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Increased thermal stability of pigment-protein complexes of pea thylakoids following catalytic hydrogenation of membrane lipids

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The membrane lipids of pea thylakoids were hydrogenated in situ using the homogeneous catalyst palladium-di-(sodium alizazine monosulphonate). Following hydrogenation, particle-free patches corresponding to phase-separated gel-phase lipids were observed in the fracture-faces of thylakoid membranes. Freeze-fracture studies on samples of hydrogenated thylakoids incubated at elevated temperatures indicated that hydrogenation reduces the tendency of the heated membranes to destack and vesiculate at higher temperatures. Measurements of chlorophyll *a* fluorescence emission and the thermal properties of hydrogenated thylakoids suggest that the hydrogenation process also leads to an increase in the thermal stability of pigment-protein complexes of the Photosystem II light-harvesting apparatus.

Introduction

Leaves and isolated chloroplasts show marked reductions in their photosynthetic activity following exposure to temperatures above about 40–45°C [1,2]. Measurements of changes in chlorophyll *a* fluorescence emission under such conditions [3–6] indicate that the light-harvesting apparatus of Photosystem II (PS II) is particularly susceptible to thermal damage. On the basis of such studies, Schreiber and Armond [6] suggested that heating leads to an irreversible functional reorganisation of PS II. Freeze-fracture studies [7,8] have since revealed that incubation of chloroplasts at elevated temperatures leads to the progressive dissociation of the supramolecular complex corresponding to the PS II light-harvesting unit and the consequent destacking of the thylakoid membrane. Using differential-scanning calorimetry (DSC), Cramer et al. [9] have identified a series of endothermic transitions corresponding to order–disorder transitions of different structural domains within the chloroplast. The lowest temperature transition, with a maximum at 42–44°C, correlates with the release of manganese from the thylakoid membrane, loss of O_2 evolution ability by the chloroplast and a decrease in the redox potential of high-potential cytochrome *b*-559. It appears to correspond to the thermal disruption of a protein component on the donor side of PS II and as such probably reflects part of the dissociation process referred to above.

A number of studies [5,10,11] indicate that acclimation of plants, particularly those native to hot desert climates, to high growth temperatures results in an increase in their threshold temperatures for thermal damage to PS II. Acclimation of plants to different growth temperatures is also known to result in changes in saturation of the

Abbreviations: DSC, differential-scanning calorimetry; $Pd(QS)_2$, palladium-di-(sodium alizarine monosulphonate); PS II, Photosystem II.

lipids of their chloroplast membranes [11,12] and/or changes in thylakoid lipid/protein ratios [13,14]. The results obtained in acclimation studies of this type, however, appear to vary greatly with different plant species and it has proved difficult to determine the relative importance of different factors in membrane stability. The situation is further complicated by the realisation that transacylation reactions, that change the pattern of molecular species present in the membrane without changing the lipid class distribution or the overall saturation of the lipids, may also play an important part in acclimation [15].

In this paper, we attempt to assess the specific role of changes in membrane lipid saturation in determining the thermal stability of chloroplasts. In order to isolate such changes from other membrane-linked changes, we have used the homogeneous catalyst $Pd(QS)_2$ (a sulphonated alizarine derivative of palladium Pd(II)) to mediate the hydrogenation of the lipids of isolated chloroplasts in situ. This catalyst differs from the Wilkinson catalyst used in earlier studies in this laboratory [16] in that it is water-soluble, and can hence be removed from the reaction mixture by a simple washing procedure. Its utility in the hydrogenation of the unsaturated lipids of algal cell membranes [17], pea chloroplasts [18], isolated plant cell membranes [19] and plant protoplasts [20] has already been established in a series of studies performed by Vigh and his co-workers. In this investigation, the catalyst is used to hydrogenate the membrane lipids of isolated pea thylakoids and the thermal stability of the hydrogenated samples is assessed using freeze-fracture, fluorescence and DSC techniques.

Materials and Methods

Chloroplast isolation

Chloroplasts were isolated from leaf tissue of 2–3 week old pea seedlings (*Pisum sativum L.* var. Feltham First) by the method of Stokes and Walker [21] and suspended in assay medium consisting of 0.33 M sorbitol/5 mM $MgCl_2$/2 mM EDTA/10 mM NaCl/1 mM $MnCl_2$/30 mM phosphate buffer (pH 6.5). They were lysed, to yield class D chloroplasts (naked thylakoids), by resuspension in a medium consisting of 2 mM EDTA/10 mM NaCl/2 mM $MgCl_2$/15 mM phosphate buffer (pH 6.5). Chlorophyll concentrations were estimated by the method of Arnon [22].

