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Structural reorganization of thylakoid systems in response to heat treatment

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Abstract

The structural reorganization of pea thylakoid systems in response to osmotic shock in a wide range of temperatures (36–70 °C) was studied. At temperatures 40–46 °C, the configuration of thylakoid systems changed from a flattened to a nearly round, whereas thylakoids themselves remained compressed. The percentage of thylakoids stacked into grana at 44 °C decreased from 71 % in the control to 40 % in experimental samples, reaching 59 % at 48 °C. At 44 °C and above, thylakoid systems ceased to respond to the osmotic shock by disordering, in contrast to what happened at lower temperatures (36–43 °C) and in the control, and retained the configuration inherent in thylakoid systems at these temperatures. At 50 °C and above, the packing of thylakoids in grana systems changed, and thylakoids formed extended strands of pseudograna. Simultaneously, single thylakoids formed a network of anastomoses through local fusions. At temperatures of 60–70 °C, thylakoid systems appeared as spherical clusters of membrane vesicles with different degree of separation.

Additional key words: osmotic shock; pea; *Pisum*; ultrastructure of thylakoid systems.

Introduction

The processes of oxygen release, photophosphorylation, and photosystem 2 (PS2) mediated electron transport in the thylakoid membrane are very sensitive to high temperatures (Molotkovsky and Zhestkova 1965, Yamashita and Butler 1968, Tikhonov and Ruuge 1978, Berry and Björkman 1980, Cramer *et al.* 1981, Gounaris *et al.* 1983, Sundby *et al.* 1986, Thomas *et al.* 1986, Shutilova *et al.* 1992). There is a great number of papers devoted to the effect of heat on the functional activity of chloroplasts, whereas the structural aspects of this problem have received little attention (Armond *et al.* 1980, Gounaris *et al.* 1983, 1984). Reviewing the results of these studies, Staehelin (1986) noted that both heat-loving and cold-resistant plants respond to heat stress in a similar manner. The response manifests itself in (*1*) a decrease in the number of membrane stacked regions due to structural changes of these regions, (*2*) a decrease in the size of particles on the EF fracture face, and (*3*) the formation of extra-membrane aggregates due to the phase separation of non-bilayer-forming lipids. The authors of these studies believe that heat stress causes a direct physical dissociation of the light-harvesting complex and the core of PS2, and the exit of lipids from the membrane due to the phase transition affects this process in one way or another. I studied structural changes in pea thylakoid systems treated with temperatures 36–70 °C and their response to the osmotic shock in this temperature range.

Materials and methods

Chloroplasts were isolated from leaves of 2–3-week seedlings of pea (*Pisum sativum* L.). Several grams of leaves were re-suspended in a tenfold volume of cold medium containing 30–40 mM phosphate buffer and 2 mM $MgCl_2$ (pH 7.4). The homogenate was squeezed through a double layer of gauze and sedimented. The sediments of chloroplasts were washed and re-suspended in the same medium. Aliquots (0.1 cm^3) of the chloroplast suspension (chlorophyll 0.5 kg m^{-3}) were incubated for 10–15 min on a water bath at a particular temperature. After heat treatment, part of samples was subjected to osmotic shock by re-suspension for 5–10 min in 10 cm^3 of distilled water and sedimented. The sediments were fixed with 2.5 % glutaraldehyde in phosphate buffer (pH 7.4) followed

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by post-fixation in a 1 % osmium tetroxide solution. Fixed samples were dehydrated in alcohols and acetone and embedded in *Epon 812*. Ultrathin sections were stained with uranyl acetate and lead citrate.

The length of thylakoid profiles and the length and width of thylakoid systems were measured on electron micrographs of sections from 20–30 chloroplasts for each experiment.

