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Mechanical and chemical injury to thylakoid membranes during freezing in vitro

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The relative contributions of membrane rupture due to osmotic stress and of chemical membrane damage due to the accumulation of cryotoxic solutes to cryoinjury was investigated using thylakoid membranes as a model system. When thylakoid suspensions were subjected to a freeze-thaw cycle in the presence of different molar ratios of NaCl as the cryotoxic solute and sucrose as the cryoprotective solute, membrane survival first increased linearly with the osmolality of the solutions used to suspend the membranes, regardless of the molar ratio of salt to sucrose. It subsequently decreased when the ratio of sucrose to salt was not sufficiently high for complete cryopreservation by sucrose. There was an optimum of cryopreservation at intermediate osmolalities (approx. 0.1 osmol/kg). This optimum of cryopreservation at a given sucrose concentration could be shifted to lower solute concentrations, if mixtures of NaCl and NaBr were used instead of NaCl alone. At suboptimal initial osmolalities, damage is attributed mainly to membrane rupture. Under these conditions, cryopreservation is not influenced by the chaotropicity of the suspending medium. At supraoptimal initial solute concentrations, solute (i.e., chemical) effects determine membrane survival. Under these conditions, increased ratios of sugar to salt increased cryoprotection. In mixtures of NaCl and NaBr at constant molar ratios of salt to sucrose, chemical membrane damage was quantitatively related to the lyotropic properties of the ions used. The degree of chemical damage becomes more pronounced with rising osmolalities of the suspending media. With NaF as the cryotoxic solute, damage was more severe than should be expected from its lyotropic properties. This may reflect a specific interaction of fluoride with the membranes. Protein release from the membranes during freezing in the presence of different anions was qualitatively comparable at identical ratios of sugar to salt. However, the total amount of protein released was correlated linearly with membrane inactivation, even when different anions acted on the membranes. Gel electrophoretic analysis of proteins released from thylakoid membranes during freezing revealed discrete bands indicative of mechanical and chemical damage, respectively.

Introduction

Different stresses are involved in the cryoinjury of plants (see Ref. 1 for a review). A major stumbling block in our understanding of the mechanisms of freeze-thaw injury has been the difficulty in distinguishing between the effects of chemical and mechanical stresses [2,3]. In many types of experiment, these stresses are difficult to separate. If, for example, suspensions of cells or isolated membrane vesicles are frozen, pure water is converted to ice and the solutes are concentrated drastically in the vicinity of the membranes. Membrane-enclosed compartments will be dehydrated and thus be stressed osmotically. At the same time, chemical membrane damage due to the accumulation of potentially cryotoxic solutes may occur. The degree of chemical damage will – according to

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the colligative theory of membrane preservation — depend on the molar ratio of potentially membrane-toxic solutes to membrane-compatible solutes and on their chemical properties.

Generally, chaotropic substances such as inorganic salts have been shown to be cryotoxic. By adding an excess of cryoprotective substances (usually sugars or sugar alcohols), the damaging effects of salts can be offset and the membranes can be protected. Protection by membrane-compatible solutes is based on their colligative properties. Briefly, the colligative theory of freezing damage and protection [4–6] demands that the ratio of cryoprotective to cryotoxic solutes determine the extent of freezing damage at a given temperature, regardless of the initial concentration of the solutes before freezing.

Small but distinct differences in the ability to protect membranes against inactivation have been described for various oligosaccharides [7–11]. They have been attributed to non-ideal activity-concentration profiles [9] or to specific, non-colligative interaction with membrane components [10–12]. Membranes suspended in low concentrations of a potentially cryoprotective solute which, however, were insufficient for providing full protection, could be further protected against freeze-thaw injury by the addition of low concentrations of various salts, which were cryotoxic at higher concentrations [12–14].

Recently, we have proposed that the colligative mechanism of protection against freezing injury is effective predominantly at high initial solute concentrations. At low initial solute concentrations, a different mode of cryoinjury predominates [15]. Under these conditions, membrane damage occurs, even in the presence of substances considered to be cryoprotective. This injury is thought to be predominantly mechanical. It appears to be caused by osmotic contraction or expansion.

In the following, we show that thylakoid membranes are more sensitive to freeze-thaw injury in the presence of strongly chaotropic salts, if they are suspended in solutions of high initial osmolarities and thus suffer colligative damage. At low initial osmolarities, solute concentration as such is the main factor determining cryoinjury, irrespective of the composition of the suspending medium.

