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THE MODULATION OF MEMBRANE FLUIDITY BY HYDROGENATION PROCESSES

III. THE HYDROGENATION OF BIOMEMBRANES OF SPINACH CHLOROPLASTS AND A STUDY OF THE EFFECT OF THIS ON PHOTOSYNTHETIC ELECTRON TRANSPORT

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Summary

A method is reported for the in situ modification of the lipids of isolated spinach chloroplast membranes. The technique is based on a direct hydrogenation of the lipid double bonds in the presence of the catalyst, chlorotris(triphenylphosphine)rhodium (I). The pattern of hydrogenation achieved suggests that the catalyst distributes amongst all of the membranes. The polyunsaturated lipids within the membranes are hydrogenated at a faster rate and at an earlier stage than are the monoenoic lipids.

Whilst addition of the catalyst to the chloroplast causes an initial 10—20% decrease in Hill activity, saturation of up to 40% of the double bonds present can be accomplished without causing further significant alterations in photosynthetic electron transport processes or marked morphological changes of the chloroplast structure as observed in the electron microscope.

Introduction

The physiological and specific properties of biomembranes are considered to be determined primarily by the protein structures and arrangements. These

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1'-dimethyl urea; DCIP, dichlorophenolindophenol.

in turn are related to the properties of the lipid bilayer matrix such as its fluidity characteristic. The fluidity characteristics of the lipid bilayer are determined by the chain length and the degree of unsaturation of the constituent lipids [1,2]. In some instances in addition to general fluidity characteristics, particular fatty acyl residues have been implicated in biological function.

Plant membranes, in particular those of the chloroplast, are characterised by the presence of large proportions of unsaturated lipids. The major acyl residue present in chloroplast lipids is linolenic acid (18 : 3). This residue typically accounts for some 70% of the total fatty acid content of higher plant chloroplast lamellae [3,4]. The functional significance of the highly unsaturated nature of chloroplast lipids is not yet fully clear. It has been suggested that they are necessary in order to ensure that the appropriate fluidity of chloroplast membranes is maintained over a wide range of environmental temperature and a considerable literature exists relating the fatty acid content of plant tissues to such properties as chilling resistance and cold hardiness [5].

One potential method of testing the possible role of particular unsaturated lipids is to examine the effect of selective modification of the constituent lipids within the biomembrane structure.

A number of methods have already been described for the modification of membrane lipids in vivo. The fatty acid patterns of prokaryotic cells, for example, can be markedly changed by supplementation of their nutrient media with exogenous supplies of particular fatty acids [6—9]. Exposure of eukaryotic cells to amino alcohols can lead to changes in the proportions of the various classes of phospholipids present in both animal [10—12] and plant cells [13—15]. Changes in lipid composition can be brought about by changes in the growth temperature of microorganisms [16—18] or by the adaptation of animals [19] or plants [5,20] to lowered environmental temperatures. The lipid patterns of plant tissues [21,22] and bacterial systems [8] can also be altered by treatment with low concentrations of herbicides or other drugs, respectively.

All of the above methods, involve changes in the metabolic pathways associated with the synthesis of new lipid rather than the modification of existing lipid within preexisting membranes. In this paper we demonstrate how the technique of homogenous catalysis can be used to modify the lipid unsaturation pattern in such membranes. This method involves the use of a rhodium catalyst which partitions into the membrane enabling the double bonds present to be hydrogenated in situ. We have described the successful hydrogenation of model lipid biomembranes in earlier papers [23,24]. The present paper describes how we have applied the technique to the hydrogenation of the biomembranes of the chloroplast and how we have examined the effect of this upon photosynthetic electron transport processes.

Materials and Methods

Chloroplast preparation. Chloroplasts were isolated from locally purchased spinach by the method of Stokes and Walker [25] and resuspended in an assay medium consisting of 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM

$MnCl_2$, 50 mM Hepes (pH 7.6). The chlorophyll content of the samples was determined spectrophotometrically by the method of Arnon [26].