Results

Chloroplasts isolated from leaves retained the architecture of the system of internal membranes, inherent in the native state. Closed and flattened membrane discs compressed to a thickness of 20 nm (with a lumen of 5 nm) were packed into stacks, grana, which were connected by single elongated thylakoids. All thylakoids formed a united system, which was also flattened and elongated as a single thylakoid (Fig. 1*A*). The degree of flattening of these systems varied depending on many factors, including temperature. Native thylakoid systems of freshly isolated chloroplasts undergo complete disordering upon incubation in distilled water (osmotic shock) and represent tightly compressed fragments of single thylakoids of different length and orientation (Semenova 2001). Reduced grana may also be present. The fragments of thylakoids remained un-swollen. This state of shocked thylakoid systems, *i.e*. disordering, unstacking, and fragmentation, will be referred below to the loss of native organization. In the present study, I tested the response of thylakoid systems to the osmotic shock in a range of temperatures of 36–70 °C.

Fig. 1*A,B* shows thylakoid systems of freshly isolated chloroplasts incubated at room temperature (18–20 °C) and their disordering in distilled water. The degree of flattening of the thylakoid systems, which is determined by the ratio of the longitudinal to transversal axes, was 2.9, and there were 71 % of thylakoids stacked into grana (Table 1). Thylakoids were compressed and had a small lumen of about 5 nm. In distilled water, thylakoid systems became entirely disordered and fragmented. Also, small grana were visible (Fig. 1*B*).

At temperatures of 36–38 °C, the structure of thylakoid systems underwent minimum changes (Fig. 1*C*). The percentage of thylakoids stacked into grana was 63 %, which is close to that in the native state. The degree of flattening of thylakoid systems changed insignificantly. The ratio of the longitudinal to transversal axes was 2.6 compared with 2.9 in the control. The osmotic shock caused a similar disordering of thylakoid systems as in the control (Fig. 1*D*).

Table 1. Morphological analysis of pea thylakoid systems incubated at different temperatures. Means of 3 to 5 experiments.

	20 °C	38 °C	42 °C	44 °C	48 °C
Length/width of the thylakoid system	2.9	2.6	1.9	1.5	2.2
Thylakoids packed into grana [%]	71	63	42	40	59

Increase in incubation temperature to 40–42 ºC lead to a considerable loss of flattening (Fig. 1*E*). The ratio of the longitudinal to transversal axes was 1.9, and the amount of thylakoids stacked into grana decreased to 42 % (Table 1). Thylakoids remained compressed. The osmotic shock caused a similar disordering of thylakoid systems as in control samples (Fig. 1*F*).

At temperatures of 44–46 °C, thylakoid systems became even more rounded (Fig. 1*G*), the ratio of the axes was 1.5, and the portion of thylakoids stacked into grana remained practically unchanged being 40 % (Table 1). At 44 °C, the osmotic shock caused no disordering in most of the thylakoid systems, and at 45–46 ºC, all thylakoid systems did not respond to the osmotic shock (Fig. 1*H*). Thylakoid systems retained the initial configuration and integrity (Fig. 1*H*). Thylakoids did not break into fragments and were only slightly swollen (Fig. 1*H*).

At 47–48 ºC, thylakoid systems had a more flattened configuration (Fig. 2*A*), the ratio of the axes was 2.2, and the percentage of thylakoids stacked into grana was higher than at 44–45 ºC, *i.e*. 59 % (Table 1). The osmotic shock caused no disordering of thylakoid systems, and they all retained the initial configuration (Fig. 2*B*).

At a temperature of 50 ºC and above, a radical structural rearrangement of the system of thylakoid membranes took place (Figs. 2*C* and 3*A–C*). Part of thylakoids in the system formed long strands of pseudograna composed of several thylakoids, which were so tightly compressed that individual thylakoids became undistinguishable (Fig. 3*C*). These pseudograna on sections were 5–6 μm long compared with 0.5–1.5 μm in the norm (Fig. 2*C*).

Single thylakoids formed either the strands of pseudograna or a unified network of anastomoses through local fusion of adjacent thylakoids. The initial stage of fusion was clearly seen after a 1-min exposure of a thylakoid system to 50 ºC (Fig. 3*A*). In local regions of thylakoids, peak-shaped extrusions were formed, which then fused to form a continuous network clearly seen at longer exposures (Fig. 3*B*). The osmotic shock did not affect the structure of thylakoid systems incubated at 50 ºC (Fig. 2*D*).

