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Structural changes in thylakoid membranes of chilling-resistant and sensitive plants after heating and glycerol dehydration as revealed by ^{31}P NMR and electron microscopy

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Abstract

^{31}P NMR spectra demonstrated that the chemical shift anisotropy of chilling-resistant peas and spinach thylakoid membranes is 110 ppm, whereas that for chilling-sensitive tomato membranes is 150–160 ppm. These data suggest different mobilities of membrane phospholipid orthophosphate moieties. When the samples of thylakoid membranes were heated (35–50°C) or incubated in an anhydrous glycerol (at 20°C) the chemical shift anisotropy gradually decreased to a level (20–40 ppm) which is an inherent feature of all model lipid systems (Cullis et al., in: Phospholipids and Cellular Regulation, Vol. 1, Structural properties and functional role of phospholipids in biological membranes, ed. J.F. Kul (CRC Press Inc., Boca Raton) p. 1). Electron microscopy of the same samples has revealed that these dynamic changes were accompanied by reorganization of membrane structure as evidenced by the alteration of size and distribution of IMPs on the EF fracture faces, the appearance of polymorphic structures and the disturbance of intramembrane grana contacts. Elevation of temperature during glycerol embedding similarly resulted in increased structural changes. We believe that both temperature and glycerol can induce similar disturbance of grana intramembrane interactions, as well as affect intermolecular bonds within membrane interface regions. The structural aspects of the thylakoid membranes are briefly discussed.

Keywords: Heat and dehydration damages; Thylakoid membranes; Change in phospholipid mobility; Membrane structure; Lipid–protein interphase interactions.

1. Introduction

The participation of "non-bilayer" lipids in bilayer–hexagonal phase transitions and their functional role in vivo has been the object of active research (see refs. [1–3]). On the other hand, it is well known that, for instance, under heating (35–45°C) a complete inactivation of photosystem [4,5] and formation of polymorphic structures [6] and dissociation of supramolecular complexes [7] occurs in thylakoid membranes. The same damages of chloroplast membranes were

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found after decreasing of pH or after treatment of thylakoid membranes by phospholipase A_2 [8].

A number of in vivo and in vitro studies indicate that adaptation of plants to high growth temperature leads to changes of saturation of the lipids of chloroplast membranes [9,10] and/or to changes of thylakoid lipid/protein ratio [11,12]. It is now established that elevation of temperature causing destabilization of bilayer organization of thylakoid membranes stimulates the synthesis of heat shock proteins (HSPs) [12,13].

As it has been recently discussed [3,14], the role of HSPs is probably to promote the refolding and reassembly of proteins that became denatured as a result of hyperthermia or other abuse. Thermal destabilization of membrane structure probably occurs as a result of the changes in the interaction of membrane proteins and lipids. It is pertinent to note that the bulk fraction of thylakoid lipids does not form a stable bilayer in the presence of chlorophylls [15] and that most of these polymorphic changes are irreversible [3]. It is likely that at elevated temperatures, protein denaturation is induced, and that this, in turn, destabilizes lipid/protein interactions and disturbs the contacts between grana thylakoid membranes. Simultaneously, it may induce thermo-dependent rupture of hydrogen bonds within lipid–protein moiety of membrane polar regions and promote phase separation of non-bilayer lipids. The same events might occur in these regions during dehydration, since the bulk of hydrophilic lipid–protein interactions takes place between the polar-glycerol backbone of lipid molecules and non-lipid components. Clearly, much is, at present, matter of speculation, as the real nature of these changes is not yet clear.

Nevertheless, it is also well known that vacuum- or low-temperature drying of membrane fractions, organelles, and even whole organisms leads to irreversible inhibition of their functions and to structural disorder of membranes [16–18]. However, a number of organisms (for instance, some type of nematodes) are able to survive after partial or total dehydration [19,20]. As it was shown, these organisms were capable to synthesize and to incorporate up to 20% of trehalose and up to 5% of glycerol into their membranes. The latter seems to ensure intactness of membrane structure and function under total dehydration [19,21]. The protective action of these components was confirmed experimentally in both in vivo and in vitro studies [22–24] that reveal that glycerol and trehalose maintained the lipid physical properties as well as membrane functional activity during freeze-drying. It was also shown that 100% glycerol retains a stable bilayer organization [25].

To obtain an experimental verification of the mechanism suggested above on thermal and dehydrational structural changes in thylakoid membranes, we undertook a comparative study of thermal and dehydrational effects on leaves and isolated chloroplasts of chilling-resistant and chilling-sensitive plants, under heating or by dehydration by 100% glycerol. ^{31}P NMR, and EM techniques were applied.

We report that the motion of the polar groups of phospholipids of intact chilling-resistant thylakoid membranes is considerably faster then that of the polar groups of phospholipids in chilling-sensitive plants. We have also found that, under heating and glycerol dehydration, the mobility of phospholipid polar groups increased gradually.