Hydrogenation procedure. The catalyst used in the experiments was chlorotris(triphenylphosphine)rhodium (I) ($Rh(PPh_3)_3Cl$) prepared as described elsewhere [23]. It was converted to a hydride form by adding the powdered catalyst to a solvent vector of degassed dimethyl sulphoxide (Me_2SO), and bubbling with hydrogen until it dissolved to yield a pale yellow solution. Aliquots of 9.5 ml of chloroplast suspension (chlorophyll content approximately 20 mg $\cdot l^{-1}$) were placed in a light-tight, high-pressure glass reaction bottle and maintained at 0°C in an ice bath. The bottle was then flushed with hydrogen for 1 h to remove all traces of oxygen. 10 min before the addition of catalyst, the reaction bottle was transferred to a thermostated water bath and equilibrated at the required reaction temperature. The reaction was initiated by adding the hydrogenated catalyst (2 mg of catalyst in 0.5 ml Me_2SO) to the reaction vessel. The final concentration of Me_2SO in all experiments was 5% (v/v). The hydrogen pressure was then increased, normally to 6 atm, and the vessel sealed and incubated in the dark, without stirring, for the desired reaction time.

Analysis of fatty acid content. Total lipid extracts of the chloroplasts were obtained using a modification of the technique of Sheltawy and Dawson [27]. Briefly, the chloroplast samples were treated with 0.4 M perchloric acid and centrifuged at $2000 \times g$ for 15 min. The sedimenting material was then extracted with a solvent mixture of chloroform/methanol/10 M hydrochloric acid (200 : 100 : 1, v/v) containing 0.005% butylated hydroxytoluene as an antioxidant. After incubation for 30 min, 1.5 vols. of 0.1 M HCl were added and the mixture centrifuged at $2000 \times g$ for 5 min. The upper aqueous layer was discarded and the lower organic layer retained. The procedure was repeated on the chloroplast debris which collected at the solvent interface and the organic layers from the two extractions combined.

The methyl ester derivatives of the fatty acids were formed from the dry lipid extracts by heating in a solution of 14% (w/v) boron trifluoride in methanol at 70°C for 10 min. The methyl esters were subsequently extracted into light petroleum ether, dried over anhydrous sodium sulphate and analysed by gas chromatography using a Pye 104 Gas Chromatograph with a column of 10% (w/w) poly(ethylene glycol adipate) on Gas Chrom Z (80—100 mesh) maintained isothermally at 175°C using nitrogen as a carrier gas. The individual fatty acids present in the original sample were identified on the basis of the characteristic retention times of their methyl ester derivatives. The relative proportions of the fatty acids were calculated from the peak areas on the recorder trace and the total lipid present estimated by comparison of the total peak area with that of an arachidic acid (20 : 0) standard added to the sample prior to the lipid extraction (see Fig. 1).

The percentage hydrogenation was calculated as the difference between the relative numbers of unsaturated bonds in the fatty acids before and after hydrogenation. To check that the hydrogenation process did not lead to deacylation the presence of free fatty acids was examined qualitatively by thin-layer chromatography on silica gel. No free fatty acids could be detected with iodine vapour, after developing the plates with a solvent of petroleum ether/

diethyl ether/acetic acid (80 : 20 : 10, v/v) in the hydrogenated or non-hydrogenated lipid extracts.

Catalyst distribution. The chloroplasts were separated from excess catalyst suspended in the reaction medium by sucrose density gradient centrifugation. Aliquots (7 ml) of the chloroplast/catalyst reaction mixture were layered over a sucrose density gradient comprised of 7 ml 60% (w/v) sucrose and 7 ml 30% (w/v) sucrose and centrifuged at 75 000 × *g* for 1 h. Different layers of the gradient were analysed for rhodium content, using a Perkin Elmer 103 Atomic Absorption spectrophotometer operating at an absorbance band of 343.5 nm with an oxidising flame.

Measurement of photosynthetic activity. The overall photosynthetic activity of the hydrogenated and non-hydrogenated chloroplasts was assayed by the photoreduction of methyl viologen by uncoupled chloroplasts. Oxygen uptake was measured using a Clark-type oxygen electrode assembly made by Hansatech (Norfolk, U.K.). The samples, maintained at 25°C, were illuminated by a tungsten lamp providing an incident light intensity of 85 000 lux. Photosystem I activity was measured by poisoning the chloroplasts with 6 μm 3-(3,4-dichlorophenyl)-1,1′-dimethyl urea (DCMU) and adding 20 μM dichlorophenol-indophenol (DCIP)) plus 10 mM ascorbic acid as a substitute electron donor. Photosystem II activity was measured by following the bleaching of DCIP at 600 nm at saturating light intensity using a Spectronic 50 spectrophotometer. Details of the individual assay media are provided in the relevant figure captions.

