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A Biochemical Approach to the Problem of Frost Injury and Frost Hardiness

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Abstract

A general outline is given how the problem of frost hardiness can be approached from a biochemical point of view. A test system containing chloroplast membranes as frost sensitive components is described, which permits the detailed investigation of factors involved in hardening. Sucrose is shown to protect photophosphorylation and electron transport, which take place in the membrane systems of chloroplasts, against inactivation by freezing. Other sugars of low molecular weight are also effective. In the presence of sucrose photophosphorylation and electron transport even survive drying over P_2O_5 . From chloroplasts of hardy spinach leaves a high-molecular factor of protein nature has been obtained, which is more effective than sucrose in protecting chloroplast membranes. Salts interfere with the protection afforded by sucrose or by the protein factor. Unphysiological compounds such as dimethyl-sulfoxide are also capable of preventing the frost inactivation of chloroplast membranes.

Introduction

Exposure of cells to subzero temperatures may or may not result in death. As the temperature is lowered, ice formation is initiated at a certain point and progresses to a final limit on further reduction of the temperature. Water is removed from the cell and ice crystals are being formed, either inside or outside the cell, at the same time. Both the removal of water and the formation of ice may have injurious effects on the cell. Intracellular ice formation, which takes place mainly during rapid freezing (the effects of ultra-rapid freezing cannot be considered here), seems to result nearly always in the death of the cell (Asahina, 1956; Levitt, 1956) most probably owing to mechanical damage caused by ice crystals, which are formed in the protoplasm. When, on the other hand, the temperature is gradually lowered as is most the case in natural environments, the formation of ice proceeds usually outside the cell, in plant material predominantly in the intercellular space. In the course of this ice formation the cell becomes dehydrated. On thawing rehydration takes place. The stage for injury can be set during both phases, dehydration or rehydration. Different causes may attribute to this. Owing to the accompanying volume changes during dehydration and rehydration the cell is exposed to mechanical stress, which may or may not lead to injury. In addition dehydration of sensitive cell constituents itself is injurious. In the former case, physical parameters such as the rapidity of freezing or thawing can be expected to influence the extent of injury, while in the latter biochemical parameters such as the presence or absence of protective substances are determining factors.

Up to the immediate past mechanical causes have been regarded as of major

importance in frost injury (Levitt, 1956). Only in recent years a clear picture of a predominant role of biochemical factors began to emerge (Lovelock, 1957; Heber, 1959 a; Levitt, 1962). The main reasons underlying the differences in view lie, as often in science, in methodical difficulties. There are not many routes of experimental approach to the problems of frost phenomena and one of them, the use of cell fractions rather than of intact cells in the investigation of hardiness problems, has only recently been opened yielding new information on the mechanism of frost injury and frost hardiness (Heber and Santarius, 1964).

The approach to the problem of plant frost hardiness most generally used consists in the analysis of hardening and dehardening phases. When a plant capable of becoming frost resistant is subjected to certain conditions including low temperature treatment an increase in frost tolerance is observed. Conversely, a hardy plant subjected to high temperatures loses its hardiness. A number of well studied biochemical changes accompany the changes in hardiness (Levitt, 1956; Sakai, 1962; Parker, 1963). The question hard to answer from these studies is whether there is a causal relationship between the observed biochemical changes and hardiness. It is obvious from the temperature dependence of metabolic reactions that a change in the environmental temperature produces changes in the biochemical composition of a cell, and these changes need by no means be related to hardiness as shown in an elegant example by Levitt (1954). In addition, the observed changes in a biochemical parameter and hardiness usually do not closely parallel one another. All this renders the interpretation of results obtained in this type of approach difficult.

Since several years another approach to the hardiness problem has been followed in our laboratory. In a living cell the experimental separation of the large number of factors determining a given metabolic state is, at least, most difficult. It thus appears nearly hopeless to investigate with living material the effect of a given factor on the cell separately from that of others. This, however, is feasible, if not a living cell but a representative constituent thereof is used. In other words, since very probably frost affects the proteins of a cell it appeared promising to investigate the effect of different factors, which are suspected to be involved in hardening, on the response of isolated cell proteins to freezing. The major difficulty in this type of study is that a direct relationship between the observed response of the isolated cell constituent and hardiness has to be established in order to give the obtained results their proper meaning.

Initially we have tentatively correlated frost precipitation of a lipoprotein fraction to frost injury of cells (Ullrich and Heber, 1958). The protein fraction characterized by its sensitivity to freezing possesses very interesting properties in that it is protected against freezing by sugars and certain proteins. However, the significance of these findings in respect to hardiness was not perfectly clear until we succeeded, in 1962, in discovering that ATP formation accompanying electron transport in chloroplasts is affected by freezing. The biochemical activities of photophosphorylation and of electron transport are located in the membrane systems of chloroplasts. Suppression of ATP formation could be correlated to frost injury of intact cells (Heber and Santarius, 1964). Furthermore, the chloroplast system has been shown to respond in a manner similar to that of intact cells to various freezing conditions. It therefore appears suitable as a simplified

model of intact cells. One of its main advantages over using intact cells lies in the fact that only the latter contain complicated permeability barriers, which limit the entrance of protective compounds added from outside. The chloroplast system, on the other hand, is easily accessible to most exogenous compounds. Its response to freezing can quantitatively be measured and gives evidence on the extent of protection afforded by added substances. Thus isolated chloroplasts appear to be a very useful system for the *in vitro* study of frost injury and frost hardiness.

