The Effect of Freezing on Thylakoid Membranes in the Presence of Organic Acids

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ABSTRACT

The effect of salts of organic acids on washed and non-washed chloroplast membranes during freezing was investigated. Thylakoids were isolated from spinach leaves (Spinacia oleracea L.) and, prior to freezing, salts of various organic acids or inorganic salts or both were added. Freezing occurred for 3 to 4 hours at -25 °C. After thawing membrane integrity was investigated by measuring the activity of cyclic photophosphorylation.

At very low NaCl levels (1 to 3 mM, washed thylakoids) salts of organic acids either could not prevent membrane inactivation in the course of freezing (succinate) or were effective only at relatively high concentrations (0.1 M or more of acetate, pyruvate, malate, tartrate, citrate). If NaCl was present at higher concentrations (e.g., 0.1 M) some organic acids, e.g., succinate, malate, tartrate, and citrate, were able to protect frost-sensitive thylakoids at surprisingly low concentrations (10 to 20 mM). Other inorganic salts such as KCl, MgCl₂, NaNO₂ could also induce protection by organic acids which otherwise were ineffective or poorly effective. For effective protection, a more or less constant ratio between inorganic salt and organic acid or between two or more organic acids had to be maintained. Departure to either side from the optimal ratio led to progressive inactivation.

The unspecificity of the protective effect of organic acids suggests that these compounds protect colligatively. There are also indications that, in addition, more specific interaction with the membranes contributes to protection. At temperatures above the freezing point, the presence of salts of organic acids decreased the rate of membrane inactivation by high electrolyte concentrations.

MATERIAL AND METHODS

Mature leaves of spinach (Spinacia oleracea L.) were harvested directly from the field or were bought at the local market. Chloroplasts were isolated in isotonic NaCl as described earlier (10, 27). After isolation they were suspended in distilled water which resulted in osmotic rupture. Freed thylakoids were then used either directly (nonwashed thylakoids) or used after two washings in distilled water to remove stroma proteins and most of the salts, sugars, and amino acids which remained in the chloroplasts during isolation (washed thylakoids). The washings were carried out in a Beckman-Spinco L-50 centrifuge, which was operated for 5 min at about 24,000g (first washing) and for 15 min at about 38,000g (second washing). Chloride concentrations in the suspension of the washed thylakoids ranged from 1 to 3 mM. Further washings with distilled water were not performed as they drastically reduced, together with the salt content, photophosphorylation activity (12).

After isolation the membranes were incubated in solutions of inorganic salts or salts of organic acids which were held at neutral pH for about 15 min at 2 °C. Thylakoids were then either stored at 0 °C for 3 hr or rapidly frozen (within a few minutes, cf. 28) to about -25 °C. kept at that temperature for 3 to 4 hr, and then thawed in a water bath at 20 °C. Subsequently, integrity of the membranes was checked by measuring the activity of cyclic photophosphorylation. Aliquots of the thylakoids containing 60 to 80 μg of chlorophyll were trans-
ferred into the following reaction medium (µmoles per ml): tris, 13; NaCl, 30; KH₂PO₄, 2.2; ADP, 2.0; MgCl₂, 4; phenazine methosulfate, 0.3; total volume in 0.5-cm cuvets about 1.4 ml, pH 7.8. Illumination was provided by two incandescent lamps (Radium 500 w, 15,000–20,000 ft-c, 2–3 min), and controls were kept in the dark. After illumination 0.13 ml of tri-chloroacetic acid was added and P1 uptake was determined according to Fiske and SubbaRow (3). Results were expressed on a chlorophyll basis; chlorophyll was determined according to Arnon (1). It should be emphasized that the inorganic salts or the salts of the organic acids which were transferred together with the chloroplast membranes into the reaction medium in which photophosphorylation took place did not significantly influence the activity of ATP synthesis in the light under the chosen experimental conditions.