Electron microscopy. Specimens fixed in 5% glutaraldehyde and 2% osmium tetroxide in cacodylate buffer, pH 7.2, were sectioned, stained with lead citrate and uranyl acetate and then viewed in a Phillips TEM 301G electron microscope.

Results

Pattern of hydrogenation

The effect of hydrogenation in fatty acid pattern of spinach chloroplasts is illustrated in Fig. 1. This figure shows typical gas chromatograms taken from recorder traces of the methyl ester derivatives of the fatty acyl residues from normal spinach chloroplasts and chloroplasts subjected to hydrogenation for 3 h and 22 h, respectively. The chromatograms show very marked reductions in the amount of linolenic acid present following hydrogenation coupled with corresponding increases in the amounts of linoleic, oleic and stearic acids. Similar patterns of hydrogenation can be seen for the C_{16} acids. The ratio of C_{16} to C_{18} acids calculated on the basis of peak area remains constant. A typical time course for hydrogenation is shown in Fig. 2a. There is an initial rapid hydrogenation over the first few hours followed by a slowing of the reaction rate to yield a plateau after about 50% of the double bonds present initially have reacted. The changes occurring in the relative proportions of the C_{18} fatty acids during different stages of the hydrogenation process are shown in Fig. 2b. They are plotted as a function of the percentage hydrogenation rather than time of reaction to minimise distortion arising from the non-linear time course of hydrogenation. There is a progressive decrease of linolenic acid throughout

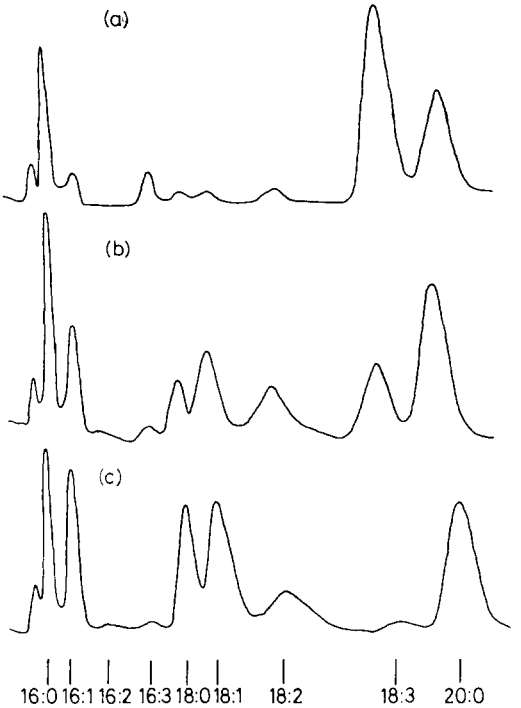

Fig. 1. Typical gas chromatograms of methyl esters of fatty acids extracted from (a) non-hydrogenated chloroplasts; (b) chloroplasts hydrogenated for 3 h, and (c) chloroplasts hydrogenated for 22 h. Hydrogenation conditions as described in Materials and Methods. The 20 : 0 peaks correspond to an internal standard added to check for lipid loss.

the hydrogenation process accompanied by increases in the relative amounts of the remaining C_{18} fatty acids. After about 50% of the double bonds initially present have been hydrogenated, the level of linoleic acid also starts to decrease as it, in turn, is hydrogenated to yield oleic acid. The loss in linoleic acid is, in

