Biochimica et Biophysica Acta, 291 (1973) 23–37

BBA 76135

EFFECTS OF FREEZING ON BIOLOGICAL MEMBRANES *IN VIVO* AND *IN VITRO*

ULRICH HEBER, LINA TYANKOVA* AND KURT A. SANTARIUS
Botanisches Institut der Universität Düsseldorf, Düsseldorf (Germany)
(Received June 29th, 1972)

SUMMARY

Membrane inactivation by freezing has been investigated using intact spinach leaves and isolated thylakoid membranes from chloroplasts of leaf cells as test material. During freezing *in vitro* in solutions containing neutral solute and a slight excess of inorganic salts such as NaCl, electron transport is stimulated while photophosphorylation is lost. Under more drastic freezing conditions damage increases, affecting dichlorophenolindophenol reduction, the rise in variable fluorescence, ferricyanide reduction and electron transport through Photosystem I, in that order. Semipolar compounds such as phenylalanine or phenylpyruvate exhibit a much higher membrane toxicity during freezing than inorganic salts. The profile of damage caused by this class of compounds is different from that caused by salts. Damage to membranes isolated rapidly from frost-killed leaves is similar to that produced by semipolar compounds during freezing *in vitro*. A few sites of damage could be identified, among them the site responsible for oxidation of water during photosynthesis. The results support the view that the sensitivity of their membranes limits the ability of cells to withstand freezing and suggest that freezing sensitivity is due to the accumulation in the cells of potentially membrane-toxic organic and norganic cell constituents.

INTRODUCTION

It is well known that frost-resistant cells are capable of surviving cooling to temperatures far below zero while sensitive cells are killed upon exposure to freezing temperatures. It appears established that cell membranes are the sites of the primary action of frost on cells[1–3]. Freezing of unprotected thylakoid membranes isolated from chloroplasts of leaf cells results in drastic changes in the permeability properties of the membranes[4]. Since a low permeability to certain ions is a prerequisite of one of the functions of these specialized membranes, *e.g.* photophosphorylation, the capability to phosphorylate ADP to ATP in the light is lost. In contrast, under some conditions of freezing *in vitro*, which inactivate photophosphorylation, electron transport reactions of the membranes do not suffer

Abbreviations: DCIP, dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide.

* Present address: Institute of Genetics and Plant Breeding, Academy of Agricultural Sciences, Sofia, Bulgaria.

damage and may even be stimulated[5]. Freezing, therefore, results in uncoupling of phosphorylation from electron transport. Loss of ATP synthesis of thylakoid membranes is a sensitive indicator of a first deleterious effect of freezing on the membrane. Damage is the result of the concentrating effect ice formation has on membrane-toxic solutes in the proximity of the membranes during freezing[6–11]. The extent of this concentration is a function of temperature[12] and of the ratio of membrane-toxic solutes to neutral or membrane-stabilizing solutes[7,8,10]. If this ratio is low, damage can be avoided or may be slight. If it is high, damage is extensive. Extending earlier work the present investigation shows that semipolar compounds may be highly membrane-toxic at the elevated concentrations reached during freezing. It will also be shown that membrane damage is not limited to alterations in permeability properties but extends to other membrane functions such as electron transport.

MATERIAL AND METHODS

Chloroplasts were isolated from fresh or briefly stored (4 °C) leaves of spinach (*Spinacia oleracea* L.) in a medium made isotonic by NaCl[5] and were shocked osmotically in water. After two washings in the centrifuge with water the final membrane suspension contained, per ml, approx. 1.5 mg chlorophyll and 4 μmoles NaCl. Equal volumes of different solutions (pH adjusted to 7.4–7.8) whose effect on the membranes during freezing was to be tested were added to aliquots of the membrane suspension. All operations were carried out at 0–4 °C. After 20 min preincubation at 0 °C the samples were transferred for usually 3 h to a freezer kept at about −20 °C. Kinetic experiments had established that freezing damage was essentially complete after 3 h freezing. Longer freezing times posed problems because of membrane alterations due to aging, which proceeded at different rates in the frozen samples and in the unfrozen controls and made interpretation of the data difficult. Rapid thawing was achieved by immersion in a water bath of 20 °C. Unfrozen controls were kept at 0 °C. Measurements were performed after thawing as rapidly as technically possible.

Cofactor of cyclic photophosphorylation was phenazine methosulfate. Photosystem II reactions were electron transport from water to dichlorophenolindophenol (DCIP)[13,14] and ferricyanide[15], from 1,5-diphenylcarbazide (DPC) to DCIP[16], and kinetics and extent of variable chlorophyll fluorescence at 686 nm[17]. The light-dependent oxidation of ascorbate *via* DCIP[18] by methylviologen was a Photosystem I reaction[13,19].

The reaction mixtures contained: For photophosphorylation: 13 mM Tris, 29 mM NaCl, 4 mM $MgCl_2$, 2.25 mM KH_2PO_4, 2 mM ADP and $6.5 \cdot 10^{-5}$ M phenazine methosulfate (pH 7.8); for the polarographic measurement of oxygen uptake by a Clark type electrode during light-dependent methylviologen reduction: 50 mM *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); for the reduction of ferricyanide: 13 mM Tris, 29 mM NaCl, 4 mM $MgCl_2$, 2.25 mM KH_2PO_4, 2 mM ADP and 0.7 mM ferricyanide; for the reduction of DCIP: 50 mM *N*-tris(hydroxymethyl)methyl glycine, 5 mM $MgCl_2$, 3 mM $NaHCO_3$ and $4 \cdot 10^{-5}$ M DCIP (pH 7.6). To replace water as electron donor $4 \cdot 10^{-3}$ M 1,5-diphenylcarbazide was added where indicated.

