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Thylakoid membrane stability in drought-tolerant and drought-sensitive plants

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Abstract. The stress stability of membranes from two drought-tolerant plants (*Craterostigma plantagineum* and *Ceterach officinarum*) was compared with that of a drought-sensitive plant (*Spinacia oleracea*) in model experiments. Thylakoids from these plants were exposed to excessive sugar or salt concentrations or to freezing. All stresses caused loss of membrane function as indicated by the loss of cyclic photophosphorylation or the inability of the membranes to maintain a large proton gradient in the light. However, loss of membrane functions caused by osmotic dehydration in the presence of sugars was reversible. Irreversible membrane damage during freezing or exposure to salt was attributed mainly to chaotropic solute effects. The sensitivity to different stresses was comparable in thylakoid membranes from tolerant and sensitive plants indicating that the stress tolerance of a plant can hardly be attributed to specific membrane structures which would increase membrane stability. Levels of membrane-compatible solutes such as sugars or amino acids, among them proline, were much higher in the drought-tolerant plants than in spinach. Isolated thylakoids suspended in solutions containing an excess of sugars remained functional after dehydration by freeze-drying. This indicates that membrane-compatible solutes are important in preventing membrane damage during dehydration of poikilohydric plants.

Key words: *Ceterach* – *Craterostigma* – Drought – Membrane stability – Resurrection plants – *Spinacia* (membrane stability).

Introduction

Poikilohydric plants, sometimes also termed resurrection plants, can tolerate loss of more than 95% of the water contained in water-saturated metabolically active tissues (Walter 1955). During dehydration, osmotic potentials increase from about 15 bar or less to more than 200 bar. In the dry state, gas exchange is no longer measurable in the tissues, but turgor is regained on rehydration and physiological processes such as respiration and photosynthesis are more or less rapidly restored.

Only a very low percentage of higher plants are desiccation-tolerant. The vast majority do not withstand severe dehydration. When water loss exceeds 60–80% of the water of saturated tissues, so-called homoiohydric plants suffer irreversible damage. Water withdrawal subjects biomembranes to multiple stresses: mechanical stresses are produced by drastic volume changes of cells and cellular compartments during dehydration and rehydration. Chemical stresses are produced by increasing cellular solute levels caused by water loss. An excessive increase in electrolyte levels is known to interfere with stabilization of thylakoids from spinach (Heber and Santarius 1964; Heber 1967; Santarius 1969). Salts act on the membranes in a Hofmeister lyotropic power series (Heber et al. 1979). At high concentrations, they cause loss of peripheral membrane proteins such as the coupling factor for ATP formation and NADP-reductase (Younis et al. 1979; Garber and Steponkus 1976; Heber et al. 1979). As a consequence of salt action, irreversible loss of membrane functions is observed. Since membranes of resurrection plants obviously tolerate mechanical and solute stress, whereas membranes of homoiohydric plants are sensitive, we wanted to know whether there are differences in stability of the membranes between the two groups of plants which can explain the differences in stress tolerance. The drought-tolerant South African *Craterostigma plantagineum* (Scrophulariaceae) (Gaff and Hallam 1974) and the mediterranean fern *Ceterach officinarum* (Oppenheimer and Halevy 1962) were used for our investigations. We have chosen to study the response of thylakoid

Abbreviation: BSA = bovine serum albumin

membranes from these plants to stress, because stress-induced alterations in biochemical activities can easily be measured.

Materials and methods

Plant material. The fern *Ceterach officinarum* Lam. et DC was collected at the Yugoslavian coast in July 1982. Plants were grown in the greenhouse at 25° C day and 21° C night temperature at varying relative humidities and light intensities (maximum 22 W m^{-2}). *Craterostigma plantagineum* Hochst. had been collected in South Africa. Plants were held at a 17° C day, 16° C night temperature regime during summer. In the fall, after some dehardening had occurred, they were transferred to a 25° C day, 21° C night temperature regime and additional incandescent light was supplied (60 W m^{-2}, 12 h photoperiod). All plants were watered regularly. Spinach (*Spinacia oleracea* L. cv. Yates) was grown in the greenhouse under short-day conditions. Light intensity was about 130 W m^{-2}. For all experiments leaves of comparable age were taken, i.e. leaves from spinach and *Craterostigma* were of similar size, those from *Ceterach* of similar dark green colour with grey to brownish scales covering the lower epidermis (young leaves are light green and possess almost white scales).

Leaf measurements. Exchange of gaseous CO_2 was measured by an infra-red technique (Dietz and Heber 1983). Simultaneously, thylakoid energization was monitored by recording apparent absorbance changes at 535 nm (Heber 1969). The slow component is caused by changes in the magnitude of the transthylakoid proton gradient (Köster and Heber 1982; Kobayashi et al. 1982), the fast component seen on illumination and darkening is caused by the electrochromic shift which peaks at 518 nm and has a shoulder at 535 nm. This signal indicates the magnitude of a light-generated membrane potential (Junge 1977).