RESULTS

Freezing of thylakoids for 3 hr at -25°C in the presence of NaCl or KCl up to a concentration of about 0.1 M results in an irreversible uncoupling of phosphorylation from electron transport; at higher salt concentrations electron transport is also affected (8, 10, 25–27). Addition of sugars, for instance, sucrose in a concentration of 0.1 M or more prior to freezing, protects the membranes against freeze inactivation (10, 25).

The effect of freezing on nonwashed thylakoid membranes in the presence of 0.1 M NaCl and various concentrations of sodium or potassium salts of different organic acids is demonstrated in Figure 1. Some of the organic acids investigated here are able to protect thylakoid membranes at unusually low concentrations during freezing. Up to 0.1 M, sodium succinate, sodium malate (not shown), sodium potassium tartrate and sodium citrate are, on a molar basis, much more effective than sucrose, sodium acetate, or sodium pyruvate (Fig. 1, b and c). At higher concentrations, adverse effects may predominate (Fig. 1a); succinate inactivates the membranes almost completely, and the protective action of tartrate and citrate decreases with increasing concentration. Acetate and pyruvate are, in this experiment, protective at concentrations up to 1.0 M; in some other experiments the protective action of acetate and pyruvate decreased also at high concentrations (26).

It should be mentioned that spinach of different origin grown at different times of the year occasionally exhibited somewhat different sensitivities to washing, salt concentration, and other experimental parameters. This can cause curves such as the ones shown in Figure 1 to be shifted somewhat along the abscissa. However, although quantitative differences in the response to freezing were observed, qualitative differences did not occur and the principal behavior was always the same.

When suspensions of washed thylakoids which contained less than 3 mM NaCl were frozen in the presence of various concentrations of organic salts or sucrose in experiments essentially similar to those described above, very different results were obtained (Fig. 2). A much lower concentration of sucrose was sufficient for the protection of the membranes. In contrast, in the absence of added NaCl, higher concentrations of sodium acetate were necessary for protection. Sodium succinate no longer acted as a protective compound. Sodium pyruvate, sodium malate (not shown), sodium potassium tartrate, and sodium citrate prevented membrane inactivation during freezing at least partially, but again only at concentrations higher than those needed in the presence of 0.1 M NaCl (cf. Fig. 1). Above 0.5 M, protection by the organic salts decreased with increasing concentration.

Figure 3 shows another aspect of membrane response towards freezing in the presence of electrolytes. Membranes were suspended in 0.1 M sucrose, which prevents inactivation during freezing in the absence of other additions. However, with increasing amounts of NaCl also present, progressive membrane inactivation is observed. Essentially similar results are obtained in the presence of organic salts, with the only difference that concentrations higher than those of NaCl are required to overcome the protection afforded by sucrose. Acetate is least toxic on a molar basis; succinate is most toxic. This is also evident from Figure 2.

From a comparison of Figures 1, 2, and 3, it follows that a salt such as NaCl needs to be present in a thylakoid membrane suspension for some salts of organic acids to realize their potential for membrane protection during freezing. This is most pronounced with succinate (Fig. 1), in which the absence of added NaCl is not protective (Fig. 2) or even quite toxic (Fig. 3), but it is also true for tartrate and citrate and even for pyruvate and acetate. In a more detailed analysis of this effect, nonwashed thylakoids were frozen for 3 hr at -25°C in the presence of 0.1 to 0.7 M NaCl and different amounts of sodium succinate (0 to about 0.5 M). After thawing, cyclic photophosphorylation was measured in a reaction medium of constant composition at a low salt level. The higher the NaCl concentration the more succinate had to be present to prevent thylakoids from being inactivated by freezing (Fig. 4a). At a given concentration of NaCl, the effect of succinate as a function of concentration follows an optimum curve. Optimal protection of nonwashed thylakoids is observed at a molar ratio of succinate to chloride of 1:2 to 1:3.

The effect of low levels of NaCl on protection against freeze inactivation by succinate can be tested with washed thylakoids (Fig. 4b). For complete protection a minimum concentration of NaCl must be present. Below the minimum level (0.1 M), only partial protection by succinate is observed.