Chlorophyll fluorescence of the membranes was excited in a medium containing 50 mM *N*-tris(hydroxymethyl)methylglycine, 5 mM $MgCl_2$ and 3 mM $NaHCO_3$ (pH 7.6) by blue light (Corning filters 4305 and 5562 and infrared filters) of 12 kergs·cm^{-2}·s^{-1} and was measured with an oscilloscope after passing through a filter system transmitting 684 nm light with a half bandwidth of 12 nm (Schott: RG 645, Corning: 2403, Balzers: Calflex C and interference filter B-40, 684 nm). The maximal slope of the fluorescence rise seen between about 100 ms and 2 s after turning on the light is defined as the rate of the rise of variable fluorescence. The extent of the variable fluorescence at steady state is given by [(steady state fluorescence)–(fluorescence at onset of illumination)] (fluorescence at onset of illumination).

Ferricyanide reduction, which was measured at 400 nm, and oxygen uptake were driven by rate saturating red light (Schott RG 630 and infrared absorbing filters), DCIP reduction (measured at 620 nm) by not fully saturating blue light (Corning filter 4303 and infrared absorbing filters). Photomultipliers used for recording fluorescence or dye reductions were protected against exciting light by suitable filter combinations. Photophosphorylation experiments were performed under high intensity white light and were terminated by trichloroacetic acid. Phosphate uptake was determined according to Fiske and SubbaRow[20].

RESULTS

Freezing of leaves

Tables I and II compare photophosphorylation, electron transport reactions and fluorescence of thylakoid membranes which were isolated from frost-killed leaves of different origin (frost-sensitive and slightly hardened) and from unfrozen controls

TABLE I

EFFECTS OF SLOW FREEZING OF INTACT LEAVES FROM FROST-SENSITIVE (NON-HARDY) SPINACH PLANTS (GROWN IN A GROWTH CHAMBER AT 15–20 °C) ON PHOTOCHEMICAL ACTIVITIES OF SUBSEQUENTLY ISOLATED THYLAKOID MEMBRANES

Freezing time 15–20 h at −20–−30 °C. Unfrozen control leaves were kept for the same time at 4 °C. Membrane activities of thylakoids which were isolated from the frost-killed leaves immediately after freezing are expressed as % of reaction shown by membranes isolated from unfrozen control leaves.

	Expt I		*Expt II*		*Expt III*	
	Unfrozen (*μmoles·mg chloro-phyll*$^{-1}$·h^{-1})	*Frozen* (%)	*Unfrozen* (*μmoles·mg chloro-phyll*$^{-1}$·h^{-1})	*Frozen* (%)	*Unfrozen* (*μmoles·mg chloro-phyll*$^{-1}$·h^{-1})	*Frozen* (%)
Photophosphorylation	1050	4	—	—	475	3
DCIP reduction	116	0	43	14	48	2
Ferricyanide reduction	380	18	185	14	185	23
Variable fluorescence, rate of rise	46*	26	63*	2	15*	2
Variable fluorescence, extent at steady state	3.2	59	2.8	31	2.0	38
Methylviologen reduction	81	80	—	—	119	58

* Arbitrary units.

TABLE II

EFFECTS OF SLOW FREEZING OF INTACT LEAVES FROM PARTIALLY HARDENED SPINACH (FIELD MATERIAL, EARLY NOVEMBER 1971) ON PHOTOCHEMICAL ACTIVITIES OF THYLAKOID MEMBRANES

Conditions as outlined in Table I.

	Expt I		*Expt II*		*Expt III*	
	Unfrozen (μmoles·mg chloro-phyll^{-1}·h^{-1})	*Frozen* (%)	*Unfrozen* (μmoles·mg chloro-phyll^{-1}·h^{-1})	*Frozen* (%)	*Unfrozen* (μmoles·mg chloro-phyll^{-1}·h^{-1})	*Frozen* (%)
Photophosphorylation	1080	46	966	26	1380	14
DCIP reduction	164	49	148	58	158	25
Ferricyanide reduction	546	42	402	68	535	31
Variable fluorescence, rate of rise	56*	100	58*	75	36*	23
Variable fluorescence, extent of steady state	3.5	89	3.0	85	1.8	54
Methylviologen reduction	336	51	139	81	186	50

* Arbitrary units.

by rapid homogenation in isotonic buffer. Slight differences in the results even within one group of experiments are not surprising in view of differences in the plant material.

Membrane damage in the unprotected leaves was very extensive. Loss of photophosphorylation was almost complete. Other activities such as DCIP reduction and the rate of the variable fluorescence rise were scarcely less affected. Unexpected and well documented, but as yet unexplained is the occasionally large difference in the sensitivity of Photosystem II-dependent ferricyanide and DCIP reduction (*cf.* pp. 29 and 30), with ferricyanide reduction as the more resistant photoprocess. Both ferricyanide and DCIP reductions were accompanied by about stoichiometric oxygen evolution. Least affected was the Photosystem I-dependent electron transport to methylviologen.

In membranes from slightly hardened material photophosphorylation exhibited the greatest loss of activity. This loss was not as extensive as with membranes from sensitive leaves. DCIP and ferricyanide reduction ranged next in sensitivity to freezing, while freezing effects on fluorescence were much less drastic than with membranes from sensitive leaves.

Several sites of freezing damage can be deducted from the data of Tables I and II. The greater loss in activity of Photosystem I-dependent photophosphorylation as compared with Photosystem I-dependent electron transport to methylviologen is consistent with the view that permeability changes, not effects on electron transport cause inactivation of photophosphorylation during freezing. Still the latter obviously suffers damage during freezing *in vivo*. There was a dramatic restoration of DCIP reduction when DPC was added as electron donor (Table III). This restored DCIP reduction was completely inhibited by $1 \cdot 10^{-5}$ M 3,4-dichlorophenyl-1,1-dimethylurea. This clearly shows that freezing affects also the site of oxygen evolution. However, recovery of DCIP reduction by DPC usually was not complete.