We also show that the increased mobility of phospholipid heads is accompanied by destabilization of lipid bilayer structures and morphological changes of the IMPs visualized on the EF of thylakoid membranes. We suggest that the data obtained may be of help to understand better the structural organization of thylakoid membranes.

2. Materials and methods

Samples and experimental procedures. Pea (Pisum Sativum), spinach (Spinacia Oleracea) and tomato (Lycopersicon Esculentum) were grown during 2–6 weeks in a greenhouse at the average temperature 22°C. Intact chloroplasts were isolated from 2–3 weeks, sprouts of pea and spinach from 4–6 weeks, tomato leaves in media contained 0.03 M PB (pH 7.5), 0.4 sucrose and 5 mM magnesium chloride. Media without sucrose were used for glycerol embedding. Small pieces of leaves and isolated chloroplasts were incubated

in thermostat at 35–50°C different periods (see Results). Glycerol dehydration was carried out by incubation of the samples in 10- to 15-fold excess of 100% glycerol at different temperature and duration. All chemicals and reactives used were reagent grade.

^{31}P NMR spectra of leaves and isolated chloroplast preparations were recorded at the appropriate temperature on a Bruker MSL-200 spectrometer (FRG, 81.01 MHz) equipped with an Aspect 3000 computer. Temperature was controlled at ±0.5°C with a standard Bruker B-VT-1000 variable temperature control unit. All chemical shift values are quoted in ppm from a pure lysophosphatidylcholine micelles (ppm), positive values referring to low-field shifts. All spectra of chloroplast dispersions (1.2 ml samples: 150–100 mg chloroplasts in $D_2D/H_2O = 1/10$) were obtained in the presence of grated broad-band decoupling (15 W input power during acquisition time). Free induction decays were obtained from up to 10000 transients. A spectral width of 100 kHz, a memory of 16 K data points, a 1 s interpulse time and a 90° radio frequency pulse were used. Prior to Fourier transformation, an exponential multiplication was applied resulting in a 100 HZ line broadening.

Electron microscopy. For thin sections leaves and chloroplast pellets were fixed with 1.25% glutaraldehyde (phosphate buffer pH 7.5, 1–2 h) and postfixed with 1% OsO_4 for 1 h. Samples were dehydrated, embedded, sectioned and stained according to standart procedures For freeze-fracture microscopy, all samples of leaves and isolated chloroplasts before or after the incubation in 100% glycerol and/or at an appropriate temperature were placed between two copper platinum covered strips which were plunged from appropriate temperature into liquid propane (−190°C) at sample cooling rates in excess of 8000–10000°C/s [26]. Up to nine frozen samples were simultaneously inserted into a modified JEE-4 freeze-fracture device (JEOL, Japan), fractured and replicated at −150°C and about 10^{-7} Torr (Pt at 45° carbon at 90°). The replicas were cleaned in Clorox, washed in water, picked up on uncoated 400-mesh grids and examined in a JEM-100 EM. The micrographs from thin sections and replicas were taken at a magnification of 10.000–50.000 ×. In all freeze-fracture micrographs, the Pt deposition direction is marked by contoured arrowheads.

Morphometric measurements of particles size and number of particles per unit of EF_s surface area were performed with an ocular micrometer mounted on a light binocular microscope. About 300 IMPs from 10 micrographs were measured for each experimental version. The average number of the IMP's per mm^2 of EF_s surface were calculated from 10 identical surfaces. The only EF_s fracture faces were used for measurements due to their more prominent changes and their concern to the grana intramembrane contacts.

3. Results

^{31}P NMR spectroscopy. ^{31}P NMR spectra of intact tomato thylakoid membranes exhibited a chemical shift anisotropy about 140–160 ppm (fig. 1A), whereas the latter for native pea and spinach thylakoid membranes was about 100–110 ppm (fig. 1B). For the dispersions of phospholipids (egg PC), it was about 40 ppm (fig. 2C). The ^{31}P NMR signals of the thylakoid membranes should be generated primarily by phosphorus acid diester of the thylakoid phospholipids. The comparison of NMR spectra (figs. 1A, 1B and 2C) suggests that all phospholipids of the thylakoid membranes have a stable bilayer structure they suggest also that free axial rotation of their polar moiety might be more restricted in tomato membranes, as compared to one of the chilling-resistant plants, the restriction being imposed by the interaction with other membrane molecular components (most likely with proteins). Upon heating (up to 35–50°C, for 0.5–3 h) of all thylakoid membrane suspensions the ^{31}P NMR signal narrowed and became more symmetric with chemical shift anisotrophy decreased to 20–40 ppm (figs. 1D, 1E). In chilling-resistant leaves and isolated tomato thylakoid membranes, this effect is delayed by 60–120 min. The incubation of pea and spinach thylakoid membranes in 100% glycerol for 2–4 h at 20°C caused the decrease of chemical shift anisotropy up to 35–40 ppm. (fig.

