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Sucrose influx and mechanical damage by osmotic stress to thylakoid membranes during an in vitro freeze-thaw cycle

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The effects of a freeze-thaw cycle on thylakoid volume were studied by micro-haematocrit centrifugation. At initial osmolalities prohibiting mechanical freezing damage thylakoids showed a volume increase after freezing, indicating solute uptake. At initial osmolalities below 0.1 osmolal thylakoids lost volume, apparently because of membrane rupture. Rupture occurred at a volume smaller than the maximum volume of unfrozen controls. Resealing of the membranes after freeze-induced rupture was demonstrated by the ability of thylakoids frozen at low initial osmolalities to act as osmometers in response to changes in the external solute concentration. From experiments conducted at 0°C the permeability coefficient for sucrose across thylakoid membranes was calculated as $P = (1.3 \pm 0.25) \cdot 10^{-10}$ m/s, independent of the applied concentration gradient. The permeability coefficient at −20°C was estimated from samples frozen in the presence of 500 mM sucrose, which yielded complete cryoprotection. A value of $P = 0.18 \cdot 10^{-10}$ m/s was calculated and from this a Q_{10} of 2.67 could be derived for the diffusion of sucrose across thylakoid membranes. From the comparison of P values at 0°C and 5°C a Q_{10} of 2.89 was calculated for diffusion under non-freezing conditions. It is suggested that mechanical freezing damage to thylakoid membranes is brought about by the diffusion of solutes into the intrathylakoid space and by loss of membrane material from the vesicles under strongly hypertonic conditions. During thawing osmotic water influx leads to thylakoid swelling and membrane rupture.

Introduction

Freezing damage to cellular systems is a complex event but it appears that membrane damage is the common denominator of injury (see Refs. 1 and 2 for reviews). Freezing damage to isolated chloroplast membranes is strongly dependent on the nature of solutes present in the suspending medium and on their concentration (see Ref. 3 for a recent review). The kind of damage occurring at high initial solute concentrations in the presence of inorganic salts has been termed chemical damage [4]. It is accompanied by the dissociation of peripheral membrane proteins such as the CF1 part of the coupling factor ATPase from the membranes [5].

Below an initial solute concentration of about 0.1 osmolal membrane survival decreases with decreasing osmolality of the suspending medium [6–8]. Damage at low initial solute concentrations has been termed mechanical freezing damage [4]. It is accompanied by the loss of the electron-carrier protein plastocyanin from the thylakoid lumen [5]. Release of plastocyanin can obviously occur only after permeability barriers have, at least temporarily, been broken down.

It is not known yet which kind of freezing

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damage prevails in vivo. It has, however, been shown recently [9] that thylakoids isolated from frost-hardy leaves show a higher resistance against mechanical freezing damage than thylakoids isolated from non-hardy leaves, while there is no difference in the susceptibility of the membranes against chemical freezing damage.

In a previous publication [5] it has been proposed that mechanical freezing injury is the consequence of osmotic membrane rupture. In the present paper a mechanism for mechanical damage is suggested: under the extremely hypertonic conditions brought about by freezing, membrane material is lost from the vesicles and solutes enter the thylakoids which under normal conditions of concentration gradient and time scale are considered to be impermeable. After solute uptake during freezing membranes rupture osmotically during thawing if osmotic water influx forces excessive expansion.

Materials and Methods

Isolation and freezing of thylakoids. Thylakoids were isolated from spinach (*Spinacia oleracea* cv. Yates) leaves as described before [8]. They were washed twice in 10 mM NaCl and frozen in the presence of added solutes as indicated for 3 h at −20°C with a cooling rate close to 0°C of approx. 0.5 Cdeg/min. The samples were thawed rapidly in a water bath at room temperature.

Measurement of packed thylakoid volume. Samples were measured after dilution with solutions containing sucrose at concentrations between 20 and 500 mM (see legends to Figs. 2 and 3 for additional details). Suspensions were centrifuged for 15 min at $12000 \times g$ in a desk-top centrifuge (Christ, Osterode, F.R.G.) in micro-haematocrit capillaries (i.d. 1.1 mm) and pellet height was measured under a magnifying glass. Three measurements were taken from each sample and averaged. No attempt was made to correct for interstitial volume.

