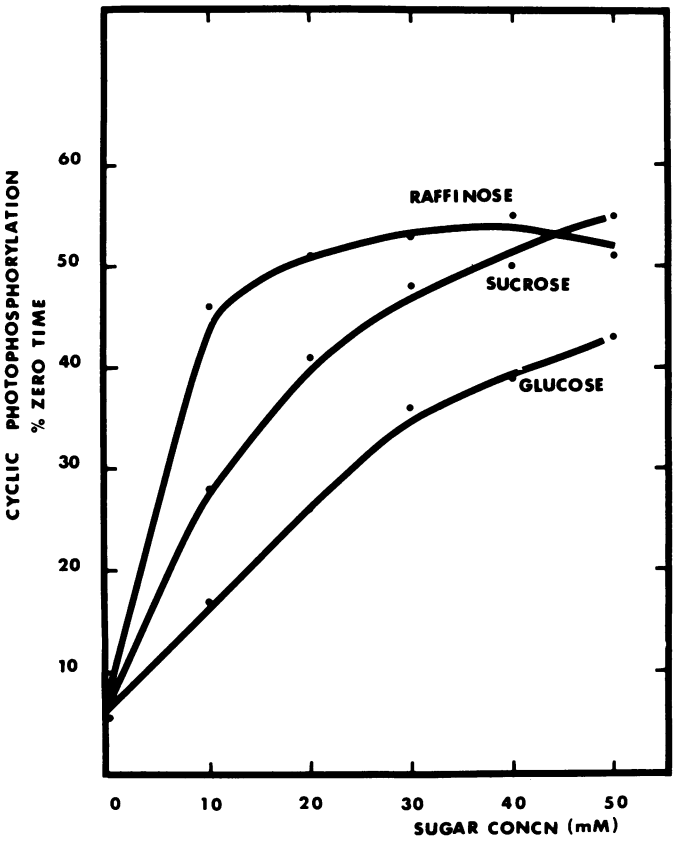

FIG. 1. Capacity for cyclic photophosphorylation of thylakoids frozen in NaCl, glucose, sucrose, and raffinose. Thylakoids were frozen to −18 C in 5 mM NaCl plus the indicated concentrations of sugar. Activity of freshly isolated controls was 1,304 μmol ^{32}Pi incorporated/mg Chl·h. Results are the average of two experiments each with two subsamples. $LSD_{0.05}$ = 10.4%.

RESULTS

Chloroplast thylakoids subjected to a slow freeze-thaw cycle in the presence of 5 mM NaCl lose the capacity for cyclic photophosphorylation (Fig. 1). The addition of 10–50 mM glucose, sucrose, or raffinose to the suspension prior to freezing protects against the loss of activity. The degree of protection is dependent upon the sugar used, and equimolar concentrations of the different sugars afford differing degrees of cryoprotection. When the activities of thylakoids frozen in 10 mM glucose, sucrose, or raffinose are compared, the protection afforded by raffinose is greater than sucrose which is greater than glucose.

The observed pattern of protection afforded by these sugars appears to be inconsistent with the proposal that thylakoid inactivation is due to exposure to elevated NaCl concentrations during the freeze-thaw cycle (91). This interpretation is predicated upon the assumption that equimolar concentrations of glucose, sucrose, and raffinose depress the NaCl concentration achieved during freezing to the same extent.

Further experiments were designed to estimate the concentration of NaCl achieved in solutions containing glucose, sucrose, and raffinose during freezing. Solution activity profiles as a function of concentration were obtained by measuring freezing point depression. Only NaCl exhibited a linear increase in activity with increasing concentration (Fig. 2). The least-squares regression equation for NaCl activity, y = 1.86 x (where y = freezing point depression (ΔT°C), and x = osmolar concentration of solute), is identical to the prediction for an ideal solute. Glucose ($y = 2.54\ x^{1.11}$), sucrose ($y = 3.35\ x^{1.34}$), and raffinose ($y = 4.97\ x^{1.52}$) deviate from ideal behavior in that activity increases exponentially rather than linearly as a function of osmolarity. The severity of deviation is greatest for raffinose and least for glucose, and sucrose exhibits an intermediate level of departure from ideality.