Materials and Methods

Isolation of thylakoids. Thylakoid membranes were isolated from spinach leaves (*Spinacia oleracea* cv. Yates) as described previously [15]. In some experiments, other washing media were used. These are described in the figure legends.

Cyclic photophosphorylation. Thylakoids were illuminated for 40 s with white light from a photographic lamp at 2000 W/m^2 (total radiation) in a medium comprising 50 mM KCl/5 mM $MgCl_2$/15 mM Tris/0.25 mM KH_2PO_4/2 mM ADP/30 μM phenazine methosulfate (pH 8.0) at 15°C. Immediately after the reaction was stopped by switching off the light, the membranes were sedimented in the cold for 5 min at $10000 \times g$. Phosphate was determined in the supernatant in the presence of 20 mM sodium acetate (pH 4.5) and 1% sodium dodecyl sulfate according to Refs. 16,17.

Osmometry. The osmolality of solutions was determined using a Knauer semimicro osmometer (Knauer K.G., Oberursel, F.R.G.).

Gel electrophoresis. Gel electrophoresis was performed as described previously [15,18]. The sensitive silver nitrate procedure of Ansorge [19] was used for protein staining.

Protein determination. Protein was determined by dye-binding according to Bradford [20], using bovine serum albumin as a standard.

Other techniques. All other techniques have been described before [15,18].

Results

In a previous publication, we have described experiments which indicated that at least two mechanisms were operative in the cryoinjury of isolated thylakoid membranes (Ref. 15, see also Introduction). This has been concluded from an optimum in cryopreservation which was observed when the membranes were suspended in solutions of different concentrations but with constant ratios of cryotoxic to cryoprotective solutes. We have proposed that at low initial solute concentrations, i.e., less than about 100 mosmol/kg, freezing damage was caused by a mechanism which was different from the mechanism causing injury at higher initial osmolalities. Injury at low initial

osmolalities was thought to be mechanical and has been attributed to the direct effect of ice crystals [11,21] or to osmotic stress [15].

At high initial solute concentrations, the classical colligative theory of cryopreservation was proposed to be valid. According to this theory, excessive salt accumulation causes membrane damage, which can be prevented by adding membrane-compatible solutes which, by colligative action, decrease the salt accumulation during freezing, when pure water is converted to ice. If our conclusion that membrane damage as a consequence of freezing in low initial solute concentrations is mechanical/osmotical is correct, the membranes should respond to the chemical composition of the medium only at high initial solute concentrations. Only then should the molar ratio of cryotoxic to cryoprotective compounds, in accordance with the colligative concept of cryoprotection, determine the degree of injury. At low initial solute concentrations, the osmolality of the medium per se and not the solute composition should be the major factor in determining the survival of the membranes. This expectation is borne out by the experiment shown in Fig. 1. Thylakoid membranes were suspended in solutions containing four different molar ratios of sucrose (as the cryoprotective solute) to NaCl (as the cryotoxic solute). When the rate of photophosphorylation is plotted as a function of the osmolality of the solutions, it becomes apparent that below about 0.1 osmolar the rate of cyclic photophosphorylation shows a linear correlation with the osmolality of the medium. At higher osmolalities, membrane protection increases, as the molar ratio of sucrose to sodium chloride increases. It can further be seen that the optimum of cryopreservation is slightly shifted towards higher osmolalities, as the ratio of sugar to salt is increased.

It has been shown previously that the chemical properties of cryotoxic solutes are a major factor in freeze-thaw injury of isolated membranes. The cryotoxicity of halogenide anions has been shown to follow the Hofmeister lyotropic power series [22], increasing from fluoride to iodide [18,23,24]. Fig. 2 shows the effect of different anions on the rate of photophosphorylation. Again, the membranes were suspended in solutions containing constant molar ratios of cryotoxic to cryoprotective solutes. It can be seen that the optimum solute

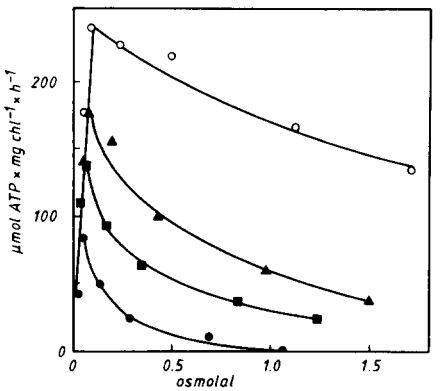

Fig. 1. The rate of cyclic photophosphorylation after a freeze-thaw cycle as a function of the osmolality of the medium. Thylakoids were suspended in solutions containing NaCl and sucrose at molar ratios of: ●, 1:1.5 (350); ■, 1:2 (351); ▲, 1:2.5 (355); ○, 1:3 (359). The values in brackets indicate the photophosphorylation rates (in µmol ATP/mg Chl per h) of the ice-bath controls at the end of the incubation period. The membranes were washed in media using the lowest concentrations at the NaCl to sucrose ratios listed above. The straight line at low initial osmolalities has been fitted by linear regression analysis ($r = 0.81$).