The experiments reported in the following deal with the chloroplast system. They pertain to the injurious effects of dehydration, which is caused by ice formation, to sensitive cell constituents, and to the protection against these effects afforded by different substances.

I. Experimental

Chloroplasts were isolated from 100 g of leaves from field grown hardy spinach as described previously (Heber and Santarius, 1964) except that 12 mmoles/l of mercaptoethanol were added to the isolation medium. Occasionally the washing in 0.35 M NaCl-buffer was omitted. The chloroplasts were osmotically ruptured by addition of 8 to 12 ml of water to the $600 \times g$ sediment. Since, contrary to earlier observations, freezing of the resulting suspension did not drastically reduce the rates of photophosphorylation, further washings were performed. The suspension was spun at 30,000 r.p.m. ($75,000 \times g$) for 10 min. The supernatant, which contained salts and soluble components of the chloroplasts (stroma material), was removed and freeze-dried. The sediment was suspended in 24 ml of water and again centrifuged at $75,000 \times g$. The supernatant was discarded, the sediment again washed with 24 ml of water and finally used. Sometimes a third washing was performed. The final sediment consists, after removal of a heavy fraction located at the bottom of the tubes, nearly exclusively of chloroplast membranes (chlorophyll content 1 to 1.5 mg/ml) in the form of osmotically swollen vesicles as shown by electronmicroscopical examination of the sedimented material. These membranes, if supplied with suitable cofactors and substrates, are still capable of supporting active photophosphorylation at rates ranging from between 300 to $900 \mu\text{moles/mg chlorophyll/hr}$. Other procedures are described in the legends to the figures.

Tris (hydroxymethyl) aminomethane (TRIS), crystalline ribonuclease and trypsin were obtained from Boehringer & Söhne, Mannheim mercaptoethanol from Calbiochem, Los Angeles, phenazine methosulfate from Sigma Chemical Company, St. Louis, Cysteine from Schuchardt, München and other chemicals from Merck Darmstadt.

II. Results

1. *Uncoupling of ATP formation from electron transport by freezing and its protection by sugars*

As reported previously (Heber and Santarius, 1964), ATP formation by isolated chloroplasts becomes uncoupled from electron transport during freezing. This is demonstrated by an increase in the rates of the light induced reduction of ferricyanide and by the loss of the accompanying ATP synthesis. Cyclic photophosphorylation catalysed

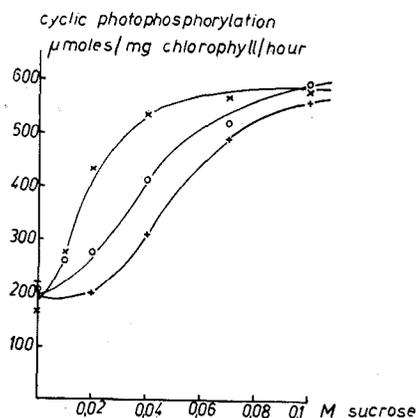


Fig. 1. Protective effect of sucrose on photophosphorylation of chloroplast membranes, which were suspended in dilute NaCl solutions. Freezing for 3 hrs at -25°C ; sucrose concentration as indicated; chlorophyll and salt concentrations: (+) Chloroplast membranes containing 1.13 mg chlorophyll per ml 2×10^{-2} M NaCl (approximate conc.). (O) Chloroplast membranes containing 0.66 mg chlorophyll per ml 1×10^{-2} M NaCl. (X) Chloroplast membranes containing 0.33 mg chlorophyll per ml 0.5×10^{-2} M NaCl. Cyclic photophosphorylation was measured as described previously, (Heber and Santarius, 1964) except that illumination was provided by two 500 Watt photo lamps and the reaction time was 2 min. Photophosphorylation of unfrozen control was $610 \mu\text{moles/mg chlorophyll/hr}$

by phenazine methosulfate behaves similar in that it also is suppressed by freezing. It is therefore considered that here also uncoupling has taken place. Sucrose is capable of preventing the deleterious effects of freezing on the system (Fig. 1). There is a characteristic lag phase in the concentration curve, which is caused by the fact that low concentrations of sucrose (or other sugars) are quite ineffective in protecting photophosphorylation. This lag has been traced to the presence of salts in the system. Salts are capable of abolishing the protection against freezing afforded by sugars in a nearly stoichiometrical fashion. One to two moles of salt are required per mole of sugar to overcome protection. This effect demonstrates that electrolytes are involved in the uncoupling process induced by freezing. Studies of Santarius (1966) on the effects of sugars and salts on the chloroplast system during drying and on the uncoupling action of very high concentrations of salts indicate that the deleterious action of salts is based on a competition between chloroplast membranes and electrolyte for structural water of the membranes. Drying, high concentrations of ions or both may result in a reorientation of structural water of lipoprotein membranes, which leads to inactivation of the system. The presence of an excess of sugars may serve to guard the membrane against the ionic influences.