TABLE III

PARTIAL RESTORATION BY DIPHENYLCARBAZIDE OF THE PHOTOREDUCTION OF DCIP BY THYLAKOID MEMBRANES ISOLATED FROM FROST-KILLED LEAVES

Freezing time 15–20 h at −20– −30 °C. Unfrozen control leaves were kept for the same time at 4 °C. Electron transport rates in μmoles DCIP reduction/mg chlorophyll per h.

	Membranes isolated from non-hardy leaves		*Membranes isolated from slightly hardened leaves*	
	Expt. I	*Expt. II*	*Expt. I*	*Expt. II*
Unfrozen control leaves, electron donor water	116	48	148	158
Plus diphenylcarbazide	145	96	141	139
Frost-killed leaves, electron donor water	5	1	86	39
Plus diphenylcarbazide	81	44	156	73

TABLE IV

EFFECT OF WASHING ON THE SUSCEPTIBILITY OF THYLAKOID MEMBRANES TO FREEZING

Thylakoids were isolated from unfrozen, partially hardened leaves. Freezing time 3 h at −20 °C. Unfrozen controls were kept for the same time at 0 °C. Reaction after freezing in % of unfrozen controls.

	Isolated thylakoids suspended in water		*Thylakoids after one washing with water*		*Thylakoids after two washings with water*	
	Unfrozen (μmoles·mg chloro-phyll^{-1}·h^{-1})	*Frozen (%)*	*Unfrozen (μmoles·mg chloro-phyll^{-1}·h^{-1})*	*Frozen (%)*	*Unfrozen (μmoles·mg chloro-phyll^{-1}·h^{-1})*	*Frozen (%)*
Photophosphorylation	875	17	732	1	561	2
DCIP reduction	133	135	133	65	133	64
Ferricyanide reduction	290	115	229	82	205	86
Variable fluorescence, rate of rise	62*	49	42*	33	51*	31
Variable fluorescence, extent at steady state	3.1	93	2.2	140	2.4	133
Methylviologen reduction	116	350	113	158	118	107

* Arbitrary units.

Freezing of isolated thylakoid membranes in water or solutions containing one solute

Table IV shows the effect of washing on the susceptibility to freezing of thylakoid membranes isolated from unfrozen and partially hardened leaves. Unwashed chloroplasts frozen in water largely lost photophosphorylation. The rate of the rise of variable fluorescence was also reduced. However, electron transport reactions were stimulated by freezing, very probably at least in part by the uncoupling of photophosphorylation. Washing the membranes with water at 4 °C reduced photophosphorylation and ferricyanide reduction by about 30% and left other activities largely unaltered. At the same time washing increased the susceptibility

of the membranes to freezing. On freezing the stimulation of electron transport by uncoupling was now more than compensated by inactivation of electron transport. Surprisingly, the extent of variable fluorescence was increased by freezing, while the rate of the fluorescence rise was decreased. A second washing did not intensify the effects observed after one washing.

If compared with the effects of freezing on membrane activities *in situ* (Tables I and II), freezing of isolated washed membranes reduced only photophosphorylation as drastically. Inactivation of electron transport reactions and of fluorescence phenomena was much less marked.

In the following only the sensitivity to freezing of water-washed thylakoids, which were suspended in different solutions, will be considered.

Fig. 1 contains information on protection or inactivation of different membrane activities during freezing of thylakoids in the presence of different compounds.

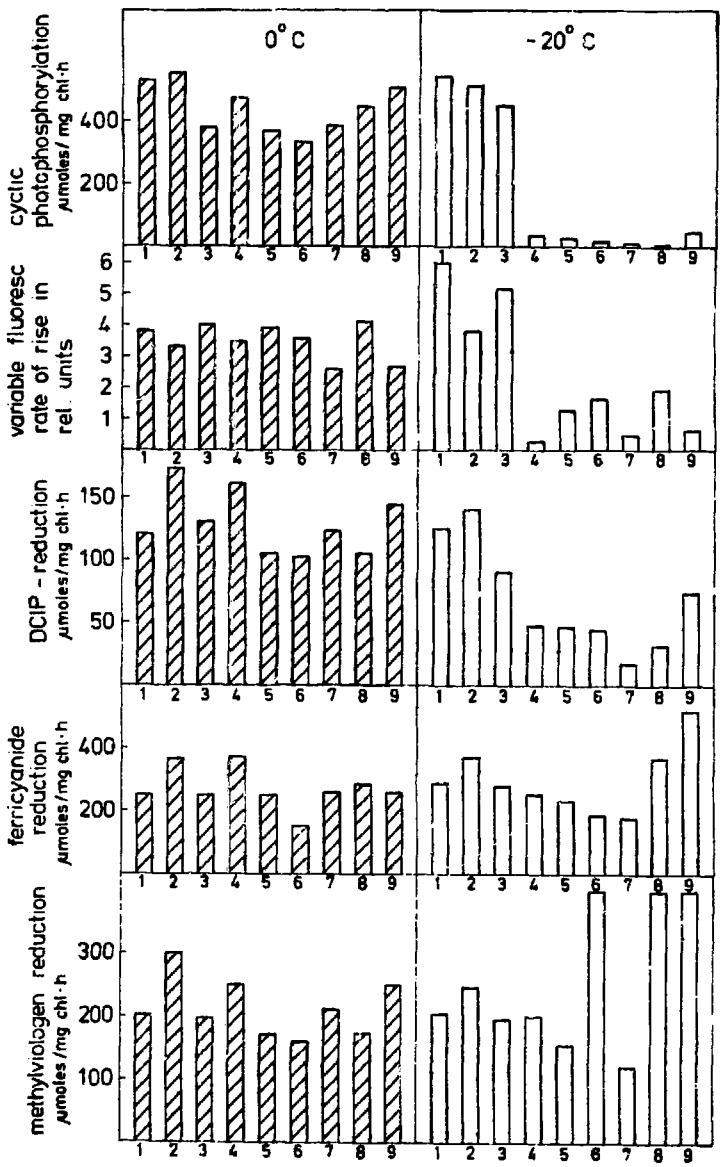


Fig. 1. Effect of 0.1 M solution of different compounds on biochemical activities of thylakoids which were kept for at least 3 h at 0 °C or were frozen for the same period of time to about −20 °C. 1, sucrose; 2, L(−)-proline; 3, β-alanine; 4 L,(−)-hydroxyproline; 5, DL-α-alanine; 6, L(+)-arginine·HCl; 7, DL-phenylalanine (0.05 M); 8, NaCl; 9, water.