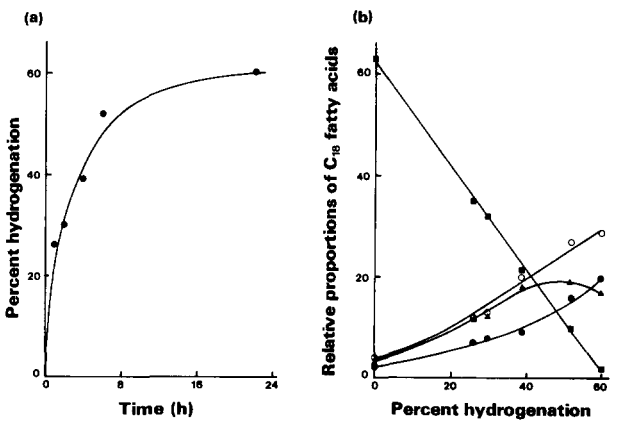

Fig. 2. (a) Time course of chloroplast hydrogenation. Sample contained chloroplasts (chlorophyll content 15 mg · l^{-1}) in assay medium. Hydrogenated with 200 mg · l^{-1} catalyst at 900 kPa at 24°C. (b) Pattern of chloroplast hydrogenation: relative amounts of 18 : 3 (■), 18 : 2 (▲), 18 : 1 (○), and 18 : 0 (●) present plotted as a function of the percentage decrease in the total number of double bonds present.

this particular experiment, almost exactly balanced by the increases in the relative amounts of the other C_{18} acids. It should, however, be noted that a nett loss of lipid (approx. 5—10%) was observed in some hydrogenation experiments. This loss may be due to lipid peroxidation following the exposure of the catalyst-treated samples to air upon completion of the hydrogenation process.

Catalyst distribution

It was apparent from the turbidity resulting from addition of catalyst to the chloroplast suspension that some of the catalyst complex did not partition into the membranes. The distribution of the catalyst in the chloroplast samples was investigated by subjecting the hydrogenated chloroplasts to sucrose density gradient centrifugation. Gradients were prepared as described in Materials and Methods. The chloroplasts sedimented to the boundary of 30% and 60% sucrose layers and excess catalyst was recovered as a pellet at the bottom of the centrifuge tube.

Chlorophyll could not be detected in the supernatant or either of the two sucrose layers. Trace quantities of chlorophyll, however, were observed in the pellet but this was negligible compared to that present in the interface between the sucrose layers. The rhodium content of the different layers was assayed by atomic absorption spectroscopy. Most of the added catalyst was found to be present in the pellet. Only a small proportion, 11%, was associated with the chloroplasts. It should be emphasized that this figure will include precipitated catalyst trapped within, or bound to the surface of, the lamellae system and is not necessarily a reflection of the amount of rhodium that exists within the membrane bilayers. The catalyst concentrations cited must, therefore, be considered as empirical measures rather than precise values for catalyst directly accessible to the lipid substrate.

Optimal hydrogenation conditions

One of the major objectives of our investigation was to establish appropriate experimental conditions to achieve maximal hydrogenation of the lipids within the chloroplast membrane whilst at the same time preserving their biological activity and structural integrity. The effects of varying hydrogen pressure, catalyst concentration and incubation temperature on chloroplast hydrogenation are summarised in Fig. 3.

In general hydrogenation is increased by increased hydrogen concentration (pressure), higher concentrations of catalyst and higher temperatures. Increasing the catalyst concentration markedly increases the rate of hydrogenation but does not significantly improve the maximum extent of hydrogenation that can be achieved. Relatively high catalyst concentrations (200—400 mg · l^{-1}) did not appreciably alter the functional activity of chloroplasts hydrogenated for 3 h but the initial loss of activity immediately following the addition of catalyst was more marked. A concentration of 200 mg · l^{-1} was therefore selected to provide an appreciable hydrogenation (approx. 35% in 3 h) together with reasonable retention of photosynthetic electron transport.

The rate of hydrogenation was found to be markedly dependent on incubation temperature as illustrated in Fig. 3c. Unfortunately, chloroplast stability also tends to decrease as the temperature is increased. The Hill activity of