Chloroplast hydrogenation.

Aliquots (20 ml) of chloroplasts (15 μg chlorphyll per ml) suspended in degassed assay medium were placed in high-pressure silicanised glass vessels. The vessels were transferred to a water bath at 4°C where the gas-phase was replaced by hydrogen at a pressure of 150 kPa. The hydrogenation catalyst $Pd(QS)_2$ was prepared by Dr. F. Joo using methods described elsewhere [23]. A stock solution of the catalyst in degassed glass-distilled water (10 $mg \cdot ml^{-1}$) was first prepared. Aliquots of this stock solution were injected into the chloroplast suspension through a silicone-rubber septum to yield the desired final catalyst concentration. The gas pressure was then increased to 250 kPa and the reaction vessels rotated at 45 rpm for the duration of the hydrogenation process. At the end of the hydrogenation procedure, the gas pressure was reduced slowly over a period of 5 min to avoid formation of gas bubbles in the reaction medium. Finally, the chloroplasts were washed three times with fresh assay medium to remove the catalyst. Appropriate controls containing oxygen-free nitrogen in place of hydrogen were used to determine the effect of catalyst on the chloroplasts.

Heat treatment

Aliquots (5 ml) of hydyrogenated, or control, chloroplasts (10 μg chlorophyll per ml) suspended in assay medium were incubated at different temperatures for 5 min. The samples were then cooled to 25°C and prepared for electron microscopy or stored on ice pending fluorescence or thermal measurements.

Electron microscopy

Samples for freeze-fracture were equilibrated with 35% glycerol, pelleted and thermally quenched from 25°C in a slurry of solid and liquid nitrogen. The samples were fractured at −115°C in a Polaron freeze-fracture device and shadowed with platinum-carbon. The replicas were cleaned, first with nitric acid and then with bleach, and examined using a Philips EM 301G electron microscope.

Fluorescence measurements

Chloroplast samples (10 μg chlorophyll · ml^{-1}) were treated with DCMU (40 μM). Their fast fluorescence induction curves were then measured using a fluorimeter system described elsewhere [24].

Differential-scanning calorimetry

Samples for calorimetry were concentrated to a pellet form by centrifugation and sealed in aluminium calorimeter pans. Thermal measurements were made using a Perkin-Elmer DSC-2 instrument with an empty pan as a reference.

Results

Fatty-acid composition

Thylakoid membrane preparations (class D chloroplasts) were hydrogenated for 0, 15 or 30 min in the presence of the water-soluble catalyst $(Pd(QS)_2$ following the procedure described by Vigh et al. [18]. Typical gas chromatograms of the methyl ester derivatives of total membrane lipid extracts of hydrogenated samples are presented in Fig. 1. They show that hydrogenation leads to very marked decreases in the proportion of linolenic acid (18 : 3), and corresponding increases in the proportions of stearic (18 : 0), oleic (18 : 1) and linoleic acid (18 : 2) fractions, present in the lipids. In order to obtain intermediate degrees of hydrogenation, the catalyst concentration was reduced from an initial value of 15 mg catalyst/mg chlorophyll to 1–2 mg catalyst/mg chlorophyll and the incubation temperature lowered from 25°C to 45°C. A typical set of hydrogenation values obtained under these conditions is presented in Table I. They indicate that approx. 30% of the double bonds of the lipids are lost after 30 min hydrogenation in the presence of 1.0 mg catalyst/mg chlorophyll.

Freeze-fracture electron microscopy

Freeze-fracture replicas were prepared from samples of non-hydrogenated control thylakoids and samples subjected to mild and extensive hydrogenation. The average number of double bonds per lipid molecule for the three samples were 5.2, 3.4 and 1.1, respectively. Four sets of replicas were prepared, one set from samples that had been