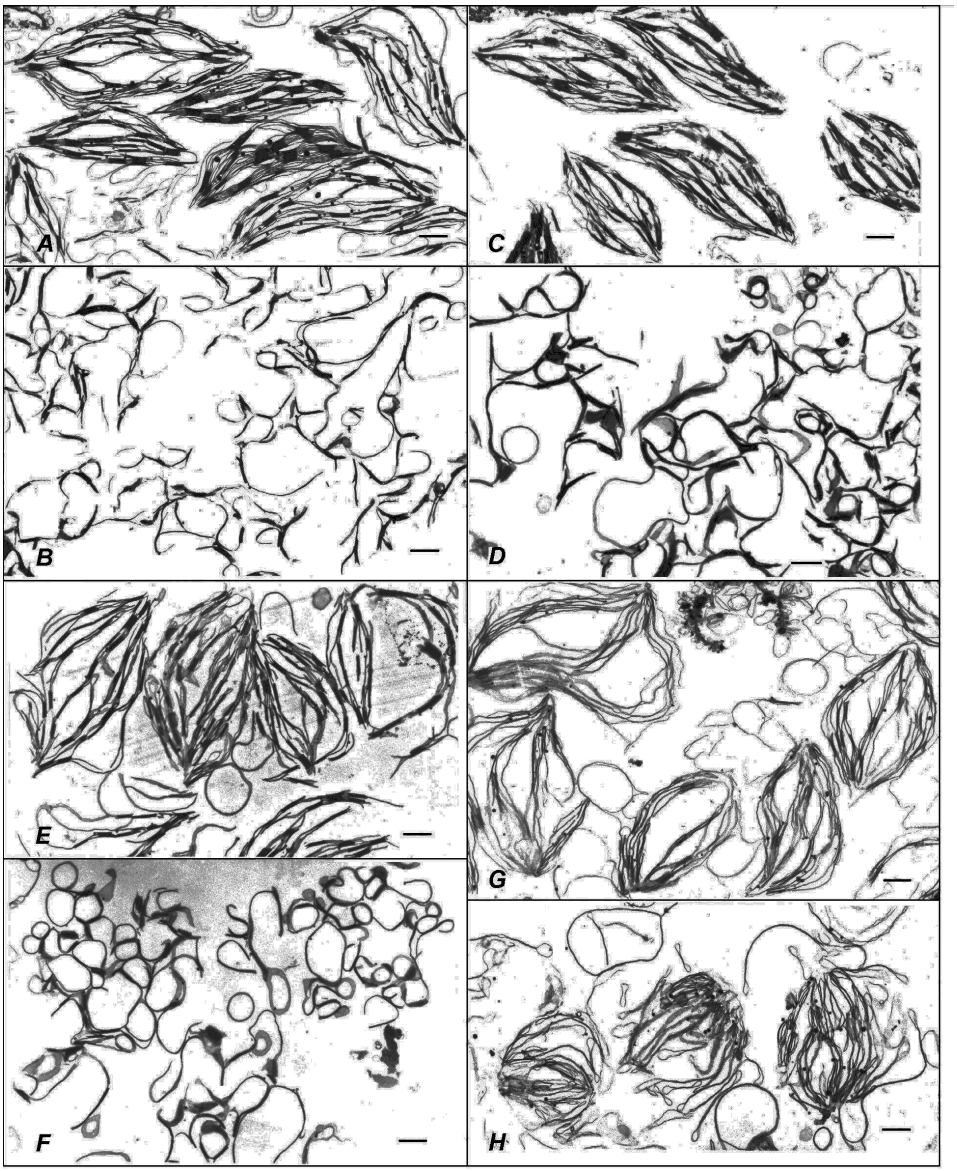

Fig. 1. Thylakoid systems of pea chloroplasts incubated for 10 min at 20 (*A, B*), 38 (*C, D*), 42 (*E, F*), or 45 (*G, H*) °C and then exposed for 10 min to a shock with distilled water (*B, D, F, H*). Extended and flattened thylakoid systems (*A*) are completely disordered in water (*B*). In *C*, thylakoid systems remain extended and flattened and the percentage of granal thylakoids is close to the control. In water all thylakoid systems are completely disordered (*D*). In *E*, thylakoid systems lose a flattened configuration and the percentage of granal thylakoids decreases. In water all thylakoid systems are completely disordered (*F*). In *G*, the configuration of thylakoid systems approaches the spherical one and the portion of granal thylakoids is minimal. No disordering of thylakoid systems in water takes place. Only a slight swelling of thylakoids occurs in *H*. *Bar*s equal 1 μm.

The incubation of thylakoid systems at temperatures 60–70 °C caused essentially the same structural changes as the incubation at 50 °C (Fig. 4*A,B*). Only a higher degree of degradation of membranes was observed. Thylakoid systems were more compact and appeared as if they were restricted by a single membrane. Strands of pseudograna were less in size or absent at all. The "cells" of the network of anastomoses decreased in size and separated from each other (Fig. 4*B*). The osmotic shock lead to a complete separation of the "cells" of the network of anastomoses and the formation of spherical clusters of single vesicles (Fig. 4*C*).