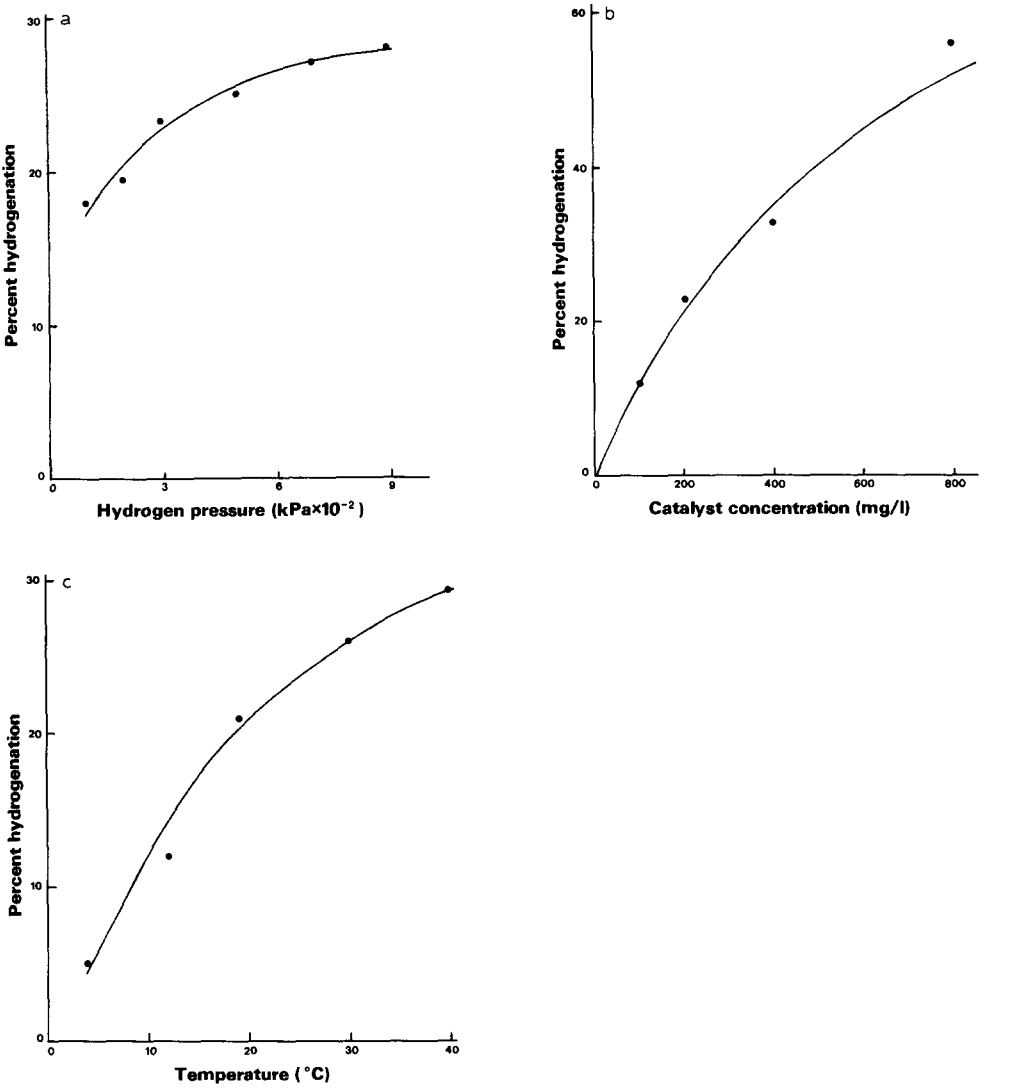

Fig. 3. Percentage hydrogenation of chloroplasts as a function of (a) hydrogen pressure; (b) amount of added catalyst, and (c) incubation temperature. The chlorophyll concentrations in each case were between 10 and 20 mg · l^{-1} and the catalyst concentration 400 mg · l^{-1}. The samples were normally incubated for 3 h at 24° C and 600 kPa.

chloroplasts in particular, is well-known to decline rapidly when temperatures of chloroplast suspensions are maintained above about 30°C [28]. The Hill activities of samples of chloroplasts kept under 1 atm of hydrogen at temperatures between 0 and 40°C for 3 h were examined. The activity was found to unaffected by incubation at temperatures below 25°C but decreased significantly with incubation at higher temperatures. All hydrogenation experiments involving measurements of biological activity were, therefore, performed on samples incubated at 25°C or less.

Photosynthetic activity

Hydrogenation appeared to have little effect on the overall rate of photosynthetic electron transport. The rates of electron transport from water to methyl viologen (i.e. non-cyclic electron transport through both Photosystem II and Photosystem I) of chloroplasts hydrogenated for 0, 1, 2 and 4 h are compared to those for non-hydrogenated chloroplasts stored at 4°C for similar periods and an anaerobic control maintained at 24°C under hydrogen at 600 kPa pressure, in Fig. 4. The most notable difference between the hydrogenated samples and the non-hydrogenated controls lies in the existence of an approximately 10—20% loss in activity in the hydrogenation sample immediately following addition of the catalyst. This initial decrease in activity does not appear to be associated with hydrogenation of the membrane lipids.