Removal of salts by washing chloroplasts with water abolishes the lag phase in protection which is observed in the presence of salts (compare Figs. 1 and 2). Whether freezing in the complete absence of salts leads to the same degree of uncoupling as in the presence of low amounts of salts cannot be tested, since photophosphorylation is inactivated by the removal of cations (Jagendorf and Smith, 1962). In the protoplasm of living cells salts are always present (for chloroplasts see Stocking, 1962).

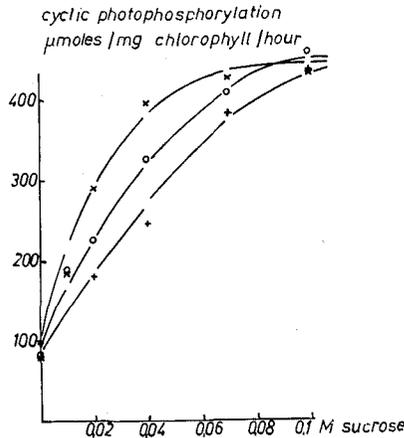


Fig. 2. Protective effect of sucrose on washed chloroplast membranes. Freezing for 3 hrs at -25°C in $ca. 4 \times 10^{-3} \text{ M NaCl}$; sucrose concentrations as indicated; chlorophyll concentrations: (+) Chloroplast membranes containing 0.85 mg chlorophyll per ml. (O) Chloroplast membranes containing 0.42 mg chlorophyll per ml. (X) Chloroplast membranes containing 0.21 mg chlorophyll per ml. Cyclic photophosphorylation was measured as described previously. Photophosphorylation of unfrozen control was 437 $\mu\text{moles/mg chlorophyll/hr}$

Even after the lag phase observed with the crude system is abolished by washing the chloroplast membranes, nearly 2 percent of sucrose is required to give full protection. On a unit weight basis, other soluble sugars are similarly effective. This concentration of sugars is often, but not always reached in hardy plant material. There are many reports in the literature on a more or less close parallelism between hardiness and sugar content (Levitt, 1956). In the light of the foregoing results it may safely be concluded that the sugar content is a very important factor in frost hardiness. However, as sugars do not protect chloroplast lamellae when separated from them by a membrane, only sugars present in the protoplasm of a cell or rather at its sensitive sites can influence hardiness.

From Fig. 2 it can be seen that the amount of chloroplast membranes present in the system is inversely correlated to the degree of protection afforded by a given concentration of sucrose within a critical concentration range. Outside this range the membrane concentration has no influence on the extent of protection.

The protective effect of sucrose is reversible. After the removal of added sucrose by washing the chloroplast membranes freezing results again in uncoupling. Chloroplasts isolated in sucrose buffer are also uncoupled by freezing after they have been washed. Obviously covalent bonding does not play a role in the protection of the chloroplast system. There is reason to assume that hydrogen bonding is involved (Heber and Santarius, 1964).

Frozen chloroplast membranes protected by sucrose can be dried in the frozen state and still retain an appreciable part of their phosphorylative activity (Fig. 3). Unprotected chloroplasts are completely inactivated by drying (Santarius, 1966). Frozen-dried chloroplast membranes, which are protected by sucrose can be stored at room temperature over P_2O_5 without appreciable loss of activity, while chloroplast suspensions are, in the absence or in the presence of sucrose, inactivated during prolonged standing at room

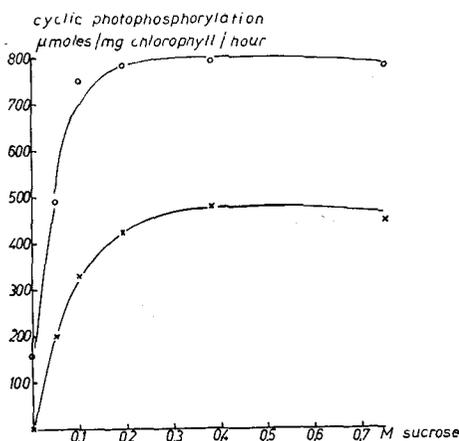


Fig. 3. Protection afforded by sucrose against freezing and against freeze-drying of chloroplast membranes. Upper curve: Rates of photophosphorylation catalyzed by chloroplast membranes, which were previously frozen for 3 hrs at -25°C . Lower curve: Rates of photophosphorylation catalyzed by chloroplast membranes of the same preparation, which were previously frozen 3 hrs at -25°C , subsequently dried *in vacuo* over P_2O_5 for 24 hrs at a temperature lower than -25°C and for another 24 hrs at $+25^{\circ}\text{C}$. Rehydration by addition of water

temperature. Thus sucrose prevents inactivation in the course of dehydration and stabilizes photophosphorylation even under very severe conditions. These effects may not only pertain to frost hardiness, but also to drought resistance (*cf.* Santarius, 1966) and to the preservation of membrane bound phosphorylation in plant organs such as seeds.

