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FREEZING

THE EFFECT OF EUTECTIC CRYSTALLIZATION ON BIOLOGICAL MEMBRANES

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SUMMARY

Thylakoids from isolated spinach chloroplasts were frozen in the presence of various concentrations of inorganic and organic salts, amino acids and sugars and the kinetics of inactivation of cyclic photophosphorylation with phenazine methosulfate and of electron transport reactions were measured as a function of temperature.

During freezing of membranes in the presence of neutral nontoxic compounds membrane damage did not occur until the eutectic temperature was reached. Then photophosphorylation became rapidly inactivated. With weakly membrane-toxic compounds there was a slow inactivation during freezing followed by rapid inactivation at the eutectic temperature. Freezing in the presence of strongly membrane-toxic compounds led to inactivation of photophosphorylation before the eutectic temperature was reached. The temperature at which eutectic crystallization occurred was dependent on the nature of the solutes present. The ratio between solute and membranes was also important: the lower the initial concentration of solutes added to membrane suspensions the lower the temperature at which eutectic solidification occurred. Some compounds such as mannitol crystallized gradually during the decrease in temperature; in this case inactivation of photophosphorylation took place parallel to the crystallization process.

In contrast to photophosphorylation, electron transport reactions were not decreased during eutectic freezing in the presence of neutral membrane-protective compounds. Rather a stimulation of electron transport was observed. However, in the presence of inorganic salts or of sodium succinate, electron transport reactions were also inactivated in addition to photophosphorylation during eutectic solidification. This inactivation seems to be a salt effect and may not directly be related to the crystallization process. Various soluble enzymes and the Ca^{2+}-dependent ATPase of thylakoids were not affected by eutectic crystallization.

The results demonstrate that eutectic crystallization which may take place during freezing is a factor in membrane damage and has to be considered as a possible cause of membrane alterations in *in vitro* studies on freezing resistance.

Abbreviation: DCIP, dichlorophenolindophenol.

INTRODUCTION

In vivo investigations on the primary cause of frost damage and frost resistance of plant and animal organisms are difficult, because organs, tissues and even single cells are highly complex systems. Therefore, attempts have been made to study the effect of freezing on systems which are simpler than intact cells. For instance, isolated mitochondria[1,2], thylakoid membranes[2–5] or enzymes[6,7] and bacteriophages[8] have been used. The freezing behaviour of these biological structures can be investigated under different conditions in simple systems consisting of water *plus* one or more solutes, *e.g.* salts, sugars *etc.* During progressive freezing of simple binary systems first only water will turn into ice until the eutectic temperature is reached where the whole system solidifies. Ternary or somewhat more complex systems will in principle behave similarly, only that before eutectic solidification takes place solutes will also start to crystallize.

The effect of eutectic crystallization on biological structures is largely unknown. Only a few investigations have been published. Lovelock[9] froze β-lipoprotein of human blood plasma in various single salt solutions for 24 h to -40 °C; he found that the higher the eutectic temperature of the suspending solution the greater the degree of damage. Asahina[10] froze sea urchin egg cells in various single salt solutions; they were instantly destroyed at the eutectic temperature. In both cases only inorganic salts, which are known to be toxic at higher concentrations for biological membranes[7] have been used. In the present work the effect of eutectic crystallization on biochemical activities of thylakoid membranes will be investigated.

MATERIAL AND METHODS

Freezing experiments were performed with thylakoid membranes which were obtained by osmotic shock from isolated spinach chloroplasts.

Chloroplast isolation

Leaves of *Spinacia oleracea* L. (100 g) were homogenated for 15–20 s in a Waring blender at 4 °C in 250 ml of a solution containing 0.05 M Tris, 0.35 M KCl, 0.01 M KH_2PO_4, 0.01 M sodium ascorbate, 0.05 M cysteine and 0.04 M β-mercaptoethanol; pH 8.0. After filtering through 8 layers of cheesecloth the homogenate was centrifuged for 5 min at 200 × *g* to sediment debris. The chloroplasts in the supernatant were sedimented at 600 × *g* by a 12 min centrifugation step and were purified by two washings in 0.35 M KCl under the same conditions. Suspension in distilled water resulted in osmotic rupture and freed the thylakoids which were stored at 0 °C.

Chlorophyll was determined according to Arnon[11].

Freezing experiments

Aliquots of the thylakoid suspension were added to solutions containing inorganic and organic salts, sugars, amino acids *etc.* as indicated. During freezing test tubes containing small volumes of the samples (0.5 ml each) were placed into a methanol bath at 0 °C and were gradually cooled with the bath to the respective low temperatures. The cooling rate was 0.5–5 °C/min. The temperature in the

samples was controlled by fine thermocouples connected to a recorder. After reaching the desired temperature the samples were thawed in a water bath of room temperature and stored at 0 °C until photophosphorylation and electron transport reactions were measured. Controls were kept for the same time at 0 °C.

The activity of cyclic photophosphorylation with phenazine methosulfate was assayed as described earlier[7,12].