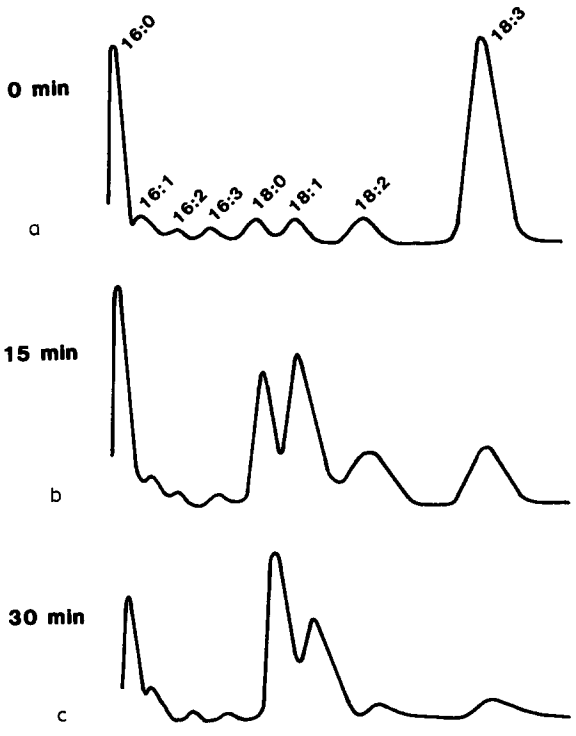

Fig. 1. Typical gas chromatograms of methyl esters extracted from non-hydrogenated thylakoid membranes (a), and from membranes hydrogenated for 15 (b) and 30 (c) min, respectively. Hydrogenation was performed at 25°C under 250 kPa H_2 in the presence of 15 mg catalyst per mg chlorophyll.

thermally quenched directly from room temperature (25°C) and the others from samples that had first been incubated at 40°, 50° or 60°C for 5 min and then recooled to 25°C prior to quenching.

In agreement with earlier studies [8,25], incubation of control chloroplasts at temperatures between 40° and about 55°C resulted in the appearance of phase-separated cylindrical-inverted lipid micelles (Fig. 2a) and a progressive loss of grana stacking (Fig. 2b). Incubation at temperatures above about 55°C led to extensive membrane vesiculation (Fig. 2c). Small regions of non-bilayer lipid structures were also seen in these vesiculated samples, but they were far less common than in samples heated to intermediate temperatures.

No obvious differences could be detected between the fracture faces of mildly hydrogenated samples thermally quenched directly from 25°C (Fig. 3a) and those of non-hydrogenated controls.