Sucrose, β-alanine and proline as examples of cryoprotective compounds were capable of preventing freeze-inactivation of all membrane activities. The higher rate of the fluorescence rise in the frozen samples as compared with the controls, which were kept at 0 °C, is a consequence of the slower aging of the frozen samples. Inactivation by aging is strongly temperature-dependent and can be retarded at subzero temperatures. If freezing does not exert harmful effects as in the presence of sufficient amounts of cryoprotective agents, it stabilizes sensitive reaction such as the fluorescence rise. Freezing in the presence of other compounds such as hydroxyproline, α-alanine or inorganic salts (NaCl) lead to inactivation of the membranes. This is predominantly caused by toxic solute effects[7,8,11]. The inactivation profile was different for different compounds. Freezing for 3 h to about −20 °C in the presence of NaCl strongly inactivated photophosphorylation, much less so the Photosystem II-dependent fluorescence rise and DCIP reduction. Photosystem II-dependent ferricyanide reduction and Photosystem I-dependent methylviologen reduction were even stimulated. Inactivation by arginine·HCl was similar to that by NaCl. In contrast, freezing in the presence of phenylalanine reduced all membrane activities including ferricyanide and methylviologen reduction. Inactivation in the presence of hydroxyproline or α-alanine was less drastic than that caused by phenylalanine. In general, cyclic photophosphorylation had the highest sensitivity to freezing. Somewhat less sensitive were the fluorescence rise and DCIP reduction, both of them Photosystem II-dependent processes. More resistant were the reduction of ferricyanide and that of methylviologen.

Freezing of isolated thylakoid membranes in solutions containing two solutes

A more detailed account of damage caused by freezing of thylakoids in the presence of some representative compounds is given in the following figures. It has been mentioned that the extent of membrane damage is a function of the freezing temperature and of the ratios of toxic to membrane-stabilizing solutes. Fig. 2 shows the influence of increasing ratios of NaCl to sucrose on stability of

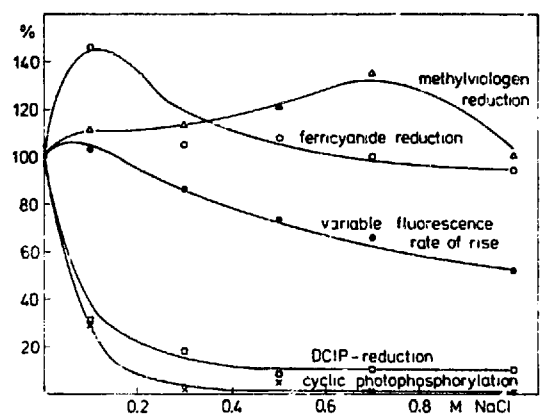
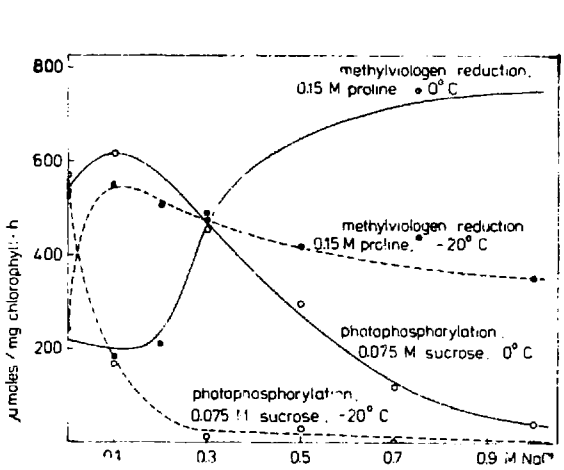


Fig. 2. Inactivation of biochemical activities of thylakoids during freezing for 3 h to about −20 °C as a function of the ratio between salt and sucrose in the suspending medium. Sucrose concentrations were 0.075 M prior to freezing; NaCl concentrations were varied as indicated. To facilitate direct comparison, the activity of frozen samples which were fully protected by sucrose in the absence of added NaCl served as a reference (100%). The rates of the references were (in μmoles/mg chlorophyll per h): P_i uptake during cyclic photophosphorylation, 575; DCIP reduction, 245; ferricyanide reduction, 500; oxygen uptake during methylviologen reduction, 430.

thylakoids during freezing to about −20 °C. The sucrose concentration in the suspension was kept constant at 0.075 M. The data are expressed as percent of the activity of frozen samples which were fully protected by 0.075 M sucrose in the absence of added NaCl. At a low concentration of NaCl sucrose prevented membrane inactivation during freezing. With increasing ratios of salt to sucrose (increasing NaCl concentration) photophosphorylation and DCIP photoreduction became progressively inactivated. In contrast to electron transport to DCIP, electron transport to ferricyanide was first stimulated. The rise in variable fluorescence followed in its freezing response the photoreduction of ferricyanide. Its extent at steady state (not shown) was not significantly influenced by freezing. The Photosystem I-dependent oxygen uptake was stimulated by freezing. An inactivation profile very similar to that shown in Fig. 2 for sucrose–NaCl was also observed in a system containing thylakoids, proline as a stabilizing (*cf.* Fig. 1) agent and NaCl.