Different inorganic salts inactivate thylakoid membranes at 0°C or during freezing at considerably different concentrations (26). For instance, bivalent cations are more toxic than monovalent cations. There is a steep toxicity gradient among the anions, starting with iodide as the most and ending with fluoride as the least toxic halogenide. Although toxic by itself, NaCl induces succinate to be protective during freezing. Figure 5 shows that KCl is as effective as NaCl in this respect. For comparison, protection of thylakoids by succrose is shown as a function of succrose concentration. In the experiments with succrose as cryoprotectant, no NaCl or KCl was added to the thylakoid suspension. It should be remembered that inorganic salts decrease protection by succrose (Fig. 3). Even though succrose was maximally effective under the chosen conditions, it was less protective at low concentrations than succinate combined with 0.1 M NaCl or KCl. Succinate alone was not only not protective but even increased damage to the membranes during freezing (cf. Figs. 1 and 2).

The chlorides of bivalent cations also cause succinate to act over a limited range of concentration as a protective agent (Fig. 6). The concentrations needed for this are lower than in the case of alkali ions (cf. Fig. 5 and Fig. 6).

Not only the cations are interchangeable. Figure 7 shows an experiment in which NaN₃O₄, in combination with sodium succinate was added to washed thylakoids prior to freezing. Again, within a given concentration ratio between inorganic salt and succinate, photophosphorylation was protected during freezing. As compared with NaCl, NaN₃O₄ is effective at considerably lower concentrations (cf. 26).

In Figure 8 the effect of combinations of different salts of organic acids on membrane survival during freezing is compared with that of the individual compounds and that of a combination between NaCl and succinate. It is apparent that any combination is more effective than an individual compound. Of the latter, succinate and NaCl not only do not pro-
Fig. 1. Effect of freezing on nonwashed chloroplast membranes which were suspended in a solution containing 0.1 M NaCl and salts of organic acids or sucrose as indicated. a: Effects of higher concentrations of additions; b and c: extended scale, effect of lower concentrations of organic acids. Abscissas: molar concentration of organic acids or sucrose. Ordinates: the activity of cyclic photophosphorylation in μmoles of P₁ uptake per mg of chlorophyll per hour after freezing. Activity of unfrozen controls around 600 (a) and 800 (b and c) μmoles of P₁ uptake per mg of chlorophyll per hour.

In previous investigations it was found that the rate of inactivation of thylakoid membranes in the presence of a given concentration of NaCl and different concentrations of sucrose at 2 C decreases with increasing sucrose concentration (26). Thus sucrose was protective even in the absence of freezing. In Figure 9 an experiment is shown in which nonwashed thylakoids were suspended at 0 C in NaCl solutions of various concentrations in the presence of different amounts of sodium succinate or sucrose. After 3 hr, cyclic photophosphorylation was measured in a reaction medium of con-

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freezing damage. Lovelock explained the protective action of glycerol by its colligative properties. If freezing proceeds slowly enough, the vapor pressure of the ice and the vapor pressure of the unfrozen solution are close to equilibrium at any freezing temperature. As the vapor pressure of the unfrozen solution is solely a function of concentration and the vapor pressure over ice of temperature, the concentration of the unfrozen solution is determined unequivocally by the temperature. If a compound is toxic to the cell or to cell membranes and damage is caused beyond a threshold concentration which is reached at a critical temperature, it follows that a second nontoxic component in the system must reduce the concentration of the toxic component at any temperature so that the threshold concentration will now build up only at a lower temperature. In other words, the nontoxic compound confers increased resistance on the system. It will, by its colligative properties, act as a protective agent. In Lovelock's experiments with red cells, protection was interpreted as being due to the fact that the nontoxic glycerol decreased the con-

**DISCUSSION**

Lovelock (20) has found that hemolysis of red blood cells which were suspended in isotonic NaCl occurred if NaCl concentration increased during freezing beyond a critical limit. In the presence of glycerol, the cells could be protected against

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**Fig. 2.** Effect of various salts of organic acids and sucrose on washed chloroplast membranes during freezing to $-25^\circ$C for 3 hr. NaCl concentration 1 to 3 mm. Abscissa and ordinate as described in Figure 1.