Light-dependent ferricyanide reduction was measured in a reaction mixture described by Santarius and Ernst[12] by recording the absorbance at 400 nm during illumination with red light[13].

Dichlorophenolindophenol (DCIP) reduction was induced by a broad band of blue light (Corning filter S 782; somewhat below light saturation) and was recorded at 620 nm; as reaction medium 0.05 M *N*-tris(hydroxymethyl)methylglycine (pH 7.6) containing 5 mM $MgCl_2$, 3 mM $NaHCO_3$ and $4 \cdot 10^{-5}$ M DCIP, was used.

Oxygen uptake with ascorbate–DCIP as electron donor and methylviologen as electron acceptor was measured polarographically with a Clark-type electrode; the reaction mixture contained 0.05 M *N*-Tris(hydroxymethyl)methylglycine, 5 mM $MgCl_2$, 3 mM $NaHCO_3$, 5 mM ascorbate, $4 \cdot 10^{-5}$ M DCIP, 1 mM KCN and 0.4 mM methylviologen (pH 7.6).

In addition to photophosphorylation and electron transport reactions the activities of ATPase and of various soluble enzymes were measured in suspensions of osmotically shocked chloroplasts or in leaf extracts.

Leaf extracts were prepared by grinding 30 g of spinach leaves in 125 ml of 0.05 M Tris buffer (pH 8.0) for 20 s in a Waring blender; the homogenate was filtered through 8 layers of cheesecloth and the juice was centrifuged for 5 min at about 2000 × *g*. The supernatant containing various soluble enzymes was frozen after addition of different salts below the eutectic temperature. After thawing enzyme activities were investigated.

Enzyme assays were performed according to Bergmeyer[14] with the exception of Ca^{2+}-dependent ATPase which was measured as described by Vambutas and Racker[15].

The temperature at which eutectic crystallization of suspensions of thylakoids in salt and sugar solutions occurred was determined by recording the time course of cooling. The first temperature jump, starting usually a few degrees below 0 °C, indicated the onset of ice formation, the second jump the eutectic crystallization of the system. It is worth mentioning that the eutectic temperatures of single salt solutions as measured in our system usually were a few degrees below the values recorded in the literature. For instance, eutectic crystallization of KCl in distilled water was found in our experiments to occur at −16 °C, of NaCl at −35–−37 °C and of sodium succinate at −16–−21 °C. Probably, during slow cooling samples "supercooled" below the real eutectic point so that when crystallization took place the heat emission was insufficient to raise the temperature to the real eutectic point. This explains some scattering in the observed eutectic temperatures.

RESULTS

Freezing of isolated thylakoids in 0.1 M solutions of various inorganic salts resulted in inactivation of photophosphorylation before the eutectic temperature

of the system was reached (Fig. 1). Eutectic crystallization of a thylakoid suspension in 0.1 M solution of NaCl was observed at approx. −36 °C, of thylakoids in 0.1 M KCl at approx. −16.5 °C, and of thylakoids in 0.1 M KNO_3 at approx. −4.5 °C; a thylakoid suspension in 0.1 M $MgCl_2$ did not show eutectic crystallization up to −40 °C. In these experiments membrane alteration is a consequence of the increased salt concentration which occurs during freezing when water is converted into ice and salts accumulate together with the membranes in the unfrozen portion of the original suspension[7,16]. It should be mentioned that sensitivity of the membranes towards freezing and increased salt concentration varied considerably by using material of different origin. In a few cases thylakoids were extremely stable and only partial inactivation of photophosphorylation occurred in the presence of NaCl or KCl before the eutectic temperature was reached. During eutectic crystallization photophosphorylation then became completely inactivated. In most instances, however, inactivation of photophosphorylation occurred during freezing long before eutectic solidification took place.

Salts of organic acids which are not as toxic as inorganic salts to thylakoids[7,17] inactivate photophosphorylation only slowly during freezing up to −40 °C (Fig. 2).