To determine whether interactions affecting predicted solute activity exist between salt-sugar or salt-sugar-thylakoid systems, the activities of concentration series of NaCl-sugar and NaCl-sugar-thylakoid systems were measured. Binary solutions containing NaCl and sugar, and ternary solutions containing NaCl, sugar, and thylakoids displayed activity profiles identical to the respective sugar solutions when plotted as a function of osmolarity (Fig. 3). The addition of NaCl to the sugar solution increased the value of the coefficient of the x variable in the activity equation, as would be predicted for a solute increasing linearly in activity without interaction (Table I). The further addition of thylakoids to NaCl-sugar solutions did not alter the activity equations, indicating that thylakoids do not possess colligative properties.

The derivation of quantitative expressions which describe solution activities of NaCl, glucose, sucrose, and raffinose as a function of temperature allows the estimation of single component contribution to solution activity at a given subzero temperature. The following calculations will demonstrate the determination of the final osmolarities of NaCl and glucose in a binary system with the understanding that the same procedure can be followed for sucrose and raffinose systems by making the appropriate substitutions of the respective activity equations. To account for the observed nonideality of sugar solutions, the conventional expression $T = 1.86\ M_f$ (after Mazur [9]) is modified to the form $T = 1.86\ M_1 + 2.54\ M_2^{1.11}$ (where T represents the absolute value of the subzero temperature in degrees C, M_f represents the final

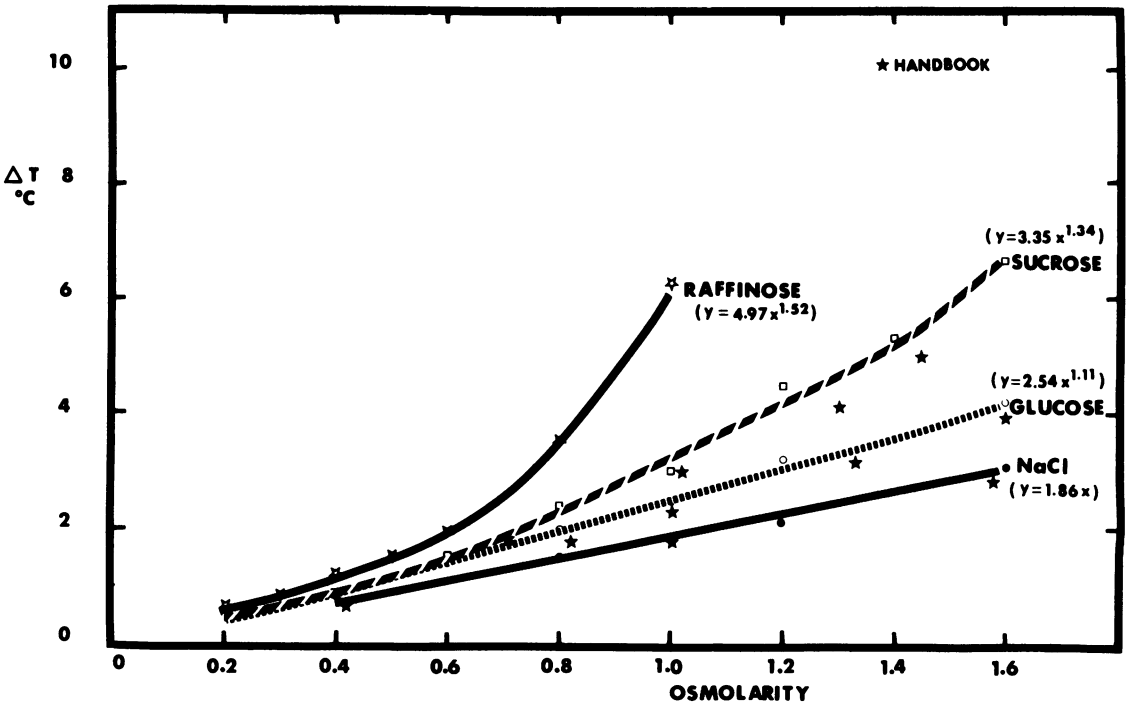

FIG. 2. Freezing point depression (ΔT°C) of a concentration series of NaCl, glucose, sucrose, and raffinose. Data are compared with those published in the Handbook of Chemistry and Physics (★) for glucose, sucrose, and NaCl.