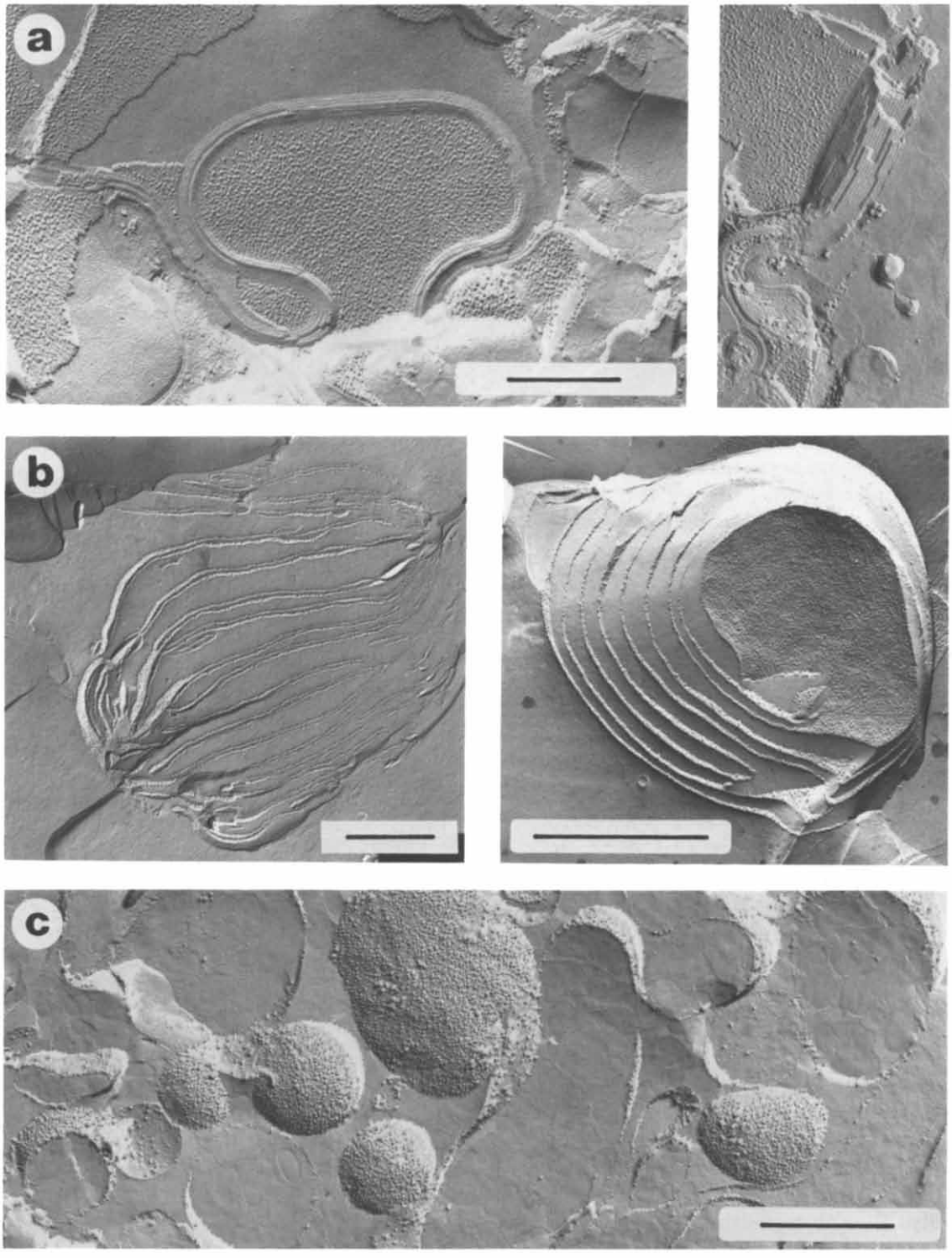

Fig. 2. Electron micrographs of freeze-fracture replicas of non-hydrogenated chloroplasts that had been incubated for 5 min at (a) 42°C, (b) 50°C and (c) 58°C, recooled and thermally quenched from 25°C. Note the presence of cylindrial-inverted micelles (CIM) in samples incubated at 42°C and the extensive vesiculation of that incubated at 58°C. The bars correspond to 250 nm, 1 μm and 500 nm in (a), (b) and (c), respectively.