The results are summarized in a diagram (Fig. 5). Two key temperature points are worth mentioning: 44–45 °C, at which the rounding of thylakoid membranes was maximal and thylakoid systems ceased to respond to the osmotic shock; and 50 °C, at which a radical structural rearrangement of both the system as a whole and single structural elements, thylakoids, took place.

Discussion

The results on the effect of heat treatment on the ultrastructure of thylakoid systems obtained in this work were similar to those of Gounaris *et al.* (1983, 1984). I showed that, at temperatures of 38–46 °C, grana were reduced in size, and the configuration of thylakoid systems changed from flattened to nearly spherical. At 48–50 °C, the membrane stacking increased, which lead to the formation of strands of pseudograna. However, membrane-membrane interactions in these pseudograna were of another origin than in the normal grana. As shown by Gounaris *et al.* (1984), above 45 °C some regions with populations of intra-membrane particles differing from those observed at room temperature are visible on fractures of thylakoid membranes. The authors called these regions "attachment sites". The contact of thylakoids in pseudograna might be accomplished just through these attachment sites.

Treatment with temperatures above 45 °C lead to the appearance of inverted micelles of membrane lipids on the fracture faces of thylakoid membranes (Gounaris *et al.* 1984). On sections, these micelles appear as large osmiophilic drops (Semenova 1999). In my experiments, after 10–15-min exposures to 38 °C and above, I observed only very small osmiophilic drops localized either on thylakoid membranes or in the lumen (Fig. 1*C*). Large