Isolation of thylakoids. The basic isolation medium contained 0.33 M sorbitol, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM KCl, 0.5 mM KH_2PO_4 and 50 mM 2-(N-morpholino)ethanesulfonic acid (Mes), pH 6.7. Immediately before use 1.25 mM sodium-ascorbate and 3.3 mM cysteine were added. For thylakoid isolations from resurrection plants, the following components were also present in the isolation medium: 0.1% bovine serum albumin (BSA), 0.5% soluble polyvinylpyrrolidone (PVP, MW 25000), 0.05% insoluble PVP (Polyclar AT) and 5 mM sodium dithionite ($Na_2S_2O_4$). Leaves (2–6 g) were homogenized in an Ultraturrax Mixer (IKA-Werk Staufen FRG, Janke and Kunkel GmbH) for 10 s (spinach leaves) and at least two times 10 s (resurrection plants). The homogenate was filtered quickly through one layer of miracloth and nylongaze (20 µm) each and sedimented for 1 min at 2000 *g*. The pellet was resuspended in a small amount of basic medium (spinach) or basic medium with 0.1% BSA (resurrection plants) and again centrifuged for 1 min at 2000 *g*. The resuspending medium contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) instead of Mes at a pH of 7.6, but was otherwise identical to the basic medium. When thylakoids were prepared for freeze-drying experiments they were shocked in a 10-fold volume of sorbitol-free resuspending medium.

Freezing. Thylakoid suspensions were diluted with equal volumes of solutions containing salt (50–500 mM NaCl) and sorbitol to give ratios of salt to cryoprotectant ranging from 0.3 to 3. They were subsequently frozen for 3 h at −18° C. After rapid thawing in a 25° C water bath phenazinemethosulfate-catalyzed cyclic photophosphorylation was measured at approx. 400 W m^{-2} actinic light (Filters RG 610 and Balzers Calflex C heat filter, Balzers, Liechtenstein) in a 1-ml Walker cuvette at 20° C by recording the pH change brought about by the reaction: $ADP^{3-} + HPO_4^{2-} \rightarrow ATP^{4-} + OH^-$. (Dilley 1972). Chlorophyll concentration was between 20 and 40 µg ml^{-1} assay medium. Loss of peripheral membrane proteins from the thylakoids during freezing (or osmotic stress) was determined as described by Mollenhauer et al. (1983).

Osmotic stress. a) NaCl. Thylakoids were diluted with salt solutions as above but salt levels were higher than in the freezing experiments. The assays were either conducted in the standard photophosphorylation medium or in a medium in which the sorbitol of the standard medium was replaced by varying salt concentrations. *b) Sorbitol.* Hyperosmotic dehydration was brought about by sorbitol. Sorbitol concentrations ranged from 0.33 to 3 M. Where indicated, photophosphorylation was also measured in the presence of varying concentrations of sorbitol. Chlorophyll concentration in the assay medium was again 20–40 µg ml^{-1}.

9-Aminoacridine fluorescence. Formation and decay of transthylakoid proton gradients were monitored by following the quenching of 9-aminoacridine fluorescence (Schuldiner et al. 1972) in a spectrofluorometer (Aminco, type SPF 500; Silver Spring, Md., USA) at red actinic light of about 200 W m^{-2} which was provided by glass-fiber optics. Fluorescence was excited at 400 nm. The photomultiplier was protected against actinic light by appropriate filters. The assay medium was identical to the resuspension medium (pH 7.6) (see isolation procedure) and contained in addition 1 mM $K_3(FeCN)_6$, 1 µM 9-aminoacridine and 10–20 µg chlorophyll in a total volume of 1.5 ml. Fluorescence quenching was recorded during a dark-light change (1 min light).

Sugar determinations. Total soluble sugars were estimated using the anthrone method after Ashwell (1957). Leaves were frozen in liquid nitrogen and homogenized with water. The resulting suspension was centrifuged and sugars were determined in aliquots of the supernatant. Glucose was used for standardization. To measure starch and related polysaccharides, the sediment was boiled for 1 h in 1 N H_2SO_4. After sedimentation of insoluble material the supernatant was assayed as described above.

Amino-acid determinations. Amino acids were determined with ninhydrin after Duggan (1957). Leaf samples were homogenized in a solution of sodium acetate (0.09 M) containing 14% glacial acetic acid (pH 5.5). The resulting suspension was centrifuged and the supernatant used for the assays. To 100 µl of the sample (supernatant of the water-soluble extract) 200 µl of reagent was added. After vigorous mixing the samples were boiled for 15 min at 100° C. The reaction was stopped by transferring the vials to an ice bath. After cooling to room temperature, 3 ml of 50% ethanol (v/v) were added to each sample and mixed thoroughly. The absorption was read at 570 nm. A mixture of alanine and glutamine was used for standardization. Proline was measured with ninhydrin according to Troll and Lindsley (1955) after extraction of the reaction product into benzene. The absorption was measured spectrophotometrically at 515 nm.