2A), whereas for tomato thylakoid membranes it was decreased up to 20 ppm (fig. 2B). Heating of glycerinated samples practically did not change ^{31}P NMR signals. Thus, NMR spectroscopy allowed us to detect the difference in motion of polar moiety of thylakoid phospholipids which is due to action of elevated temperature or glycerol dehydration. But these data do not give direct information with regard to the nature of molecular events which led to enhancement of lipid polar moiety motion. To seek additional information to correlate the data described above and thylakoid membrane morphology required electron microscopic examination.

Electron microscopy freeze-fracture replicas of the chilling-resistant thylakoid suspensions incu-

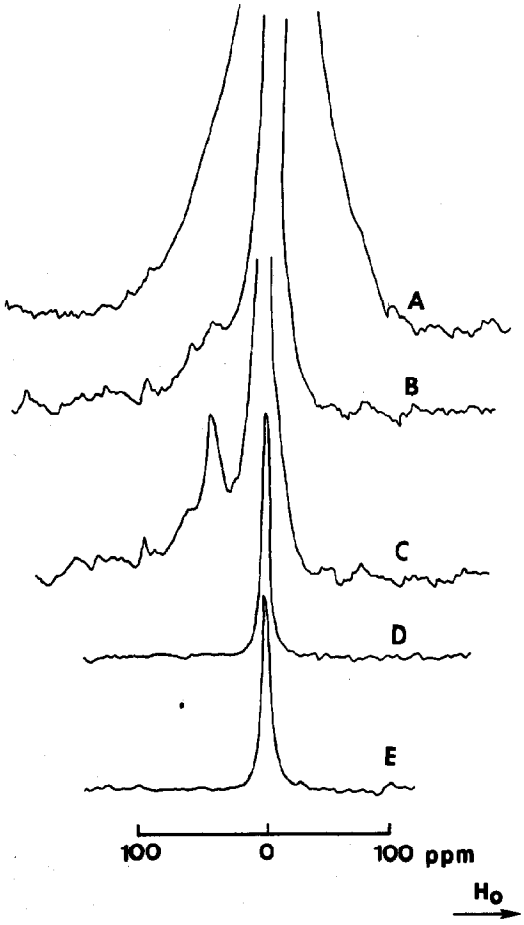


Fig. 1. 81.01 MHz 31P NMR spectra of aqueous suspensions of: (A) intact tomato thylakoid membranes at 20°C (a chemical shift anisotropy is about 140–160 ppm; (B) intact pea thylakoid membranes; (C) intact spinach thylakoid membranes after incubation at 20°C (4 h); (D) aqueous suspensions of pea thylakoid membranes after incubation at 45°C (2 h); (E) tomato mem-membranes after incubation at 50°C (3 h). Signals narrowed and became more symmetric with chemical shift anisotropy 20–40 ppm.

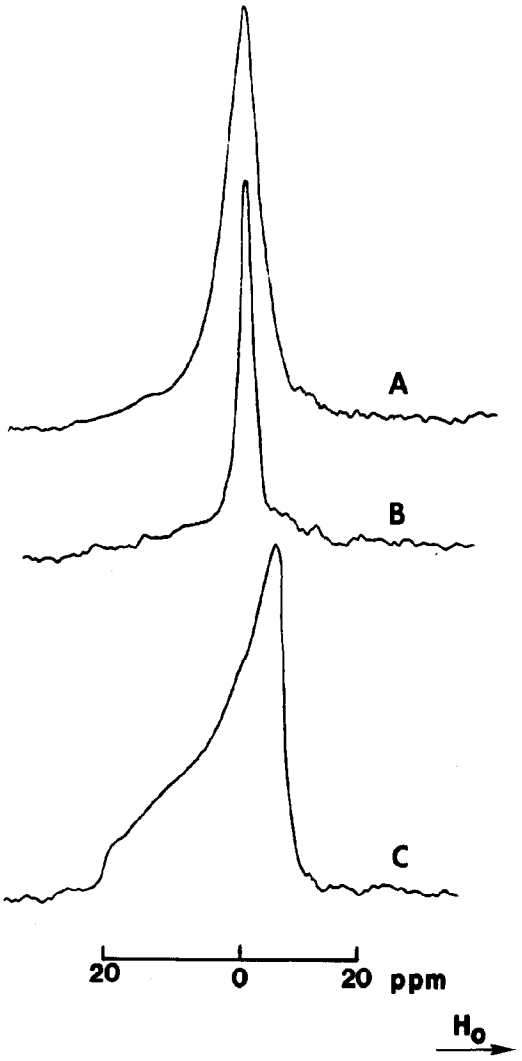


Fig. 2. 81.01 MHz 31P NMR spectra of: (A) suspensions of pea or spinach thylakoid membranes in 100% glycerol at 20°C (incubation for 4 h); a chemical shift anisotropy is about 35–40 ppm. (B) suspension of glycerinated tomato thylakoid membranes: a chemical shift anisotropy 20 ppm. (C) dispersion of phospholipid (egg PC) at 20°C: chemical shift anisotropy 40–45 ppm.