2. Influence of freezing on the electron transport chain of chloroplasts

It has not yet rigorously been proven that the whole electron transport chain of chloroplasts remains intact during the uncoupling of phosphorylation, which is induced by freezing. Ferricyanide, which has mostly been used in our experiments, is an unphysiological electron acceptor and it is not yet definitively known at which point it draws electrons from the chain (*cf.* Biggings and Sauer, 1964; Witt *et al.*, 1965). The physiological electron acceptor is NADP. As is shown in Fig. 4, in a chloroplast system protected by sucrose ADP is still phosphorylated to ATP after freezing and ferricyanide and NADP are both reduced in the light. This reduction is accompanied by the evolution of oxygen indicating that the complete electron transport chain is operative. As the sucrose protection is gradually abolished by the addition of salt ATP formation becomes uncoupled from electron transport during freezing. This is demonstrated by the decrease in the rate of ATP formation and by the corresponding increase in the rate of ferricyanide reduction. A further increase in the concentration of salt not only uncouples phosphorylation from electron transport but also impairs the electron transport chain itself as evidenced by a drastic decrease in the rates of ferricyanide and NADP reduction. It thus depends on the conditions whether an increase or a reduction in the rate of electron transport is observed as a consequence of freezing. However, only phosphorylation is affected primarily. As has been observed by Santarius (1966), rigorous removal of water by drying leads first to uncoupling of phosphorylation. Subsequently photoreduction is

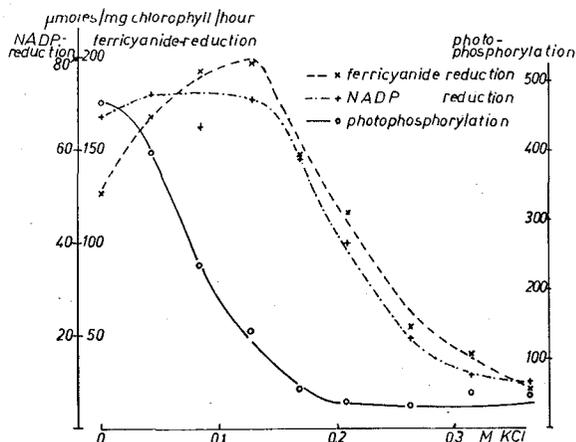


Fig. 4. The effect of freezing in the presence of different concentrations of KCl on photo-phosphorylation and electron transport of chloroplast membranes, which were protected by 0.075 M sucrose. Freezing of the chloroplast membranes for 3 hrs at -25°C . NADP reduction: Chloroplast fragments ($70 \mu\text{g}$ chlorophyll) were illuminated ($100,000 \text{ Lux}$) for 1 min in a reaction mixture containing 0.05 M TRIS, 1.2×10^{-3} M ADP, 1.2×10^{-3} M phosphate, 2×10^{-2} M NaCl, 2×10^{-3} M MgCl_2 , 5×10^{-4} M NADP and aqueous chloroplast extract equivalent to 0.4 mg of chlorophyll; total volume 1.4 ml, pH 7.8. The reaction was followed at $340 \mu\mu$. Cyclic photophosphorylation and ferricyanide reduction were determined as described previously except that illumination was provided by two 500 Watte photo lamps and the reaction time was 2 min

also inactivated. Thus the effects of freezing and drying on chloroplast membranes are similar. Chloroplasts isolated from non-hardy spinach immediately after frost-killing of the leaves not only have lost the capability to synthesize ATP (Heber and Santarius, 1964), but also to reduce ferricyanide. Thus freezing can affect electron transport in addition to phosphorylation not only *in vitro*, but also *in vivo*.

In mitochondria of spinach leaves, electron transport is also severely damaged by freezing in the presence of salts (Heber and Santarius, 1964).

3. The protection of the chloroplast system by a high-molecular factor contained in chloroplasts

In the present study it was noticed that chloroplasts from hardy spinach leaves, which were prepared as outlined previously (Heber and Santarius, 1964), were only slightly uncoupled by freezing. In the earlier experiments mainly non-hardy spinach was used and excellent uncoupling had been observed using the same procedure (Heber and Santarius, 1964). It is likely that the differences in the results are caused by differences in the degree of hardiness in the material used rather than by some other factor. For in the spring, when the spinach became frost-sensitive, chloroplasts were again easily uncoupled by freezing. The question had to be answered, why hardy spinach chloroplasts are less easily uncoupled than non-hardy ones. A possible explanation would be that chloroplasts from hardy leaves, which are rich in soluble sugars, still contain sufficient sugar to protect phosphorylation against freezing. However, this possibility was not borne out by the results of sugar analyses, which clearly showed that the sugar content of chloroplasts isolated from hardy leaves was insufficient to protect phosphorylation to

Table 1. Sugar content of chloroplast suspensions and effect of freezing on photophosphorylation of osmotically ruptured unwashed chloroplasts from hardy leaves