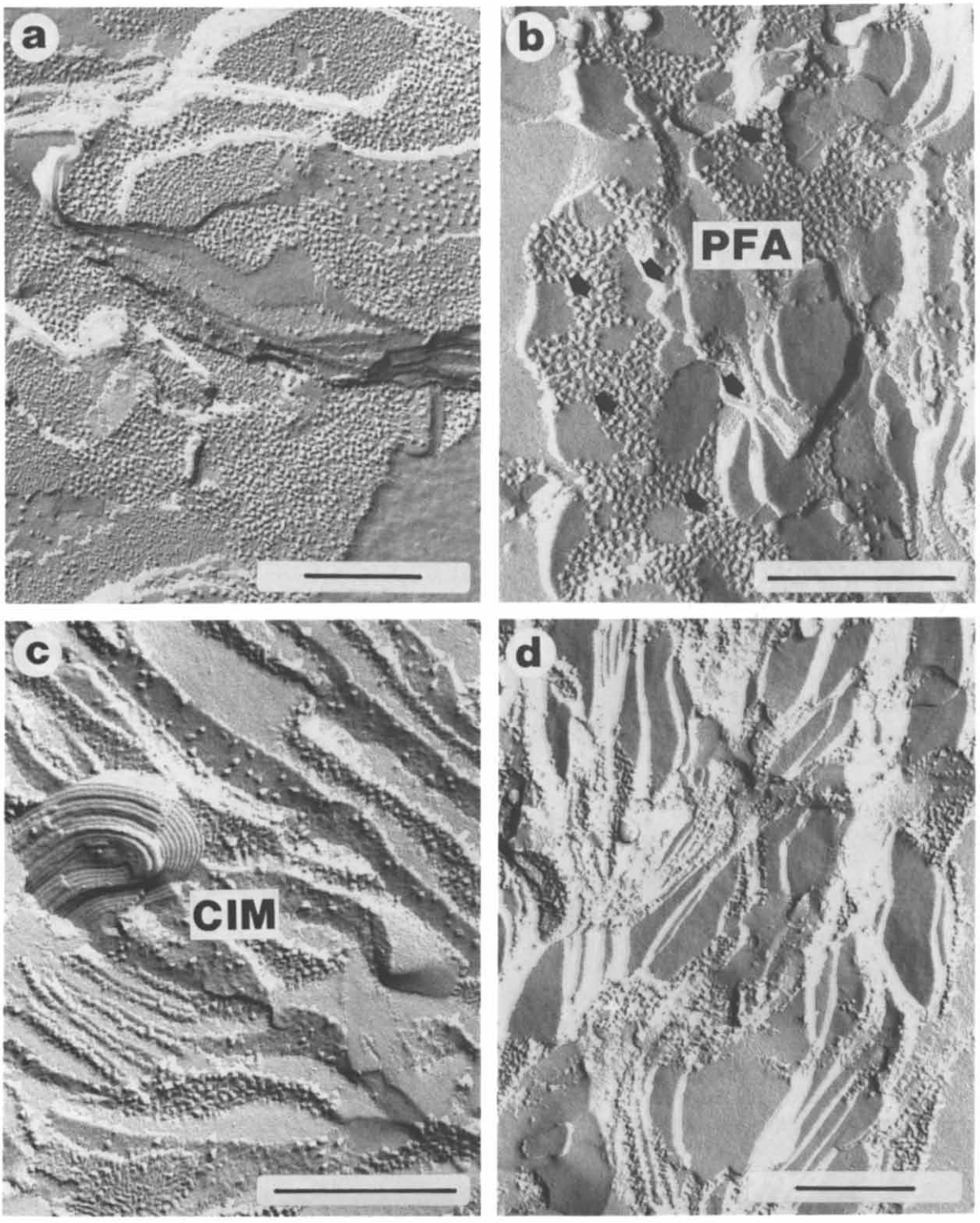

Fig. 3. Electron micrographs of freeze-fracture replicas of hydrogenated chloroplasts incubated for 5 min at 25°C (a) and (b) or 50°C (c) and (d) prior to thermal quenching from 25°C. Samples (a) and (c) were subjected to mild hydrogenation and samples (b) and (d) to extensive hydrogenation. The average number of double bonds per lipid molecule decreased from 5.2 to 3.4 in the former and from 5.2 to 1.1 in the latter samples. Note the presence of particle-free areas (PFA) in the more heavily hydrogenated samples. The bars correspond to 250 nm in each case. CIM, cylindrical-inverted micelles.

The fracture faces of the corresponding sample subjected to extensive hydrogenation (Fig. 3b), however, were quite different in appearance. They were characterised by the presence of large numbers of smooth paricle-free areas. These were particularly noticeable in the protoplasmic fracture-faces of the membranes which normally contain large numbers of close-packed 9–11 nm diameter particles. Their presence in the corresponding exoplasmic fracture faces, which contain rather fewer particles, was less obvious. These particle-free regions closely resemble similar patches seen in replicas of blue-green alga *Anacystis nidulans* thermally quenched from temperatures below that of the main gel-to-liquid crystal phase transition of its membrane lipds [26–28]. X-ray diffraction and DSC measurements performed on lipid extracts of chloroplast preparations hydrogenated under similar conditions utilised in these experiments are reported elsewhere [29]. They indicate that significant fractions of the lipids present in such samples are in the gel-phase at room temperature strongly supporting the view that the particle-free patches seen in the hydrogenated chloroplasts correspond to phase-separated regions of gel-phase lipid.

The effects of incubation at 50°C for 5 min on the membrane organisation of hydrogenated chloroplasts are illustrated in Fig. 3c and 3d. Mildly hydrogenated samples showed no obvious losses of grana stacking of the type seen in non-hydrogenated controls. Phase-separated regions of cylindrical inverted micelles were, however, common in such samples. In general, the fracture faces resemble those of control chloroplasts that have been incubated at 40–45°C suggesting that hydrogenation tends to enhance the overall thermal stability of the thylakoid organisation.

Extensively hydrogenated samples thermally quenched from 50°C (Fig. 3d) are characterised by extensive regions of tightly appressed particle-free sheets that closely resemble the open planar sheets seen in hydrogenated total polar lipid extracts of chloroplasts [30] or highly saturated samples of monogalactosyldiacylglycerols [30–32]. The presence of these tightly appressed sheets make it difficult to assess whether or not the thylakoids still contain conventional grana stacks. The particle-free areas are clearly much larger than those seen in the non-heated samples, but it is not certain whether this reflects the presence of additional lipid arising from non-bilayer forming lipids that have phase-separated on heating and then entered the gel-phase on recooling the sample to room temperature, or simply an annealing process involving the fusion of smaller pre-existing gel-phase patches of the type shown in Fig. 3b.

Samples of mildly and extensively hydrogenated chloroplasts incubated at 60°C yielded replicas very similar to those obtained from the corresponding samples incubated at 50°C. In neither case, was there any sign of membrane vesiculation of the type seen in non-hydrogenated samples incubated at high temperatures (Fig. 2c). This again confirms the increased thermal stability of the thylakoid following hydrogenation.

