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QUELQUES ASPECTS DU ROLE DES LIPIDES MEMBRANAIRES DANS LA FONCTION PHOTOSYNTHETIQUE

THESE

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AGING OF THE PHOTOSYNTHETIC APPARATUS
V. CHANGE IN pH DEPENDENCE OF ELECTRON TRANSPORT AND RELATIONSHIPS TO ENDOGENOUS FREE FATTY ACIDS

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SUMMARY

Aging of isolated spinach chloroplasts results in an acid shift of the pH optimum for electron flow through both photosystems or through either photosystem (PS) alone. Added linolenate has similar effects on the pH dependence of electron transport. Unsaturated fatty acids, predominantly linolenate, are released during aging and the changes in electron flow activity are correlated to the release of fatty acids. The formation of free fatty acids (FFA) is thus at least one cause of the alteration in the electron transport activities of aged chloroplasts.

INTRODUCTION

Chloroplast aging as a tool for studying photosynthetic electron transport was first recognized by Vernon and Zaugg [1] and has been intensively studied in this laboratory [2—7] and by others [8—13]. Aging in vitro of isolated spinach chloroplasts leads to an alteration of the structure and function of the thylakoid membranes which was attributed to a release of FFA (especially linolenic acid, $C_{18:3}$) following hydrolysis of galactolipids during the aging process [14,15]. This hypothesis has been indirectly verified: both unsaturated fatty acids and aging in vitro induce chloroplast swelling and inhibit light-induced shrinkage [2,4,7]. Also, both treatments caused a sequential inhibition of photosystems II and I electron flows and of their associated photophos-

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*Some of this work was carried out by A.R. as part of a doctoral program in this laboratory. Abbreviations: DAD, diaminodurene; DBMIB, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; FFA, free fatty acids; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid; MV, methylviologen; PD and PDox, reduced and oxidized *p*-phenylene diamine; PS, photosystem; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

phorylations [3,4,6,16,17] and increased *o*-diphenoloxidase activity [4,5] in the same way.

It is known that the inhibition of electron flow by fatty acids is pH-dependent [7,14,18]. For instance, linolenic acid causes a shift in pH optimum toward acidity. This phenomenon was shown to be related to the thylakoid membrane integrity as suggested by the action of exogenous linolenic acid on the ΔpH, pHi and proton uptake [18]. The question, therefore, was whether aging in vitro would as well induce a shift in the pH optimum and be related to the endogenous level of FFA during this process. Such an approach has already been adopted by McCarty and Jagendorf [14]. The present investigation extends their study to various electron transport systems and correlates the pH dependence of electron flow with kinetics of FFA release during aging. The present results confirm that the effects of aging in vitro and of added fatty acids stem from the same mechanism.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea*, var. Nobel) was grown in a growth chamber [18]. Thylakoid membranes were prepared as described previously [18] and resuspended in 25 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES) (pH 7.6) and 0.35 M sucrose to 2 mg chlorophyll per ml or in the aging medium (25 mM HEPES, pH 7 or 8, and 175 mM NaCl) to 1 mg chlorophyll per ml. Thylakoid membranes were dark-aged at 20°C and aliquots were taken at various times for the determination of electron flow activities or FFA content. All isolation operations were carried out at 4°C and in dim light. Chlorophyll was determined spectrophotometrically [19].

Electron transport for PS II + PS I (from H_2O to methylviologen (MV)) was measured polarographically (O_2 electrode, Clark type) by the consumption of oxygen. This type of electron flow was also assayed spectrophotometrically at 420 nm in the presence of $K_3Fe(CN)_6$ or oxidized *p*-phenylene diamine (PDox) [20], or at 340 nm in the presence of $NADP^+$ [17] as electron acceptors. Electron transport through PS I only (from diaminodurene (DAD), reduced 2,6-dichlorophenolindophenol (DCPIP) or *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine (TMPD), to MV) was measured polarographically as described above. Electron transport through PS II only (from H_2O to PDox in the presence of dibromothymoquinone (DBMIB)) was measured either polarographically (oxygen evolution) or spectrophotometrically (at 420 nm).