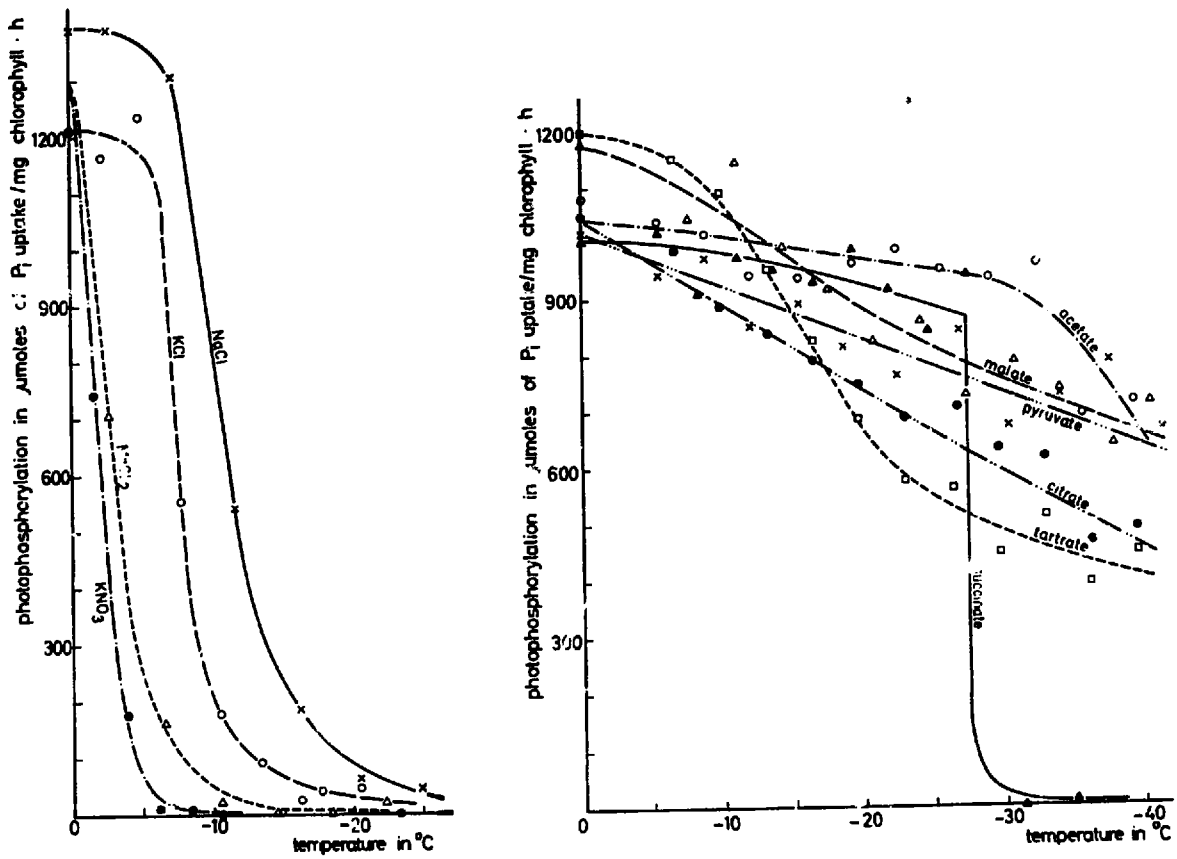

Fig. 1. Activity of cyclic photophosphorylation after freezing of isolated thylakoid membranes as a function of the freezing temperature. Membranes were frozen in the presence of 0.1 M solutions of KCl, NaCl, KNO_3 and $MgCl_2$.

Fig. 2. Activity of cyclic photophosphorylation after freezing of isolated thylakoid membranes as a function of the freezing temperature. Membranes were frozen in the presence of 0.15 M solutions of salts of various organic acids such as sodium acetate, sodium pyruvate, sodium DL-malate, sodium succinate, potassium-sodium tartrate and sodium citrate. Eutectic crystallization in the suspension containing sodium succinate took place at about −27 °C.

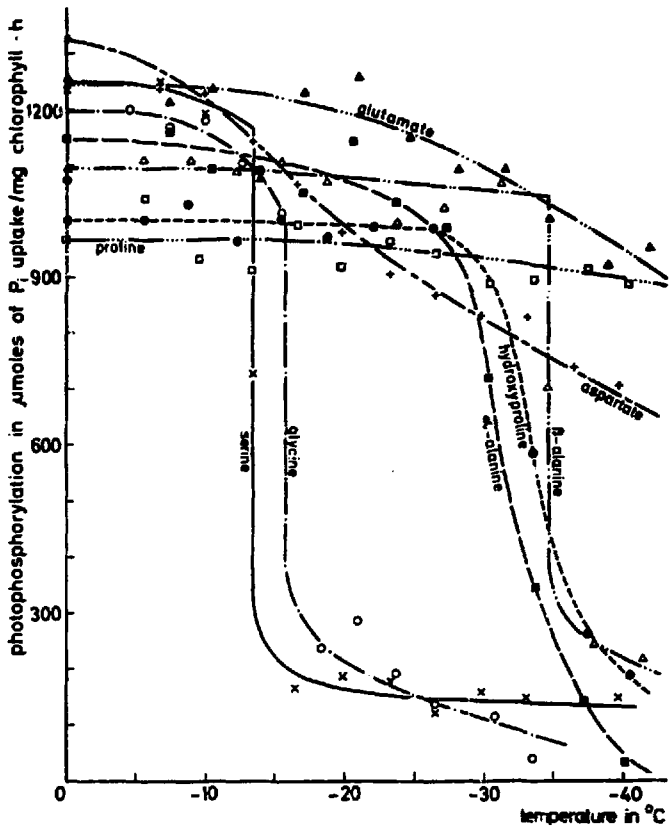

Fig. 3. Activity of cyclic photophosphorylation after freezing of isolated thylakoid membranes as a function of the freezing temperature. Membranes were frozen in the presence of 0.15 M solutions of various amino acids such as glycine, DL-α-alanine, β-alanine, L(−)-serine, the sodium salts of L(+)-aspartic and L(+)-glutamic acid, L(−)-proline and L(−)-hydroxyproline. Eutectic crystallization in the suspension containing serine, glycine or β-alanine took place at about −13, −16 and −35 °C, respectively.

