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STABILIZATION AND INACTIVATION OF BIOLOGICAL MEMBRANES DURING FREEZING IN THE PRESENCE OF AMINO ACIDS

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SUMMARY

As a contribution to the understanding of frost tolerance of cells and organisms the effect of amino acids on thylakoid membranes during freezing was investigated.

Freezing inactivates photophosphorylation of thylakoids by irreversibly altering essential membrane properties. Washed thylakoids, frozen in the presence of some amino acids such as proline, threonine, γ-aminobutyric acid or lysine·HCl, were protected against inactivation by freezing. Membrane-toxic compounds such as inorganic salts reduced the protection.

Other amino acids such as glycine, alanine, serine, hydroxyproline, sodium or potassium aspartate or glutamate were unable to prevent the alteration of washed membranes by freezing. In fact, membranes protected by sucrose or by cryoprotective amino acids became inactivated during freezing when an excess of these amino acids was also present. Although individually unable to provide protection, in certain combinations with one another these amino acids became protective. Even in combination of amino acid with a membrane-toxic inorganic salt such as NaCl was protection observed. For effective protection a suitable ratio between membrane-toxic compound and amino acid had to be maintained. Departure from this ratio to either side resulted in inactivation.

A third group of amino acids, among them phenylalanine, tyrosine, valine, leucine, isoleucine, methionine and arginine·HCl, did not prevent freeze inactivation of thylakoid membranes either in the absence or in the presence of inorganic salts. Membranes protected against inactivation by a cryoprotectant such as sucrose became inactivated during freezing if one of these amino acids was also present.

Membrane inactivation during freezing is due to the accumulation in the unfrozen part of the system of potentially membrane-toxic compounds such as inorganic salts or amino acids possessing apolar side chains, and in some instances perhaps also to eutectic solidification of the complete system. Protective compounds protect during freezing partly by colligative action, *i.e.* by their unspecific ability to reduce the concentration of toxic solutes below the limit of toxicity. Further, specific interaction between cryoprotectants and membranes plays an important role in membrane preservation during freezing.

The results indicate that the accumulation of potentially toxic cell com-

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ponents, such as inorganic but also organic cell compounds, which become concentrated up to toxic levels during extracellular freezing, are the cause of injury during freezing of frost-sensitive cells. In frost-hardy cells, protection is provided by compounds that reduce non-specifically the concentration of toxic substances or that protect specifically by membrane stabilization.

INTRODUCTION

Both in the plant and the animal kingdoms a number of organisms can withstand the effects of subzero temperatures, while others are killed by freezing. Resistance of cells to low temperatures may be acquired and can be lost. While many plants and animals are always sensitive to freezing, others are sensitive only during the summer. Even cells of poikilothermic organisms, which are killed during exposure to cold, can survive freezing in the presence of cryoprotectants. This shows that resistance to freezing may be a function of the composition of cell contents and medium surrounding the cells. In recent years evidence has accumulated that injury to cells during freezing is caused by the susceptibility of cell membranes[1–5]. During freezing, cells become dehydrated owing to the crystallization of water. As a consequence, the concentration of cell constituents increases dramatically. Depending on their composition, concentration levels of individual compounds may be reached where interaction with cell membranes takes place and leads to membrane alterations that result in cell death. For red cells LOVELOCK[6] has demonstrated that concentration of salts during freezing is responsible for cell death.

However, a detailed investigation of the effects of individual compounds on cell stability during exposure to cold is made difficult by the permeability barriers separating intact cells from the surrounding space which often prevent additives from entering the cells. For this reason a simpler system was sought, representative of the cell in its freezing response, but open to factor analysis and not subject to its limitations. Of the different membranes of the cell that appear to be subject to freezing damage not all are equally suited as test systems, since, apart from isolation problems, not for all of them can functional integrity and its loss be easily demonstrated. Isolated membranes from chloroplasts of leaf cells are biochemically highly active and use light energy to synthesize ATP from added ADP and phosphate. ATP synthesis depends on the morphological and functional integrity of the membranes. Only slight alterations in membrane properties result in the loss of phosphorylation even though electron transport accompanying phosphorylation may remain unaffected.

Freezing of thylakoid membranes causes inactivation of phosphorylation[7,8]. Cryoprotective compounds, which protect intact cells against freezing, also protect thylakoid membranes against inactivation of phosphorylation[8–11]. For these and other reasons it is thought that thylakoid membranes are part of the membrane systems of the cell that are responsible for the sensitivity of cells to cold. In order to elucidate the effects which elevated concentrations of some cell constituents might exert on the cell and its membrane systems during freezing, isolated thylakoids were frozen under different conditions in the absence and in the presence of different amino acids. A brief account of some aspects of this work has been given recently[12].

EXPERIMENTAL

Chloroplasts were isolated from field-grown leaves of spinach (*Spinacia oleracea* L.) in a medium containing NaCl as described previously[8]. After isolation, they were ruptured in water. Thylakoid membrane systems were freed from soluble chloroplast components by two washings with water[11]. Owing to osmotic water uptake by the membrane vesicles the final sediment was semifluid and contained approx. 4 mM NaCl. All operations were carried out at 4°. After the addition of equal volumes of solutions containing amino acids and/or NaCl or KCl to aliquots of the semifluid sediment the resulting membrane suspensions were preincubated for 20 min at 0° and then frozen for 4 h at −25° if not indicated otherwise. After rapid thawing in a water bath at room temperature, photophosphorylation of the membranes with phenazine methosulfate as a cofactor was determined as described previously[8,11]. Controls were kept for 4 h at 0° and were also assayed for photophosphorylation activity. Care was taken to ascertain that additives carried over from the freezing experiments into the medium used to assay photophosphorylation did not influence the rate of photophosphorylation. Thus, changes in the rate of photophosphorylation after freezing reflect effects of freezing, not of other parameters. Results are expressed as μmoles of phosphate uptake per mg chlorophyll per h.