When linolenic acid, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), DAD (dissolved in ethanol) and DBMIB (dissolved in methanol) were added to these reaction mixtures, appropriate controls were made with ethanol (0.5%) and methanol. Light intensity was approximately $5 \cdot 10^5$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1} and the temperature was 20°C.

The determination of FFA was carried out as follows: at the desired time, 1 ml of chloroplast suspension (1 mg chlorophyll per ml) was mixed with sufficient chloroform/methanol (1:2, v/v) to obtain one phase. Sephadex G-25

[21] was then added to the mixture under nitrogen and the slurry evaporated. The resulting powder was resuspended in diethylether and the slurry was filtered on a small column through a sintered glass under nitrogen. This dehydrated solution was passed on Amberlite IRA-400 according to the procedure of Savary and Desnuelle [22] except that diethylether was used instead of ethylene oxide. After washing the column with diethylether, FFA were recovered under N_2 with 2% formic acid in diethylether, evaporated to dryness and methylated in methanolic H_2SO_4. Methylesters were extracted with pentane and separated by gas-liquid chromatography at 160°C on a 3% DEGS column with N_2 as carrier gas. Quantitative analysis was made for each individual available methylester by a standard curve.

RESULTS

The pH optimum of the electron flow rate decreased from 9.0 in unaged chloroplasts to 8—7.5 in chloroplasts aged at pH 7 and 8, respectively (Fig. 1A). The shift resulted from a decrease in electron transport activity at pH 9 and an increase at pH 7.5. Fig. 1A shows also that compared to the effect of aging at pH 7, aging chloroplasts at pH 8 accentuated both the shift of pH optimum toward acidity and the inhibition of the electron flow activity. The shift in pH optimum can be brought about by fatty acids (Fig. 1B) as has already

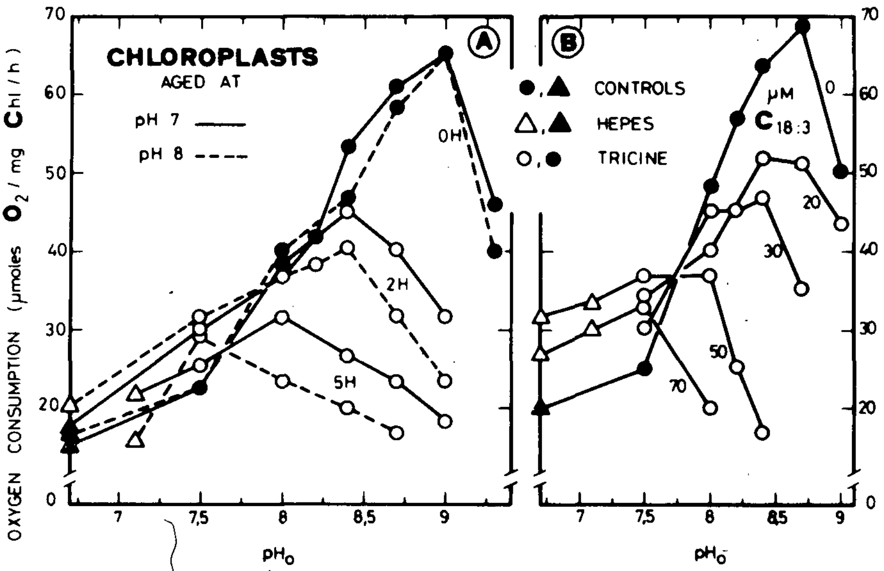

Fig. 1. Influence of aging (A) and linolenic acid (B) on PS II + PS I electron flow (from H_2O to MV) as a function of pH. The reaction mixture contained: 50 mM of buffer (HEPES or Tricine) at various pH values, 35 mM NaCl, 0.15 mM methylviologen, 2 mM NaN_3 and chloroplasts (20 μg chlorophyll per ml). Other conditions as described in Materials and Methods.

been shown in other electron flow systems [16,18]. As in the case of aging linolenic acid (the major fatty acid in thylakoid membrane lipids) inhibited the electron flow activity in fresh chloroplasts only in the alkaline range of pH whereas it stimulated it in the more acidic range (Fig. 1B).