A similar loss of activity is observed if the non-hydride form of the catalyst is added to the chloroplast suspension. The loss is proportional to the amount of catalyst added, suggesting that it is due to an interaction between the rhodium catalyst and the chloroplast. Addition of the equivalent amount of dimethylsulphoxide solvent in the absence of catalyst has no effect on chloroplast activity. Apart from this initial decrease in activity the changes in the rate of photosynthetic electron transport in the hydrogenated chloroplasts (the relative extent of hydrogenation of which are shown in the inset to Fig. 4) closely parallel those observed for the anaerobic preparation.

Appreciable variations were observed in the temperature stability of individual chloroplast preparations. Some preparations retained up to 90% of their initial activity over a 4 h hydrogenation period, whilst other deteriorated more rapidly. Any loss of activity in the hydrogenated samples, apart from the initial loss due to the catalyst itself, was invariably associated with a similar decrease

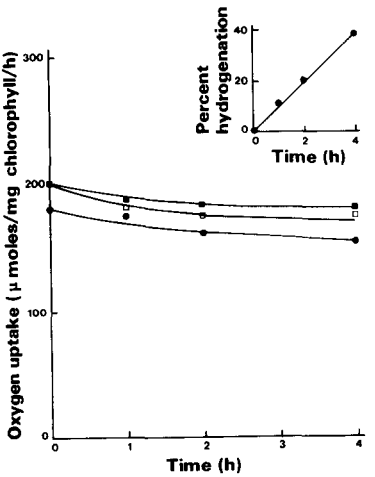

Fig. 4. Rates of photosynthetic electron transport from water to methyl viologen as a function of hydrogenation time for chloroplasts hydrogenated under standard conditions (●), anaerobic control without catalyst (□) and refrigerated control without catalyst (■). Electron transport was measured from initial rates of oxygen uptake on chloroplast samples (chlorophyll content 25 mg · l^{-1}) suspended in 3 ml of assay medium containing 80 μM methyl viologen, 1.6 mM sodium azide and 30 μM NH_4cl. Insert shows hydrogenation time course.

in activity in anaerobic controls indicating that such losses were the result of physiological deterioration of the chloroplasts on storage, rather than to specific changes induced by the hydrogenation process.

The corresponding plots for changes of Photosystem II activity (as reflected in the Hill reduction of DCIP) and Photosystem I activity (as measured by the transfer of electrons from reduced DCIP to methyl viologen) on hydrogenation are shown in Fig. 5a and b. The initial decrease in Hill activity following the addition of the catalyst (Fig. 5a) is similar to the effect observed for electron transport from water to methyl viologen (Fig. 4). In contrast, the plots for Photosystem I activity (Fig. 5b) show an enhanced rate of photosynthetic electron transport in the hydrogenated samples. The rate of electron transport from reduced DCIP to methyl viologen is about 25% greater immediately following the addition of catalyst and increases to about 75% above the initial rate after 2 h. Increases in Photosystem I activity are often observed in aged chloroplasts [29]. Indeed, a similar but appreciably slower rate of increase in Photosystem I activity is detectable in the anaerobic and refrigerated preparations shown in Fig. 5b. The larger increase observed in the hydrogenated sample suggests that the addition of catalyst may cause some change in chloroplast morphology which makes the electron donor $DCIPH_2$ more accessible to Photosystem I. This need not infer, however, that the catalyst-induced changes are necessarily identical to those associated with ageing.

Chloroplast structure

The structure of hydrogenated chloroplasts was examined under the electron microscope. Samples subjected to hydrogenation tended to contain fewer