Expt. no.	Cyclic photophosphorylation (μ moles/mg chlorophyll/hr)		Sugar content (M)
	Unfrozen	Frozen	
1	657	516	2.1×10^{-3}
2	844	642	2.05×10^{-3}
3	700	593	4.1×10^{-3}

Contrary to the procedure otherwise employed in this investigation chloroplasts were not washed with water after isolation. Chloroplasts from 100 g of leaves were sedimented in NaCl-buffer at $600 \times g$, resuspended in the same medium and again centrifuged at $600 \times g$. The sediment was suspended in 12 ml of water and used for the freezing tests. Freezing time was 3 hrs at -25°C . After sedimentation of the chloroplast fragments sugars were assayed in the supernatant by an anthrone method (Morris, 1948). Sucrose was used as a reference

any appreciable extent. This is evident from a comparison of the sugar concentrations observed in the chloroplast system (Table 1) with those required to give good protection (Figs. 1-3). Washing of the chloroplasts with water resulted in the removal of the factor responsible for the protection of phosphorylation. Chloroplast fragments washed twice with water were readily uncoupled by freezing, with or without addition of salts. The main components in the washings were, besides salts, soluble proteins. The washings were concentrated by freeze-drying. Readdition of the soluble factor to washed chloroplast membranes yielded variable results. Either no or only slight protection was found. Never was the protection afforded by the soluble factor as great as observed with the unwashed chloroplasts, from which the factor had not yet been removed. Dialysis of the soluble factor against dilute buffer resulting in the removal of salts and other low molecular components increased its effectiveness as a protective agent, but still in some instances little protection of the chloroplast system against freezing was observed.

Attempts to fractionate the components of the washing were successful. Addition of acid to pH 3 or 3.5 resulted in the precipitation of most of the proteins. Retitration to pH 8 and subsequent removal of the precipitated proteins by centrifugation have led to a fraction containing only about 15% of the proteins present in the starting material. After dialysis this fraction was more active than the starting material in protecting washed chloroplast fragments against the effects of freezing (Fig. 5, note the differences in the protein concentrations required to give the indicated protection). The precipitated proteins exhibit no or only weak protection of the chloroplast system after they have been dissolved and dialysed (Fig. 6). The extent of protection by the active fraction depends on a preincubation period together with chloroplast fragments. Without preincubation there is usually much less protection than after a preincubation period of some hours (Fig. 7). But even after preincubation the system is not completely protected by the applied amounts of protective agent. This may be explained by sterical factors as discussed later on or by an inhibitor present in the protective fraction. In some experiments excess of protective agents not only did not further protect photophosphorylation but actually resulted in a decrease in the observed reaction rates. Since this decrease

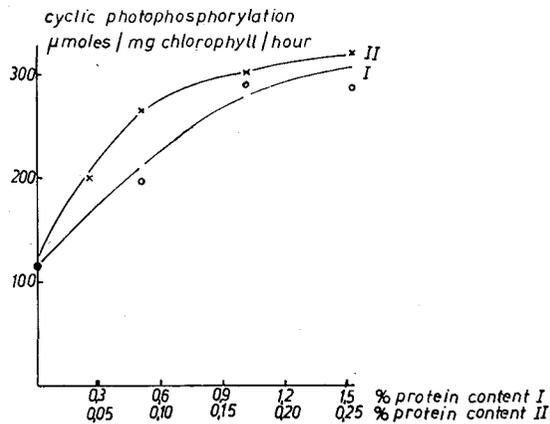


Fig. 5. Effect of different amounts of dialysed stroma material (I) and of a protective fraction derived therefrom (II) on photophosphorylation of previously frozen chloroplast membranes. Note the different concentrations of I and II indicated at the abscissa. Dialysed stroma material (I): 60 mg of freeze-dried stroma material per ml of water were dialysed against 5×10^{-3} M TRIS pH 7.9 for 18 hrs. The resulting solution (protein content *ca.* 3% as determined spectrophotometrically) was diluted to give the concentrations indicated above. Protective fraction (II): Freeze-dried stroma material was dissolved in water (60 mg/ml) and acidified by the addition of HCl under continuous stirring to pH 3. After retitration to pH 8 precipitated protein was removed by centrifugation and the supernatant dialysed against 5×10^{-3} M TRIS pH 7.9. The resulting solution (protein content *ca.* 0.5%) was diluted to give the concentrations indicated above. Immediately afterwards freezing for 3 hrs at -25°C . Photophosphorylation of unfrozen controls was $500 \mu\text{moles/mg chlorophyll/hr}$