Freeze-drying. Thylakoids isolated as described above were diluted with varying amounts of sucrose, glucose, fructose and sorbitol or with proline or a combination of sucrose and proline. Effects of BSA addition were also investigated. The activity

of cyclic photophosphorylation of thylakoids was measured immediately after isolation. Samples were then frozen at −18° C and part of them subsequently freeze-dried in a lyophilizer for one week. During freeze-drying, the temperature did not exceed −15° C. Control samples were kept at −18° C. After storage for one week, they were thawed and photophosphorylation was measured. Freeze-dried thylakoids were suspended in a solution containing 0.05 M NaCl and 0.1 M sorbitol and cyclic photophosphorylation was recorded. Occasionally, ferricyanide reduction and 9-aminoacridine fluorescence quenching were also recorded.

The osmotic potentials of leaves and solutions were measured cryoscopically in a Semi-Microosmometer (Knauer KG, Oberursel FRG). Chlorophyll was determined according to Arnon (1949).

Results and discussion

1. Isolation of biochemically active thylakoids from resurrection plants. Conventional methods (Cockburn et al. 1968, with modifications as described above) used for the isolation of chloroplasts or thylakoids did not yield active thylakoid preparations from leaves of *Craterostigma* and *Ceterach*. Attempts to reduce thylakoid inactivation during isolation by binding phenolic constituents of the leaves or other interfering material released on homogenization to compounds such as polyvinylpyrrolidone or bovine serum albumin (BSA) were unsuccessful as long as chloroplast-isolation procedures were carried out under aerobic conditions. Active thylakoid preparations were obtained when phenoloxidases were inhibited by low concentrations of sodium dithionite (Anderson 1968).

2. Sensitivity of thylakoid functions to dehydration in vivo. Spinach is a mesophyte which in contrast to the resurrection plants is sensitive to dehydration. At room temperature, thylakoid functions in leaves as measured by the formation of a transthylakoid proton gradient (=light scattering) and a light-generated membrane potential (=electrochromic shift) decrease drastically when about 80% of the water of water-saturated leaves has been lost. In the initial stage of water withdrawal, the light-scattering change was stimulated before it finally disappeared after drastic wilting. The stimulation is caused by closure of the stomata which leads to an increase in leaf energization in the light owing to the decrease in energy consumption by photosynthesis (Fig. 1; Dietz and Heber 1983). Electron transport was similarly impaired by loss of water from leaves of spinach and of the resurrection plants, but whereas thylakoid functions were lost irreversibly in spinach, they were restored on rehydration in the resurrection plants. Figure 2 shows the decline of photosynthetic CO_2 uptake during wilting of a leaf of *Craterostigma* and of spinach. Rehydration did not restore photosynthesis in spinach because the leaf had not survived. In *Craterostigma*, the restoration of respiratory CO_2 production (not shown) preceded that of photosynthesis.

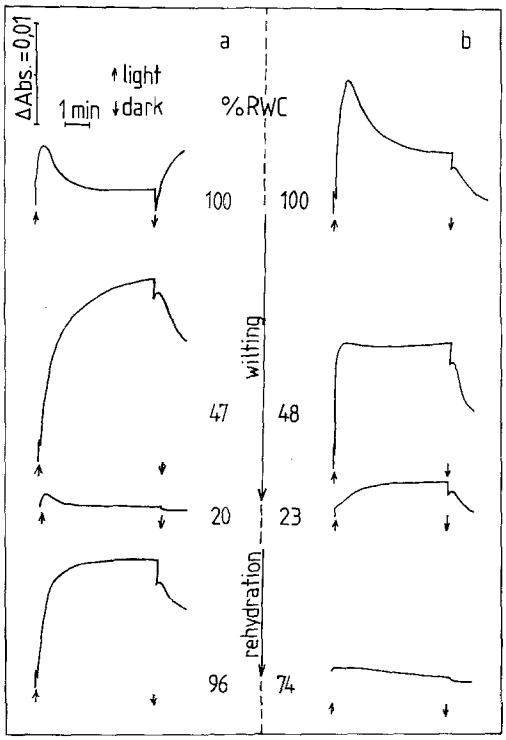

Fig. 1a, b. Apparent absorbance change (light scattering) at 535 nm after 5 min illumination at 50 W m^{-2} (slow changes) and 518 nm membrane potential (fast changes) of a leaf of *Craterostigma* (**a**) and of spinach (**b**) at different relative water contents (RWC) during wilting and rehydration (100% RWC = water-saturation). Numbers in the figure indicate the water status of the leaves

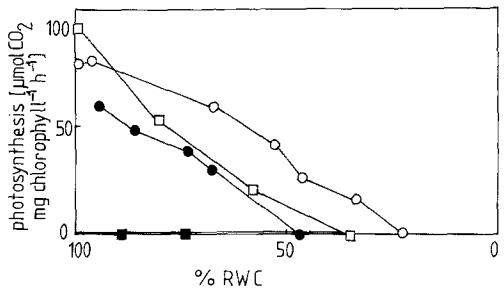

Fig. 2. Net photosynthesis of a leaf of *Craterostigma* (□) and of spinach (○) during wilting (*open symbols*) and reswelling (*closed symbols*) as a function of relative water contents (RWC). Leaves were rehydrated when photosynthesis had disappeared