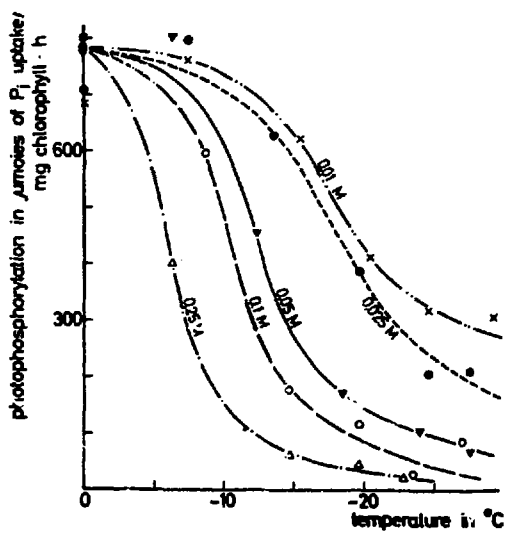

Fig. 4. Activity of cyclic photophosphorylation after freezing of isolated thylakoid membranes in the presence of various concentrations of NaCl as a function of the freezing temperature. Eutectic crystallization of the membrane suspension in 0.5 M NaCl occurred at about −32 °C, in 0.25 M NaCl at about −34 °C and in 0.1 M NaCl at about −37 °C.

However, photophosphorylation of thylakoids suspended in sodium succinate became completely inactivated at the temperature where eutectic crystallization occurred. In some other experiments inactivation during slow progressive freezing in sodium succinate occurred before the eutectic temperature was reached; in these cases also a higher sensitivity of the membranes seemed to be responsible for the different behaviour.

A sudden and almost complete inactivation of photophosphorylation during freezing of thylakoids in the presence of amino acids (Fig. 3) was also an indication of eutectic crystallization irrespective of whether or not the amino acids were slightly toxic (serine, glycine, α-alanine and hydroxyproline) or non-toxic (β-alanine) to the membranes (*cf.* ref. 18). At freezing temperatures above the eutectic point the membranes were only slowly alterated when slightly toxic amino acids were present; β-alanine did not cause membrane alterations during freezing above the eutectic temperature. In the presence of proline, glutamate and aspartate no eutectic crystallization could be found up to −40 °C. Highly toxic amino acids such as phenylalanine or methionine[18] inactivated photophosphorylation at relatively high temperatures. It should be mentioned that α-alanine and hydroxyproline did not show a clear discontinuity in the freezing curve when inactivation of photophos-

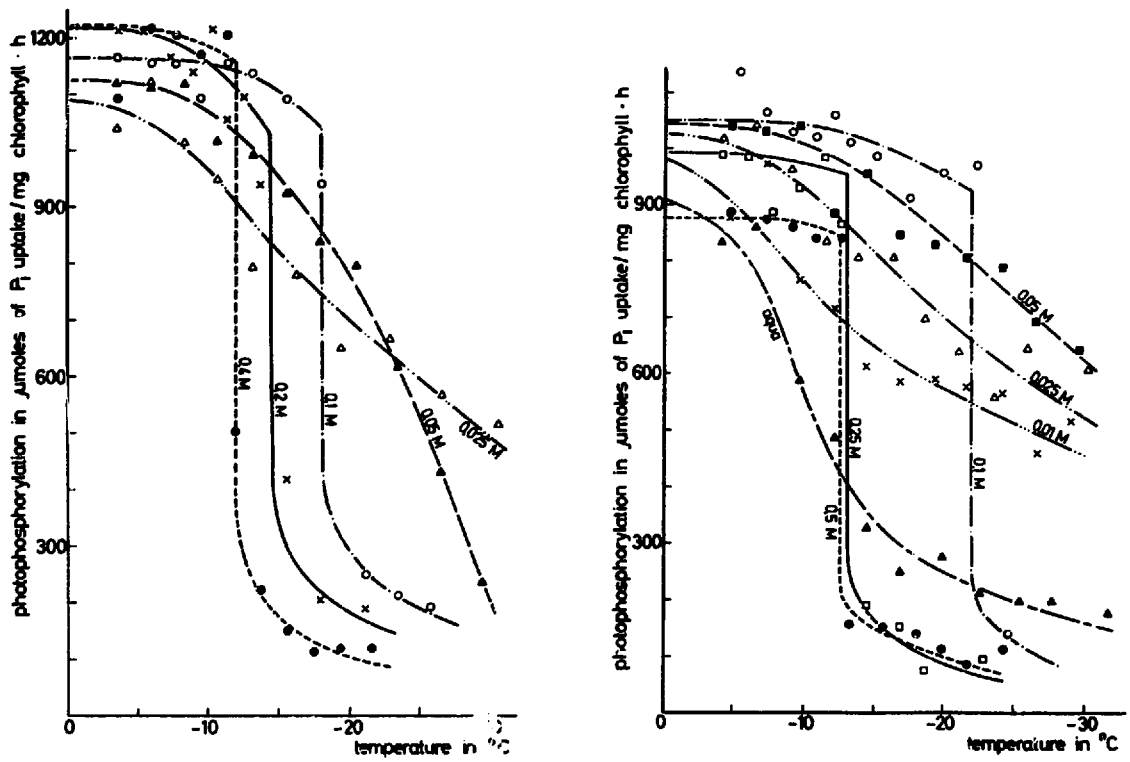

Fig. 5. Activity of cyclic photophosphorylation after freezing of isolated thylakoid membranes in the presence of various concentration of glycine as a function of the freezing temperature. Eutectic crystallization of the membrane suspension in 0.4 M glycine occurred at about −12 °C, in 0.2 M glycine at about −14 to −15 °C and in 0.1 M glycine at about −18 °C.