All other methods have been described before [4,8].

Results

The response of a suspension of vesicles surrounded by semipermeable membranes to changes in external osmolality can be described by the Boyle–van't Hoff equation:

$$V - b = (1/p)\,RTn$$

with V = total vesicle volume; b = apparent non-osmotic volume; p = osmotic pressure of the suspending medium in osmol/kg H_2O; R = gas constant; T = absolute temperature; n = number of moles of solute within the volume of $V - b$. A plot of V vs. 1/osmolal will yield a straight line with slope RTn and an intercept with the ordinate at b. The apparent non-osmotic volume includes all solids such as membranes and solutes plus the interstitial volume at infinite solute concentration. For better comparison all figures show $V - b$. The slope of the straight line (RTn) at a given temperature is determined by the amount of osmotically active material inside the vesicles. It can only change when solutes enter or leave the vesicles. For a more detailed discussion of the parameters of the Boyle–van't Hoff equation see Ref. 10.

Thylakoid membrane vesicles have been reported to act as ideal osmometers over a wide range of externally applied concentrations of solutes such as NaCl or sucrose [11]. Fig. 1 shows the osmotic response of thylakoids kept at 0°C for 3 h in solutions containing different sucrose concentrations. Sucrose is thought to be a non-penetrating solute with a reflection coefficient within 1% of 1.0 [12]. The volume of unfrozen thylakoids increased linearly when plotted as a function of reciprocal osmolality as expected from the Boyle–van't Hoff relation. When thylakoids were frozen for 3 h after suspending them in the different solutions, the expected linear response was observed only at high sucrose concentrations. At these concentrations, thylakoid volumes were larger after than before freezing. The larger volumes of thylakoids observed after freezing can be explained only if these thylakoids contained more osmotically active material. By the same line of reasoning it must be concluded that the decrease in the volume of frozen thylakoids observed at decreased sucrose concentrations (i.e. increased reciprocal concentrations; right part of Fig. 1) is caused by solute loss.

Gain by and loss of osmotically active solutes from frozen thylakoids are explored in more detail

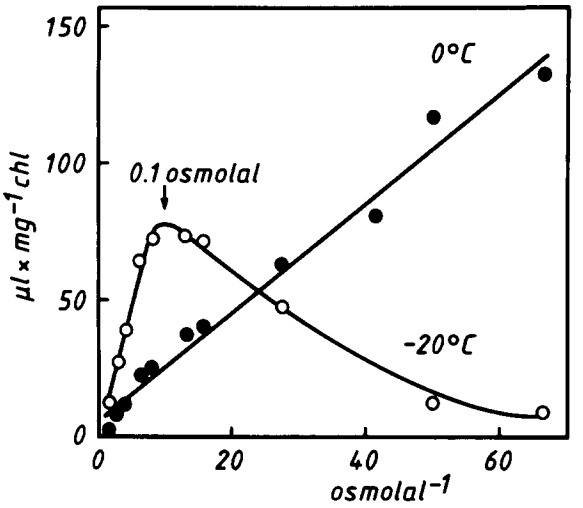

Fig. 1. Thylakoid volume in response to different osmolalities of the suspending medium after 3 h at 0 °C or −20 °C. The samples contained 5 mM NaCl and additional sucrose resulting in total concentrations between 0.015 osmolal and 0.64 osmolal. Packed thylakoid volume is plotted as a function of the reciprocal osmolality of the suspending medium (Boyle–van't Hoff plot). The straight lines were fitted to the data by linear regression analysis (0 °C: $r = 0.99$; −20 °C at 1/osmolal < 10: $r = 0.99$).