3. Sensitivity of thylakoids to dehydration in vitro. When isolated thylakoids from spinach are frozen,

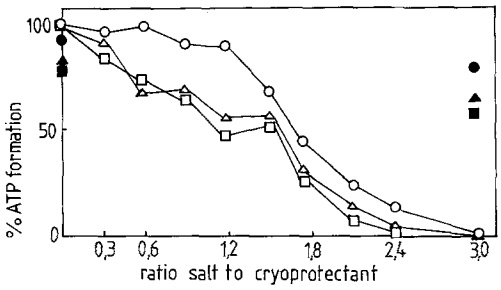

Fig. 3. Cyclic photophosphorylation of spinach (o), *Craterostigma* (□) and *Ceterach* (△) thylakoids after 3 h freezing at −18° C as a function of the ratio of NaCl to sorbitol. All measurements were conducted in the standard photophosphorylation medium. *Closed symbols* represent 0° C controls with and without salt measured after 3 h incubation. Values are given as percentage of the unfrozen controls which were 90, 140 and 250 μmol ATP mg chlorophyll^{-1} h^{-1} for *Ceterach, Craterostigma* and spinach, respectively

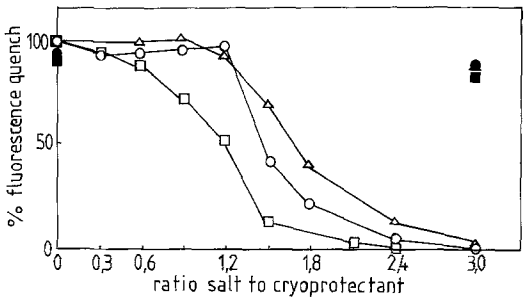

Fig. 4. Light-dependent quenching of 9-aminoacridine fluorescence as measured after 1 min of actinic illumination of frozen-thawed thylakoids. Values are given as percentage of the quenching of 0° C controls. Symbols and conditions otherwise as in Fig. 3

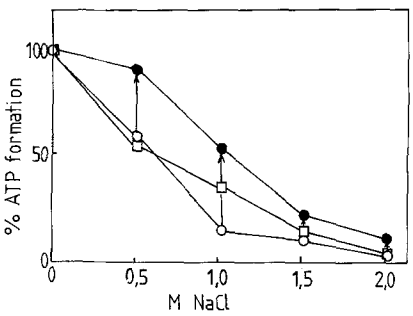

Fig. 5. Cyclic photophosphorylation of spinach (o) and *Ceterach* (□) thylakoids after exposure for 0.5–1 h to different concentrations of NaCl. For spinach, values are shown which were measured during stress (i.e. high salt concentration present during the assay, *open symbols*) and on release of stress (i.e. in the absence of high salt concentration in the assay medium, *closed symbols*). For *Ceterach* both assay modes gave identical results

drastic dehydration occurs as water is converted to ice. When the ratio of potentially cryotoxic solutes (for instance NaCl) to membrane-compatible solutes (for instance soluble sugars and sugar alcohols) in the medium is low, complete protection against the stresses produced by freezing and thawing is observed. When the ratio is high, membrane function is lost as exemplified by the loss of photophosphorylation.

We sought to answer the question whether membranes from resurrection plants can tolerate dehydration and salt stress produced by freezing which would inactivate spinach thylakoids. The sensitivity of thylakoids from spinach to freezing in the presence of various salt/sugar ratios and from resurrection plants is compared in the following figures. Since ATP synthesis depends on the morphological and structural integrity of the membrane, it was used as a criterion for protection or damage. Figure 3 shows photophosphorylation of thylakoids after freezing and thawing in media containing NaCl and sorbitol at increasing molar ratios. Under the conditions of the experiment, spinach thylakoids were practically fully protected against damage up to a NaCl/sorbitol ratio of 1. Increasing this ratio further led to incomplete recovery of photophosphorylation and finally to its complete loss which was usually accompanied by a decline in the rate of electron transport. Thylakoids from *Craterostigma* and *Ceterach* failed to show increased resistance to freezing stress compared with spinach thylakoids. Results similar in principle were obtained when 9-aminoacridine-fluorescence quenching was used to indicate the magnitude of the transthylakoid proton gradient formed by actinic illumination of frozen-thawed thylakoids (Fig. 4). However, the proton gradient appeared to be somewhat more resistant to salt stress than photophosphorylation. When the membranes were dehydrated by freezing in the presence of high concentrations of sugars such as sucrose or sugar alcohols such as sorbitol, no deleterious effects of dehydration became apparent. Unfrozen controls kept at 0° C with and without salt showed no appreciable decrease in activity during the time interval used for freezing indicating that sodium chloride did not interfere with the phosphorylating system at the low concentrations used for the freezing experiments. However, when the osmotic conditions encountered during a freeze-thaw cycle were mimicked at room temperature by exposing the membranes to high salt concentrations, there was a decrease in activity similar to the one observed after freezing and thawing (Fig. 5). Stress was varied by increasing the NaCl concentrations from 0.5 to 2 M while sorbitol was kept constant at 165 mM. After incubating the thylakoids in the stress medium, photophosphorylation was mea-