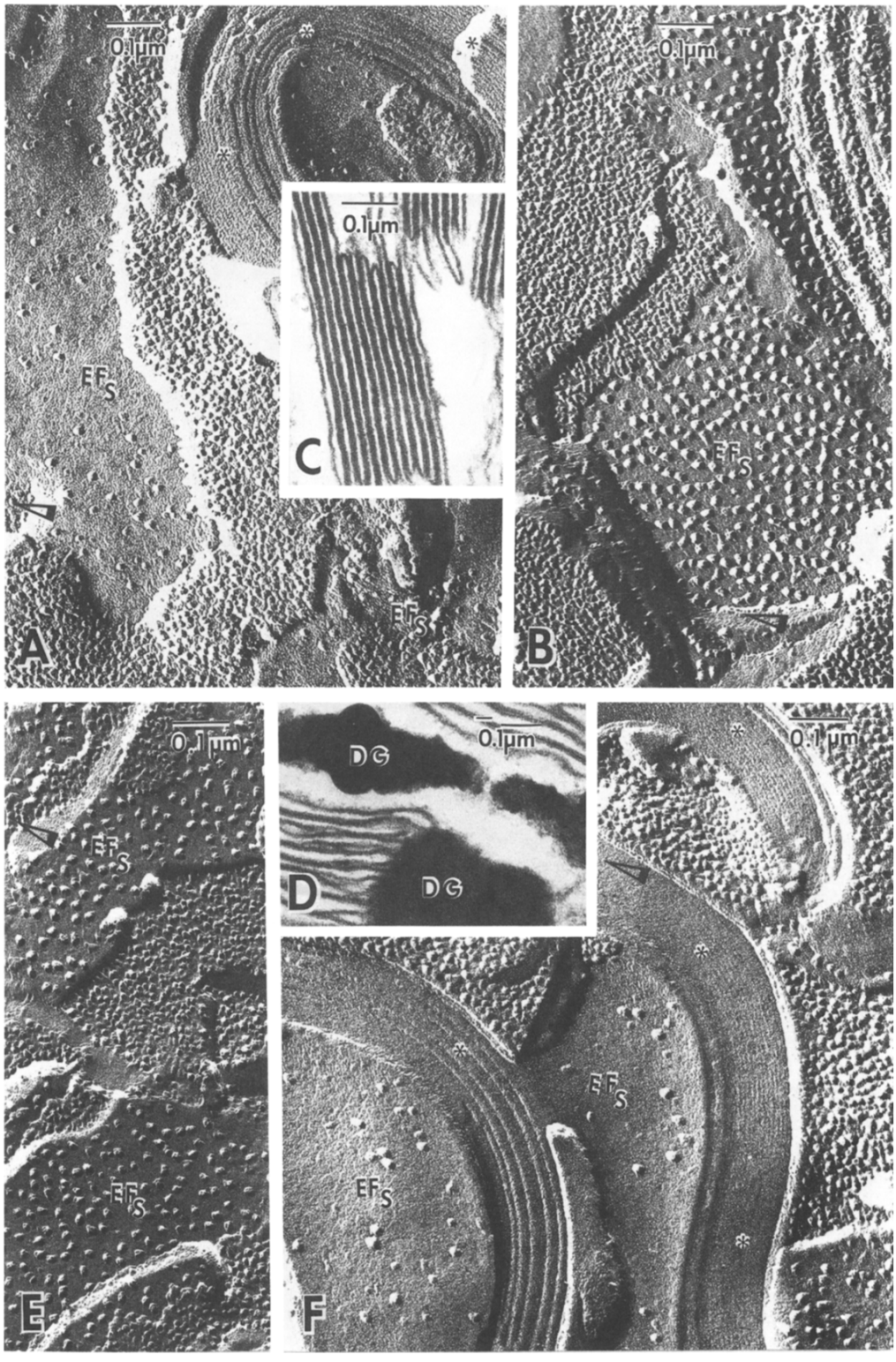


Fig. 3. Electron micrographs of freeze-fracture replicas (A, B, E, F) and thin-sections (C, D) of aqueous suspension of thylacoid membranes: (A) of pea incubated at 37 C (1 h) and quenched from the same temperature and (B) qenched from 20°C (control); (F) of spinach incubated at 50°C (35 min) and quenched from the same temperature; (E) control quenched from 20°C.