Fig. 6. Activity of cyclic photophosphorylation after freezing of isolated thylakoid membranes in the presence of various concentrations of δ-amino-*n*-valeric acid as a function of the freezing temperature. Eutectic crystallization of the membrane suspension in 0.5 M amino acid occurred at about −12 to −13 °C, in 0.25 M amino acid at about −13 °C and in 0.1 M amino acid at about −22 °C.

phorylation took place; possibly crystallization was so slow that heat emission could not be noticed with our registration technique (*cf.* also Fig. 7).

The data presented in Figs 1–3 clearly show that eutectic crystallization inactivates photophosphorylation of thylakoid membranes rapidly irrespective of the nature of the solute present.

Inactivation of photophosphorylation as a function of solute concentration and temperature is shown in Fig. 4 for NaCl. Surprisingly inactivation proceeded faster and at higher temperature when the initial solute concentration was high (*cf.* also ref. 16). With decreasing salt concentration inactivation was slower and occurred at lower temperatures. Rates of cooling were identical in these experiments. As demonstrated in Fig. 1, highly membrane-toxic solutes inactivated photophosphorylation before eutectic crystallization occurred. Differences in the ratio between toxic solutes and thylakoids are responsible for the different inactivation rates and inactivation temperatures seen when membranes were suspended and then frozen in different concentrations of the same compound[16].

Slightly toxic compounds (such as glycine[18] or succinate[17]) and non-toxic solutes (such as δ-amino-*n*-valeric acid[19] or β-alanine[18]) alike inactivated photophosphorylation rapidly when the eutectic temperature was reached (Figs 5 and 6). As this temperature was shifted to lower values by changed ratios of thylakoids to added solute or, in other words, by decreased initial concentrations of the added solute at constant thylakoid concentrations, inactivation took place at lower temperatures. For instance, in Fig. 5 thylakoids in 0.4 M glycine showed eutectic crystallization at approx. −12 °C, but the same system in 0.2 M glycine crystallized at approx. −14—15 °C, and therefore, inactivation of photophosphorylation

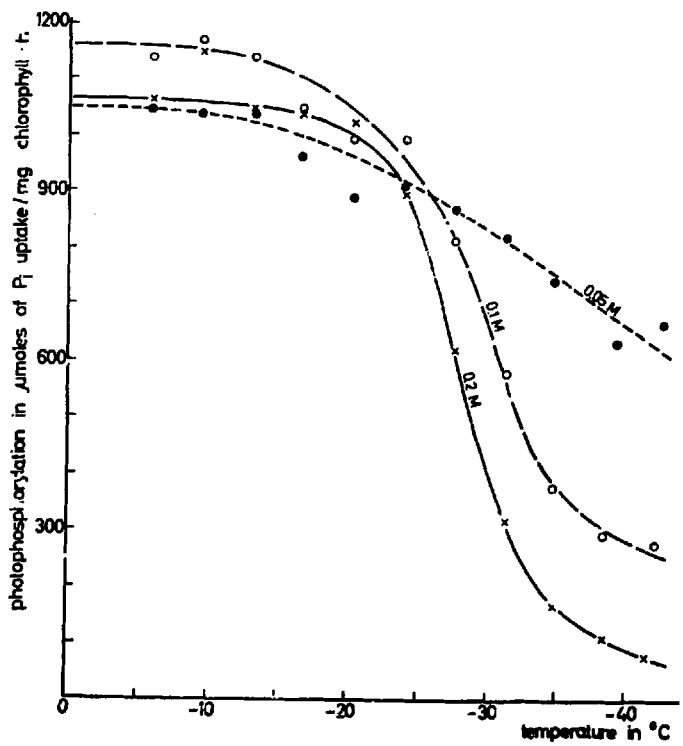

Fig. 7. Activity of cyclic photophosphorylation after freezing of isolated thylakoid membranes in the presence of various concentrations of D(−)-mannitol as a function of the freezing temperature.

occurred at that temperature. Fig. 6 shows that with decreasing solute concentration at constant thylakoid concentration the stabilizing effect of the amino acid on the membranes decreases and inactivation curves approach the freezing behaviour of thylakoids in distilled water. With increasing solute concentration, on the other hand, the eutectic temperature rises and eutectic inactivation takes place.