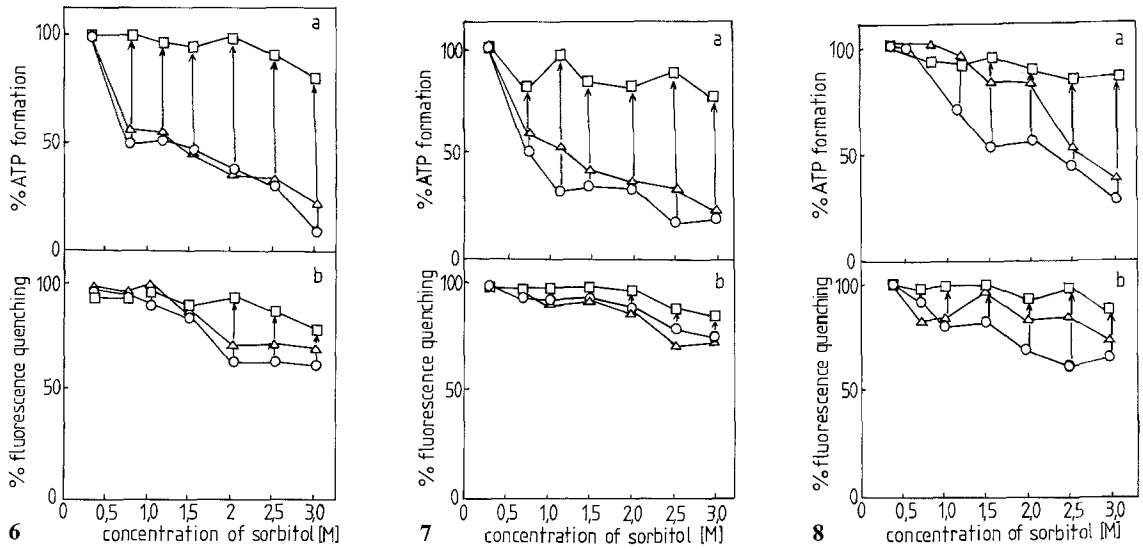

Fig. 6. a Cyclic photophosphorylation of spinach thylakoids under different osmotic-stress treatments, i. Membranes were immediately transferred to assay media which contained sorbitol as indicated (△). ii. Membranes were incubated for 30–60 min in media containing sorbitol as indicated and were then measured as above (○). iii. Membranes were incubated for 30–60 min in media containing sorbitol as indicated and were then transferred to an assay medium which contained 0.1 M sorbitol only (□). 100% phosphorylation rate was 305 μmol ATP mg chlorophyll^{-1}h^{-1}. **b** Quenching of 9-aminoacridine fluorescence in the light and the effects of increasing dehydration of spinach thylakoids. Conditions and material otherwise identical to **a**

Fig. 7. a Cyclic photophosphorylation of *Craterostigma* thylakoids under osmotic stress. Treatments and symbols are the same as in Fig. 6. 100% rate was 210 μmol ATP mg chlorophyll^{-1}h^{-1}. **b** 9-Aminoacridine-fluorescence quenching in the light (after 1 min) of *Craterostigma* thylakoids. Conditions and symbols as already described in Figs. 6a, b

Fig. 8. a Cyclic photophosphorylation of *Ceterach* thylakoids exposed to osmotic-stress treatments as described in Fig. 6a. 100% value was 142 μmol ATP mg chlorophyll^{-1}h^{-1}. **b** Light-dependent 9-aminoacridine-fluorescence quench of *Ceterach* thylakoids. For other conditions and symbols see Figs. 6a, b

sured both under stress conditions, i.e. in the presence of increased salt levels, or after transfer of the thylakoids from the stress medium to the standard assay medium. Partial recovery of photophosphorylation was seen in spinach but not in *Ceterach* when the salt stress was relieved. The extent of recovery depended on the length of the incubation period with high salt. When sorbitol instead of salt or freezing was used to lower the water potential in the membrane suspensions, the results shown in Figs. 6–8 were obtained. In the presence of high levels of sorbitol, photophosphorylation of spinach thylakoids was decreased (Fig. 6a) and electron transport was stimulated. Inhibition of photophosphorylation was not greatly dependent on the time of preincubation in hypertonic media. Transfer of the membranes from the stress medium to a hypotonic assay medium resulted in practically complete restoration of ATP formation in the light. Apparently, sorbitol inhibition was fully reversible. Similar results have been obtained before by Santarius and Ernst (1967) and Santarius and Heber (1967) with sucrose and other sugars. Proton-gradient formation in the light as measured by 9-aminoacridine-fluorescence quenching (after 1 min) decreased in hypertonic sorbitol only when photophosphorylation was already appreciably depressed (Fig. 6b), i.e. at osmotic potentials higher than 100 bar (corresponding to approx 1.5 M sorbitol).