Table 1

Particle density and size distribution of total particle number (%) of EF_s fracture face of pea thylakoid membranes

Incubation	Particules per μm^2	Particle size (nm)		
		10–14	14.5–20	20
control (20°C)	1200	15	75	10
heating (50°C)	400	70	30	–
100% glycerol	600	46	54	–
100% glycerol (20°C) + heating (50°C)	600	70	30	–

Table 2

Particle density and size distribution of total particle number (%) of EF_s fracture face of tomato thylakoid membranes

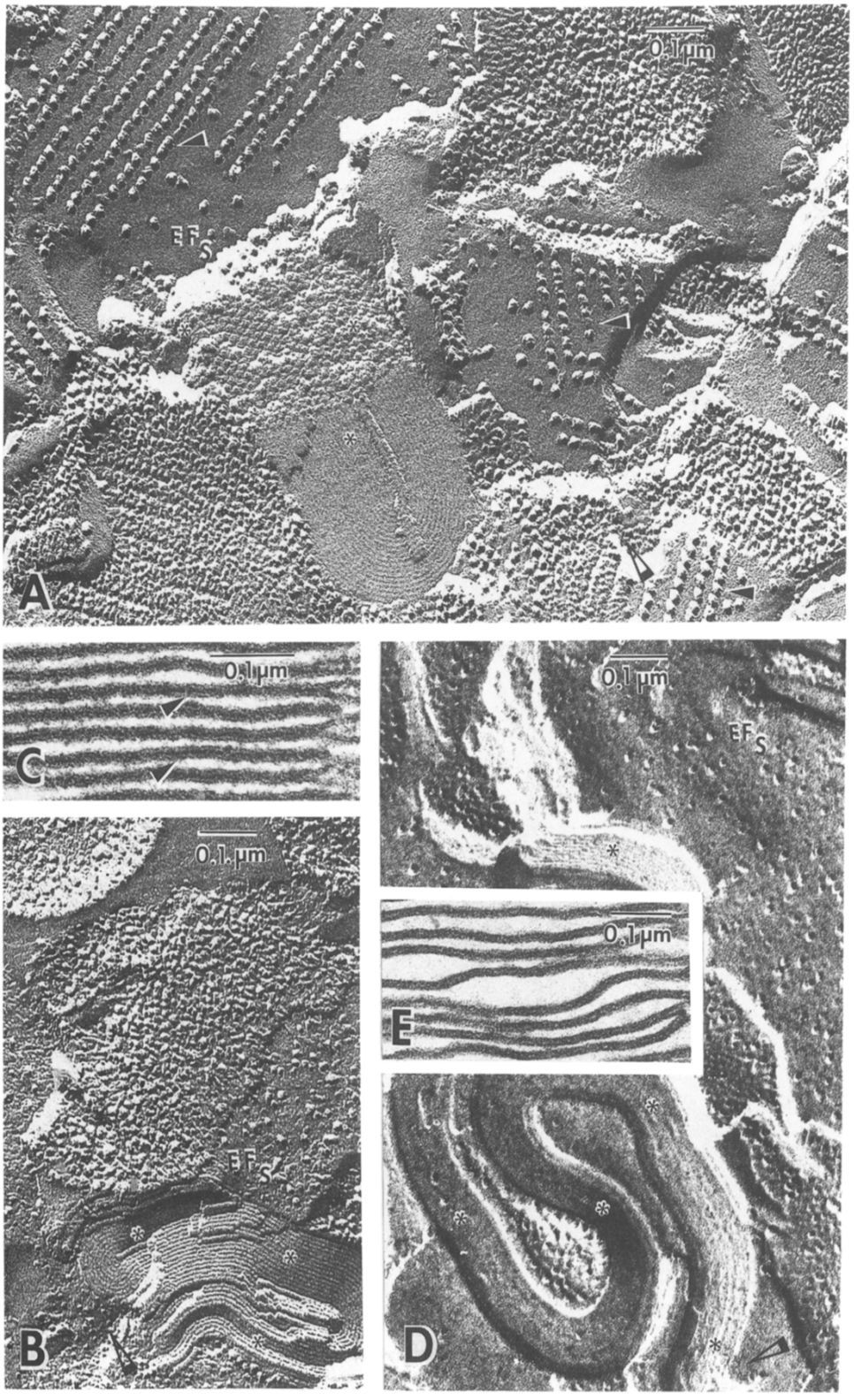
Incubation	Particles per μm^2	Particle size (nm)		
		10–14	14.5–20	20
control (20°C)	1400	20	60	20
heating (50°C)	700	50	50	–
100% glycerol	1600	–	25	75
100% glycerol (20°C) + heating (50°c)	600	36	44	20