In earlier experiments on the susceptibility of membranes to drying it was noticed that some carbohydrates such as ribose, arabinose, raffinose and mannitol which were added to the membranes as protective agents tended to crystallize during the drying process. This crystallization resulted in inactivation of photophosphorylation[20]. In Fig. 7 an experiment is demonstrated in which thylakoids have been frozen in the presence of various concentrations of mannitol. In this case inactivation of photophosphorylation occurred over a broad temperature range, probably parallel to the crystallization process in the unfrozen solution of the suspension. With decreasing concentration of mannitol inactivation was again shifted to lower temperatures.

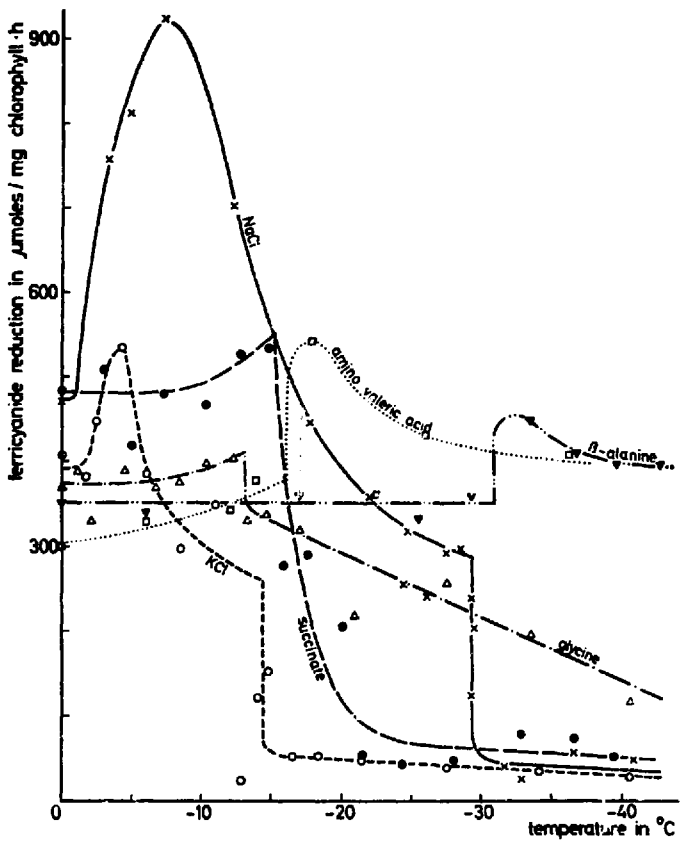

Fig. 8. Activity of light-dependent ferricyanide reduction after freezing of isolated thylakoid membranes in the presence of 0.4 M solutions of various compounds as a function of the freezing temperature. Eutectic crystallization of the membrane suspension took place at the following freezing temperatures: in KCl at about −14 − −15 °C, in NaCl at about −29 − −33 °C, in sodium succinate at about −17 − −19 °C, in glycine at about −12 − −13 °C, in β-alanine at about −31 °C and in δ-amino-*n*-valeric acid at about −16 °C.

In the above experiments photophosphorylation of thylakoids has been used as the sole criterion of membrane integrity. Figs 8–10 demonstrate that light-dependent electron transport of thylakoids is also influenced by eutectic solidifi-

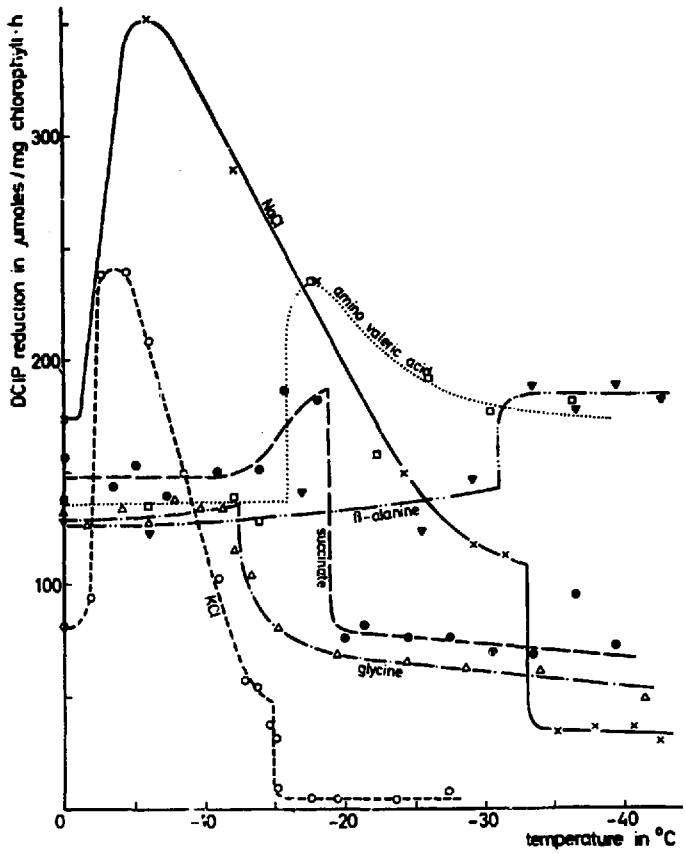

Fig. 9. Activity of light-dependent dichlorophenolindophenol reduction after freezing of isolated thylakoid membranes in the presence of 0.4 M solutions of various compounds as a function of the freezing temperature. The DCIP reduction was not light saturated. Eutectic crystallization see legend to Fig. 8.