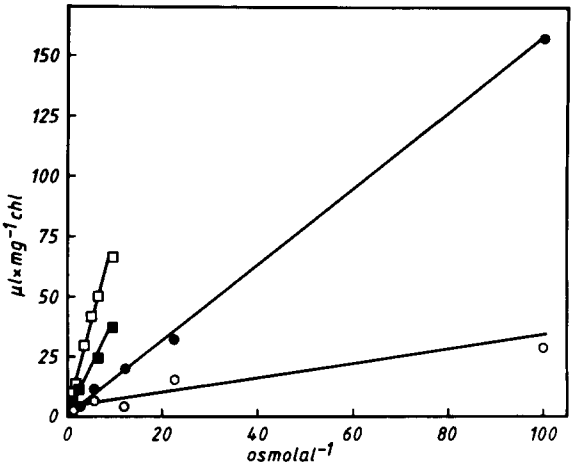

Fig. 2. Osmotic response of thylakoid vesicles. Suspensions were either kept for 3 h at 0 °C in the presence of 5 mM NaCl/10 mM sucrose (●) or 5 mM NaCl/500 mM sucrose (■) or in the same solutions for 3 h at −20 °C (corresponding open symbols). Aliquots of the samples were then diluted 5-fold with sucrose solutions of different concentrations. Packed thylakoid volume is plotted vs. the reciprocal of the osmolalities of the resulting solutions. The lines were fitted to the data by linear regression analysis: ●, $r = 0.99$; ■, $r = 0.95$; ○, $r = 0.80$; □, $r = 0.99$.

in Fig. 2. Thylakoid membranes were incubated in the presence of either 5 mM NaCl/10 mM sucrose or 5 mM NaCl/500 mM sucrose. Samples were either frozen-thawed or kept under ice-bath conditions as controls. After 3 h aliquots of the samples were diluted 5-fold with sucrose solutions of different concentrations and thylakoid volume was measured. Samples frozen in the presence of low solute concentrations showed a smaller volume change than controls incubated at the same concentration (Fig. 2).

Samples frozen in the presence of high sucrose concentrations, which afford complete cryoprotection [8], showed the steepest slope in Fig. 2, indicating solute uptake during freezing. The slope of unfrozen control samples kept at the high sucrose concentration was less steep than that of the frozen samples, but still significantly steeper than that of the samples held at 0 °C in the presence of low solute concentrations. This shows that sucrose slowly penetrated the thylakoid membranes even in the absence of freezing.

The influx of sucrose into the thylakoid vesicles at 0 °C is dependent on time and concentration (Fig. 3). The changes in the slope of the lines in Fig. 3 are further analysed in Fig. 4. Solute influx was linearly correlated with the concentration of the incubation medium at any given incubation time (Fig. 4a). Solute influx also proceeded linearly with time at all solute concentrations applied (Fig. 4b). Only in the presence of 10 mM sucrose was no change in the slopes detectable (Fig. 3), apparently because resolution was insufficient at this concentration.

From the data presented in Figs. 3 and 4 it is possible to calculate the permeability coefficient (P) for sucrose through thylakoid membranes at 0 °C. It is calculated from

$$J/c_0 = -P$$

with J = solute influx in mol/cm^2 per s; c_0 = concentration of solutes in the suspending medium, assuming that the sucrose concentration inside the thylakoid vesicles was zero at the start of the experiment. From the data presented in Fig. 4b a