RESULTS

Washed thylakoid membranes, suspended in a solution containing 5 mM or less NaCl, were frozen for 4 h at −25°. These membranes showed either a large reduction in or, at higher concentrations of salt, even loss of their ability to form ATP in the light from ADP and phosphate. Addition of increasing amounts of protective additives progressively preserved photophosphorylation. Sugars, glycerol, some organic acids, dimethylsulfoxide and other compounds may serve as protectants[8–11]. Protection was abolished by an excess of inorganic salts. The higher the concentration of cryoprotectant, the more salt was required to inactivate the membranes during freezing.

Photophosphorylation rates of washed chloroplast membranes as used in this work were, at the start of the experiment, usually 600–750 μmoles per mg chlorophyll per h. Loss of activity during 4 h storage at 0° due to aging was sometimes negligible (less than 5 %), sometimes significant. In the latter event fully protected chloroplast membranes had, after freezing, higher photophosphorylation activity than the unfrozen controls owing to the extremely slow aging of protected membranes at −25°.

Different amino acids acted very differently on the membrane system during freezing. Depending on their effects they may be arranged in three different groups.

Group 1

Fig. 1 shows the phosphorylation capacity of washed thylakoids after freezing for 4 h at −25° in the presence of different amounts of proline, threonine, γ-aminobutyric acid, arginine succinate, lysine·HCl, and of sucrose. In this experiment, little phosphorylation survived freezing in the absence of protective compounds. However, with increasing amounts of the amino acids or of sucrose present, phosphorylation increased until frozen samples had the same activity as unfrozen controls. Although

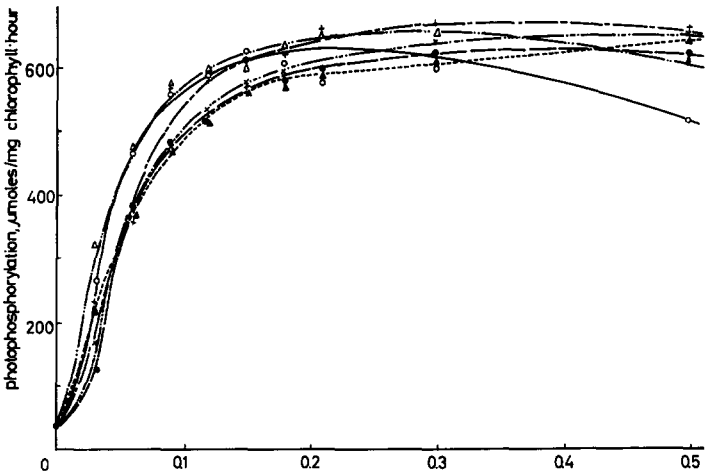

Fig. 1. Protection of washed thylakoid membranes against freezing by amino acids of Group 1 as a function of amino acid concentration (M). Protection by sucrose is shown for comparison. Freezing time 4 h at −25°. Photophosphorylation at a rate of about 650 μmoles per mg chlorophyll per h indicates complete protection. ▲---▲, proline; ×-·-·-×, threonine; ●--●, γ-aminobutyric acid; △-·-·-△, arginine succinate; ○-○, lysine·HCl; +---+, sucrose.

proline and γ-aminobutyric acid appeared to be somewhat less active than sucrose and threonine, no large differences were seen in the protection by the individual compounds.

Salts decreased the protection afforded by the amino acids. In Fig. 2 low concentrations of threonine, while sufficient to protect significantly in the absence of added NaCl, were unable to provide protection in the presence of higher concentrations of

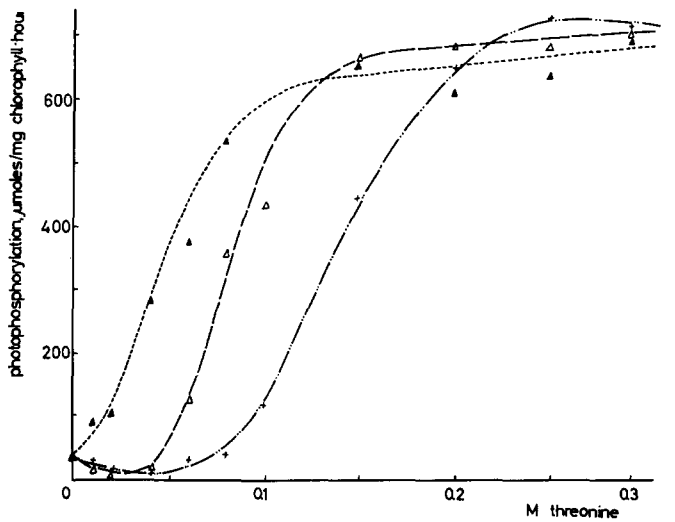

Fig. 2. Protection of washed thylakoid membranes against freezing by threonine in the presence of about 3, 100 and 150 mM NaCl as a function of threonine concentration. Freezing time 4 h at −25°. Photophosphorylation at a rate of about 720 μmoles per mg chlorophyll per h indicates complete protection. ▲---▲, threonine; △ — — △, threonine + 0.1 M NaCl; +-·-·-+, threonine + 0.15 M NaCl.

salt. The more salt present, the more threonine was required for protection. There appeared to be an almost stoichiometric relationship between salt concentration and the level of threonine needed for protection. Similar relations also existed for other amino acids of Group 1, for instance proline[12], and for sucrose (*cf.* ref. 8).