When the same experiments were performed with thylakoids from *Craterostigma,* similar results were obtained (Fig. 7a, b), although proton gradient formation appeared to be somewhat less affected than in spinach thylakoids. In *Ceterach* thylakoids the degree of inhibition of photophosphorylation was somewhat lower at moderate stress levels than in the other two species (Fig. 8a, b). Differences in the shape of the inhibition curves can be explained by the varying physiological state of the plant material. The effects were reversible on transfer of the thylakoids to hypotonic conditions, and the general response to high sorbitol concentrations was similar in all three species.

The data obtained in the dehydration experiments described above permit the following general interpretation. In all species investigated, electron transport was highly resistant to solute stress. The proton gradient was less sensitive than photophosphorylation. In the freezing experiments, the

proton gradient was not much diminished up to salt-to-sugar ratios of about 1.2. At room temperature, sorbitol was tolerated up to 1.5 M. At these limits, photophosphorylation was already decreased by 50% or more, whereas electron transport was stimulated. When the membranes were sedimented after freezing and the supernatants were subjected to sodium-dodecylsulphate gel electrophoresis, release of peripheral membrane proteins was found which increased as salt-to-sugar ratios increased. Among the released proteins, subunits of the chloroplast coupling factor were prominent. Results of these experiments are not shown here because they are very similar to those recently published for spinach thylakoids by Mollenhauer et al. (1983). Thus uncoupling of photophosphorylation from electron transport and loss of detachable proteins are responsible for initial membrane damage. Excessive salt stress produced by freezing also causes an irreversible alteration in the semipermeability properties of the membranes. Leakiness explains the loss of the proton gradient. These factors taken together account for the final complete loss of photophosphorylation. Whereas electron transport had largely disappeared after freezing with high salt-to-sugar ratios it was still active under severe osmotic dehydration at room temperature which merely resulted in reversible inhibition. Even at the highest osmotic potentials, inhibition was accompanied by the loss of fewer peripheral membrane proteins than observed at irreversible freeze damage. However, Figs. 6–8 illustrate that membrane leakiness was also partly responsible for the decline of activity.

4. Levels of solutes. Apparently, even a drastic lowering of the water potential in thylakoid suspensions did not cause irreversible membrane damage when solutes such as sorbitol decreased the water potential, whereas a chaotropic salt such as NaCl caused irreversible membrane damage when present at elevated concentrations. In vivo, the solute environment of biomembranes is highly complex. Ions are known to be responsible for a major part of the osmotic potential in most plants, and the chaotropicity of different ions varies according to their position in the lyotropic power series of Hofmeister (Heber et al. 1979). The balance between membrane-compatible and chaotropic solutes appears to play an important role in the sensitivity or resistance of cellular systems to water stress. Chloroplasts were found to be capable of accumulating sugars, and the overall sugar content of plant cells rises when they become frost-hardy (Heber and Santarius 1964).

Table 1a. Levels of total soluble sugars of both saturated and partly desiccated leaves from spinach, *Ceterach* and *Craterostigma*. Values are given on a dry-weight basis (DW) as well as concentration in cell sap in case of fresh tissues. The data represent values of at least two determinations each of six different leaves of each species

Species	Water loss (%)	Total soluble sugars	
		mg g^{-1}DW	mM in cell sap
Spinacia	0	23.1	11.7
	61.4	119	
Ceterach	0	189.1	195
	77.3	240.4	
Craterostigma	0	187.9	94.3
	87	264.6	

Table 1b. Sugar contents of spinach, *Ceterach* and *Craterostigma* leaves (freshly harvested) before and after hydrolysis and ratios of starch (in glucose equivalents) to sugar

Species	Sugar content (mg g^{-1}DW)		Ratio starch to sugar
	Before hydrolysis	After	
Spinacia	38.7	79.9	2.06
Ceterach	176.9	73.8	0.42
Craterostigma	220.2	42.9	0.19

Table 2. Levels of total amino acids and of proline of saturated, partly and severely desiccated leaves of spinach, *Ceterach* and *Craterostigma*. The data are means of at least two determinations of two different leaves of each species. Concentrations are based on the dry weight (DW) of the tissue and on cell-sap volume as regards saturated leaves

Species	Water loss (%)	Total amino acids		Proline	
		mg g^{-1} DW	mM in cell sap	mg g^{-1} DW	mM in cell sap
Spinacia	0	8.74	8.77	0.078	0.015
	22	10.28		0.33	
	71.3	23.93		1.79	
Ceterach	0	19.59	44.2	1.63	0.71
	47.7	27.67		1.74	
	76.4	20.6		1.03	
Craterostigma	0	5.7	5.82	0.165	0.031
	67	17.4		0.133	

Since not only sorbitol and sucrose, but also a variety of other soluble sugars (and certain amino acids, see below) are known to protect thylakoids against inactivation during freezing, it was of interest to determine sugar levels in resurrection plants. Table 1a shows determinations of soluble