Fluorescence studies

Given the increased stability of thylakoid membrane organisation following hydrogenation, it is clearly of interest to determine whether this increased stability extends to the organisation of PS II which is known to be particularly heat-labile. Direct measurements of changes in PS II-mediated electron-transport rates proved to be impracticable. The electron transport efficiency of our preparations, both of controls and hydrogenated samples, were found to be too low and too variable to allow reliable comparisons. We, therefore, used fluorescence measurements to assess the final stability of the PS II light-harvesting apparatus.

The ratio of variable chlorophyll *a* fluorescence (F_V) to maximal fluorescence (F_M) measured under conditions in which PS II-mediated electron transport is blocked by the inhibitor DCMU has been widely used as an index of the photochemical trapping efficiency of PS II [33]. Measurements of fast fluorescence induction curves of hydrogenated and non-hydrogenated chloroplasts prior to heat-stress yielded ratios of F_V/F_M of 0.4–0.6. These values whilst appreciably lower than those normally obtained for intact chloroplasts are not unreasonable for class D chloroplasts, particularly in view of the incubation time and repeated washes associated with the hydrogenation procedure.

Hydrogenation led to appreciable reductions in the values of F_0, F_V and F_M. As shown in Fig. 4, the extent of these reductions was determined by the amount of catalyst used to hydrogenate the

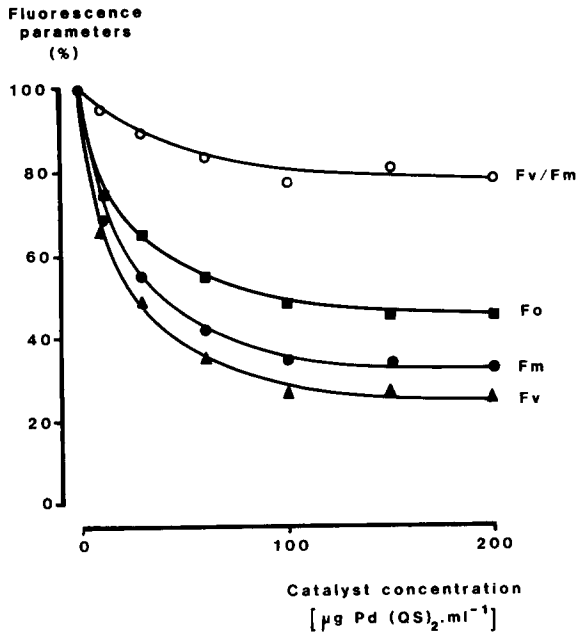

Fig. 4. Effect of the concentration of catalyst used in hydrogenation on the values of F_0 (■), F_M (●), F_V (▲) and F_V/F_M (○). Samples were hydrogenated for 30 min at 4°C and then washed three times with fresh assay medium prior to measurement of fluorescence parameters. All samples contained 40 μM DCMU.

samples. Similar results (not shown) were obtained for controls in which nitrogen was used in place of hydrogen indicating that the reductions reflect a quenching effect of residual catalyst remaining after washing rather than a direct effect of hydrogenation. The values of F_V/F_M were found to be largely unchanged by this process.

A plot of the relative values of F_V/F_M for hydrogenated and nonhydrogenated control chloroplasts subjected to 5 min incubations at different temperatures prior to measurement at room temperature is presented in Fig. 5. Non-hydrogenated samples show a sharp drop in the value of F_V/F_M over the temperature range 35–45°C with an almost complete loss of F_V at temperatures above 45°C. Hydrogenation results in a marked protection against the loss of variable fluorescence. In the heavily hydrogenated samples, in which the average number of double bonds per lipid molecule decreased from 5.2 to about 0.5, little or no decrease in the value of F_V/F_M was seen at temperatures below 45°C (Fig. 5a). Mild hydrogenation involving the reduction of the average number of double bonds per lipid molecule from 5.2 to 4.7, however, provided little or no protection (Fig. 5b). Samples treated with catalyst in the presence of N_2 rather than H_2 show changes similar to those seen for control preparations [34] indicating that the protective effect is associated with hydrogenation and not simply to exposure to the catalyst.