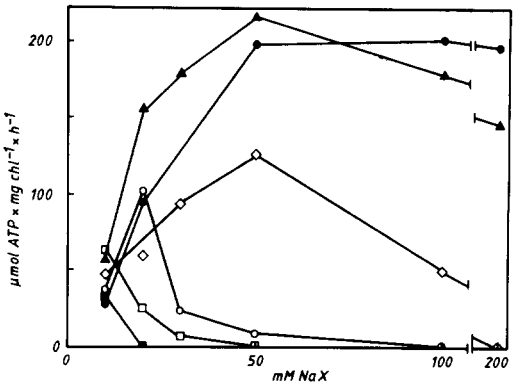

Fig. 2. The rate of cyclic photophosphorylation after a freeze-thaw cycle in the presence of different anions. All samples contained salt and sucrose at molar ratios of 1:2.5. The following salts were used: ●, NaF; ▲, NaCl; ■, NaBr; ◇, NaCl and NaBr at a molar ratio of 3:1; ○, NaCl and NaBr at a molar ratio of 1:1; □, NaCl and NaBr at a molar ratio of 1:3. The thylakoids were washed in ●, 10 mM NaF, 25 mM sucrose (300); ▲, ◇, ○, 10 mM NaCl, 25 mM sucrose (372); □, ■, 10 mM NaBr, 25 mM sucrose (396). The values in brackets give the photophosphorylation rates (in µmol ATP/mg Chl per h) of the ice-bath controls at the end of the incubation period.

concentration for cryopreservation was strongly dependent on the chemical properties of the ions tested. As the chaotropicity of the anion increased, the optimum was shifted towards lower solute concentrations. In solutions containing predominantly NaBr as the cryotoxic solute, an optimum in cryopreservation could no longer be resolved. In solutions containing sodium iodide and sucrose, no activity was recovered at the sugar-to-salt ratio used in Fig. 2.

The lyotropic properties of salts have been investigated in detail by Voet [25]. He could show that, among other effects, the ability of salts to dissolve (or precipitate) proteins can be treated quantitatively in relation to sodium sulfate and sodium chloride, which were chosen as standards. Voet was able to assign a number, N, to every ion. The higher the number, the stronger was the 'salting-in' effect of a given ion, i.e., its ability to keep proteins in solution. This ability can be explained by the influence of these ions on water structure. By breaking the structure of water to a different extent, they allow different amounts of proteins to stay in solution (for a comprehensive review see Ref. 26). The effects of mixtures of different ions were found to be additive if expressed by N. Membrane survival as expressed by the rate of photophosphorylation after freezing thylakoids in the presence of different anions is shown in relation to the lyotropic number, N, in Fig. 3. It can be seen that, for a given initial osmolarity, the photophosphorylation rate was linearly dependent on N if chloride and bromide are considered. If fluoride and chloride are compared, a different slope is obtained (Fig. 3; see Discussion). The sensitivity of the membranes to the chemical properties of solutes as a function of initial osmolarities

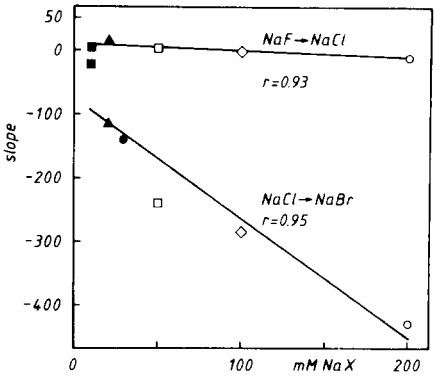

Fig. 4. The slopes of the lines NaF-NaCl and NaCl-NaBr were taken from Fig. 3 and plotted against the initial salt concentrations. Where applicable, the lines were fitted by linear regression analysis. The coefficients of regression for the lines NaCl-NaBr were: 10 mM NaX, 25 mM sucrose, $r = 0.61$; 20 mM NaX, 50 mM sucrose, $r = 0.88$; 30 mM NaX, 75 mM sucrose, $r = 0.92$; 50 mM NaX, 125 mM sucrose, $r = 0.96$; 100 mM NaX, 250 mM sucrose, $r = 0.97$.