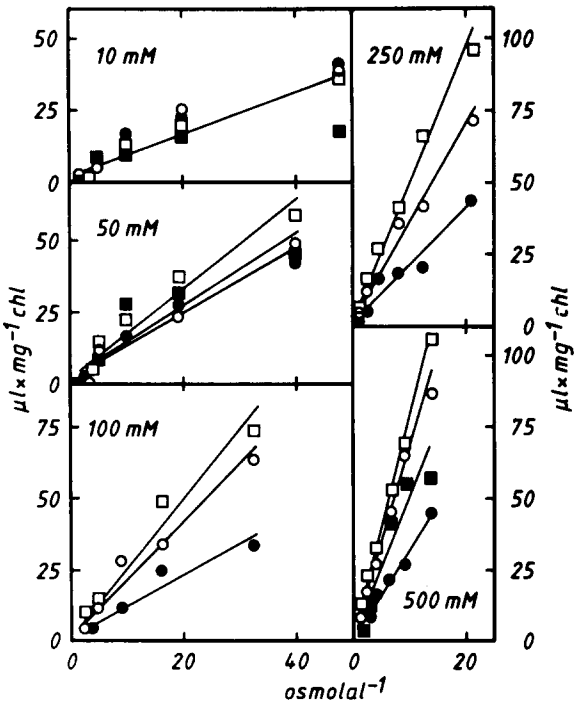

Fig. 3. Uptake of sucrose by thylakoids incubated in sucrose solutions of different concentrations. The thylakoid suspensions contained 5 mM NaCl and the sucrose concentrations indicated in the panels. Samples were kept at 0 °C throughout the experiment. After 0.1 h (●), 2 h (■), 4 h (○) and 6 h (□) aliquots were taken from the samples and diluted 10-fold with sucrose solutions of different concentrations. Packed thylakoid volume is plotted vs. the reciprocal of the osmolalities of the resulting solutions. The steepness of the slopes indicates the extent of sucrose penetration into the intrathylakoid space during incubation of the thylakoids in sucrose solutions. All lines were fitted to the data by linear regression analysis ($r = 0.88$–0.99).

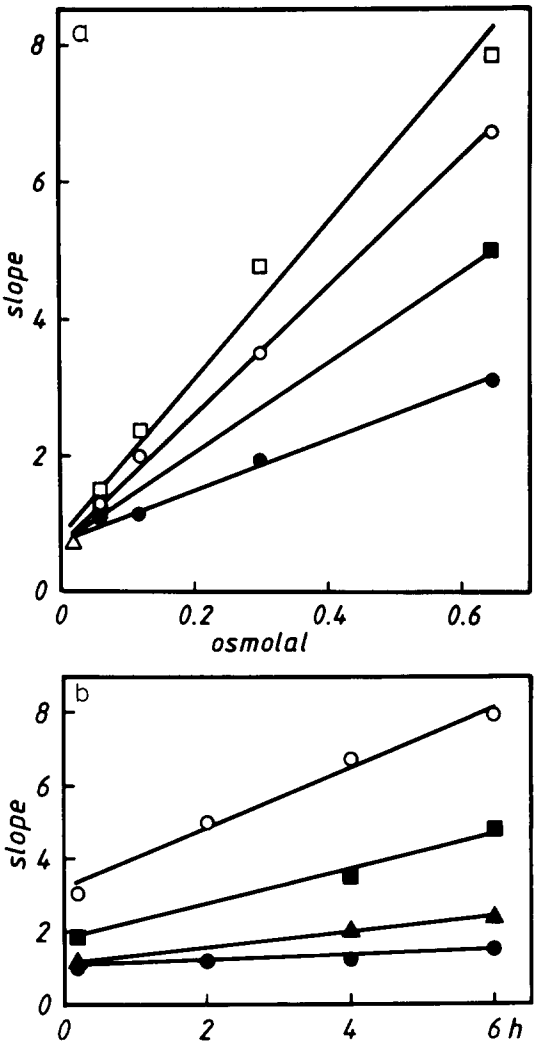

Fig. 4. (a) The slopes of the lines in Fig. 3 are plotted against the osmolalities of the solutions in which the samples were incubated. The symbols are the same as in Fig. 3. The lines were fitted to the data by linear regression analysis ($r = 0.99$ in all four cases). △ indicates the slope of the samples incubated at 10 mM sucrose (compare Fig. 3). (b) The slopes of the lines in Fig. 3 are plotted against the incubation time of the samples (see legend to Fig. 3 for details). The lines were fitted to the data by linear regression analysis: ●, 50 mM sucrose, $r = 0.92$; ▲, 100 mM sucrose, $r = 0.99$; ■, 250 mM sucrose, $r = 0.99$; ○, 500 mM sucrose, $r = 0.99$. The samples suspended in 10 mM sucrose were omitted from this graph, since there was no measurable volume change with time at this concentration (compare Fig. 3).

permeability coefficient of $P = (1.3 \pm 0.25) \cdot 10^{-10}$ m/s was calculated assuming a thylakoid area of 16.7 $cm^2/\mu g$ chlorophyll [13,14]. Since the membranes had been hypotonically stressed prior to the experiment by washing them twice in 10 mM NaCl, control experiments were performed with membranes washed isotonically (400 mM sucrose), which yielded a value of $P = 0.9 \cdot 10^{-10}$ m/s.