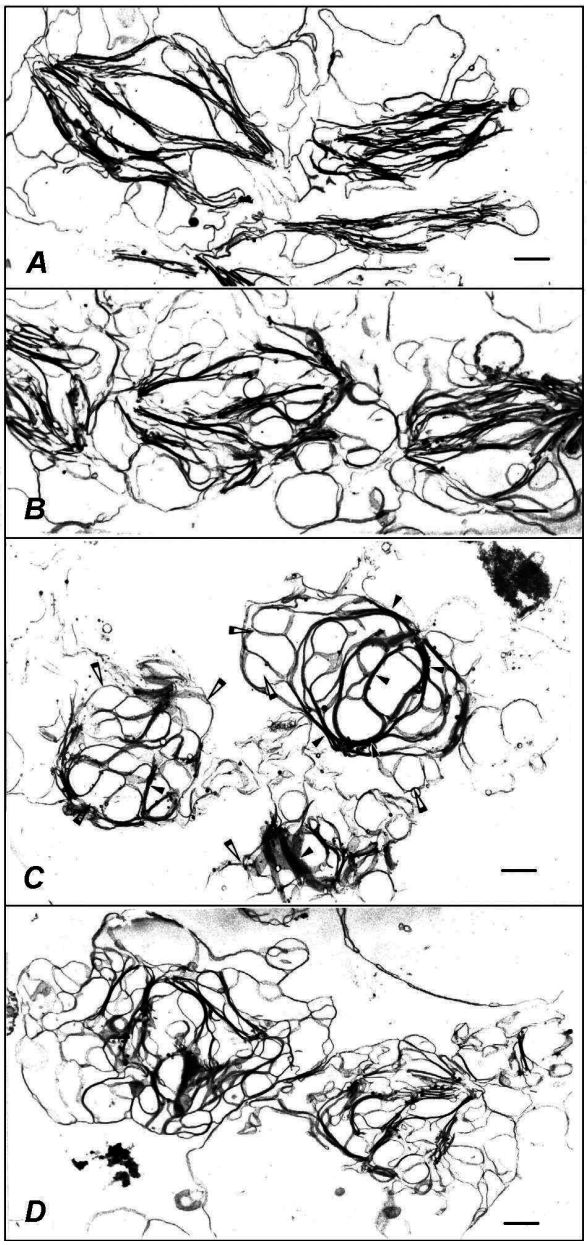

Fig. 2. Thylakoid systems of pea chloroplasts incubated for 10 min at 48 (*A, B*) or 50 (*C, D*) °C and then exposed for 10 min to a shock with distilled water (*B, D*). Thylakoid systems are more flattened, the percentage of granal thylakoids is higher than at 45 °C (*A*), and in water (*B*) no disordering of thylakoid systems takes place, but thylakoids are slightly swollen. In *C*, thylakoids form strands of pseudograna (*dark arrows*) and a network of anastomoses (*light arrows*). In water no disordering of thylakoid systems takes place (*D*). *Bars* equal 1 μm.

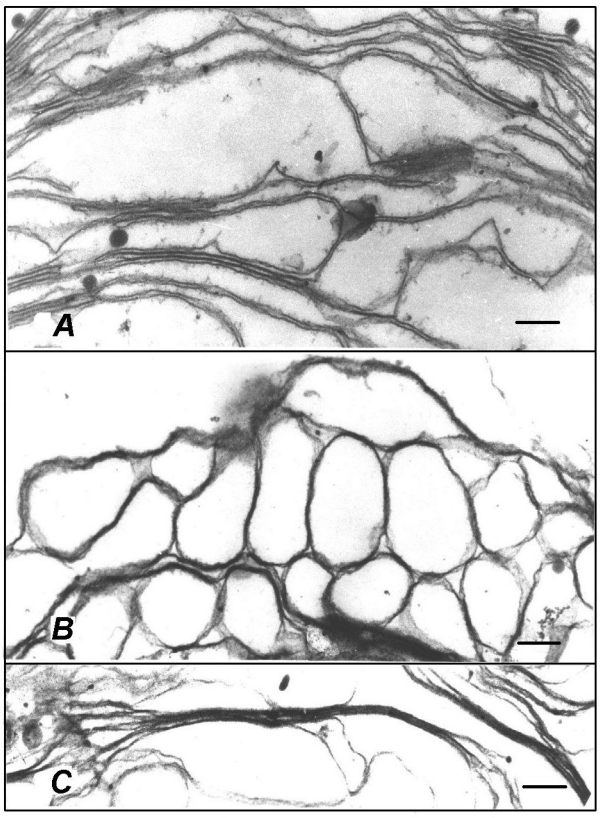

Fig. 3. Initial stage of local fusions of single thylakoids shown at a large magnification (50 °C, 1 min). Peak-shaped outgrowths of thylakoid membranes are seen (*A*), which fuse after a longer exposure (50 °C, 20 min) to form a network of anastomoses (*B*). Thylakoids of the pseudograna at a large magnification (*C*). *Bars* equal 0.2 μm.

drops like those that were observed in thylakoids incubated for 35 min at 50 °C (Semenova *et al.* 1994) were not observed in this work. Presumably, 10–15-min exposures were insufficient for the formation of large lipid drops.