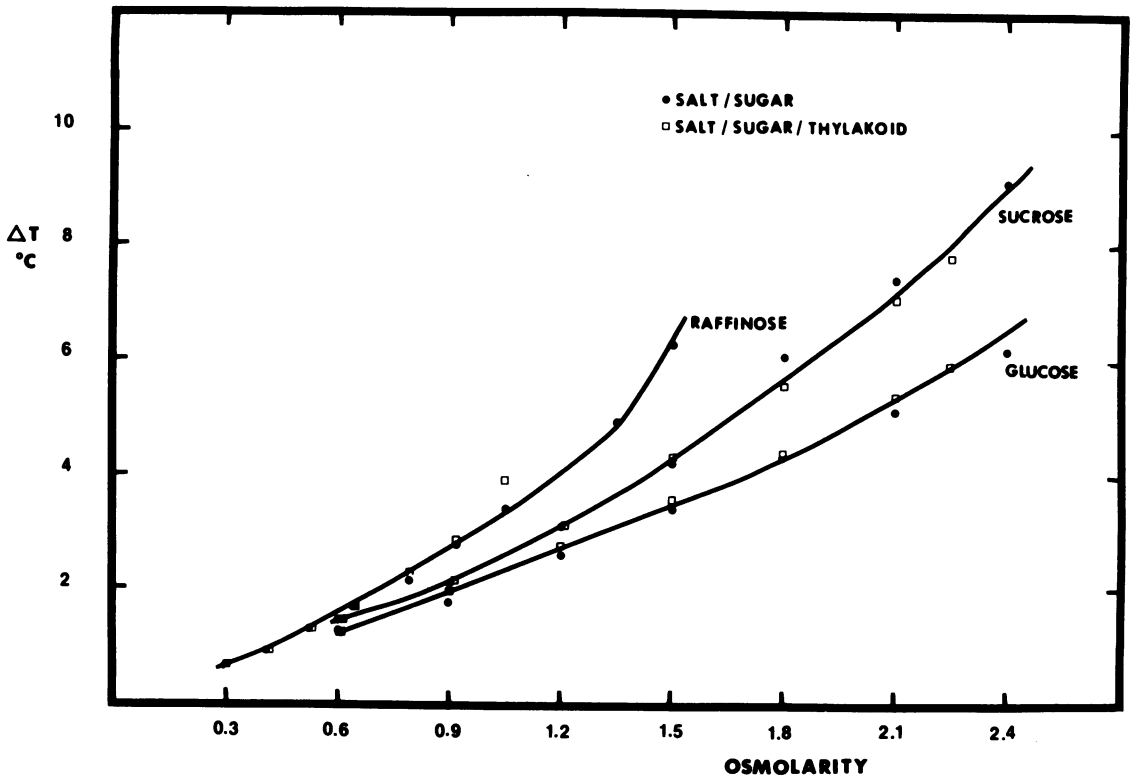

FIG. 3. Freezing point depression (ΔT°C) of binary solutions (1:4 molar ratio NaCl to sugar) and ternary solutions containing fixed ratio of NaCl, sugar, and thylakoids (1.0 M sugar to 250 mM NaCl to 500 μg/ml Chl).

osmolarity of solute, M_1 represents the final osmolarity of NaCl, and M_2 represents the final osmolarity of glucose). Since the ratio of the osmolarities of glucose and NaCl is constant, the expression can be reduced to one unknown so that $T = 1.86\ M_1 + 2.54\ a\ M_1^{1.11}$ where $a = M_2/M_1$. After the value of M_1 is determined at a given T by an iterative procedure, then the final osmolarities of NaCl (M_1) and glucose ($M_2 = a\ M_1$) are defined. The fraction of water unfrozen at this T g (q_T), is then the ratio of the initial NaCl osmolarity (M_i) to the final NaCl osmolarity (M_1). (After Mazur, $T = 1.86\ M_1$ and $1.86\ M_i/q_T$, then $(1.86\ M_i/q_T) = 1.86\ M_1$ and

q_T = (1.86 M_i/1.86 M_1), where T, q_T and M_1 are previously defined, and M_i = initial osmolarity of NaCl). The mole fraction of NaCl in the unfrozen solution is equal to the moles NaCl present divided by the sum of the moles water unfrozen, moles sugar present, and moles NaCl present.