Whereas different amino acids of Group 1 were, on a molar basis, about equally effective in protecting washed thylakoids against freezing damage, resistance to injury was different when the membranes were frozen in the presence of salt. Fig. 3 shows survival, after freezing, of membranes that had been suspended in solutions containing 0.1 M KCl and various amounts of amino acids or sucrose. Without added amino acid, the membranes were damaged during freezing. With increasing concentration of amino acids or of sucrose present, the membranes frozen in the presence of threonine were best protected. Arginine succinate was somewhat less, γ-aminobutyric acid and lysine·HCl considerably more effective than sucrose. Least protective was proline under these conditions.

Group 2

When washed thylakoids were frozen in the presence of different concentrations of serine, actually less phosphorylation survived freezing than in the absence of serine. Similar observations were made with hydroxyproline, which in contrast to proline, was not only not protective if added to washed thylakoids before freezing but added to freezing damage. Unfrozen controls with serine and hydroxyproline showed unimpaired phosphorylation indicating that these amino acids at the concentrations used did not interfere with the phosphorylation system. No protection, and in fact increased freezing damage, was also seen in the presence of glycine, alanine, aspartate and glutamate. However, when a third component such as NaCl, which by itself adds to freezing damage[6,8,13], was added to the system, protection was observed. This is

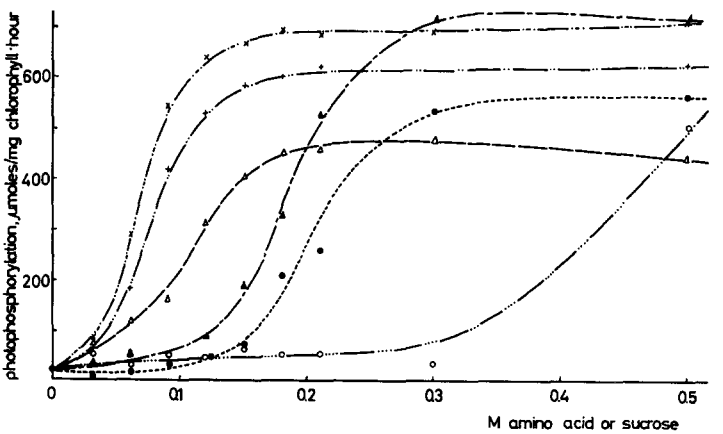

Fig. 3. Protection of washed thylakoid membranes against freezing by amino acids of Group 1 in the presence of 0.1 M KCl as a function of amino acid concentration. Protection of sucrose is shown for comparison. As compared with Fig. 1, protection is decreased by KCl in all cases, although to different extents. Freezing time 4 h at $-25°$. Photophosphorylation at a rate of about 700 μmoles per mg chlorophyll per h indicates complete protection. ○-···-○, proline + 0.1 M KCl; ×–·–·–×, threonine + 0.1 M KCl; +···+, γ-aminobutyric acid + 0.1 M KCl; ●---●, arginine succinate + 0.1 M KCl; △–△, lysine·HCl + 0.1 M KCl; ▲---▲, sucrose + 0.1 M KCl.

shown in Fig. 4. Washed thylakoids suspended in 0.15 M sucrose, and with no other additive present, were protected against freezing damage. With increasing amounts of NaCl, phosphorylation was progressively inactivated during freezing until at a ratio of more than 1 between salt and sucrose little phosphorylation remained[8]. In contrast,

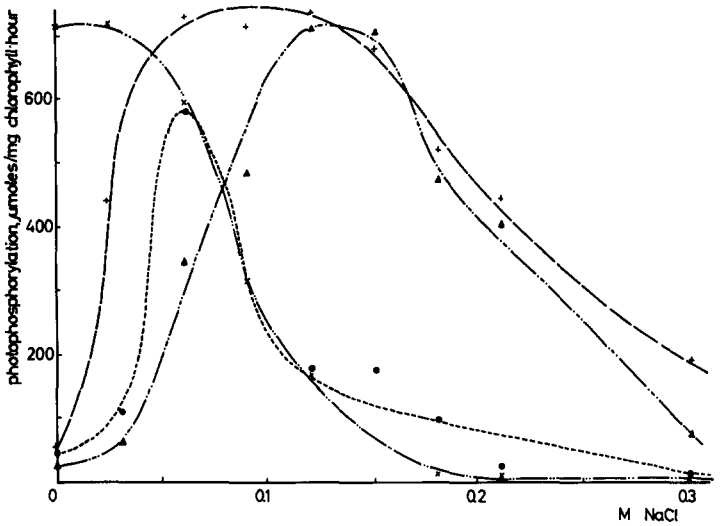

Fig. 4. Protection of washed thylakoid membranes against freezing by amino acids of Group 2 and by sucrose as a function of NaCl concentration. Freezing time 4 h at −25°. Photophosphorylation at a rate of about 720 μmoles per mg chlorophyll per h indicates complete protection. +−−+, 0.15 M α-alanine; ▲−·−·−▲, 0.15 M serine; ●−−−●, 0.15 M hydroxyproline; ×−·−·×, 0.15 M sucrose.