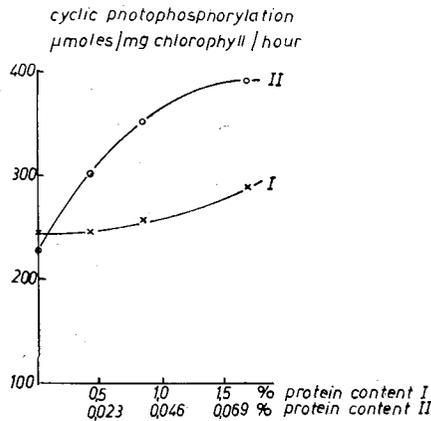


Fig. 6. Protective effect of two fractions derived from freeze-dried stroma material on photophosphorylation. Note the different concentrations of I and II indicated at the abscissa. Acid precipitate (I): During the preparation of the protective fraction a precipitate is formed, which is, after washing with water, dissolved in dilute alkali and dialysed against 5×10^{-3} M TRIS pH 7.9 for 18 hrs (protein content *ca.* 5%, RNA content 0.06%). Protective fraction (II): Prepared as outlined in legend to Fig. 5 (protein content *ca.* 0.23%, RNA content 0.03%). Preincubation of chloroplast membranes with the fractions for $2\frac{1}{2}$ hrs at 0°C , then freezing for 3 hrs at -25°C . Photophosphorylation of unfrozen control $430 \mu\text{moles/mg chlorophyll/hr}$

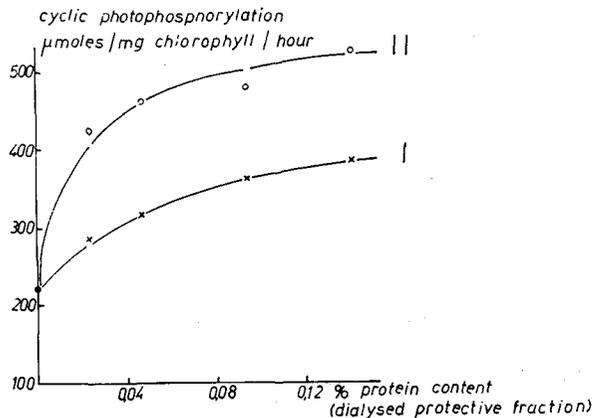


Fig. 7. Effect of preincubation of chloroplast membranes with the protective fraction on the extent of protection against freezing. (I) samples frozen for 3 hrs at -25°C directly after addition of the indicated amounts of protective fraction. (II) chloroplast samples preincubated for $1\frac{1}{2}$ hrs at 0°C with the indicated amounts of protective fraction and then frozen for 3 hrs at -25°C . Preparation of the protective fraction as outlined in the legend to Fig. 5 (protein content 0.29%, RNA content 0.09% as determined spectrophotometrically). Photophosphorylation of unfrozen control 610 $\mu\text{moles}/\text{mg}$ chlorophyll/hr

was obtained both with the frozen chloroplast system and with unfrozen controls it probably resembles an inhibition caused by some component of the protective fraction.

The question must also be considered whether, instead of protecting chloroplast membranes against freezing, the active fraction rather stimulates the "background" phosphorylation surviving freezing. However, this possibility can be ruled out, since phosphorylation of unfrozen chloroplast membranes is stimulated by the addition of the protective fraction only to a small extent indicating probably some protection also against the slow inactivation of the system during standing. A similar small effect on photophosphorylation of unfrozen controls, which are kept at 0°C , has been observed with sucrose and other protective agents.

The protection afforded by the stroma factor is abolished by the addition of salt (Fig. 8). A very similar behaviour towards salt of a chloroplast system protected by sucrose is discussed in section II, 2 (*cf.* Fig. 4).

The UV-spectrum of the dialysed fraction displays an absorption maximum at 260 to 262 $m\mu$ and a rather broad shoulder to longer wavelengths. It thus resembles the spectrum of a nucleoprotein or of a mixture of proteins and nucleic acids (Fig. 9). The ratio of protein to nucleic acid in the protective fraction as calculated from two-wavelength-methods (Layne, 1957; Heber, 1963)* ranges from between about 3/1 to 10/1. The spectrum of the precipitated protein shows an absorption maximum at 270 to 276 $m\mu$. The shoulder at lower wavelengths is indicative of the presence of only minor amounts of nucleic acids (ratio of protein to nucleic acids *ca.* 100/1).

* The protein content as determined by a micro-Kjeldahl procedure is 25 to 50% lower than that indicated by the optical measurements. A considerable error may also reside in the results of the nucleic acid determinations, since accurate absorption coefficients of the protein moiety are not known. However, on a comparative scale the applied optical methods yield useful results.

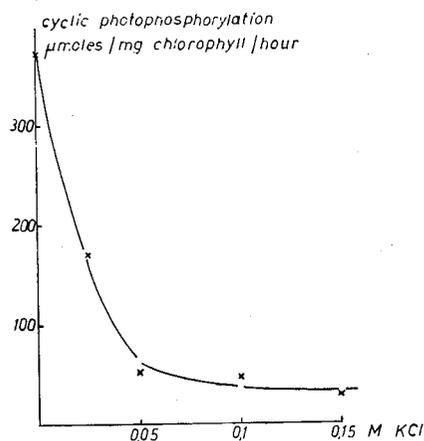


Fig. 8. Effect of different concentrations of salt on a chloroplast system protected by a stroma fraction against freezing. Washed chloroplast membranes containing 0.6 mg chlorophyll/ml were preincubated with dialysed stroma material (9 mg of protein/ml) and the indicated amounts of KCl for 2 hrs at 0°C and were then frozen for 3 hrs at -25°C. Subsequently, photophosphorylation was measured. Photophosphorylation of unfrozen controls was 600 μmoles/mg chlorophyll/hr