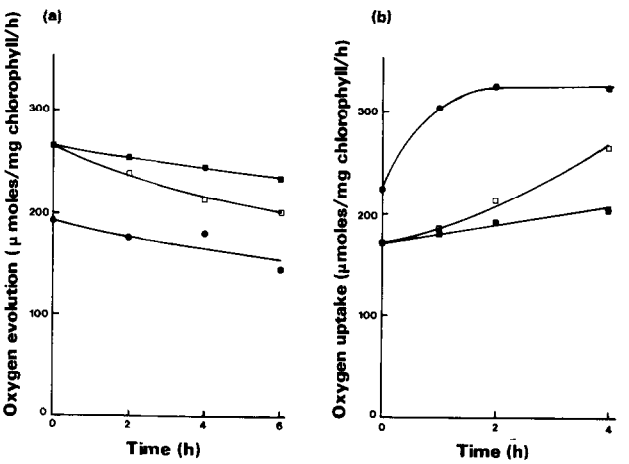

Fig. 5. (a) Photosystem II activity measured as the initial rate of oxygen evolution as a function of hydrogenation time. Activity measurements were made on samples suspended in assay medium containing 30 μM DCIP with 30 μM NH_4Cl as an uncoupler. Samples consisted of chloroplasts (chlorophyll content 18 mg · l^{-1}) hydrogenated under standard conditions (●), an anaerobic control without catalyst (□), and a refrigerated control without catalyst (■). (b) Plots of Photosystem I activity measured as the initial rate of oxygen uptake by DCMU (6 μM)-poisoned chloroplasts (chlorophyll concentration 25 mg · l^{-1}) suspended in 3 ml of assay medium containing 80 μM methyl viologen, 1.6 mM sodium azide, 30 μM NH_4Cl, 20 μM DCIP and 10 mM sodium ascorbate.

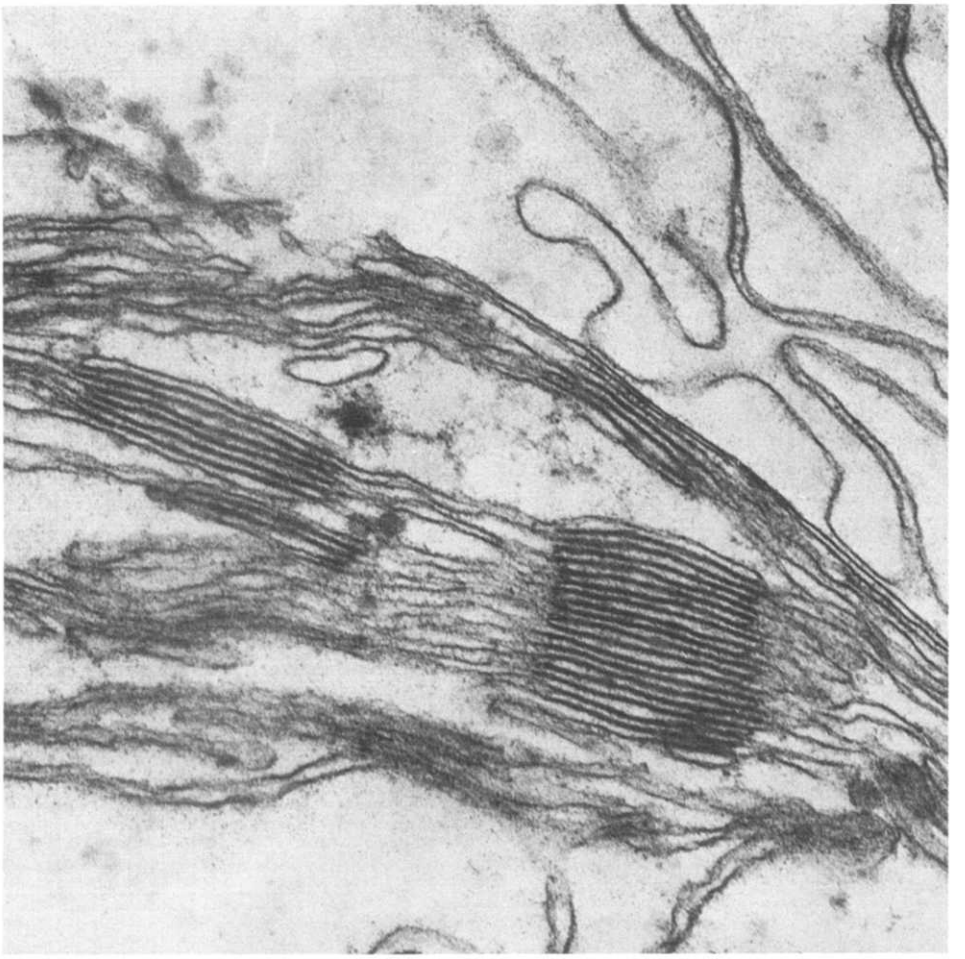

Fig. 6. Electron micrograph showing preservation of grana stacking in spinach chloroplasts hydrogenated for 3 h at 24° C and 600 kPa using 400 mg · l^{-1} catalyst. Approximate magnification X50 000.

intact chloroplasts than the corresponding anaerobic controls. Although the percentage of broken chloroplasts increased in the hydrogenated samples, the basic structure of the chloroplasts remained unchanged and grana stacking was preserved (see Fig. 6).