A differential protective effect of glucose, sucrose, and raffinose is not manifested when the effects of freezing on thylakoid cyclic photophosphorylation capacity are analyzed with respect to the mole fraction of NaCl achieved during freezing (Fig. 4). Having accounted for the dissimilarities in deviation from ideal behavior exhibited by glucose, sucrose, and raffinose, the extent of protection afforded by the various sugars against thylakoid inactivation appears to be the same.

The capacity for cyclic photophosphorylation is a complex, composite function dependent upon the functional integrity of the thylakoid with respect to impermeability to protons, the ability to generate a proton gradient, and the maintenance of chloroplast-coupling factor (CF_1) on the thylakoid membrane (6). Since a systematic approach has been used in characterizing the freeze-induced lesions resulting in uncoupling of photophosphorylation (2, 3), a similar approach can be used in describing the effects of cryoprotection on these lesions.

Glucose, sucrose, and raffinose protect thylakoid vesicles against the freeze-induced loss of light-induced proton uptake capacity (Fig. 5). A single response curve for the extent of protection *versus* final osmolarity of NaCl achieved can be plotted irrespective of the identity of the sugar used. The increasing protection afforded by decreasing the NaCl activity during freezing is similar in magnitude to the response demonstrated for cyclic photophosphorylation (compare Figs. 4 and 5).

Table I. *Freezing Point Depressions of Concentration Series of Sugars, Binary Solutions Containing Salt and Sugar, and Ternary Solutions Containing Sugar, Salt, and Thylakoids*

Activity curves are least-squares fit power curve estimates, where y is the freezing point depression and x is molar sugar concentration. The binary solutions were composed of a 1:4 molar ratio of salt (NaCl) to sugar. The ternary solutions were composed of the ratio of 0.25 M NaCl to 1.0 M sugar to 500 μg/ml Chl. *r* is the correlation coefficient.

Solute(s)	Concentration Range	Activity Curve	*r*
	M		
Glucose	0.2–2.0	$y = 2.54\ x^{1.11}$	1.00
Glucose/NaCl	0.4–1.5	$y = 3.65\ x^{1.12}$	1.00
Glucose/NaCl/thylakoids	0.4–1.5	$y = 3.65\ x^{1.12}$	0.99
Sucrose	0.2–1.8	$y = 3.35\ x^{1.34}$	0.99
Sucrose/NaCl	0.4–1.5	$y = 4.38\ x^{1.30}$	0.99
Sucrose/NaCl/thylakoids	0.4–1.5	$y = 4.37\ x^{1.30}$	0.99
Raffinose	0.2–1.0	$y = 4.97\ x^{1.52}$	0.98
Raffinose	0.2–0.8	$y = 4.17\ x^{1.36}$	0.99
Raffinose/NaCl	0.2–.75	$y = 5.47\ x^{1.32}$	0.99
Raffinose/NaCl/thylakoids	0.19–0.7	$y = 5.47\ x^{1.32}$	0.99