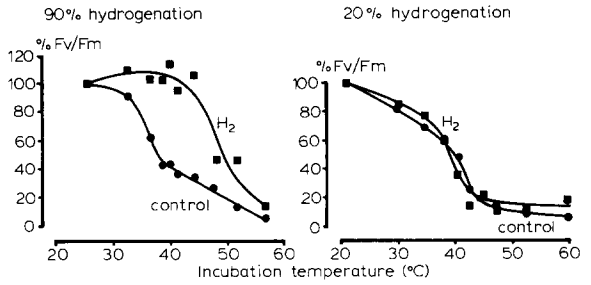

Fig. 5. Effect of heat-stress on the F_V/F_M values of hydrogenated chloroplasts. Hydrogenated (■) and non-hydrogenated chloroplasts (●) were incubated for 5 min at elevated temperatures prior to measurement of fluorescence induction at 20°C. The mildly and extensively hydrogenated samples were exposed to 1.0 mg and 14.0 mg catalyst/mg chlorophyll, respectively. Both samples were hydrogenated at 4°C under 250 kPa hydrogen for 30 min. The non-hydrogenated control samples were exposed to catalyst under a nitrogen atmosphere and subjected to a similar washing regimen to the hydrogenated samples.

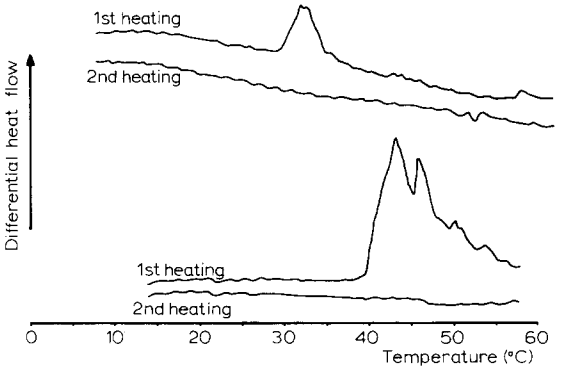

Fig. 6. Typical thermograms showing the first and second heating scans obtained for non-hydrogenated and hydrogenated chloroplasts. Hydrogenation was performed at 4°C under 250 kPa hydrogen in the presence of 15 mg catalyst/mg chlorophyll. The average number of double bonds per lipid molecule before and after hydrogenation were 5.29 and 1.17, respectively.

TABLE I

FATTY-ACID ANALYSIS OF LIPIDS EXTRACTED FROM PEA CHLOROPLASTS HYDROGENATED AT DIFFERENT CATALYST LEVELS

The figures represent mol% at each catalyst concentration. The incubation time was 30 min.

Fatty acids	Catalyst concentration (mg/mg chlorophyll)			
	0 mg	1.00 mg	1.33 mg	1.5 mg
16:0	14.3	15.8	19.4	21.3
16:1	4.2	8.5	6.9	5.0
16:2	4.7	3.6	4.4	5.0
16:3	6.7	5.1	2.9	2.6
18:0	4.0	7.0	11.2	37.6
18:1	5.0	21.8	24.9	20.6
18:2	7.5	20.8	19.5	4.3
18:3	53.6	17.3	10.8	3.9
Double bond index				
Total	2.15	1.46	1.21	0.64
C_{18}	2.58	1.72	1.45	0.62
C_{16}	1.13	1.06	0.73	0.67
% Hydrogenation				
Total	–	32	44	70
C_{18}	–	33	44	76
C_{16}	–	6.2	35	41

Differential-scanning calorimetry

Examples of differential scanning calorimeter traces obtained for hydrogenated and non-hydrogenated control chloroplasts are presented in Fig. 6. Three sets of thermograms were measured for four independent samples of control chloroplasts and three sets for each of three samples of chloroplasts hydrogenated for 15, 30 and 120 min, respectively. In the case of the 30 min hydrogenated sample, the average number of double bonds per lipid molecule was 1.17, indicating that substantial hydrogenation was taking place.

While there was a great deal of variation in the individual traces obtained in this investigation, all the traces showed certain common characteristics. Firstly, in all cases, the traces showed measurable endotherms only on initial heating. They showed no exotherms and the initial endotherm was not restored on long-term storage. This behaviour is typical of changes associated with the irreversible denaturation of proteins [35]. Secondly, the threshold temperatures for these transitions, independent of the detailed shape of the endotherm, was invariably in the range 39–41°C for the hydrogenated and 27–31°C for the non-hydrogenated samples suggesting that lipid hydrogenation raised the threshold temperature for these protein-linked changes by about 10°C. Comparison with earlier studies performed by Cramer et al. [9] suggest that this threshold temperature corresponds to the occurrence of irreversible changes in the organisation of PS II.