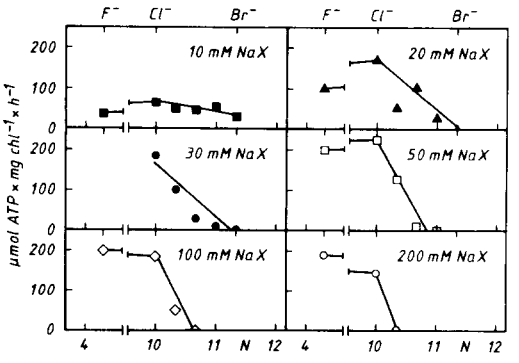

Fig. 3. The rate of cyclic photophosphorylation after a freeze-thaw cycle in the presence of different anions as a function of the lyotropic number, N (Ref. 25, see text). All samples contained salt and sugar at a molar ratio of 1:2.5. The data were taken from Fig. 2 and plotted in different panels for the respective initial concentrations (in mM salt) indicated in the figure.

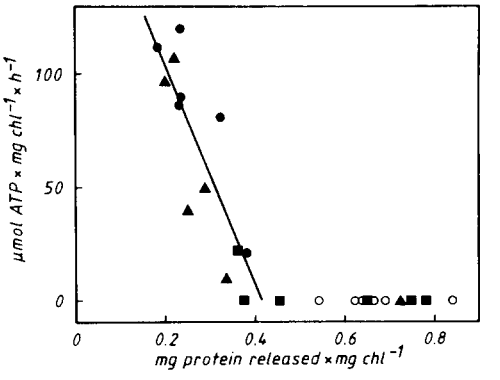

Fig. 5. Correlation of the rate of cyclic photophosphorylation with protein release after freezing to −20°C. All samples contained salt and sucrose at a molar ratio of 1:2.5. The salts used were: ●, NaF; ▲, NaCl; ■, NaBr; ○, NaI. The line was fitted to the data using linear regression analysis ($r = 0.83$). Only data from samples that were not completely inactivated were included in the computation.