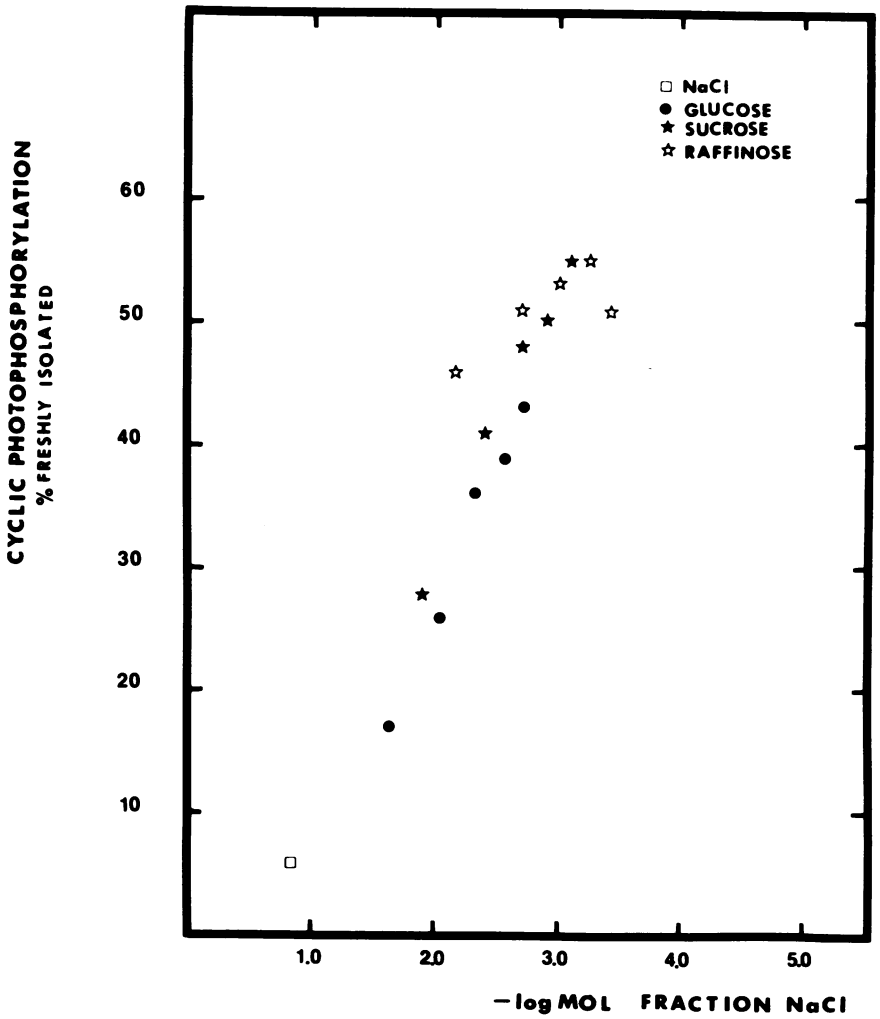

FIG. 4. Relationship between the capacity for cyclic photophosphorylation and the predicted mol fraction of NaCl to which thylakoids are exposed at −18 C. Data are replotted from Figure 1. Data can be fitted to the line $y = 20.34 \times -9.95$ with the correlation coefficient $r = 0.94$.