with 0.15 M alanine, serine or hydroxyproline present, and in the absence of salt, the membranes did not survive freezing. Only after the addition of NaCl (or other salts) was protection observed. In this event, NaCl appeared to act over a limited range of concentrations as a protective substance. There were differences in the ratio of salt to amino acid that was optimal for protection. For hydroxyproline the ratio was about 0.5, for alanine about 0.75 and for serine about 1. An increase in the concentration of NaCl beyond the optimal ratio decreased the stability of thylakoids during freezing. Resistance against salt inactivation was similar when thylakoids were protected by 0.15 M hydroxyproline and 0.15 M sucrose. Thylakoids frozen in the presence of 0.15 M serine or alanine had a significantly higher resistance against inactivation by increased levels of NaCl. A similar result was obtained with some organic acids[11].

Survival of membranes suspended in salt and amino acid mixtures and subsequently frozen has also been measured as a function of amino acid concentration[12]. Whereas photophosphorylation of thylakoids, which were frozen in the presence of NaCl only, did not survive freezing, addition of increasing concentrations of amino acids of Group 2 (such as sodium aspartate, sodium glutamate, serine, alanine, hydroxyproline) first increasingly protected and, at excessive concentrations, finally decreased photophosphorylation. Again protection was observed only at certain ratios between salt and amino acid. For sodium aspartate and sodium glutamate the range of protective ratios was broad, for hydroxyproline narrow, and for other amino acids of Group 2 intermediate.

For different concentrations of alanine, protection and final inactivation of thylakoids as a function of salt concentration is shown in Fig. 5. The similarity to protection by the amino acids of Group 1, as demonstrated in Fig. 2, is obvious. A significant difference is the absence of protection in the absence of NaCl, *i.e.* inactivation by an excess of protective agent. An excess of salt also resulted in inactivation.

When amino acids of Group 2 were added in increasing concentration to washed thylakoid membranes that were partially protected by sucrose or amino acids of Group 1, first further protection and then, at higher concentrations, inactivation took place during freezing. Similarly, when washed thylakoids were frozen in the presence of 0.1 M serine and various concentrations of alanine, inactivation was observed at low concentrations and in the absence of alanine. At higher concentrations the interplay of both amino acids resulted in protection (Fig. 6). Increasing the concentration of alanine beyond an optimum again led to inactivation.

The combination of two inorganic salts of considerable toxicity, such as NaCl and KNO_3, always resulted in inactivation of the membranes irrespective of the concentration ratio. Only when the toxicity of one of the salts was considerably lower than that of the other, as with alkali fluorides as compared with chlorides, was some, but not very significant protection observed by a combination of the salts within a small range of concentration ratios.

Group 3

Valine, leucine, isoleucine, methionine, tyrosine and phenylalanine are representatives of a third group of amino acids. The behavior of phenylalanine in freezing experiments with and without NaCl is compared with that of amino acids belonging to Groups 1 and 2 in Figs. 7–9.

When washed thylakoids were frozen in the presence of sucrose, lysine (Group 1), alanine (Group 2) or phenylalanine (Group 3), the membranes supplied with sucrose

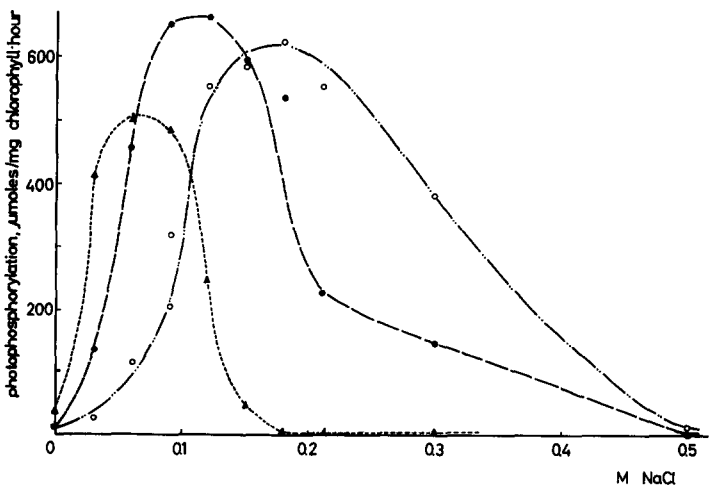

Fig. 5. Protection of washed thylakoid membranes against freezing in the presence of different concentrations of α-alanine and NaCl. Freezing time 4 h at −25°. Photophosphorylation at a rate of about 660 μmoles per mg chlorophyll per h indicates complete protection. ▲---▲, 0.1 M α-alanine; ●––●, 0.2 M α-alanine; ○·····○, 0.3 M α-alanine.