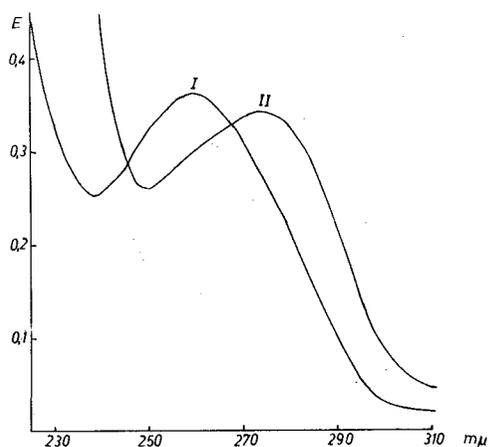


Fig. 9. UV-spectra of the fractions obtained by acid treatment of stroma material from chloroplasts of spinach leaves. Spectrum (I) protective fraction (obtained as outlined in the legend to Fig. 5 and properly diluted with 5×10^{-3} M TRIS pH 7.9): Approximate protein content 50 μg/ml, RNA content 11 μg/ml. Spectrum (II) precipitate formed on addition of acid to a solution of stroma material (see legend to Fig. 5), dissolved by the addition of alkali, dialysed for 18 hrs against 5×10^{-3} M TRIS and properly diluted: Approximate protein content 275 μg/ml, RNA content 3.5 μg/ml

Heating of the active fraction to 90°C for 2 min neither leads to appreciable precipitation of protein nor to a decrease in the protective action on the chloroplast system (Table 2). However, precipitation by trichloroacetic acid results in a very marked decrease in protection or in the loss of it. Trichloroacetic acid removes about 80% of the (nucleo) protein as shown by the UV-spectrum of the neutralized and dialysed TCA treated fraction.

Table 2. Effect of heating on the properties of the protective fraction derived from stroma material

Protein of protective fraction in the chloroplast system (%)	Photophosphorylation (μ moles/mg chlorophyll/hr)		
	Protective fraction added		Without protective fraction
	Unheated	Heated to 90°C for 2 min	
0.14	530	515	230
0.14	385	378	170
0.05	273	283	160

Chloroplast membranes 0.3 ml were preincubated with protective fraction (heated or unheated) or with water (0.3 ml) and were then frozen for 3 hrs at -25°C

Preliminary experiments with enzymes also point to a protein nature of the protective compound(s). Dialysis of the active fraction overnight in the presence of trypsin drastically reduces its protein content and its protective action. Surprisingly, treatment with ribonuclease effects under the same conditions also a marked decrease or a loss in the protection of the chloroplast system. Whether this is caused directly by the hydrolysis of nucleic acids or more indirectly, cannot yet be decided. The preparation of ribonuclease used in these experiments reduces under the applied conditions also the protein content of the active fraction during dialysis.

The content of free or bound sugars in the active fraction is very low as evidenced by sugar determinations with the anthrone/sulfuric acid reagent (2 to 4×10^{-4} M using sucrose as a reference). Most probably it cannot account for the protective properties of the fraction.

The results of these experiments indicate that the active principle present in the protective fraction is a protein, which possesses rather unusual properties in that it is heat stable. The sensitivity towards treatment with ribonuclease and the optical properties of the active fraction further suggest that the protective protein may in fact be a nucleoprotein. Further experimentation is required to throw more light on the nature of the protective protein and to clarify its function *in vivo*.

4. Protective effects of unphysiological compounds

Besides soluble sugars and, as outlined above, special proteins, other compounds are capable of preventing the inactivation of the chloroplast system by freezing. Of special interest is the behaviour of dimethyl-sulfoxide for two reasons. Dimethyl-sulfoxide permeates into intact cells and is often used for the protection of blood cells against freezing. It does not, as other protective compounds, contain hydroxyls and its affinity to water may be explained on the grounds that free electron pairs of the oxygen atom are engaged in hydrogen bonding. Fig. 10 shows that dimethyl-sulfoxide is capable of protecting photophosphorylation against freezing. On a unit weight basis, it is similarly effective as sucrose. As should be expected dimethyl-formamide, which is less hygroscopic than dimethyl-sulfoxide, is also less effective in preserving photophosphorylation.

There is little doubt that a systematic search will lead to the detection of other interesting compounds capable of protecting membrane systems against the effects of freezing.