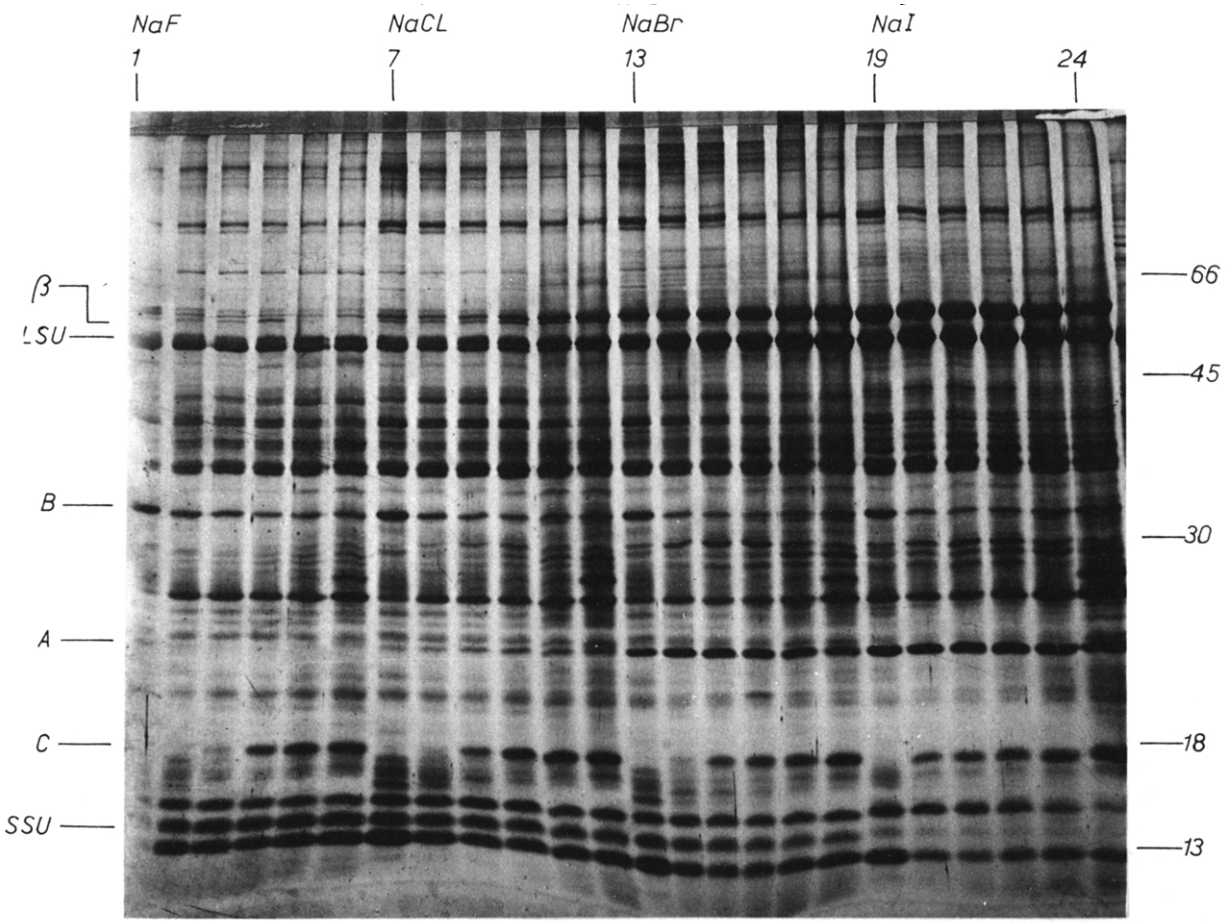

Fig. 6. Electrophoretic analysis of proteins released from thylakoids after a freeze-thaw cycle in the presence of different anions. The membranes were washed in 10 mM NaX/25 mM sucrose. The positions of molecular weight standards are indicated. Molecular mass is given in kilodaltons. LSU, SSU: Large and small subunit of ribulosebisphosphate carboxylase; beta indicates the position of a subunit of the CF1 coupling factor. The bands marked A, B, C are discussed in the text.

Lanes	mM NaX	mM sucrose	Lanes	mM NaX	mM sucrose
1, 7, 13, 19	10	25	4, 10, 16, 22	100	250
2, 8, 14, 20	20	50	5, 11, 17, 23	200	500
3, 9, 15, 21	50	125	6, 12, 18, 24	300	750

can be assessed from the slopes of the curves in Fig. 3. It can be seen that the susceptibility to chemical injury is linearly dependent on the initial osmolarity of the suspending medium.

Freezing damage to thylakoid membranes is accompanied by a loss of membrane proteins [18,24,27]. The electrophoretic patterns of proteins released by freeze-thaw treatment in the presence of NaCl are very similar to the patterns of proteins released by washing thylakoids in solutions containing EDTA [28]. Protein release is linearly correlated with membrane inactivation when sodium chloride and sucrose are used as solutes [15]. As has been shown previously [18], the total amount of protein released from the membranes increases with the cryotoxicity (or the lyotropic number N) of the anion used. In the presence of different anions, freeze-thaw inactivation of thylakoid mem-

branes is correlated linearly with protein release, as long as the membranes are not inactivated completely (Fig. 5).

The gel electrophoretic patterns of proteins released from the membranes after a freeze-thaw cycle in the presence of different anions and at different initial osmolarities is shown in Fig. 6. The general pattern of protein release was similar at different initial osmolarities, regardless of the solutes present. Some proteins show approximately the same intensities regardless of the freeze-thaw regime they were subjected to. There are, however, proteins (e.g., band A), which strongly respond to the lyotropic properties of the anions present. They are preferentially released in iodide but not in fluoride. The release of some proteins is influenced by the initial solute concentration of the suspending medium. This was also found for solutions containing iodide, which inactivated the membranes completely during a freeze-thaw cycle. The band marked B shows a protein which is preferentially released at low initial osmolalities, while the band marked C indicates a protein preferentially released at high initial solute concentrations.

A few bands are found in lower intensities in supernatants in the presence of iodide, most notably the small subunit of ribulosebisphosphate carboxylase. This is thought to be due to the presence of iodide in the solutions used to wash the thylakoids before freezing, which should be expected to lead to a better solubilization of loosely bound proteins [29]. For the large subunit of ribulosebisphosphate carboxylase this effect is not as clearly seen, because the beta subunit of coupling factor, which is detached in large amounts during freezing in iodide, obscures this band.