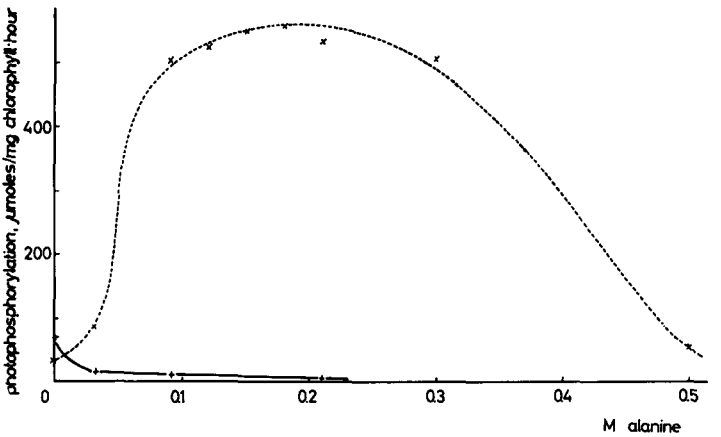

Fig. 6. Protection of washed thylakoid membranes against freezing by combinations of two amino acids of Group 2, *i.e.* serine and α-alanine. Serine or α-alanine alone could not prevent inactivation of the membranes by freezing. Freezing time 4 h at −25°. Photophosphorylation at a rate of about 550 μmoles per mg chlorophyll per h indicates complete protection. Data from two different experiments. ×---×, α-alanine + 0.1 M serine; +—+, α-alanine alone.

and lysine were extensively protected (Fig. 7). Alanine and phenylalanine even promoted inactivation.

In the presence of 0.1 M NaCl only phenylalanine was not protective (Fig. 8). Amino acids of Groups 1 and 2 (threonine and serine) often protected at concentrations where sucrose was not yet effective. However, with increasing concentrations of amino

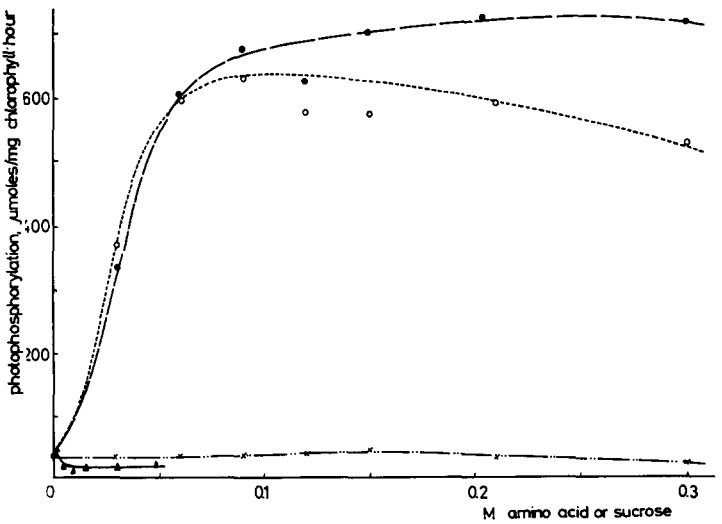

Fig. 7. Photophosphorylation of thylakoid membranes after freezing in the presence of various concentrations of phenylalanine (Group 3), α-alanine (Group 2), lysine·HCl (Group 1), and sucrose. Freezing time 4 h at −25°. Photophosphorylation at a rate of about 730 μmoles per mg chlorophyll per h indicates complete protection. ▲—▲, phenylalanine; ×-·-·×, α-alanine; ○---○, lysine·HCl; ●--●, sucrose.

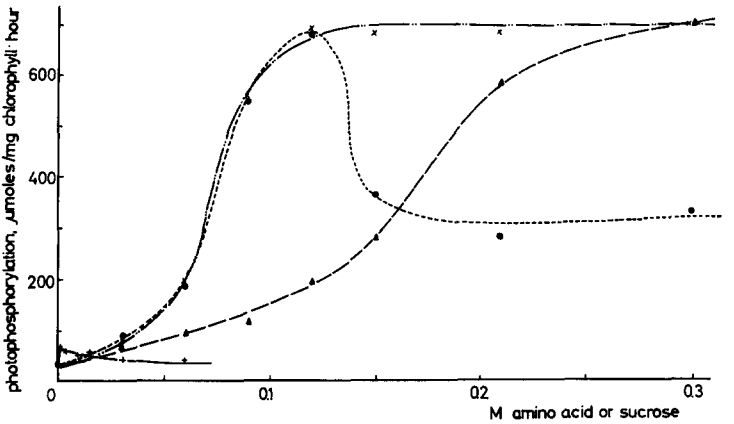

Fig. 8. Photophosphorylation of thylakoid membranes after freezing in solutions that contained in addition to 0.1 M NaCl various concentrations of phenylalanine (Group 3), serine (Group 2), threonine (Group 1), and sucrose. Freezing time 4 h at −25°. Photophosphorylation at a rate of about 700 μmoles per mg chlorophyll per h indicates complete protection. +—+, phenylalanine + 0.1 M NaCl; ●---●, serine + 0.1 M NaCl; ×-·-·×, threonine + 0.1 M NaCl; ▲--▲, sucrose + 0.1 M NaCl.

acids of Group 2 (serine) protection was lost. At high concentrations only amino acids of Group 1 (threonine) and sucrose were effective protectants.

Washed thylakoids protected by 0.1 M sucrose became progressively inactivated during freezing even at low concentrations of phenylalanine (Fig. 9). The addition of amino acids of Group 2 (serine) to the protected thylakoids decreased the protection at much higher concentrations. Amino acids of Group 1 (threonine) added to the protection provided by sucrose even at high concentrations.