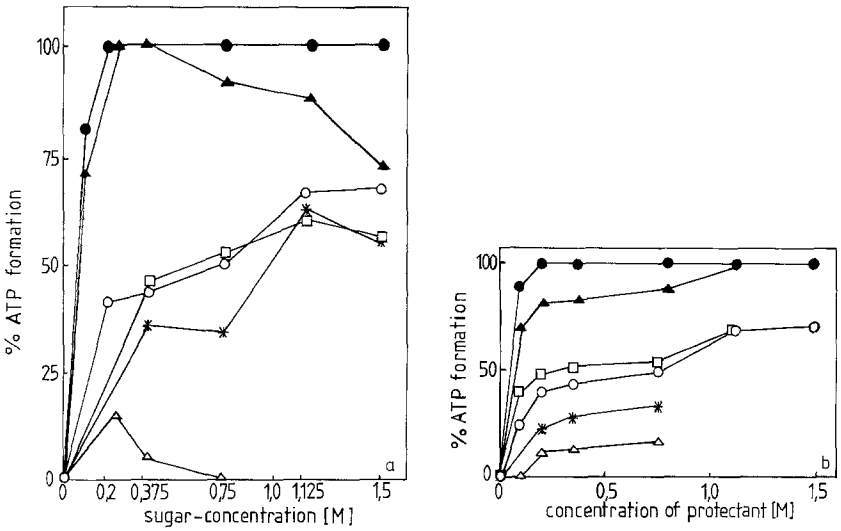

Fig. 9. a Recovery of photophosphorylation activity of spinach thylakoids after freezing (*closed symbols*) and freeze-drying (*open symbols*) as a function of increasing concentrations of sorbitol (△), glucose (*), fructose (□) and sucrose (○). Values are shown as percentage of the 0° C controls which was 352 μmol ATP mg chlorophyll^{-1}h^{-1}. **b** Recovery of photophosphorylation of spinach thylakoids in the presence of increasing concentrations of sucrose (○), proline (△), a combination of sucrose + 0,1 M proline (□) and of sucrose + 5% BSA (*) after freezing (*closed symbols;* only shown for sucrose and proline and after freeze-drying (*open symbols*). 100% rate was 375 μmol ATP mg chlorophyll^{-1}h^{-1}

sugars in water-saturated and drastically wilted tissues. There was a large difference in sugar content between water-saturated leaves of the mesophyte spinach and of the resurrection plants. On a dry-weight basis, the latter contained about eight times as much as soluble sugar as spinach. During slow wilting, starch is known to be hydrolysed, and hydrolysis of polymeric carbohydrates may account for the increased contents of soluble sugars observed in wilted spinach and dried *Craterostigma* and *Ceterach* leaves. The stress-induced carbohydrate degradation was much less pronounced in resurrection plants than in spinach. Recalculation of the data on a concentration basis reveals sugar levels in water-saturated leaves of resurrection plants between 94 and 195 mM as against 12 mM in spinach, when the simplifying assumption is made that glucose is the only soluble sugar. Since sucrose often predominates, this is an overestimation. As is also apparent from the increase in soluble sugars during wilting of leaves, measurements of starch (Table 1 b) revealed starch contents which were higher in spinach than in the resurrection plants. However, it needs to be kept in mind that starch levels vary considerably with the physiological state of the leaves and with the time of day. An increase in the concentration of some amino acids, has been reported to occur on exposure of plants to low temperatures and low atmospheric water potentials. Proline has been known for a long time to increase its concentration in response to water stress (Levitt 1980). It is an effective cryoprotectant. Also, for some resurrection plants, elevated proline levels were reported (Hsiao 1973, Tymms and Gaff 1978).

Levels of soluble amino acids are generally not high in leaf cells. When they increase during wilting, protein breakdown may be responsible. Table 2 shows amino-acid levels in water-saturated spinach, *Ceterach* and *Craterostigma* leaves at different stages of wilting. In water-saturated tissue, the total amino-acid concentration was about 44 mM in *Ceterach* as against 9 mM in spinach. With 0.7 mM in *Ceterach* and 0.015 mM in spinach, proline levels were also very different. The concentration of proline was higher in *Ceterach* than in spinach by a factor of 50 and that of other amino acids by a factor of 5. During wilting, amino acids did not much increase in *Ceterach*, whereas a large increase was observed in spinach. This increase may be attributed to protein breakdown. In *Craterostigma*, in contrast, amino-acid levels did not appear to be increased. The amount of proline found in *Craterostigma* was only about twice as much as in spinach (see also Tymms and Gaff 1978). Thus there are differences in solute content between tissues of different resurrection-plant species.