It was of interest to compare the permeability coefficient calculated for membranes at 0 °C with the permeability coefficient at the freezing temperature (−20 °C). For this purpose the data presented in Fig. 2 were used, but some assumptions had to be made. Temperature measurements with thermocouples had shown that the samples needed approx. 30 min to reach the final temperature

(data not shown). During this time the membranes experience intermediate temperatures and solute concentrations, which are difficult to assess in a quantitative manner. I have therefore calculated the permeability coefficient at −20°C with a time of 2 h 40 min at a final solute concentration of 12 molal (estimated according to Ref. 6). This results in a value of $P = 0.18 \cdot 10^{-10}$ m/s. From this a $Q_{10} = 2.67$ is obtained for the diffusion of sucrose across thylakoid membranes under freezing conditions. A comparison of the permeability coefficients at 0°C and 5°C yielded a Q_{10} of 2.89 for diffusion under non-freezing conditions.

The predictive value of these calculations was tested by comparing calculated volume changes during freezing with the data presented in Fig. 1. The calculation was based on the regression lines of Fig. 4a and b and on Fig. 5, which shows that the slopes of the regression lines in Fig. 4a change linearly with incubation time. Using this relationship it is possible to calculate the slope for any given combination of external solute concentrations and incubation times. The slope for a sample kept at 0°C for 2 h 40 min in the presence of a 0.64 osmolal sucrose solution is 5.47. Assuming that a frozen sample was kept at −20°C for the same time and using the Q_{10} of 2.67 this results in a calculated slope for the frozen samples of 12.53. The linear part of the −20°C curve in Fig. 1 has a slope of 9.45, indicating that experimental results can be predicted with reasonable accuracy.

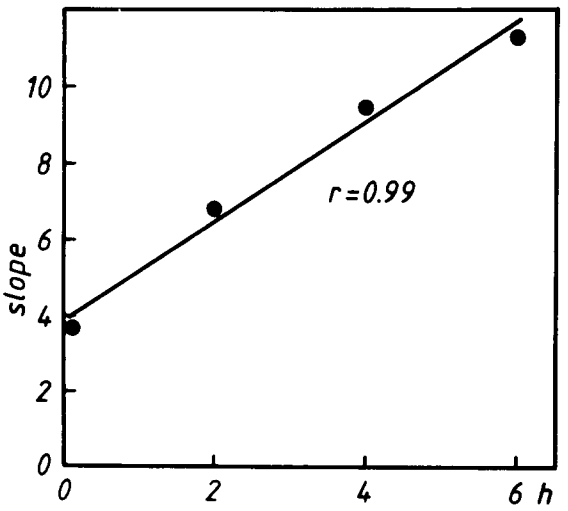

Fig. 5. The slopes of the lines in Fig. 4a, indicating the dependence of volume changes of thylakoids on the concentration of the suspending medium at different incubation times, are plotted as a function of incubation time. The line was fitted to the data by linear regression analysis.

Discussion

Mechanical freezing damage in vitro is accompanied by the loss of the lumenal electron-carrier protein plastocyanin [5]. Loss of an intrathylakoid protein is best explained by membrane rupture. This is demonstrated in Fig. 1, which shows that thylakoids frozen-thawed at low initial solute concentrations had smaller volumes than thylakoids kept at 0°C at low initial solute concentrations. Apparently, they had lost internal solutes after thawing. At high initial sucrose concentrations a linear Boyle–van't Hoff plot was obtained. It broke off at about 0.1 osmolal, the concentration below which mechanical freezing damage occurs [4]. The slope of the linear part of this curve at high initial solute concentrations is significantly steeper than the slope obtained from the ice-bath controls (Fig. 1), indicating solute uptake during freezing. Thylakoids frozen-thawed at low initial osmolalities did not regain the same volume that the controls reached, indicating loss of membrane material, as will be discussed below.

Although thylakoid membranes damaged by freezing at low initial osmolalities had lost most of their osmotically active solutes (Fig. 1), they were capable of maintaining a proton gradient in the light [5]. That semipermeability is indeed restored is also demonstrated in Fig. 2. Samples frozen at low initial osmolalities still responded to changes in the external solute concentration as ideal osmometers, but showed a gentler slope than the control samples. When the solute concentration was sufficient for complete cryoprotection (500 mM sucrose), the slope of the Boyle–van't Hoff plot was steeper in the frozen-thawed samples than in the unfrozen controls. An increase in thylakoid packed volume during freezing has also been found for samples frozen in the presence of NaCl [15] or sorbitol [16].