**Fig. 3.** Effect of freezing on washed chloroplast membranes which were suspended in a solution containing 0.1 m sucrose and salts of organic acids or NaCl as indicated. Abscissa: molar concentration of organic acids or NaCl. Ordinate: the activity of cyclic photophosphorylation as percentage of the unfrozen controls.

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**Fig. 4.** Effect of freezing on chloroplast membranes which were suspended in solutions containing NaCl and sodium succinate as indicated. a: Nonwashed chloroplast membranes, higher concentrations of succinate and NaCl; b: washed chloroplast membranes, lower concentrations of succinate and NaCl. Abscissa: molar concentration of succinate. Ordinates: the activity of cyclic photophosphorylation in $\mu$moles of $P_i$ uptake per mg of chlorophyll per hour.
concentration of the toxic NaCl during freezing so that the NaCl concentration did not reach the critical limit.

Similar considerations apply also to the thylakoid system. Freezing of thylakoids in the presence of NaCl results in membrane inactivation. The addition of a compound which is not toxic at higher concentration, such as sucrose or acetate, decreases the NaCl concentration during freezing at any one temperature. If sufficient sucrose or acetate are added prior to freezing, the NaCl concentration remains below the critical limit during freezing and inactivation does not occur. The same could even be possible when different salts are combined which alone are toxic in the absence of other compounds provided their mode of membrane inactivation is different. If in the presence of only one potentially toxic compound during freezing the tolerance limit is exceeded, membrane inactivation will be observed. Freezing in the presence of a second, also potentially toxic compound to the same temperature will produce the same total concentration in the unfrozen part of the system. However, individual concentrations of the two toxic components are different. If both of them do not reach the toxic level, protection will be observed. These relations explain part of the observations reported above. Figure 4 shows that membrane protection during freezing in the presence of succinate and NaCl is only observed within certain concentration ratios between organic and inorganic salt. In the presence of a low concentration of succinate, membrane inactivation during freezing is due to the accumulation of NaCl beyond the critical concentration. At high succinate

![Figure 5](image1.png)

**Fig. 5.** Effect of freezing on washed chloroplast membranes which were suspended in a solution containing 0.1 M NaCl, 0.1 M KCl and/or succinate as indicated. Abscissa: sodium succinate, molar concentration. Protection by sucrose is shown for comparison. For the sucrose curve the abscissa shows sucrose, not succinate concentrations. Ordinate: the activity of cyclic photophosphorylation in μmoles of P<sub>i</sub> uptake per mg of chlorophyll per hour.

![Figure 6](image2.png)

**Fig. 6.** Effect of freezing on washed chloroplast membranes which were suspended in a solution containing MgCl<sub>2</sub> and sodium succinate as indicated. Abscissa and ordinate as described in Figure 5.

![Figure 7](image3.png)

**Fig. 7.** Effect of freezing on washed chloroplast membranes which were suspended in a solution containing NaNO<sub>3</sub> and succinate as indicated; as control, 0.1 M NaCl instead of NaNO<sub>3</sub> was used. Abscissa: sodium succinate, molar concentration. Ordinate: the activity of cyclic photophosphorylation in μmoles of P<sub>i</sub> uptake per mg of chlorophyll per hour.
In addition to colligative action, a more direct and specific interaction between membranes and protective agents appears to be involved in protection during freezing. This is indicated by results of experiments on membrane protection at 0°C. In Figure 9 it was shown that succinate and sucrose were able to protect thylakoid membranes from inactivation by high concentrations of electrolytes, e.g., NaCl at 0°C. Contrary to the situation encountered during freezing, in this case increasing the succinate or sucrose concentration relative to that of NaCl did not significantly change the NaCl concentration in the system; nevertheless protection occurred. This cannot be explained by the colligative concept and points to rather specific effects of the protective compounds. From the available data it cannot be decided how much protection during freezing is due to colligative action and how much is due to specific effects the various ions contribute to it.