Discussion

The present results show that the unsaturated fatty acyl residues of biomembrane lipids can be substantially hydrogenated within membrane structures. Hydrogenation of 30—40% of the double bonds present in membrane lipids of spinach chloroplasts can be achieved without significant change in appearance in the electron microscope or loss in their capability for photosynthetic electron transport. Higher amounts of hydrogenation (50—60%) were obtained by prolonging the reaction times, or by the use of increased concentrations of

catalyst but chloroplasts subjected to such treatments tended to show marked losses in Photosystem II-mediated photosynthetic electron transport. This was partly a consequence of the presence of high catalyst concentrations and partly a reflection of natural deterioration. Prolonged incubation of chloroplast samples, at room temperature, even in the absence of catalyst was often observed to lead to a marked deterioration of the organelles.

The difficulties associated with achieving higher percentage hydrogenations may reflect differences in the relative susceptibilities of the tri-, di- and monoenoic acyl residues to hydrogenation rather than differences in the accessibility of individual classes of lipids or their existence in different domains. Measurements of the time course and the pattern of hydrogenation, for example (Fig. 2a and b), indicate that virtually all of the linolenic acid (18 : 3) residues of the chloroplasts are susceptible to hydrogenation. As these residues are known to be present in approximately equal proportions in the lipids of both the stroma lamellae and the grana stacks [4], this suggests that the catalyst complex is capable of reaching both the major internal membranes of the chloroplast and catalysing the hydrogenation of their constituent lipids.

Earlier studies in our laboratories [24] of the pattern of hydrogenation of liposomes formed from soybean lecithin showed that hydrogenation tended to proceed in a stepwise manner involving first a reduction of linolenic acid residues (18 : 3), to linolenic (18 : 2), to oleic (18 : 1), and finally to stearic acid residues (18 : 0). Examination of the data presented here suggests that the same general pattern of hydrogenation exists in biomembrane systems. The major difference between the dispersed lipid substrate and the chloroplast system is the rate at which the lipid is hydrogenated. Despite the addition of considerably higher catalyst concentrations to the chloroplast suspension compared to those used in the model membrane experiments, the rate of hydrogenation was significantly slower. Preliminary experiments suggest that both the rate and extent of hydrogenation of total lipid extracts of chloroplast dispersed in water are broadly similarly to those obtained with the original chloroplasts. This would suggest that the differences lie in the type of lipids rather than in the presence of membrane proteins. It is possible that the bulky hydrated sugar groups of the galactolipids, which are present in particularly high proportions in chloroplasts [4], either interfere with the partition of the catalyst into the hydrophobic membrane phase or alter the packing of the acyl chains in some way that hinders orientation of the catalyst about the bonds undergoing reaction. The accessibility and susceptibility of different lipid classes are currently being investigated.

The addition of catalyst even at relatively high concentrations does not appear to have any marked effect on the overall rate of photosynthetic electron transport of the chloroplasts. There is a small, but consistent loss of Photosystem II activity directly following the addition of catalyst (see Fig. 4) but the extent of this loss does not appear to increase with prolonged incubation. There is an accompanying increase in Photosystem I activity but this is only observed of the substitute electron donor $DCIPH_2$ is added as the turnover rate of Photosystem II rather than Photosystem I is rate limiting under the high light intensities employed in our experiments.

The functional measurements were all performed immediately following the

hydrogenation procedure and the long-term effects of lipid saturation on chloroplasts stability was not assessed. Detailed physical studies of the different chloroplast membranes after hydrogenation will be required to show to what extent membrane fluidity and permeability are altered by the procedure.

One feature of the present hydrogenation procedure is that the rhodium catalyst is practically insoluble in water and remains associated with the membrane at the end of the reaction. This is an unsatisfactory feature of the process. We are currently attempting to overcome this aspect by developing a new class of water-soluble catalysts which can be substantially washed out of the membrane system on completion of the hydrogenation process, which we will report in future papers.

Acknowledgments

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