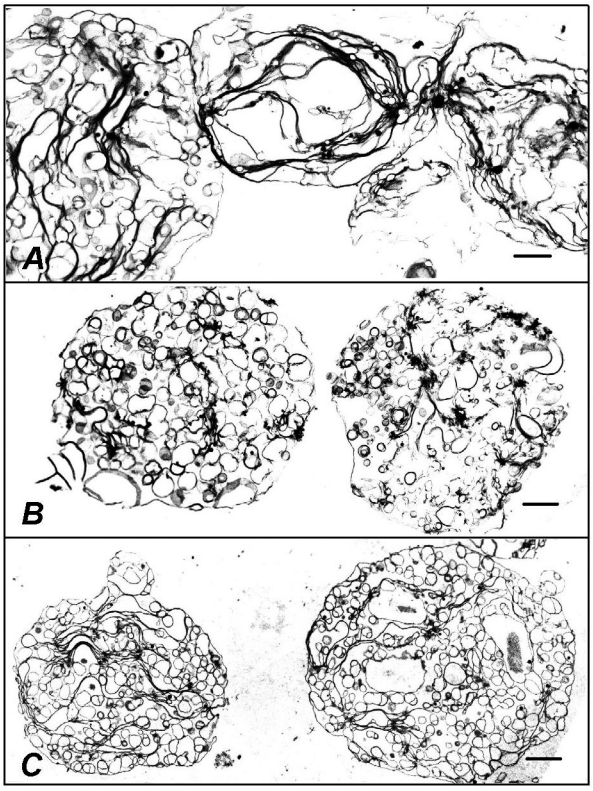

Fig. 4. Thylakoid systems of pea chloroplasts incubated for 10 min at 60 (*A*) or 70 (*B*) °C and then exposed for 10 min to a shock with distilled water (*C*). The configuration of thylakoid systems is close to spherical. Pseudograna are reduced, thylakoids are decomposed into single vesicles (*A*), and this process is aggravated at 70 °C (*C*). In water no disordering of thylakoid systems takes place. Single thylakoid vesicles are even more separated one from another (*C*). *Bars* equal 1 μm.

I show in this work that the formation of membrane vesicles at temperatures above 50 °C results not from the swelling of thylakoids but from the local fusions of neighbouring thylakoids. Similar local fusions of thylakoid membranes occur at room temperature after the action of dibucaine or tetracaine (Semenova *et al.* 1996).

A target of the inhibiting action of heat treatment in chloroplasts is the thylakoid membrane and its most sensitive component, the system of oxygen-evolving activity of PS2 (Berry and Björkman 1980, Thompson *et al.* 1989, Shutilova *et al.* 1992, Yamane *et al.* 1998, Shutilova 2000). The complete loss of the photochemical activity of PS2 was registered by the EPR method in bean chloroplasts at 45 °C (Tikhonov and Ruuge 1978) and in wheat leaves at 43 °C (Lyutova and Tikhonov 1983). The electron transport through PS2 is completely inhibited at 45 °C (Gounaris *et al.* 1983). The complete inhibition of oxygen release by thylakoids occurs at 43–44 °C according to Cramer *et al.* (1981), at about 47 °C according to Sundby *et al.* (1986), and at 44 °C according to Shutilova *et al.* (1992).

Thompson *et al.* (1989) showed that at temperatures of 42–48 °C none of the proteins incorporated into PS2 undergoes de-naturation, and the thermal transition on the calorimetric curves A_2 is of another nature than protein de-naturation. I showed that the loss of the native state of thylakoids when they cease to respond to osmotic shock occurred just in this temperature range in which the oxygen-evolving activity was completely inhibited. At 44 °C, a partial loss of ability for disordering occurred, and at 45 °C and above none of the thylakoid systems showed disordering and fragmentation of membranes. This reaction can be explained only by complete rearrangement of intermolecular interactions in the membrane towards their enhancement.