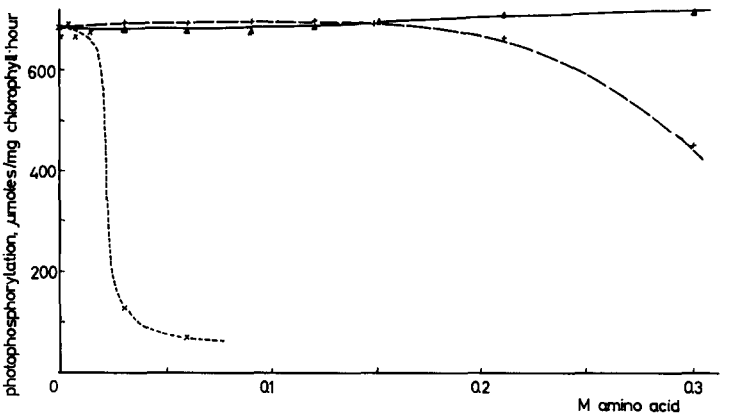

Fig. 9. Photophosphorylation of thylakoid membranes after freezing in solutions that contained 0.1 M sucrose for complete protection and in addition various concentrations of phenylalanine (Group 3), serine (Group 2), and threonine (Group 1). Freezing time 4 h at −25°. Photophosphorylation at a rate of about 680 μmoles per mg chlorophyll per h indicates complete protection. ×---×, phenylalanine + 0.1 M sucrose; +--+, serine + 0.1 M sucrose; ▲—▲, threonine + 0.1 M sucrose.

Thus phenylalanine was not only incapable of exerting significant protection but effectively increased damage. Similar effects were observed with other amino acids of Group 3. A small protective effect at unusually low concentrations will not be considered here.

Thylakoid membranes are stable at pH values not far from neutrality[14]. Therefore, basic and acidic amino acids have to be neutralized before they can be added to the membranes. Arginine and lysine are examples showing that the position of basic (or, for that matter, of acidic) amino acids within a group is not only a function of their molecular properties but also depends on the counter ion. Arginine succinate and lysine·HCl belong to Group 1 since they were capable of protecting washed thylakoids against freezing in the absence and in the presence of added salt. On the other hand, neither arginine·HCl nor lysine nitrate gave protection. Even when present in low concentrations during freezing, they inactivated thylakoid membranes that were protected by 0.1 M sucrose. Thus, their effects on thylakoid membranes during freezing were similar to those produced by Group 3 amino acids.

The following observations show that ice formation is no prerequisite for membrane damage during freezing. Rather, solute concentration is a decisive parameter. Thylakoids incubated in solutions of inorganic salts at 0° lost their photophosphorylation activity with increasing salt concentration and with increasing incubation time[13,14]. The same held true for amino acids of Group 3. The concentrations that were effective in inactivating membranes were comparable with those of NaCl. In contrast, amino acids of Group 2 were, on a molar basis, much less toxic at 0° than amino acids of Group 3. At higher concentrations approaching the limit of solubility they also caused membrane inactivation. The behavior of amino acids of Group 1 was similar to that of sugars. They were non-toxic up to high concentrations.

DISCUSSION

Membrane toxicity

During freezing of cells or simpler biological systems such as isolated membrane vesicles, suspended in suitable solutions, several effects occur simultaneously: the temperature decreases, water crystallizes, the system becomes dehydrated and the concentration of solutes in the unfrozen part of the solution increases. Which of these effects lead to cell death or to the inactivation of isolated cell membranes?

Lowering the temperature cannot be responsible, as it is known that supercooling of cells that, if frozen, are killed, is without effect. Thylakoid membranes suspended in sucrose solutions can be brought to liquid nitrogen temperature without inactivation.

There is excellent evidence reviewed elsewhere that the mechanical stress produced by growing ice crystals does not result in injury to cells as long as ice formation occurs extracellularly as it usually does during slow "physiological" freezing[5,15–17]. Inactivation of isolated thylakoid membranes by freezing is the more extensive the higher the concentration of the salt solutions used for suspending the membranes, whereas the extent of ice formation is inversely related to solute concentration[14]. Also the kinetics of inactivation are different from those of ice formation[14]. This shows that in normal circumstances mechanical effects of ice formation cannot be responsible for inactivation. However, there may be conditions for which this statement does not

apply. If the temperature during freezing reaches the eutectic of the system and if there is no barrier to solute crystallization, complete solidification will occur. Under these conditions mechanical effects produced by crystal growth may contribute to inactivation, as seen by different kinetics of inactivation of thylakoid membranes suspended in NaCl solutions at very low temperatures[14].

LOVELOCK[6] has shown that haemolysis of red cells occurred as a consequence of salt concentration during freezing. Thylakoid membranes are also sensitive to the elevated electrolyte concentrations to which they are exposed during freezing. There are specific effects of different inorganic anions and cations, which become apparent not only during freezing but also during incubation of high concentrations with the membranes at 0° (ref. 13).

As seen from their effects on thylakoid membranes during freezing and at 0° (*cf.* p. 587) amino acids of Group 3, which are also normal constituents of cells, have a potential toxicity, which is similar to or even higher than that of inorganic salts. Amino acids of Group 2 are toxic to thylakoids only at higher concentrations, whereas amino acids of Group 1 do not inactivate membranes. Thus, there is considerable variation in the membrane toxicity of individual amino acids.

A detailed interpretation of toxicity appears impossible as long as the molecular architecture of the membranes is poorly understood. However, it is noteworthy that Group 3 contains valine, leucine, isoleucine, phenylalanine and a number of amino acids having apolar side chains. It is possible to speculate that the concentration of the system brought about by freezing permits these amino acids to form van der Waals bonds with apolar residues of the membranes thereby altering the membrane structure. Damage to membranes caused during freezing by amino acids of Group 2 may be caused in a related way, whereas inorganic salts may be assumed to act on the membranes by suppressing at a suitable ionic strength internal charge–charge interactions. From these considerations it is to be expected that amino acids and salts do not interact at the same sites with the membranes but may, individually or in groups, have different points of attack.