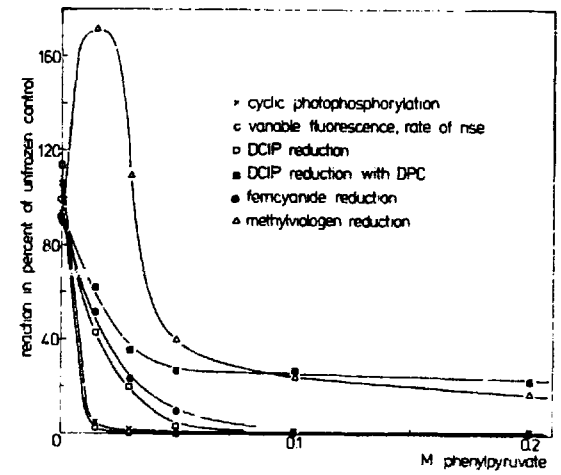


Fig. 3. Cyclic photophosphorylation and methylviologen reduction of thylakoids kept for 3 h at 0 °C and about −20 °C as a function of the NaCl concentration in the suspending medium. Sucrose and proline were also present at a constant concentration and served to prevent membrane damage during freezing in the absence of NaCl.

Fig. 4. Inactivation of biochemical activities of thylakoids during freezing for 3 h at about −20 °C as a function of the ratio between phenylpyruvate (sodium salt) and sucrose in the suspending medium. Sucrose concentrations were 0.075 M prior to freezing; phenylpyruvate concentrations were varied as indicated. Values are expressed as percent of the activities of unfrozen controls which were suspended in the same medium that was used for the freezing experiments. Rates of the fully protected thylakoids in the absence of phenylpyruvate after storage for 3 h at 0 °C (in μmoles/mg chlorophyll per h): P_i uptake during cyclic photophosphorylation, 620; DCIP reduction, 93; DCIP reduction in the presence of DPC as electron donor, 115; ferricyanide reduction, 362; oxygen uptake during methylviologen reduction, 113.

Sensitivity of photophosphorylation to salts is not only expressed during freezing. At concentrations, which are higher than those required for inactivation during freezing, photophosphorylation is slowly lost also at 0 °C (Fig. 3). Other membrane activities were also adversely affected by very high NaCl concentrations at 0 °C. Their resistance was generally much higher than that of photophosphorylation. Oxygen uptake was always stimulated by high NaCl concentrations at 0 °C (Fig. 3).

As shown in Fig. 1, freezing in the presence of phenylalanine caused extensive membrane damage. Since phenylalanine is not sufficiently soluble and may reach

the limit of solubility during freezing, sodium phenylpyruvate which is probably similar in its effect on thylakoid membranes, but much more soluble, has been used instead in the experiment shown in Fig. 4. In the absence of phenylpyruvate the membranes were completely protected against freezing by 0.075 M sucrose. The same concentration of sucrose was also present when phenylpyruvate was added to the system. Very low concentrations of phenylpyruvate were sufficient to overcome the stabilizing effect of sucrose and to inactivate photophosphorylation during freezing. Variable fluorescence (both the rate of the fluorescence rise seen on illumination and the extent at steady state) was completely abolished already by low concentrations of phenylpyruvate. The photoreduction of DCIP and of ferricyanide were also highly sensitive. Oxygen uptake was first increased beyond that of unfrozen controls, but inactivated at higher concentration ratios of phenylpyruvate to sucrose. It is remarkable that DCIP reduction was significantly restored by donating electrons to Photosystem II from diphenylcarbazide (Fig. 4, see also restoration after freezing of leaves, Table III). After inactivation by freezing in the presence of NaCl DCIP photoreduction was stimulated by DPC only occasionally and then to a smaller extent.

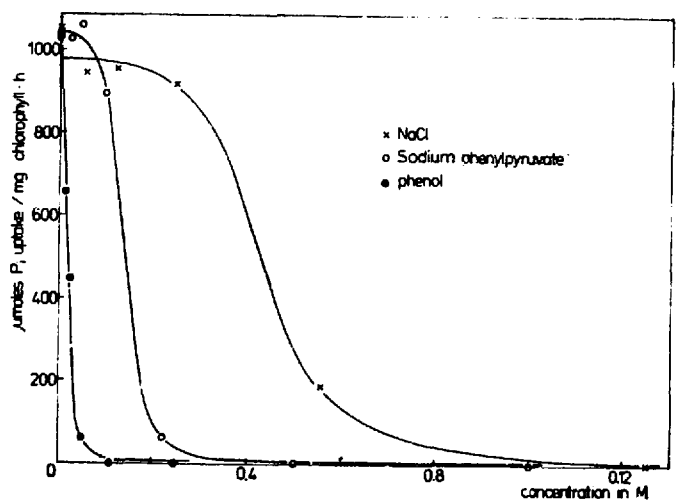


Fig. 5. Sensitivity of cyclic photophosphorylation during freezing of thylakoids for 3 h to about −20 °C in solutions containing sucrose and NaCl, phenylpyruvate (sodium salt) or phenol. Sucrose concentration prior to freezing was 0.1 M; concentrations of NaCl, phenylpyruvate or phenol were varied as indicated.

Thus the inactivation by salt (Fig. 2) and by phenylpyruvate (Fig. 4) are different. During freezing, thylakoids are considerably more sensitive to semipolar compounds, of which phenylpyruvate or phenylalanine are representative examples, than to salts. In Figs 5 and 6 the sensitivity of cyclic photophosphorylation and of ferricyanide reduction to freezing in the presence of NaCl, sodium phenylpyruvate or another semipolar compound, phenol, is compared. Sucrose was also added to the membrane suspension in a concentration sufficient to completely protect the membranes against freezing in the absence of NaCl, phenylpyruvate or phenol. Very low concentrations of phenol (less than $1 \cdot 10^{-2}$ M) already resulted in extensive membrane destruction during freezing. Controls kept at 0 °C were unaffected by these concentrations. Phenylpyruvate did not influence the membranes during freezing at the same concentrations that caused damage by phenol. However at

somewhat higher concentrations membrane damage occurred as is evident from the inactivation of both photophosphorylation and ferricyanide reduction. The membranes were least sensitive to NaCl. Ferricyanide reduction was even drastically stimulated while, at rather high NaCl concentrations, photophosphorylation was inactivated. In other experiments a small, but still conspicuous increase of ferricyanide reduction was also observed during the inactivation of photophosphorylation in the presence of phenylpyruvate or phenol.