bated at 35–37°C for 60 min (or at 45–50°C for 10 min) and subsequently quenched (or fixed) usually revealed large areas composed of non-bilayer, tightly packed polymorphic structures (figs. 3A, 3F), which we interpreted to correspond to the dense granules in thin section micrographs (fig. 3D). The dimensions and morphology of these polymorphic structures were quite similar to those of structures that were found in total thylakoid lipid suspensions containing chlorophylls [15]. The size and number of the IMPs per μm^2 of EF_s fracture face was decreased as compared to that of control samples (figs. 3B, 3E, also see table 1). Longer heating of the samples for 30–60 min at 45–50°C resulted in an overall increase of the structural changes (fig. 3F) and the appearance of unstacked grana membranes (fig. 3D, control fig. 3C). It is of interest to note that the storage of isolated pea thylakoid membranes for up to 72 h at 0°C also lead to the appearance of the same polymorphic structures and to a peculiar change of IMPs distribution on EF_s fracture faces (fig. 4A). However, more prolonged storage at −196°C preserved membrane structure well. These results might indicate that the structural well. These results might indicate that the structural changes induced by heating and dehydration are similar to those that involve membrane lipid phase transitions. Time-dependent accumulation of lipid phase transitions might be responsible for observed structural changes after storage of pea thylakoids at 0°C. The thermo-induced structural changes in leaves of chilling-resistant plants usually appeared within the same time delay as it was detected for ^{31}P NMR spectra.

Incubation of pea and spinach membranes in 100% glycerol at 20°C resulted in identical structural changes (fig. 4B). However, grana intramembrane contacts became more condensed (fig. 4C). Heating of glycerol-embedded samples induced an overall increase of structural changes of the thylakoid membranes and in unstacking of grana membranes (fig. 4E).

Incubation of tomato membrane suspensions at 45–50°C for 1–24 h did not induce any polymorphic transition. However, after heating for more than 2 h, some morphological changes (see Table 1) of EF_s particles became detectable (fig. 5B), as compared to the control samples (fig. 5A). Unstacked tomato grana membranes could only be visualized after more than 2 h incubation at 45–50°C. The incubation of tomato leaves for periods not longer than 2 h at 50°C and cooling to 20°C led to reversible and insignificant structural changes of thylakoid membranes, whereas

Fig. 4. Electron micrographs of freeze-fracture replicas (A, B, D) and thin-sections (C, E) of aqueous suspension of: (A) pea thylakoid membranes incubated at 0°C (72 h) polymorphic structures (asterisks) and a peculiar (linear, arrowheads) distribution of particles on EF_s fracture faces are seen; (B) spinach thylakoid membranes incubated in 100% glycerol (4 h) at 20°C and quenched from the same temperature, the same structural changes (see fig. 3, are seen, but grana intramembrane contacts became more condensed (C); (D) the same as in (B), but incubated at 45°C (2 h) the heating is accompanied by an overal increase of structural changes and by the appearance of unstacked grana membranes (E).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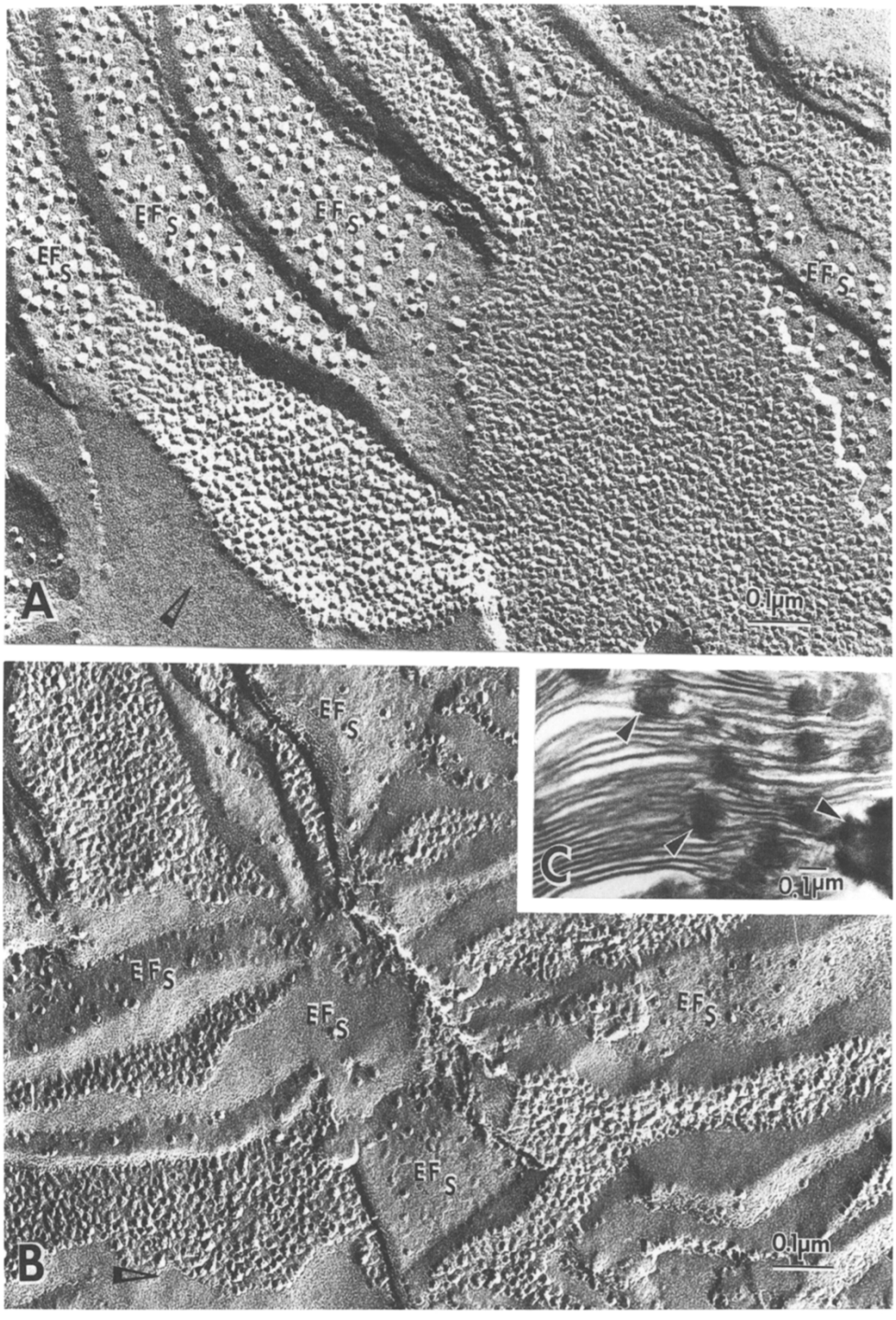