Discussion

The general pattern of changes in fatty-acid saturation of chloroplast membrane lipids reported in this study is very similar to that reported by Restall et al. [16] in their experiments employing Wilkinson's catalyst and the more recent work of Vigh et al. [18], using the same water-soluble palladium catalyst as employed here. The overall efficiency of hydrogenation is, however, much higher than in either of the earlier studies. This probably reflects the fact that both these studies utilised unbroken chloroplasts in which the extent of hydrogenation is limited by the rate of penetration of the catalyst through the chloroplast envelope. Naked thylakoid preparations were preferred in the present study as their use avoids problems associated with sample heterogeneity and ensures a more uniform hydrogenation of the thylakoid membranes.

Studies in this laboratory, in which either isolated chloroplast lipids have been hydrogenated using Adam's catalyst [30] or chloroplast lipids have been hydrogenated in situ using the water-soluble $Pd(QS)_2$ catalyst [29], show that even relatively mild hydrogenation (involving reductions in the average number of double bonds per lipid molecule of approx. 30%), results in the conversion of the isolated lipids from their normal liquid crystalline state at room temperature to the gel state. The formation of particle-free patches, similar to those seen in the fracture faces of membranes of thermophilic blue-green algae thermally quenched from temperatures below that of their main gel-to-liquid crystal phase transition, indicate that similar conversions can take place in the thylakoid. The fact that patches of this type are only seen at higher degrees of hydrogenation (Fig. 3b and d) suggests that the disordering effects of membrane proteins may interfere with their for-

mation in the native membrane.

The main point of interest in the present study is whether or not hydrogenation leads to an increase in the threshold temperature for heat-induced damage to the light-harvesting apparatus of PS II. Schreiber and Armond [27] have previously shown that the value of F_0 (the fluorescence yield of chloroplasts measured under conditions when all PS II traps are open) increases on heating isolated chloroplasts whilst that of F_M (the corresponding yield when all PS II traps are closed) decreases. As a result, the value of the variable component of fluorescence ($F_V = F_M - F_0$) falls rapidly over the temperature range 35–50°C. Our measurements of the variation of F_V/F_M, the normalised value of F_V corresponding to the trapping efficiency of PS II, indicate that hydrogenation results in a marked increase in the threshold temperature for heat-induced losses of PS II trapping capability (Fig. 5).

Gounaris et al. [8] showed that the structural reorganisation of the chloroplast thylakoid resulting in losses of grana stacking at temperatures above 40–45°C can be explained in terms of a physical dissociation of the PS II light-harvesting apparatus. The observation that hydrogenation leads both to an increased ability to resist thermal destacking and subsequent membrane vesiculation (cf. Figs. 2 and 3) and to an increase in the thermal denaturation temperature of key elements of the PS II light-harvesting apparatus (Fig. 6) adds further support to the idea that increases in lipid saturation favour the stabilisation of PS II to thermal stress.

The results reported in this investigation all suggest that increased membrane lipid saturation leads to an increased stability of thylakoid organisation and that hydrogenation mimics the effect of high-temperature acclimation. The molecular basis of this phenomenon, however, are far from clear. Raison et al. [11] have suggested that acclimation is associated with changes in the fluidity of the thylakoid membrane. Measurements of the mobility of electron spin resonance [11] and fluorescence [36] probes indicate that the fluidity of thylakoid membranes, as reflected in the motion of such probes, remains effectively constant for plants grown at different temperatures. It is not at all clear, however, how the preservation of a particular membrane viscosity might serve to stabilise membrane structures.

The thylakoid membrane of the chloroplast contains a high proportion of the non-bilayer forming lipid monogalactosyldiacylglycerol. This is thought to be required to ensure efficient sealing, and hence the stabilisation of large supramolecular complexes, such as the PS II light-harvesting unit, within the membrane bilayer [37,38]. Non-bilayer forming lipids tend to phase-separate from mixtures of bilayer and non-bilayer forming lipids at higher temperatures [39] and chloroplast membranes subjected to heat-stress [8,25]. In the case of chloroplasts, they give rise to non-bilayer structures of the type shown in Fig. 2b and 3c which clearly disrupt thylakoid organisation. Increases in lipid saturation brought about by hydrogenation raises the threshold temperature at which such phase-separations occur [29,30]. It is this reduced tendency for phase-separation to occur that, in our view, underlies the increased thermal stability of the thylakoid membranes of hydrogenated chloroplasts.

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