5. Freeze-drying of thylakoids. The data of the Figs. 3–8 do not give support to the hypothesis that thylakoids from resurrection plants are more resistant to dehydration stress than thylakoids from the drought-sensitive mesophyte, spinach. They do show, however, that polyhydroxy compounds such as sorbitol and related solutes are tolerated by the membranes which can be protected in their presence against dehydration injury brought about by freezing. The data of Tables 1 and 2 show that the concentration of soluble polyhydroxy compounds and of amino acids which are also known to be able, at least in part, to protect thylakoids against freeze-inactivation, are unusually high in resurrection plants. In order to test

the hypothesis that soluble sugars and amino acids are involved in membrane protection during dehydration of leaves, thylakoids were subjected to freeze-drying. During lyophilization, electron transport was lost when cryoprotectant was absent. It was stimulated by lyophilization when the protectant was in low concentrations which could not prevent uncoupling (data not shown). In the experiments of Fig. 9a, different sugars (glucose, fructose, sucrose, sorbitol) were added in increasing concentrations to spinach thylakoids. Photophosphorylation of 0° C control samples was measured immediately. Aliquots of the mixtures were frozen at −18° C for freeze-dried. After 7 d, photophosphorylation of resuspended freeze-dried material was compared with that of frozen and then thawed control samples. Recovery of frozen samples was 100% except when sorbitol was present as cryoprotectant. At the highest sorbitol concentration used (1.5 M), photophosphorylation was decreased to 75% of the activity of the unfrozen control sample which had been assayed 7 d ago. In the freeze-dried aliquots all sugars were protective, and the extent of protection depended on concentration. The failure of sorbitol to be protective is probably the result of partial crystallization at high concentrations. The best recovery was measured in the presence of 1.5 M sucrose. Sucrose does not crystallize during freezing. When proline was added to sucrose-containing media, enhanced protection could be observed at lower concentrations (Fig. 9b). Proline protected best of similar amino acids tested. Addition of BSA to sucrose-containing media decreased protection which is in compliance with Garber and Steponkus (1976) who found that soluble proteins are not very effective cryoprotectants. The experiments show that it is possible to recover membrane functions which depend on membrane integrity, such as photophosphorylation, after completely drying thylakoids, provided an excess of membrane-compatible solute is present.

Conclusions

This study focuses on a particular cellular membrane, the thylakoid. To draw conclusions about the drought-tolerance of a plant from studies of a particular membrane requires justification. Although it is evident that the thylakoid system is not necessarily the primary site of drought injury, and that other membranes may be more vulnerable to stress than the thylakoids, the fact that thylakoids prove sensitive to dehydration in one plant and tolerant in others makes them a suitable object for study. We have shown that the resistance of thylakoids from two resurrection plants cannot be explained by a particular membrane structure which makes the membrane insensitive to dehydration stress. Comparable sensitivities of thylakoids from spinach and thylakoids from the resurrection plants indicate that survival of the latter during drying of the leaves must be attributed, at least in part, to the composition of the membrane surroundings. Indeed, crude cell-sap analysis showed that the resurrection plants contained much more membrane-compatible solutes than the drought-sensitive mesophyte, spinach. We do not believe that there is a particular solute in resurrection plants which makes them desiccation-tolerant. Rather, the action of a multitude of membrane-stabilizing agents appears to prevent irreversible breakdown of membrane structures during dehydration. Some amino acids are also known to protect membranes. Several act optimally in the presence of some salt (Heber et al. 1971). They might well be suited for protection in vivo. At low concentrations, chaotropic solutes which are normal constituents of the cells contribute to membrane stabilization, but dehydration may increase their concentration to toxic levels. Apparently, membrane-compatible solutes can counter their effects. Indeed, thylakoids freeze-dried in the presence of both sugars and low concentrations of salts largely retained the ability to phosphorylate ADP in the light. This reaction requires, in addition to a highly complex biochemical machinery, a closed membrane system with intact permability properties. Nevertheless, it would still be feasible that some hardening occurs during wilting of whole leaves, i.e. that drying is accompanied by alterations in the membrane system which cannot be detected in the in-vitro system used in this study.

It has been mentioned in the introduction that two main stresses can damage membranes during these processes. Chemical stress by chaotropic reagents can cause membrane damage and this work shows how such damage can be avoided. Mechanical stresses to membranes will arise both during drying and swelling: if the surface area of the membranes is reduced during dehydration it must increase during rehydration. During surface area reduction sequestration of membrane material must occur. Expansion on rehydration would lead to breakage of the bilayer structure if sequestered material does not become available again. Osmotically dehydrated chloroplasts were found to rupture when they were returned to isotonic conditions. Apparently, they had lost envelope material

during shrinking. Indeed, during osmotic dehydration loss of solutes has been observed, which might be related to the detachment of envelope material. Dehydrated chloroplasts appeared to have intact, i.e. sealed envelopes. In vivo rupture of chloroplasts does not occur (Kaiser and Heber 1981), presumably because the lens shape permits dehydration and rehydration without much surface-area reduction.

During extensive dehydration of cells, a reduction of surface area of membranes can only be avoided by membrane folding. In thylakoids, membrane folding is facilitated by constituents such as mono- and digalactosylglycerides. Proper spacing of their head groups permits membrane curvature. It remains to be seen how the membrane system of resurrection plants adapt to the need for either surface-area reduction or folding during dehydration and membrane expansion or unfolding on rehydration.

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