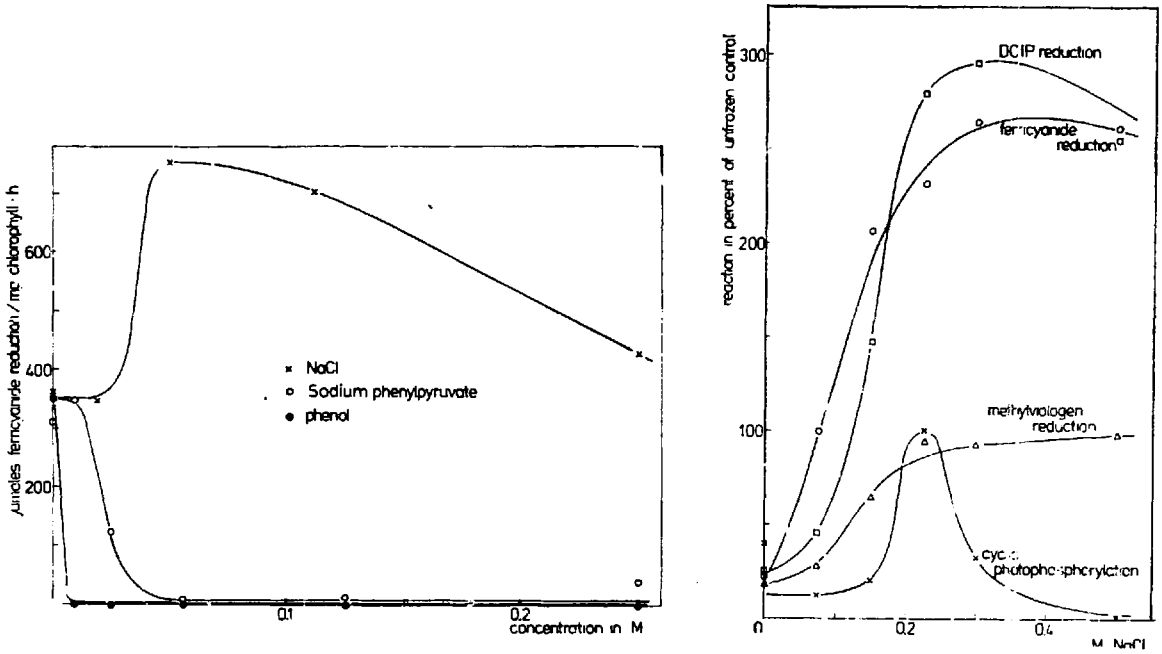


Fig. 6. Sensitivity of ferricyanide reduction during freezing of thylakoids for 3 h to about −20 °C in solutions containing sucrose and NaCl, phenylpyruvate (sodium salt) or phenol. Sucrose concentration prior to freezing was 0.075 M; concentrations of NaCl, phenylpyruvate or phenol were varied as indicated.

Fig. 7. Inactivation and protection of biochemical activities of thylakoid membranes during freezing for 3 h to about −20 °C as a function of the ratio between sodium succinate and NaCl in the suspending medium. The concentration of sodium succinate was 0.15 M prior to freezing; the concentration of NaCl was varied as indicated. Data are expressed as percent of the activities of unfrozen controls which were suspended in 0.15 M sodium succinate without added NaCl and were stored for 3 h at 0 °C (100%). Rates of the unfrozen thylakoid membranes (in µmoles/mg chlorophyll per h): P_i uptake during cyclic photophosphorylation, 111; DCIP reduction, 68; ferricyanide reduction, 182; oxygen uptake during methylviologen reduction, 595.

It does not appear to be accidental that the type of membrane injury produced during lethal freezing *in vivo* (Tables I and II) resembles more the damage caused by compounds such as phenylpyruvate (Figs 4–6) than that caused by inorganic salts (Figs 2 and 6).

In previous work it has been shown that phosphorylation of thylakoids which were frozen in the presence of a number of compounds such as sodium succinate, α-alanine or glycine is inactivated[7,8]. However, if a second component such as NaCl is also present in the system in a certain concentration ratio phosphorylation is preserved. Beyond the protective range of concentration ratios excess of NaCl again results in inactivation. A similar experiment is shown in Fig. 7. Sodium succinate was present at a constant concentration of 0.15 M, while the concentration

of NaCl was varied. At ratios of succinate to NaCl higher than 2 all tested membrane activities except the extent of variable fluorescence (not shown) were drastically affected by freezing. It should be noted that the eutectic temperature was not reached so that mechanical effects of solidification cannot be responsible for damage to the membranes[6]. Obviously succinate is membrane-toxic at the elevated concentrations reached during freezing. Only if a second component such as NaCl is present at a concentration high enough to significantly suppress the rise in the succinate concentration during freezing can damage be prevented. This is the case at a ratio of succinate to NaCl of slightly below 1. A further increase in the concentration of NaCl finally resulted in salt damage to the membranes The two types of damage to the membranes are readily apparent from the different inactivation profiles at either side of the optimal ratio of succinate to salt. Damage caused by excessive concentrations of succinate was similar to that caused by freezing in the presence of phenylpyruvate. However, on a concentration basis succinate was much less toxic than phenylpyruvate. Damage by excess NaCl on the other side of the optimal concentration ratio resembles, as should be expected, the salt damage profile shown in Fig. 2. Results which were similar in principle to those shown in Fig. 7 for combinations of succinate and NaCl were also obtained for α-alanine–NaCl combinations.