At present most studies on the heat-induced inhibition of PS2 are devoted to photochemical processes measured by fluorometric methods and to changes in the structure of pigment-protein complexes (Thompson *et al.* 1986, 1989, Bilger and Schreiber 1990, Cao and Govindjee 1990, Yamane *et al.* 1997, 1998, Matos *et al*. 2002, Kreslavskiï and Khristin 2003). The decrease in the level of variable fluorescence during thermal treatment may be caused by the separation of the light-harvesting complex 2 from the PS2 core complex (Yamane *et al.* 1997). High temperatures (above 42 °C) induce structural changes and the degradation of proteins and pigment-protein complexes, such as CP43 and CP47, the proteins of the PS2 reaction centre, D1 and D2, and the 33-kDa protein (Thompson *et al.* 1989, Yamane *et al.* 1998). According to Thompson *et al.* (1989) and Yamane *et al.* (1998), the thermal inactivation of the oxygen-evolving complex of PS2 is due to the extremely low stability of the manganese-protein complex involved in the water oxidation system. Heat treatment results in the disordering of the protein complex of PS2 and the separation of the 32-kDa protein, which leads to the loss of the manganese cluster that catalyzes oxygen evolution.

However, the disordering of protein complexes during heat treatment may be a consequence of more intricate processes. Presumably, the disordering of protein ensembles is caused by changes in intermolecular interactions due to the loss of lipids from the membrane interior as a result of the phase transition, as shown by Gounaris *et al.* (1984) and Semenova *et al.* (1994). Weak hydrogen bonds and van der Waals interactions are replaced by stronger chemical bonds, which just results in the loss of the water oxidation function.

Presumably, the water oxidation system functions only at very weak intermolecular interactions, and the formation of strong bonds inhibits the work of the system. The occurrence of the direct correlation between the

inhibition of the oxygen-evolving activity of thylakoids and the complete loss of the intrinsic capacity of thylakoids for disordering in salt-free medium was shown during prolonged storage of thylakoids (Semenova and Khorobrykh 2001).

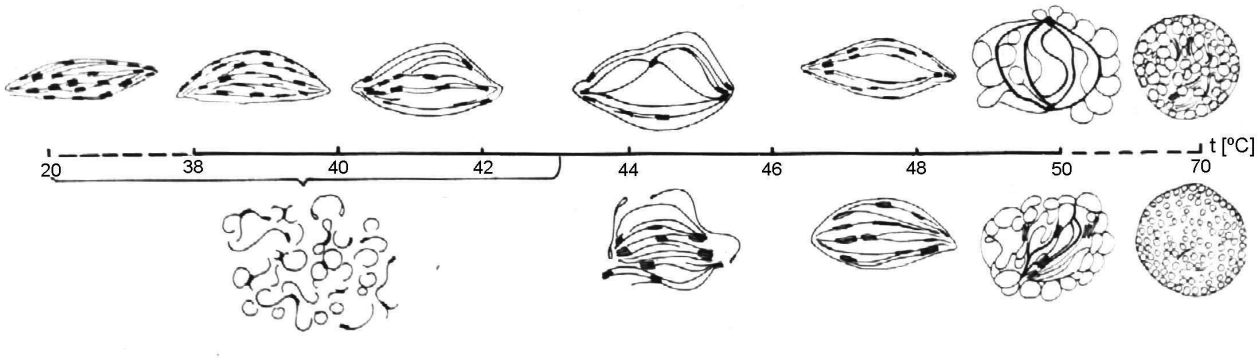

Fig. 5. A diagram showing structural rearrangement of thylakoid systems at different temperatures (*top*) and upon incubation in distilled water after heat treatment (*bottom*).

Thomas *et al.* (1985) and Ignat'ev *et al.* (2001) showed that the oxygen-evolving activity of thylakoid membranes is completely inhibited at pH 4.0–4.5. I showed in this study that, at the same pH values, the thylakoid system loses the ability for disordering (Semenova 2002).

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