Fig. 3 shows that a substance such as sucrose, to which thylakoids are generally believed to be impermeable, penetrates thylakoid membranes

even under non-freezing conditions. The permeability coefficient for sucrose across thylakoid membranes was calculated as $P = (1.3 \pm 0.25) \cdot 10^{-10}$ m/s. This value is appreciably higher than the value reported for the plasmalemma of spinach mesophyll protoplasts ($P = 0.3 \cdot 10^{-10}$ m/s [18]).

It has been speculated [15,16] that due to the dehydration of the membranes during freezing transient membrane breakage leads to solute influx. Williams and Meryman [16] have proposed that the vesicles have a minimum tolerable volume below which they resist further shrinkage. A hydrostatic pressure would then develop across the membrane which would lead to transient membrane rupture and solute influx. This 'minimum critical volume hypothesis' has been challenged by several authors (see e.g. Ref. 2 for a review).

It was therefore of interest to analyse the permeability properties of thylakoid membranes in order to evaluate whether such a mechanism is required to explain solute uptake during freezing or whether diffusion alone could be responsible for the observed volume changes. From the data presented in Fig. 2 it was possible to calculate the permeability coefficient for sucrose across thylakoid membranes at $-20°C$ ($P = 0.18 \cdot 10^{-10}$ m/s) and by comparing it to the permeability coefficient at 0°C a Q_{10} of 2.67 was calculated. The Q_{10} for the diffusion of sucrose under non-freezing conditions was determined from permeability measurements at 0°C and 5°C as $Q_{10} = 2.89$. These values are quite similar and also in good agreement with Q_{10} values reported for the diffusion of different substances through human erythrocyte membranes at non-freezing temperatures, which ranged between 2.3 and 3.0 (reviewed in Ref. 18). This indicates that freezing temperatures do not drastically change the permeability properties of the membranes and argues against membrane breakage during freezing as proposed in Refs. 15 and 16.

By comparing volume changes during freezing calculated under the assumption of diffusion as the only factor responsible with measured volume changes (Fig. 1), it can be seen that changes were less drastic than expected from the calculations. Thus it appears that membrane breakage during freezing is not only unlikely (see above) but also unnecessary to explain the experimental data.

Osmotic damage to thylakoid membranes during an in vitro freeze-thaw cycle corresponds to the kind of damage which has been termed expansion-induced lysis in protoplasts (see Ref. 2 for a review). However, only little influx of solutes was found during freezing of isolated protoplasts [19]. Dowgert and Steponkus [20] reported that protoplasts lyse at a volume smaller than the initial volume after a freeze-thaw cycle. The same has been reported for unilamellar liposomes [21]. Frozen-thawed thylakoids challenged with dilute solutions after freezing were unable to reach the volume the control samples attained (Fig. 1). While the Boyle–van't Hoff plot of the suspensions kept at 0°C was linear over the measured concentration range and reached a volume of 133 µl/mg chlorophyll, the frozen-thawed samples had a maximum volume of only 73 µl/mg chlorophyll. In protoplasts such as reduction is explained by the appearance of membrane vesicles formed from the plasma membrane during hypertonic shrinkage. Upon thawing the vesicles are not reincorporated at a sufficiently high rate to compensate for the osmotic swelling [22]. Although vesiculation during freezing has never been demonstrated directly in thylakoids, the data presented in Fig. 1 indicate that the mechanisms of freezing damage may at least be similar in different membrane systems.

Slow permeation of solutes into thylakoids has been reported, but to my knowledge has never been analysed in detail, for a variety of substances such as sorbitol [16], NaCl [15], KCl [23] and several amino acids [24]. Some of these substances are also present in different concentrations in the chloroplast stroma. Whether the freezing damage thylakoids suffer in vivo is, at least in part, of the same nature as osmotic membrane damage in vitro remains to be determined.

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