Fig. 5. Electron micrographs of freeze-fracture replicas (A, B) and thin-section of tomato leaves (C) and aqueous suspensions of isolated thylakoid membranes quenched from 20°C (A) and quenched from 50°C after incubation at the temperature more then 6 h (B); (C) tomato leaves incubated at 50°C during 4 h: numerous dense granules (arrowheads) are seen; the appearance of these granules coincided with unreversible polymorphic lipid transition.

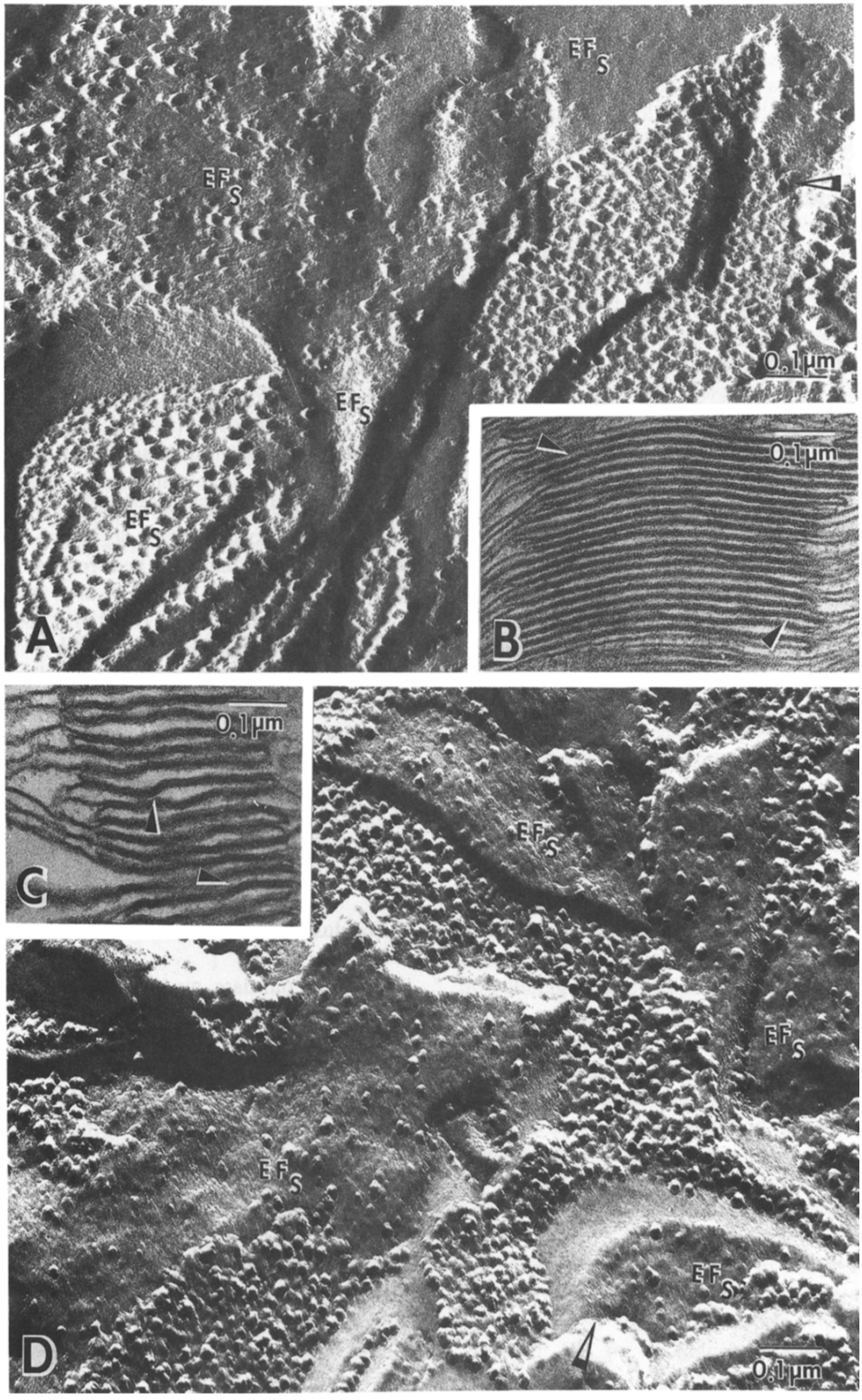

Fig. 6. Electron micrographs of freeze-fracture replicas (A, D) and thin sections (B, C) of tomato thylacoid membranes: (A, B) embedded into 100% glycerol (at 20°C, 4 h); (C, D) glycerinated sample was incubated at 50°C during 1 h (see text and table 2).

the rising temperature longer than 2 h coincided with an unreversible polymorphic transition (fig. 5C, dense granules). Embedding of tomato thylakoid membranes into 100% glycerol at 20°C for 2–4 h, induced the increasing of size of EF_s IMPs (compare fig. 5A, also table 2) as well as their number per μm^2 of EF_s (fig. 6A, see also table 2). There was a definite correlation of those changes with the increasing of electron density of grana intramembrane contacts (fig. 6B). This correlation disappeared under heating of glycerol embedded tomato samples: grana membrane contacts were well preserved (fig. 6C, arrowheads), but the size of EF_s IMPs and their number per mm^2 of EF_s faces decreased significantly (fig. 6D, see also table 2).

4. Discussion

Our results confirm that elevated temperature and dehydration of thylakoid membranes destabilize membrane structure by similar molecular mechanisms. This is possibly due to protein denaturation and to disturbance of hydrogen bonds within lipid–protein moiety of membrane polar regions. In contrast to temperatures which might directly denaturate membrane proteins, glycerol seems to first dehydrate membrane interface regions which might in turn lead to conformational changes of proteins.

Our ^{31}P NMR and electron microscopy data show that elevation of temperature and glycerol embedding induce an identical rate of mobility of lipid orthophosphate moiety. Lipid motion gradually increases and proportionally coincides with membrane structural polymorphic transition, except in isolated (but not in leaves) tomato thylakoids: in spite of the sharp decrease of their chemical shift anisotropy (up to 20 ppm in glycerinated samples) neither isotropic signals in NMR spectra and polymorphic structures in electron microscopy were observed.