The effects of aging and $C_{18:3}$ were also studied in two other types of electron flow as a function of pH (Fig. 2). The basic features described in Fig. 1 were again observed: (a) a shift of the pH optimum of the electron flow from

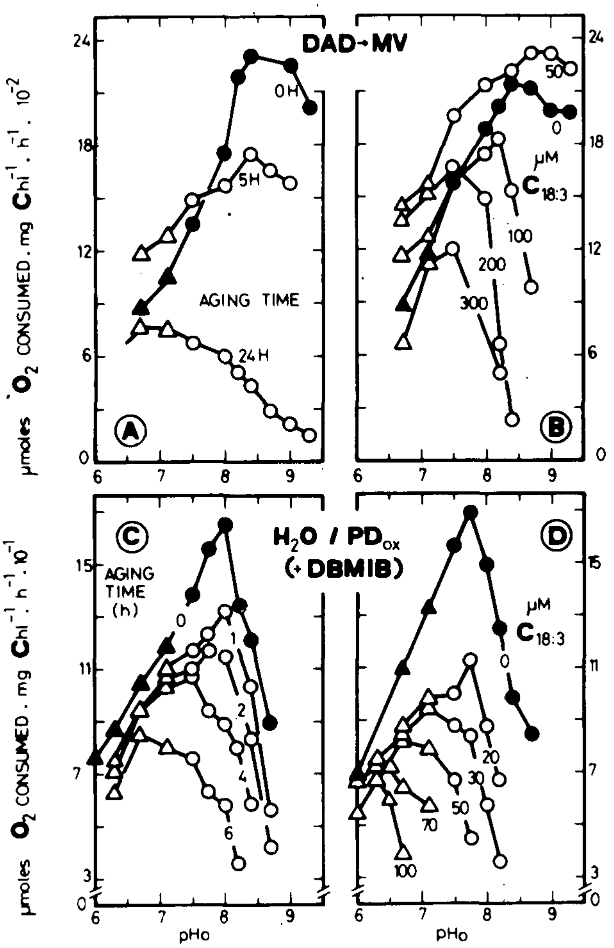

Fig. 2. Influence of aging (A, C) and linolenic acid (B, D) on PS I (from DAD to MV, see Figs. A and B) and PS II electron flow (from H_2O to PDox in the presence of DBMIB, see Figs. C and D) as a function of pH. The reaction mixture for PS I was the same as in Fig. 1 except that 10 µM DCMU, 300 µM DAD, 4 mM sodium ascorbate were added; for PS II, it contained 50 mM of buffer at various pH, 35 mM NaCl, 0.2 mM reduced *p*-phenylene diamine (PD), 1.2 mM $K_3Fe(CN)_6$, 1 µM DBMIB and chloroplasts (20 µg chlorophyll per ml). ▲,●, controls; ▲,△, HEPES; ●,○, Tricine. Chloroplasts were aged at pH 7. Other conditions as in Materials and Methods.

either DAD to MV or H_2O to PDox toward acidity; (b) a rapid inhibition of the activity in the alkaline range as a function of aging (Figs. 2A and C) and $C_{18:3}$ concentrations (Figs. 2B and D); (c) either no change or a slight activation of the activity in the more acidic range. In addition, the electron transport through PS II was much more sensitive to aging and to $C_{18:3}$ than that through PS I. As an example, the same sensitivity profiles were obtained after 24 h of aging or 300 μM $C_{18:3}$ in PS I and only after 6 h or 50—70 μM $C_{18:3}$ in PS II electron flows. All these results have been verified in other electron flow conditions (not shown): (a) from H_2O to ferricyanide or PDox (PS II + PS I); (b) from reduced DCPIP or TMPD to MV in the presence of DCMU (PS I); (c) from H_2O to ferricyanide or DCPIP in the presence of DBMIB (PS II). In conclusion, aging in vitro of chloroplasts and linolenic acid have similar effects on the activity and pH dependence of the photosynthetic electron flow.