DISCUSSION

Physical effects of freezing

The following events take place during progressive freezing: Lowering of the temperature results in ice formation within or outside of the system. The latter is usually the case during physiological freezing of intact cells or tissues. Removal of water to ice crystals concentrates the system. Solutes and suspended particles will accumulate in its unfrozen part until water vapor equilibrium is reached between ice and solution. If during the concentration of solutes the limit of solubility of a component of the system is reached, crystallization will occur on further lowering of the temperature. At the eutectic temperature the system will solidify. The latter events may take place in model systems such as used in part of the present investigation. Crystallization of solutes during freezing rarely seems to occur *in vivo* and eutectic behaviour of cells or tissues has not been observed[21]. Since it appears to play no role *in vivo*, it is desirable to avoid it *in vitro*. This is possible by suitable control of the freezing temperature or by adding components which reduce the eutectic temperature or which do not readily crystallize[6]. In most of the experiments reported eutectic freezing did not occur as is evident from the known eutectic temperatures of the added solutes and, in some cases, from the thermal analysis of the freezing process. In the absence of eutectic freezing, therefore, the observed membrane inactivation cannot be explained by mechanical effects. It is also not caused by the temperature change *per se*. The only alternative remaining is the action of accumulated solutes on the membranes. In fact, at excessive salt concentrations membrane inactivation is observed without freezing at 0 °C (Fig. 3; *cf.* also refs 6, 7, 11, 12).

The total concentration of an unfrozen solution existing in equilibrium with ice is a function of temperature[12]. If several compounds are present during freezing

their colligative action has to be taken into consideration[7,8,10]. If the ratio of a potentially membrane-toxic solute to a neutral or membrane-stabilizing solute is high, damaging levels may be reached during freezing and membrane inactivation will take place. A low ratio will at the same total concentration of solute produced during freezing result in a much smaller concentration of the toxic compound. If this is not yet damaging, protection will be observed. Even a combination of two individually toxic solutes may be protective, if the two compounds act differently on the membranes. In Fig. 7 damage caused by a high ratio of succinate to NaCl is caused by succinate. At a concentration ratio of about 0.7 neither succinate nor NaCl reach damaging levels during freezing and protection is observed. Beyond this ratio NaCl rises to damaging concentrations causing salt inactivation. These relations are also reflected in the profile of damage on either side of protection.

Effects on phosphorylation and electron transport

During normal photosynthesis water is oxidized and electrons are transferred sequentially *via* Photosystems II and I to the physiological electron acceptor[23]. The latter can be replaced by artificial electron acceptors such as DCIP or ferricyanide. Although both of them can accept electrons from the reduced side of Photosystem I[13,24], they appear to interact with the chain mainly at a site between Photosystem II and I[14,15]. Diphenylcarbazide can donate electrons between water and Photosystem II bypassing water oxidation[16]. If no other electron acceptor is present, oxygen will in the light slowly accept electrons from the chain. Under these conditions electron carriers will accumulate in the reduced form on illumination. In the oxidized state the intermediate Q, which accepts electrons from Photosystem II, quenches fluorescence keeping it low[25]. During its reduction fluorescence rises to a maximum. The difference between quenched and maximal fluorescence is defined as variable fluorescence. The rate of the fluorescence rise should be expected to be proportional to electron flow to Q and to be inversely proportional to reoxidation of Q. Bypassing Photosystem II, electrons can also be fed *via* reduced DCIP into the chain. In the light they can be transferred in a Photosystem I reaction to an electron acceptor such as methylviologen. Reoxidation of reduced methylviologen gives rise to oxygen uptake. In the presence of phenazine methosulfate electrons can be shortcircuited into a cyclic pathway incorporating Photosystem I. ATP synthesis is coupled to this electron transport.

In earlier work membrane injury during freezing has been characterized by measurements of light-induced proton transport across thylakoid membranes, of thylakoid shrinkage and of osmotic membrane responses[4]. These measurements demonstrated that loss of photophosphorylation during freezing was not caused by inactivation of the phosphorylating enzyme but was the direct consequence of the loss of semipermeability properties of the membranes. Electron transport to NADP or ferricyanide, which provides the energetic basis for phosphorylation, was shown to be unaffected or even stimulated under conditions that already cause inactivation of phosphorylation[11,12]. Inactivation of electron transport during freezing required salt–neutral solute ratios which were considerably higher than those sufficient to damage phosphorylation. Thus, freezing damage may be viewed as a stepwise process with each step requiring more drastic inactivation conditions. This can be shown by experiments which permit a gradual development of damage.

In Figs 2–6 membranes were first stabilized by a suitable protective agent (sucrose), which does not crystallize during freezing. By adding increasing amounts of a second destabilizing compound protection during freezing was gradually abolished. Such experiments permit the comparison of the extent of damage caused during freezing by different potentially membrane-toxic compounds. In addition, the experiments give evidence on the profile of damage. They further permit an estimate of the different steps of membrane alteration which cause progressive loss of membrane function.

Under the influence of NaCl (Fig. 2), freezing first uncoupled phosphorylation from electron transport. Direct evidence of uncoupling in addition to that already reported[5] was the increased rate of electron transport to ferricyanide. At first sight loss of DCIP reduction, a Photosystem II process, appears to contradict this conclusion. The simultaneous stimulation of ferricyanide reduction and loss of DCIP reduction, which marked a second stage of injury as seen more clearly during freeze-inactivation of proline-protected membranes than from Fig. 2, is difficult to explain on the basis of conventional theory. The tentative attempt to explain loss of DCIP reduction by the development of steric hindrance, which prevents the electron acceptor DCIP, but not ferricyanide from reaching the site of reduction on the membranes, appears to fail since DCIP reduction could in a number of instances (Table III, Fig. 4) be considerably stimulated by diphenylcarbazide which should not alter the site of electron transfer to DCIP. Also feeding of electrons by reduced DCIP into the chain for methylviologen reduction proceeded at rates much higher than those observed for DCIP reduction.