Discussion

In this and the preceding publication [15], we have tried to study freeze-thaw injury over a constant background of chemical stress by suspending the membranes in solutions of constant molar ratios but different concentrations of cryotoxic to cryoprotective solutes. An optimum in cryopreservation at intermediate osmolarities has suggested two types of injury, proposed to be predominantly mechanical at low initial solute concentrations and predominantly chemical at high initial solute concentrations [15]. Chemical injury is caused by the dissociation of peripheral membrane proteins [15,18,24]. Further investigations are necessary to define mechanical injury to membranes properly. It might be either caused by the action of ice crystals, by hypertonic breakage during dehydration, or by osmotic rupture during rehydration. Williams and Meryman [30] have observed the uptake of the normally non-penetrating cryoprotectant sorbitol into thylakoid vesicles under the strongly hypertonic conditions reached during freezing. On thawing, osmotic swelling would then result in membrane rupture.

Figs. 1 and 2 show that for a given pair of cryotoxic and cryoprotective solutes (NaCl and sucrose in Fig. 1), membrane survival after freezing is linearly dependent on the osmolality of the medium at low solute concentrations. At high initial solute concentrations, chemical damage prevails. Chemical damage is determined by the ratio of cryotoxic to cryoprotective solutes (Fig. 1) and by the cryotoxicity of the solutes (Fig. 2). Chemical damage is enhanced by higher initial solute concentrations. Since the final concentration of solutes is only determined by the freezing temperature, higher osmolarities of the suspending medium lead to increasing volumes of unfrozen solution in coexistence with ice. These larger volumes are thought to shift the association-dissociation equilibrium of peripheral membrane proteins towards dissociation and thus lead to a higher degree of freezing damage [15]. The optimum in cryopreservation at intermediate osmolarities can be shifted by varying the composition of the medium (Figs. 1 and 2). By using extremely chaotropic solutes, it can be suppressed totally (Fig. 2).

The chemical damage during freezing can be quantitatively related to the lyotropic properties [25] of the ions used (Fig. 3), which have initially been determined at non-freezing temperatures. The slopes of the lines in Fig. 3 between $N = 10$ and $N = 11.3$, i.e., between chloride and bromide can be read to indicate the relative contributions of mechanical and chemical injury (Fig. 4). The higher the slopes of these lines, the stronger is the influence of the chaotropicity of the suspending medium on membrane survival during freezing. In the presence of 10 mM salt, cyclic photophos-

phorylation is hardly influenced by the chemical properties of the solutes as expressed through N. The sensitivity to chemical damage increases linearly with increasing initial osmolarity (Fig. 4). This result is in accordance with the previous observations that membranes frozen in very low sucrose concentrations are damaged, but are well protected when suspended in high sucrose concentrations [11,15,27]. It also agrees with the fact that the degree of cryoprotection by higher membrane concentrations is linearly dependent on the initial osmolarity of the suspending medium. Higher membrane concentrations are thought to protect thylakoids against chemical damage by shifting the association-dissociation equilibrium of peripheral membrane proteins towards association [15].

Fluoride is more cryotoxic than chloride at low initial concentrations but less cryotoxic (as should be expected from its rank in the Hofmeister lyotropic power series) at high initial solute concentrations. The differences between fluoride and chloride in relation to their lyotropic number are far less pronounced than those between chloride and bromide (Fig. 3). This becomes also apparent in the release of membrane proteins as shown in Figs. 5 and 6. The band intensities between fluoride and chloride are not drastically different. The influence of the initial osmolarity on the sensitivity of the membranes to chemical damage is much less pronounced between fluoride and chloride, as shown by the slope in Fig. 4. The discontinuity in the freeze-thaw toxicity between fluoride, chloride and bromide as related to the lyotropic number is not readily explicable. It may indicate a specific interaction between the membranes and one of the ions tested. When membranes are washed in solutions containing fluoride and kept at ice-bath temperature, photophosphorylation is lower as compared to media containing chloride and bromide (see legend to Fig. 2). Specific effects of various carbohydrates on thylakoid membranes during freezing have been described (see references cited in the Introduction).

Gel electrophoretic analysis of the proteins released at different initial osmolalities and in the presence of different sodium salts has revealed individual bands which respond to the initial concentration of the suspending medium (e.g., bands B and C in Fig. 6). Other bands respond strongly to the chemical properties of the solution (e.g., band A in Fig. 6). We hope that the identification of such proteins will lead to a better understanding of the mechanisms of freezing injury to biomembranes.

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