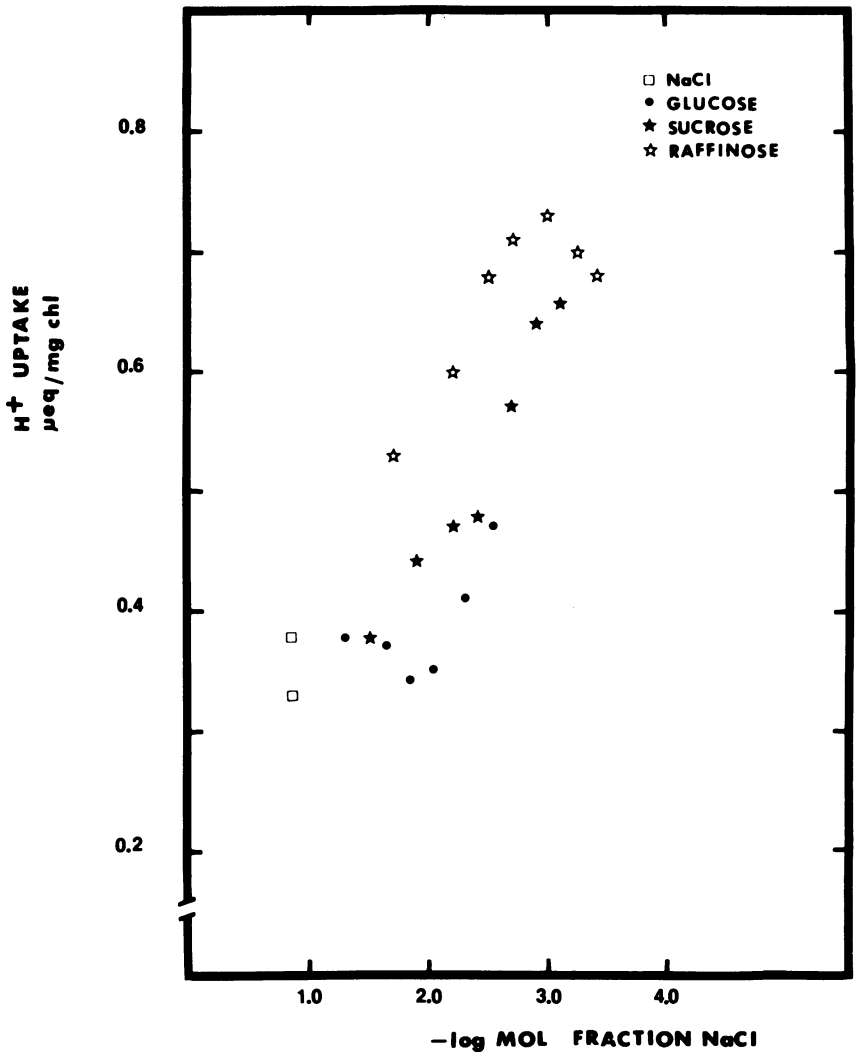

FIG. 5. Capacity for light-induced proton uptake of thylakoids exposed to increasing mol fractions of NaCl during freezing at −18 C. Proton uptake of freshly isolated suspensions was 1.31 μeq H^+/mg Chl. Data can be fitted to the line $y = 0.17 \times +0.14$ with the correlation coefficient $r = 0.82$.

Two of the three freeze-induced lesions to light-induced proton uptake capacity demonstrated by Garber and Steponkus (2) can be quantitated independently. An estimate of thylakoid osmotic responsiveness following freezing was obtained by measuring the particle-packed volumes of suspensions subjected to NaCl solutions of increasing osmolarity. Increasing values of the slope of the van't Hoff plots (particle-packed volume plotted against reciprocal of osmolarity of added solute) were interpreted as indicative of maintenance of osmotic responsiveness and indicative of retention of membrane semipermeability. The magnitude of cryoprotection afforded by glucose, sucrose, and raffinose to maintenance of thylakoid osmotic responsiveness is dependent upon the reduction achieved in the final concentration of NaCl to which the membranes were exposed (Fig. 6). Thylakoids were inactivated to a greater extent at any given predicted final NaCl concentration when frozen to −5.3 C compared to −18 C. Apart from the difference in the level of cryoprotection at the two temperatures, these data are consistent with the observation that glucose, sucrose, and raffinose may protect via a reduction in the NaCl concentration achieved during freezing.

A second freeze-induced lesion, the loss of CF_1 from the thylakoid membrane (2), can be demonstrated by determining the loss of Ca^{2+}-dependent ATPase from the membrane fraction of previously frozen thylakoid suspensions. When analyzed with respect to the final NaCl concentration achieved during freezing, the pattern of protection afforded by glucose, sucrose, and raffinose is indistinguishable (Fig. 7). In contrast to the protection afforded to osmotic properties, protection against the loss of Ca^{2+}-dependent ATPase activity is independent of the freezing temperature. Results obtained by freezing to −5.3 C and −18 C can be plotted as one curve, and activity is thus dependent only on the reduction in NaCl concentration achieved by the cryoprotectants.

DISCUSSION

The differential protective effect of equimolar concentrations of glucose, sucrose, and raffinose against the freeze-induced inactivation of the chloroplast thylakoid system has been previously interpreted as evidence contrary to a colligative mechanism of cryoprotection (12, 13). Further investigation into the cryoprotective effect of these neutral sugars has revealed that departures from ideal behavior with respect to increasing activity with increasing concentrations may account for the differential protective effect noted when inactivation data are plotted as a function of molarity. The ability to account for differential increases in protective solute activity during freezing allows a more accurate assessment of the concentration of toxic solute (NaCl) to which

the thylakoid vesicles are exposed at any given combination of freezing temperatures and initial solute concentrations.

These results are in agreement with earlier observations concerning the uncoupling of photophosphorylation by freezing in the presence of dilute NaCl solutions (4). Correlative decreases in the capacity for light-induced proton uptake, loss of osmotic responsiveness, and disappearance of Ca^{2+}-dependent ATPase activity from thylakoid vesicles subjected to freezing and thawing are manifested (2). The pattern of protection afforded by glucose, sucrose, and raffinose against freeze inactivation of these functions is consistent with the proposal that the elevated concentration of NaCl is the driving force for inactivation. The disparity between the levels of protection afforded to maintenance of osmotic responsiveness when thylakoids are frozen to −18 C compared to −5.3 C demonstrates that factors other than the absolute concentration of NaCl achieved may interact to determine the extent of inactivation. The rate of inactivation may be slower with decreasing temperature, but the possibility that a systematic error in determining the absolute mole fraction of NaCl achieved at the different temperatures cannot be excluded. When membrane-bound Ca^{2+}-dependent ATPase activity was determined on thylakoids exposed to elevated NaCl concentrations in the supercooled or frozen state, a similar disparity in absolute sensitivity was observed (13). Steponkus *et al.* attributed this difference to the lower stability of hydrophobic bonds at relatively high subzero temperatures rather than to a temperature effect on rate of inactivation.