cation. Interestingly, different effects are observed. During freezing in the presence of inorganic electrolytes such as KCl and NaCl first a stimulation of ferricyanide reduction, DCIP reduction and oxygen uptake was observed which is caused by the uncoupling of phosphorylation; at lower temperatures progressive inactivation with decreasing temperature is due to the increasing salt concentration[7,21,22]. At the eutectic temperature electron transport is suddenly and drastically decreased. The same holds true for sodium succinate which is not as toxic as KCl and NaCl[7,17]. Glycine, as a mildly membrane-toxic compound does not produce fundamental changes in electron transport during eutectic crystallization. On the other hand, protective compounds such as β-alanine and δ-amino-*n*-valeric acid caused an increase in electron transport during eutectic crystallization, most probably a result of the inactivation of photophosphorylation. These findings are rather surprising as a uniform effect of solidification on the membranes had been expected.

Finally, the effects of eutectic crystallization on the membrane ATPase and on various soluble enzymes have been investigated. As can be seen in Table I, all enzymes investigated here are not influenced by freezing down to and below the eutectic temperature. Therefore, it appears that a main effect of eutectic crystallization on biological systems is the alteration of membranes rather than that of individual enzymes.

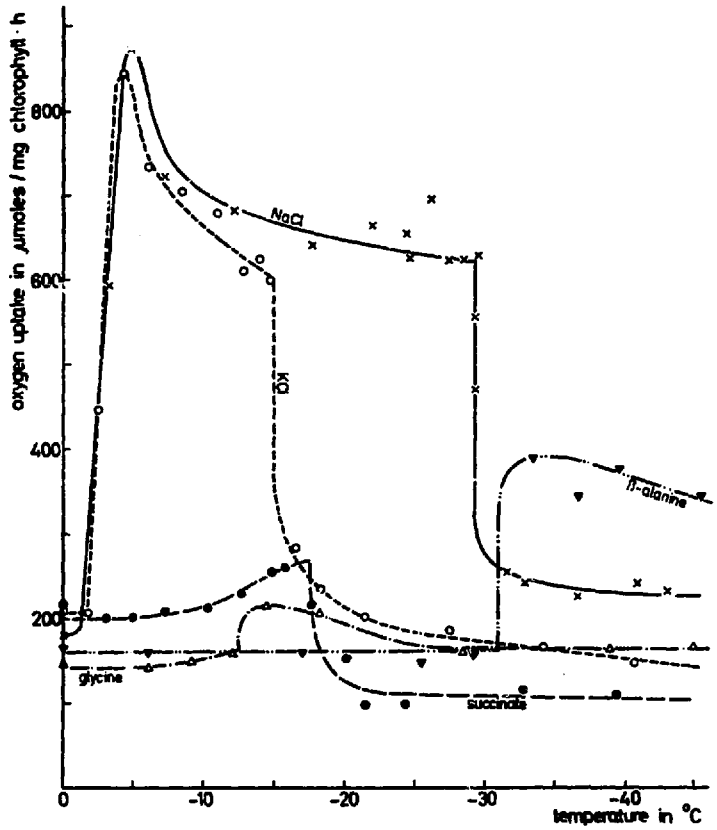

Fig. 10. Activity of light-dependent oxygen uptake after freezing of isolated thylakoid membranes in the presence of 0.4 M solutions of various compounds as a function of the freezing temperature. Eutectic crystallization see legend to Fig. 8.

TABLE I

THE ACTIVITY OF Ca^{2+}-DEPENDENT ATPASE AND VARIOUS SOLUBLE ENZYMES AFTER FREEZING TO TEMPERATURES A FEW DEGREES CENTIGRADE BELOW THE EUTECTIC CRYSTALLIZATION

Enzyme activities are expressed in percent of the unfrozen controls. For the estimation of Ca^{2+}-dependent ATPase, malic dehydrogenase, aldolase and glutamate oxaloacetate transaminase shocked isolated chloroplasts were frozen; glutamate pyruvate transaminase, pyruvic kinase and glutathione reductase were investigated in leaf extracts.

Suspending medium	*0.4 M KCl*	*0.4 M NaCl*	*0.4 M sodium succinate*
Ca^{2+}-dependent ATPase	82	55	110
Malic dehydrogenase	124	112	113
Aldolase	97	114	9
Glutamate oxaloacetate transaminase	117	117	109
Glutamate pyruvate transaminase	92	96	100
Pyruvic kinase	95	107	110
Glutathione reductase	116	103	94

DISCUSSION

During freezing of biological membranes suspended in an aqueous solution first water is converted into ice. As the ice crystals are formed, solutes and mem-

branes become concentrated in the remaining liquid. If the solutes are potentially toxic, the increase in their concentration may result in membrane damage[7,8,16,23]. Further lowering of the temperature progressively removes water from the system until the solubility limit of a solute is reached. In simple binary systems containing also membranes eutectic crystallization occurs at this point and the whole system solidifies. In more complicated systems crystallization of individual solutes together with ice precedes eutectic solidification. There is also the possibility that crystallization of solutes does not occur and, therefore, eutectic solidification cannot take place at all.