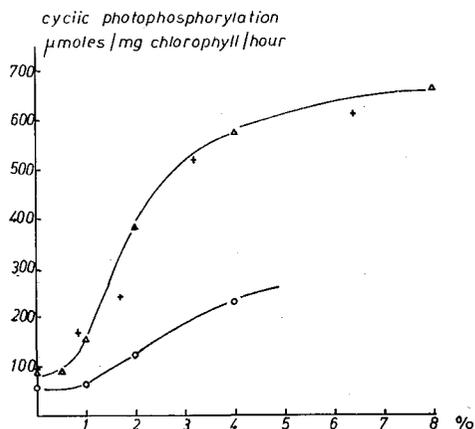


Fig. 10. Protective effect of dimethyl-sulfoxide and dimethyl-formamide as compared with that of sucrose. Freezing of the chloroplast system 3 hrs at -25°C . Δ : Dimethyl-sulfoxide, \circ : Dimethyl-formamide, +: Sucrose

III. Discussion

The results presented in this and in a previous investigation (Heber and Santarius, 1964) show that dehydration of the protoplasm or, more precisely, of sensitive components of the protoplasm occurring during ice formation can be tolerated provided the cell is able to accumulate sufficient amounts of protective compounds. The mechanism, by which protection is exerted, is not perfectly clear, but it seems that hydrogen bonding is involved. A theory capable of explaining the observed phenomena has been put forward (Heber and Santarius, 1964) and will be developed in more detail in a subsequent communication.

Injury set by dehydration appears to pass through several stages as evidenced by the results presented in Fig. 4. Relatively weak dehydration of chloroplast membranes results in the uncoupling of phosphorylation from electron transport and in a number of other phenomena, which will be described elsewhere. Electron transport itself is not affected. However, dehydration under electrostatic stress in the presence of rather high amounts of ions leads to major alterations in the membrane structure resulting also in the inactivation of electron transport.

Sucrose and other sugars are capable of preventing the uncoupling of phosphorylation and the inactivation of electron transport. The protective effects of sucrose have already been dealt with (Heber and Santarius, 1964). Rather high concentrations are required to give complete protection. Such concentrations are often, but not always observed *in vivo* with hardy plant material. Results presented in section II, 3 reveal the existence of another protective compound. Its properties point to a protein nature. At the present state of knowledge it can only be speculated on the function of this compound *in vivo*. However, two points should be considered. The first is the low concentration required to give protection. As demonstrated in Figs. 5 to 7, a protein concentration of 0.1% in the fraction containing the compound is sufficient to give appreciable protection. With sucrose as protective compound similar effects are obtained with concentrations of more

than 1% (Fig. 10). It thus appears that, on a unit weight basis, the protective protein is at least ten times as effective as sucrose. The usual chlorophyll concentration in the test system was 0.6 mg/ml. The ratio of membrane protein to chlorophyll in the chloroplast membranes is roughly 6-8. It follows that an amount of protective protein equivalent in weight only to a fraction of the sensitive membrane is sufficient to give protection.

Another point worth to be discussed is the dependency of protection on a preincubation period (Fig. 7). Most probably preincubation is required for diffusion of the high-molecular factor to the sites, which are to be protected. The fact that it has not been possible to observe complete protection of the system by the protein factor (cf. Fig. 6) may similarly be explained. In the *in vitro* system part of the sensitive membranes may be unavailable to the high-molecular factor and cannot be protected. The need for preincubation to obtain good protection demonstrates beautifully that a close contact between protective compound and sensitive structure is required. Only if located at specific sites can a potentially protective compound really exert its action. In a great number of *in vivo* experiments no close correlations or deviations from otherwise good correlations between hardiness and the content in the cell of sugars or soluble proteins have been observed (for an extensive review of the literature see Levitt, 1956). On these grounds a major importance of these compounds in hardiness has been doubted or even rejected. However, living cells are rather complicated systems. A cell of a higher organism contains a number of compartments, which are separated from one another by membranes. These act, in the form of chloroplast and mitochondrial membranes, of the nuclear membrane, of the endoplasmic reticulum and of the inner and outer membranes of the protoplasm, as permeability barriers. Membranes constitute as much as 40 to 60% of the total protein of many cells. It is not difficult to visualize that protective compounds, especially if high-molecular in nature, may be unequally distributed in the cell, may the latter be in the hardened or in the dehardened state. Now the least protected part of a cell, which is subjected to freezing, will decide on death or survival. Experimentally an uneven distribution of protective compounds with all its consequences on survival will usually not be apparent. Special methods such as the isolation of cell organelles in nonaqueous media are required to demonstrate the predominant accumulation of protective compounds in certain cell organelles during hardening (Heber, 1959 b).

The relations outlined above illustrate the difficulties encountered in the interpretation of data from *in vivo* experiments. They have prevented the general recognition of the fact that accumulation of sugars and other protective compounds at sites sensitive to frost or, more precisely, at lipoprotein structures is of primary importance in the process of hardening.

As has been shown in section II, 4, not only compounds occurring naturally in the cell are capable of protecting membranes against the effects of freezing. Any compound fulfilling some basic requirements should be expected to exert protection. Since the membrane system is injured by the removal of water, a protective compound should be able to either retain the water required for the maintenance of membrane function in the system (Meryman, 1960) or to substitute it. It should further exert no secondary effect on the system interfering with its biological function and it should be available

at the sensitive sites. From this it appears promising to search for protective compounds, which are more effective than those used presently to influence hardiness and to protect cells against freezing.

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