The decrease of chemical shift anisotropy in this case may be attributed to the breakage of lipid–protein links, which might be related to changes of IMPs morphology on EF_s fracture faces. It may be concluded that during the isolation of tomato thylakoids the stability of their lipid bilayers is significantly increased. Moreover, the heating of glycerinated samples does not induce any additional changes in the ^{31}P NMR signals, but it causes the additional reorganization of membrane structure: the bulk of polymorphic structures becomes enlarged and it affected the morphology of IMPs and, in addition, induced membrane unstacking. These thermo-induced changes in glycerinated samples probably occurred due to the disturbance of intramembrane grana contacts.

It is not impossible that tomato (and probably other chilling-sensitive plants) grana membranes possess particular intramembrane links between hydrophilic molecular components. Those might be responsible for the more restricted motion of phospholipid polar moiety of intact thylakoids and for the providing with heat-resistant properties.

We believe that both the temperature and glycerol by itself can induce similar disturbance of grana intramembrane interactions, as well as affect intermolecular bonds within membrane interface regions. This link breakage promotes both lipid rotational freedom (the appearance of polymorphic structures) and affects the physical state of lipid–protein complexes, which in turn influences IMP morphology.

5. Conclusion

Based on our data, we conclude that the structural (and probably functional) organization of all thylakoid membranes is intact near the phase transition temperature of their phospholipids (25–30°C). We suggest that thylakoid membranes in intact leaves of pea and spinach are more resistant to elevated temperatures, as compared to isolated ones.

We believe our study to be the first where a correlation between the mobility of polar moiety of membrane phospholipids and membrane morphological changes of thylakoid membranes of chilling-resistant and sensitive plants has been demonstrated. Also new is our demonstration that the different restriction of lipid polar group

motion of intact thylakoid membranes of different plants. We wish to emphasize that the interactions between different molecular components near and within membrane interphase regions are quite important for the maintenance of structural and functional integrity of most biological membranes.

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