The experiments described above show that thylakoid membranes which have not been inactivated by high solute concentrations during freezing suddenly loose phosphorylation activity when eutectic crystallization takes place. Although their eutectic points are rather high, sugars such as glucose (eutectic point −5.1 °C) or sucrose (eutectic point −13.9 °C) do not crystallize during freezing down to the temperature of liquid nitrogen. This is probably due to the relatively large water binding capacity of these sugars[20]. Also in the course of drying of biological material in the presence of glucose or sucrose no crystallization was observed although about 99% of the water was removed[20]. In experiments of Lovelock[9] β-lipoprotein became inactivated during freezing in the presence of NaCl when the eutectic point of the solution was reached; the addition of methanol, glycerol, glucose or sucrose prior to freezing prevented freezing inactivation. It was even possible to freeze-dry β-lipoprotein without appreciable denaturation in the presence of sucrose. Freezing of electrolyte solutions in the presence of dimethylsulfoxide also eliminated eutectic crystallization. This is presumed to be the result of the ability of dimethylsulfoxide to prevent water from freezing and of the capacity of the resulting complex to serve as a solvent for electrolytes[24]. On the other hand, mannitol which crystallizes during drying[20] could not prevent inactivation of photophosphorylation of thylakoid membranes in the course of freezing to low temperatures (Fig. 7).

The question arises whether inactivation of photophosphorylation of thylakoid membranes during eutectic solidification is the result of an inactivation of the electron transport pathway. However, the experiments with isolated thylakoids demonstrated in Figs 8–10 have shown that ferricyanide reduction and DCIP reduction, which are Photosystem II reactions, and oxygen uptake with ascorbate–DCIP as electron donor which is a Photosystem I reaction are not inactivated by eutectic solidification if the latter occurred in the presence of membrane-protective compounds. The increase in electron transport during eutectic crystallization is the result of an uncoupling effect. Therefore, the decrease of electron transport during eutectic solidification in the presence of inorganic salts or succinate seems to be a rather specific salt effect which is related to eutectic crystallization in an as yet unknown manner.

A number of soluble enzymes and the Ca^{2+}-dependent ATPase of chloroplasts did not show significant changes after freezing to temperatures below the eutectic crystallization (Table I). Therefore, of the investigated reactions, only photophosphorylation of thylakoid membranes drastically decreased during eutectic solidification.

The observation that eutectic crystallization leads to inactivation of biological structures is in agreement with results of Asahina[10] on the freezing sensitivity of

sea urchin eggs. Asahina also found that during freezing of cells in electrolyte solutions destruction of the biological material did not occur before eutectic crystallization was observed.

The question arises how does eutectic crystallization alterate biological structures. It is commonly agreed that during intracellular freezing of intact cells the formation of larger intracellular ice crystals causes death mechanically by disrupting structural elements within the cells[24–28]. However, ice formation in suspensions of isolated thylakoid membranes does not in the presence of protective agents result in inactivation. Therefore, the simple formation of ice crystals in the surroundings of the isolated thylakoids cannot be responsible for the observed damage. During eutectic crystallization a complete removal of water from the contacting medium suddenly occurs; this may exert destructive effects on the surface of the biological structures[10]. In addition, complete solidification is accompanied by volume changes of the system which may cause mechanical membrane disruption.

Thus the alteration of isolated membranes during freezing results from two factors: (1) it is a function of the nature and the concentration of toxic compounds, of the exposure time and of the temperature at which the membranes are kept in the frozen state before the eutectic temperature is reached[16], and (2) it takes place in any case during eutectic crystallization. This holds true for non-toxic (protective) and toxic compounds.

In binary systems containing a single solute in addition to water, the eutectic point is independent of the initial concentration of the solute. The more complex systems investigated in this paper contain several components such as thylakoid membranes, a low percentage of stroma proteins and some KCl (from the last washing, concentration about 0.01 M) in addition to the added solute. In such systems the eutectic temperature depends also on the concentration ratios of the individual components: the lower the initial concentration of the added solute in relation to the other constituents the lower the temperature at which eutectic solidification occurs. Mixtures of several solutes in water often have no real eutectic point but a eutectic zone[29] in which a slow and progressive crystallization occurs on lowering the temperature (*cf.* Figs 3 and 7).

In vivo — within the living cell — frost sensitive structures are always surrounded by a mixture of various compounds. These highly complex systems contain solutes such as sugars and proteins which either do not or do not readily crystallize when concentrated and which are capable of preventing the crystallization of other compounds of the system. Therefore, under natural conditions, eutectic behaviour has not been observed in cells[29]. It therefore appears that only during experimental studies *in vitro* eutectic behaviour of suspensions containing biological structures and different solutes has to be taken into consideration.

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