Williams and Meryman (31) assume that the reduction in the volume of the chloroplast membranes due to the removal of water in the course of freezing is responsible for membrane injury. Therefore, any compound capable of penetrating thylakoid membranes should be protective by preventing reduction in volume beyond a tolerable minimum under hypertonic conditions during freezing. From our data it cannot be concluded that a reversible membrane leak is necessary for protection. The fact that two toxic compounds when present in a suitable combination are also able to protect thylakoid membranes during freezing rather suggests that the colligative action of the compounds in connection with specific effects are responsible for protection.

Heber (5) has shown that inactivation during freezing is due to changes in permeability of biological membranes. Furthermore, it is well known that electrolytes influence mem-

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**Fig. 8.** Effect of freezing on washed chloroplast membranes which were suspended in a solution containing organic acids or combinations of 60 mM succinate and acetate, tartrate, citrate, or NaCl as indicated. Abscissa: molar concentration of organic acids or NaCl. Ordinate: the activity of cyclic photophosphorylation in μmoles of P_i uptake per mg of chlorophyll per hour.

concentrations, the NaCl is unable to prevent succinate accumulation during freezing beyond the damaging level, and inactivation results from the deleterious effects of succinate. If this interpretation of the data is correct, it is necessary to postulate that NaCl and succinate inactivate membranes at high concentrations specifically and independently of one another.

The unspecificity of protection of thylakoid membranes during freezing by different compounds can be considered as additional evidence that protection of the membranes is due, at least in part, to colligative action. As seen in Figures 4 to 7, different inorganic salts affect the membranes in the presence of salts of organic acids in a similar manner. The same holds true if salts of different organic acids are combined (Fig. 8).

Some observations, while not contradicting the colligative concept, cannot be explained by it, and it appears necessary to invoke additional effects. Figures 1, b and c show that relatively low concentrations of succinate, tartrate, or citrate (20 mM) are sufficient to counteract the deleterious effects of a rather high concentration of NaCl (0.1 M) providing protection. However, at equimolar concentrations of NaCl and succinate, protection by succinate breaks down (Fig. 4a), even though Figure 3 shows that NaCl is much more toxic to the membranes than succinate. Also, if protection were purely colligative, sodium acetate and sodium pyruvate, both salts of monobasic acids, should be equally effective in the presence of NaCl, but this is not the case (Fig. 1a). Similar observations have also been made in other cases.

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**Fig. 9.** Protective effect of succinate and sucrose on nonwashed chloroplast membranes which were suspended for 3 hr at 0°C in NaCl solutions of different concentrations. Abscissa: NaCl, molar concentration. Ordinate: the activity of cyclic photophosphorylation in μmoles of P_i uptake per mg of chlorophyll per hour after salt treatment.
brane properties. It appears possible that salts of organic acids stabilize sensitive membranes without changing physiological and biochemical functions. It is concluded that colligative action and specific effects on sensitive structures cause protection of biological membranes by salts of organic acids during freezing.

There is little doubt from the in vitro data that salts of organic acids contribute, in addition to sugars and specific proteins, to the frost resistance of plant cells in vivo. An increase in the concentrations of citric and malic acids in the winter months was found in needles from conifers (24) and in leaves of Sempervivum (17). In contrast, changes in the common organic acids could not be found during cold acclimation in the bark of Cornus stolonifera (19). No information is available on the distribution of the organic acids within the frost-resistant cells. For efficient protection, an increase in the concentration of organic acids would have to occur in the vicinity of frost-sensitive membranes, e.g., in the chloroplasts and mitochondria. An increase in the concentration of protective compounds in the vacuole, for instance, would not result in protection.

Kappen and Ulrich (16) found that in frost-resistant halophytes the concentration of electrolytes within the chloroplasts is much higher than that of sugars. Isolated thylakoids of halophytes are as sensitive to increased levels of salt as thylakoids from other plants. In this case the deleterious effects of increased salt concentrations during freezing have to be counteracted by compounds other than sugars. Salts of organic acids are, among other protective material, likely to be involved in the protection of this plant material.

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LITERATURE CITED