A third stage of injury occurring at high ratios of salt to sugar was indicated by the decrease of electron transport to ferricyanide, which was accompanied by a decrease in the rate of the fluorescence rise. Both processes should be expected to behave similarly as both are Photosystem II reactions. The only photoreaction not significantly decreased during freezing even at high NaCl concentrations was the Photosystem I-dependent methylviologen reduction (Figs 2 and 7). The occasionally large stimulation of this reaction by freezing (Figs 1 and 4) may be attributed in part to the uncoupling of phosphorylation, in part perhaps also to changes in the accessibility of the membrane sites donating and accepting electrons. However, in the presence of excessive succinate (Fig. 7), phenylalanine (Fig. 1) or phenylpyruvate (Fig. 4) even the highly resistant methylviologen reduction was inactivated during freezing indicating another step of membrane injury.

As has been mentioned (p. 26), a few sites of injury can be identified. Restoration of electron transport to DCIP by DPC shows that the water splitting site is affected by freezing. Another site of freezing is on the path to DCIP beyond the site accepting electrons from DPC, a third one in or close to Photosystem I. Uncoupling of phosphorylation is an indication of membrane permeability alterations. It is remarkable that similar damage has been caused by freezing to thylakoid membranes *in situ* in the leaf (Tables I–III) and to isolated thylakoids (Figs 1–7) even though environmental conditions were different. This is additional evidence that the *in vitro* experiments are relevant to the problem of freezing injury of complex cells.

Even though profiles of salt damage and of damage caused by phenylpyruvate and succinate were different, in all cases photophosphorylation was the first mem-

brane activity affected by freezing. Next were DCIP reduction and the rise in variable fluorescence (Fig. 2) or *vice versa* (Fig. 1). Least sensitive were ferricyanide reduction and oxygen uptake during reoxidation of reduced methylviologen. These membrane reactions were occasionally only slightly or not at all inactivated even at high ratios of salt to neutral solute. In contrast, freezing in the presence of only somewhat elevated concentrations of phenylpyruvate affected ferricyanide reduction and even electron transport through Photosystem I to methylviologen not much less than the highly sensitive photophosphorylation. The profile of membrane damage after frost-killing of cells resembled more that caused by *in vitro*-freezing of isolated membranes in phenylpyruvate–sucrose solutions than in salt–sucrose solutions. This is especially significant in the experiments with partially hardened leaves. In these cases a considerable fraction of photophosphorylation survived freezing indicating that protective compounds had already accumulated during hardening[21]. Other membrane activities were also strongly affected, although not as much as photophosphorylation. Under the conditions of partial protection salt damage should have left electron transport in Photosystems I and II largely intact. In fact, stimulation due to uncoupling should have been observed. Damage caused by semipolar compounds such as phenylpyruvate, on the other hand, affects both photophosphorylation and electron transport, the latter perhaps to a somewhat smaller extent. The data suggest that besides salts also semipolar compounds may be responsible for damage to biological membranes during freezing of cells which have not become sufficiently protected during hardening or which are unable to develop freezing resistance by the accumulation of cryoprotectants.

ACKNOWLEDGEMENTS

The competent assistance of Miss I. Strusch and Mrs U. Krüner is gratefully acknowledged. This research was supported by the Deutsche Forschungsgemeinschaft. One of us (L.T.) held a fellowship of the Alexander-von-Humboldt-Stiftung.

REFERENCES

1 U. Heber, *Cryobiology*, 5 (1968) 188.
2 P. Mazur, *Annu. Rev. Plant Physiol.*, 20 (1969) 419.
3 G. E. W. Wolstenholme and M. O'Connor, *The Frozen Cell*, Ciba Found. Symp., Churchill, London, 1970
4 U. Heber, *Plant Physiol.*, 42 (1967) 1343.
5 U. Heber and K. A. Santarius, *Plant Physiol.*, 39 (1964) 712.
6 K. A. Santarius, *Biochim. Biophys. Acta*, 291 (1973) 38.
7 K. A. Santarius, *Plant Physiol.*, 48 (1971) 156.
8 U. Heber, L. Tyankova and K. A. Santarius, *Biochim. Biophys. Acta*, 241 (1971) 578.
9 J. E. Lovelock, *Biochim. Biophys. Acta*, 10 (1953) 414.
10 J. E. Lovelock, *Biochim. Biophys. Acta*, 11 (1953) 28.
11 K. A. Santarius, *Planta*, 89 (1969) 23.
12 K. A. Santarius and U. Heber, *Cryobiology*, 7 (1970) 71.
13 H. T. Witt, B. Skerra and J. Vater, in J. B. Thomas and J. C. Goedheer, *Currents in Photosynthesis*, A. D. Donker Publ., Rotterdam, 1966, p. 273.
14 K. Sauer and R. B. Park, *Biochemistry*, 4 (1965) 2791.
15 J. Kelly and K. Sauer, *Biochemistry*, 7 (1968) 882.
16 T. Yamashita and W. L. Butler, *Plant Physiol.*, 44 (1969) 435.
17 W. L. Butler, in D. R. Sanadi, *Current topics in Bioenergetics*, Vol. 1, Academic Press, New York, 1966, p. 49.
18 L. P. Vernon and W. S. Zaugg, *J. Biol. Chem.*, 235 (1960) 2728.

19 M. Avron, in D. R. Sanadi, *Current topics in Bioenergetics*, Vol. 2, Academic Press, New York, 1967, p. 1.
20 C. H. Fiske and Y. SubbaRow, J. Biol. Chem., 81 (1929) 629.
21 J. Levitt, in H. T. Meryman, *Cryobiology*, Academic Press, London, 1966, p. 495.
22 K. A. Santarius and U. Heber, *Planta*, 73 (1967) 109.
23 H. T. Witt, B. Rumberg, P. Schmidt-Mende, U. Siggel, B. Skerra, J. Vater and J. Weikard, *Angew. Chem.*, 77 (1965) 821.
24 B. Ke, *Plant Physiol.*, 42 (1967) 1310.
25 L. N. M. Duysens and H. E. Sweers, *Studies on Microalgae and Photosynthetic Bacteria*, Jap. Soc. Plant Physiol., The University of Tokyo Press, Tokyo, 1963, p. 353.