Protection of membranes against inactivation by freezing

There appear to be several possible ways of protecting cells or their membranes against the effects of freezing. From the foregoing it is clear that a major factor in membrane inactivation during freezing is the concentration of solutes to a toxic level in the unfrozen solution surrounding the membranes.

The simplest mode of protection is to prevent a potentially toxic compound during freezing from reaching a toxic level in the unfrozen solution with the aid of a second colligatively acting compound. The total concentration of any unfrozen solution in equilibrium with ice is unequivocally determined by the temperature, since the vapor pressure of ice, which at constant pressure is solely a function of temperature, must be identical with the vapor pressure over the solution, which is a function of solute concentration. Therefore at any freezing temperature above the eutectic temperature the molar concentration of a toxic compound in the presence of the same concentration of a non-toxic substance must be half that found in its absence if solutes show ideal behavior. Since this is usually not so, some deviation from the expected reduction in the concentration may occur. If a non-toxic compound is added to a potentially toxic substance before freezing in a concentration sufficient to reduce the

level of the latter during freezing below the limit of toxicity, no inactivation of the membranes will be observed, although in the absence of the non-toxic compound inactivation may have been complete. The non-toxic compound will thus appear as a protective agent.

LOVELOCK[18] has convincingly demonstrated that glycerol protects red cells, suspended in isotonic NaCl, against freezing damage colligatively by preventing the salt from reaching a critical threshold value of 0.014 mole fraction in the unfrozen solution, which is in equilibrium with ice. The data in Fig. 1 demonstrate that several compounds, rather unrelated in their molecular structure, protect washed thylakoid membranes against freezing damage to a strikingly similar extent. An approx. 0.15 M concentration is required for full protection. The extent of protection of thylakoids against freezing damage is not only a function of the concentration of protective agents but also of the electrolyte level in the suspension, as shown in Fig. 2. The relation between electrolyte and protective effect of a given concentration of protective agent suggests that the latter serves to reduce the concentration of the salt below the toxic level. To do this at a given subzero temperature, a fixed ratio between protective agent and salt has to be maintained. In fact, for all but the lowest concentrations of NaCl the molar ratio between threonine and NaCl at the point of 50 % stabilization of the membranes is approx. 0.85 (Fig. 2). Similar fixed ratios between protective agent and salt have been shown for protection by sucrose (ratio 1)[8], by proline (ratio 3)[12] and by salts of organic acids[11]. The increase in the ratio to higher values that is observed in the presence of very low concentrations of NaCl (Fig. 2) can be explained on the grounds that under these conditions factors different from NaCl concentration, such as membrane concentration, contribute to inactivation during freezing.

While amino acids of Group 1 are protective above a limiting concentration, which is determined by temperature, salt concentration and membrane concentration, an amino acid of Group 2, if added at various concentrations to washed thylakoids, not only does not protect against freezing damage, but rather adds to injury. However, in the presence of salts or of members of its own group or of Group 1 it is very effective as a protective agent. How can this be explained?

Amino acids of Group 2 in high concentrations are toxic and they inactivate membranes. If thylakoids are suspended in a dilute and, therefore, non-toxic solution of an amino acid of Group 2 and are frozen, the concentration of the amino acid increases in the unfrozen portion of the system and will at a sufficiently low temperature reach the limit of toxicity. Membrane inactivation will occur and is in fact observed. If, before the system is frozen to the same temperature, salt is added at a low concentration, it will reduce colligatively the concentration of the amino acid. In the presence of enough salt, the toxicity limit of the amino acid will not be reached during freezing. As a consequence, protection will be observed. Increasing the salt level further will result in a still lower concentration of the amino acid at the freezing temperature. At the same time the salt accumulates during freezing and may itself reach and then exceed its limit of toxicity. When it does, salt inactivation will occur. These relations are shown for several amino acids of Group 2 in Figs. 4 and 5. Their colligative action makes salts and amino acids of Group 2, respectively, appear as protective agents at a ratio between salt and amino acid usually of 0.5–1, while salt toxicity is expressed at higher and amino acid toxicity at lower ratios. This clearly demonstrates the non-specificity of colligative protection.

From the foregoing it follows that inactivation and protection of thylakoid membranes by an amino acid of Group 2 is a function of salt concentration. But the combination of two amino acids of Group 2 such as alanine and serine (Fig. 6) also yields results similar to those described for the system amino acid *plus* inorganic salt. This has important implications.

If different compounds of comparable toxicity affect the membranes in an identical manner, for instance by interacting with the same site within the membrane, colligative protection by a combination of these compounds should be impossible. Even though addition of a second component reduces the final concentration of a first component during freezing, the total concentration of both compounds reached during freezing remains unaffected. As they act in the same way on the membrane, changes in their relative concentrations do not result in changes of the stress to which the membranes are exposed and inactivation occurs. Colligative protection can only be observed by non-toxic compounds, by compounds differing significantly in their membrane toxicity, and by compounds of comparable toxicity if they act on different sites of the membranes. If colligative protection is seen in experiments involving the combination of two equally toxic substances this testifies that their mode of affecting the membrane is different.