Chloroplast thylakoids exhibit a pronounced sensitivity to hypertonic salt solutions at 0 C. In the absence of freezing, exposure to concentrated NaCl solutions rapidly diminishes the capacity for light-induced proton uptake and inactivates membrane associated Ca^{2+}-dependent ATPase activity (13). Exposure of thylakoid suspensions to hypertonic NaBr has been used to obtain subunits of CF_1 from the vesicular membrane (7). The proposition that elevated NaCl concentrations may be the driving force for inactivation during freezing is consistent with the observed sensitivity of the thylakoid system to hypertonic NaCl. These data do not exclude the possibility that crypoprotection may be afforded by mechanisms other than a colligative reduction of NaCl concentration as has been proposed by Santarius (12). Parameters which increase parallel to the sugar activities during freezing include the concentration of —OH groups and the fraction of water remaining unfrozen. Both parameters have been postulated to be stabilizers of membrane structure so that cryoprotection may be due to increased membrane stability rather than decreased solute toxicity (1, 9).

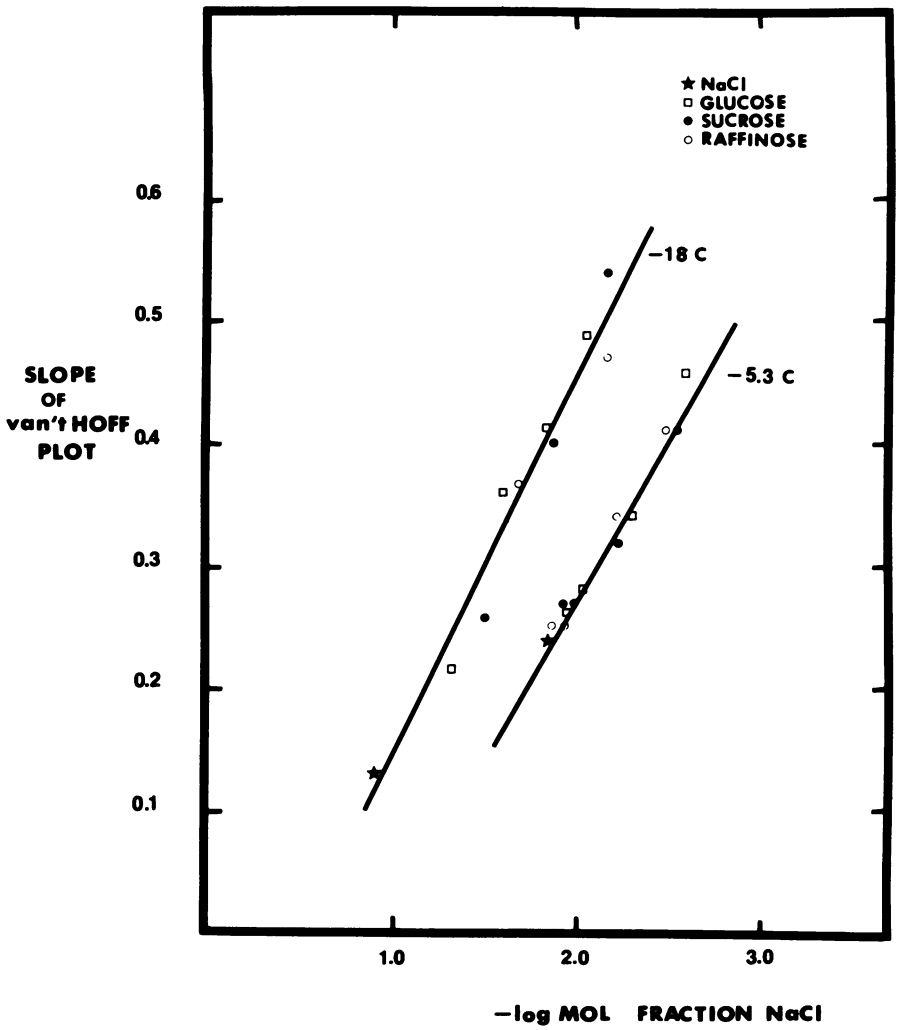

FIG. 6. Relationship between maintenance of osmotic responsiveness following freezing and the predicted mol fraction of NaCl to which thylakoids are exposed at −18 C and −5.3 C. The value of the control suspension held at 0 C for 4 h was 0.51. Data for −18 C can be fitted to the line $y = 0.3 \times -0.15$ with $r = 0.98$ and data for −5.3 C can be fitted to the line $y = 0.27 \times -0.27$ with $r = 0.98$.

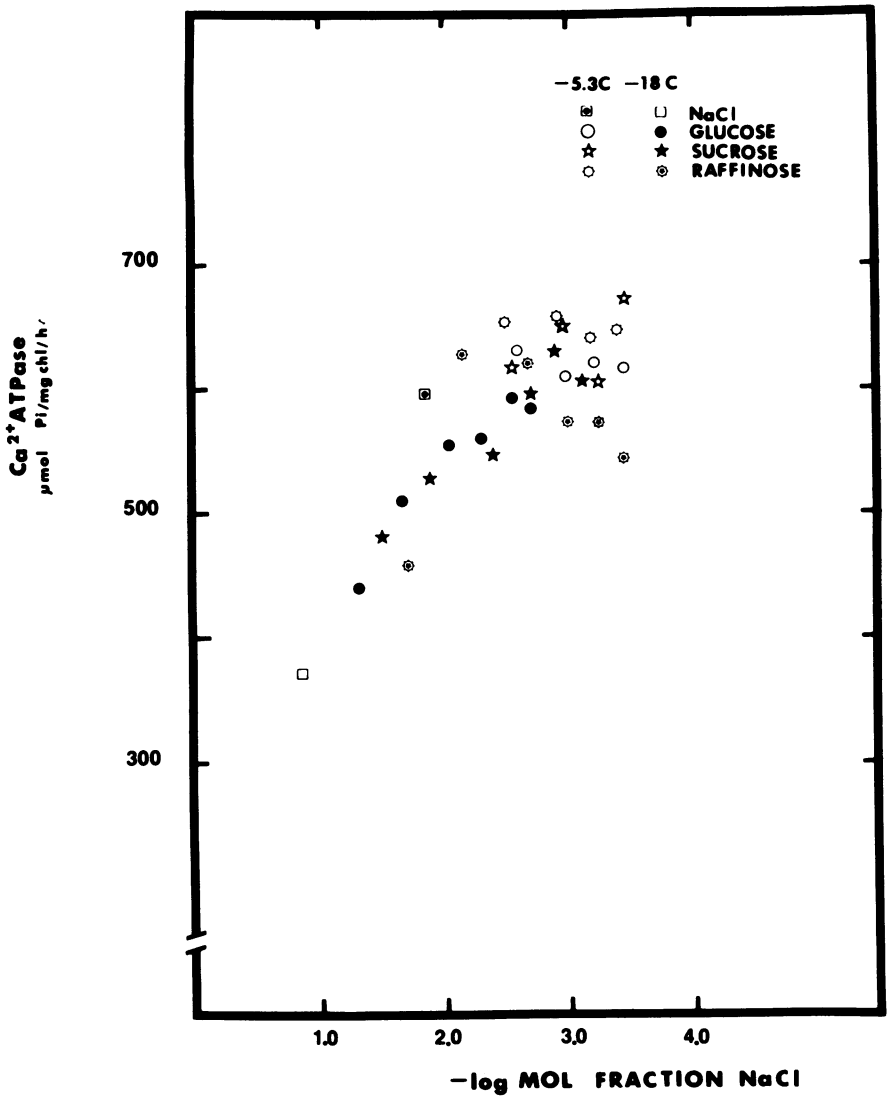

FIG. 7. Ca^{2+}-dependent ATPase activity remaining on the thylakoid membrane following exposure to increasing mol fractions of NaCl at −18 C and −5.3 C. Data for both −18 C and −5.3 C can be fitted to the line $y = 78 \times +379$ with $r = 0.78$.

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