It must, therefore, be concluded that serine and alanine specifically interact with the membranes. Their points of attack are different from one another and from that of inorganic salts. Inorganic salts, on the other hand, inactivate the membranes by some common mechanism, as a combination of inorganic salts usually does not lead to colligative protection. Only very slight protection is found if inorganic salts differing widely in their membrane toxicity, such as NaF and KCl, are combined.

In an alternative interpretation of the data, it may be assumed that the damage to membranes produced during freezing in the presence of amino acids of Groups 2 and 3 may be caused by eutectic solidification of the system rather than by amino acid toxicity. The combination of one of these amino acids with others or with salts would shift the eutectic temperature and protect the system by preventing solidification. However, this interpretation does not take into account the observed toxicity at 0°. Further, it has been shown that sodium phenylpyruvate, which is considerably more soluble than the structurally related phenylalanine, but of comparable toxicity, behaves in freezing experiments similarly to phenylalanine. Moreover, if added to thylakoids which are protected by 0.1 M sucrose, amino acids of Groups 2 and 3 at excessive concentrations suppress the protective effect of sucrose and inactivate the membranes during freezing (Fig. 9). Under these conditions eutectic solidification of the system appears impossible as sucrose does not crystallize during rapid freezing. Still, partial crystallization of amino acids during freezing may also be expected to contribute to the observed effects.

Although the efficiency of different non-toxic amino acids in protecting washed thylakoid membranes against freezing is similar (Fig. 1), the resistance of membranes to salt inactivation during freezing is markedly different in the presence of different amino acids of Group 1 (Fig. 3). Even though amino acids of Group 2 are themselves toxic to membranes, some of them (alanine, serine, aspartate and glutamate) render the membranes more insensitive than does sucrose (*cf.* Fig. 4). At first sight this seems to be inconsistent with protection of the membranes on a colligative basis. Different non-toxic compounds, if present in isoosmolar concentrations, should exert the same

protective effect on the membranes. Toxic compounds such as amino acids of Group 2 should always be less, not more, effective than the non-toxic sucrose. However, this view is oversimplifying. It neglects deviations from ideal behavior, which at the high solute concentration reached in the unfrozen portion of the system during freezing may be considerable. Moreover, it does not take into account interactions between membranes and protective agents. From the protection of membranes observed in combination experiments involving two potentially toxic amino acids it has been inferred that their toxicity is caused by specific interaction. The extent of unstabilization of the membranes by specific interaction with different amino acids is different. It is now postulated that, if specific interaction from a certain concentration limit may unstabilize membranes, membrane stabilization should also be possible. In fact, protection of thylakoids against inactivation by inorganic salts in the presence of salts of organic acids was observed not only at freezing temperatures, but also at 0°, although under these conditions no reduction in the concentration of the inorganic salt by the organic acid was possible[11]. Experiments demonstrating the existence of membrane stabilization by some amino acids will be described in a separate publication.

It is likely, therefore, that the increased salt resistance of membranes in the presence of some amino acids is caused by a stabilizing influence on the membranes, even when at high concentrations of these amino acids unstabilizing effects may predominate and result in the inactivation of the membranes. Such stabilizing effects also have to be assumed for the protective action of high molecular weight compounds such as specific proteins[2,9,19,20], which are effective at unusually low concentrations.

The experiments described above were performed with isolated thylakoid membranes. The question arises whether the results are pertinent to the problem of frost hardiness of intact cells. It is well known that some amino acids occur in significant amounts within plant cells. To be effective either as cryoprotectants or as injurious agents they have to be in contact with the frost-sensitive membranes. Chloroplasts isolated by a nonaqueous fractionation procedure from leaves of different plants were found to contain levels of amino acids higher than would be expected from a uniform distribution in the cells[21]: there appeared to be an accumulation of some amino acids in chloroplasts *in vivo*. An increase in the concentration of some amino acids, especially proline, has been reported to occur on exposure of plants to low temperatures[22–26]. Moreover, the addition of amino acids, *i.e.* proline, to plants increased cold resistance[27]. As proline is protective irrespective of the composition of the system (*cf.* p. 580) it must be assumed that it contributes to resistance in those plants where it accumulates. The same considerations apply for other protective amino acids, particularly for those that have a high effectiveness in the presence of inorganic salts.

KAPPEN AND ULLRICH[28] measured Cl^- concentrations up to 0.6 M in chloroplasts from frost-hardy halophytes. Sugar concentrations in the chloroplasts were too low to account for protection. Since thylakoids even from halophytes are senstive to NaCl, it must be assumed that other substances also contribute to protection. GOAS[29,30] found a high level of some amino acids, *i.e.* proline, alanine and γ-aminobutyric acid, in halophytes. The properties of some amino acids as described above and of organic acids[11] make these compounds particularly suited for protection in the presenceof NaCl.

On the other hand, injurious effects of amino acids must also be considered. The action of Group 3 amino acids on thylakoid membranes during freezing leaves little room for doubt that, *in vitro*, toxic amino acids contribute, together with other

potentially toxic cell components, to the frost sensitivity of cells. Their action has to be counterbalanced by cryoprotectants if a cell is to survive during freezing.

In previous publications it was shown that dehydration of thylakoid membranes by drying also results in membrane inactivation[10,31]. Sugars, if present in sufficient amounts, are able to protect the membranes completely during drying. It may be worth mentioning that increased levels of amino acids, *e.g.* proline, were also found in plant cells that had acquired drought resistance[32–34], and the addition of proline to plants decreased the sensitivity against desiccation[35,36]. Obviously, certain amino acids contribute to the resistance of different plants not only against freezing but also against drought.

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