The evolution of the endogenous FFA pools in thylakoid membranes during aging in vitro should give a more direct evidence of the involvement of these acids in the above photochemical changes. Since we found that the rate of FFA release from chloroplasts isolated from old leaves was rather slow (especially at pH 8), we chose to correlate the inhibition of electron flow activity with the release of FFA in chloroplasts isolated from young leaves which have a much higher rate of fatty acid release. The same chloroplast suspension was used for measuring $NADP^+$ photoreduction and FFA levels (Fig. 3). The amount of total and unsaturated FFA (namely $C_{18:3}$ which represented around 80% of FFA) increased rapidly up to 2 h of incubation at pH 7 and then more slowly (Fig. 3A). The level of saturated FFA was almost constant during the aging period. The content of total and unsaturated fatty acids (especially $C_{18:3}$) increased in a linear fashion between 0 and 3 h of incubation; then the rate of hydrolysis accelerated between 3 and 4 h of aging at pH 8 (Fig. 3B). Saturated fatty acids remained almost constant. Moreover, the initial values for free unsaturated fatty acids were higher at pH 8 (0.73 μmole/mg chlorophyll) than at pH 7 (0.54) and the total amount of FFA accumulated after 4 h of aging was greater at pH 8 (1.67) than at pH 7 (1.37). However, the spectra of the released FFA was qualitatively the same for both incubation pHs and the amounts of these acids were in the decreasing order: $C_{18:3}$ (c) >> $C_{16:3}$ (c) > $C_{16:2}$ (c) > $C_{18:2}$ (c) > $C_{16:0}$ > $C_{18:1}$ (c) > $C_{16:1}$ (t) > $C_{18:0}$. Fig. 3 shows also that the photoreduction of $NADP^+$ was inversely correlated with the release of FFA. At both pH 7 and 8, the photoreduction of $NADP^+$ decreased. However, the decrease in activity was less at pH 7 (Fig. 3A) than at pH 8 (Fig. 3B). These results confirm those obtained in Fig. 1A with the H_2O to MV system and show that both the electron flow and FFA release during aging were correlated in a pH-dependent process.

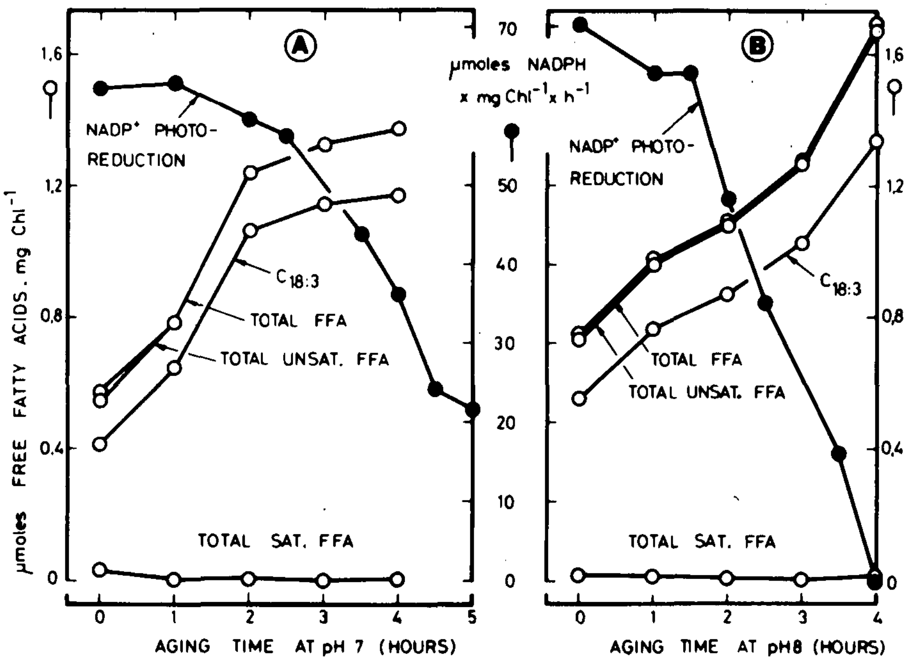

Fig. 3. Variation of electron flow activity (H_2O to $NADP^+$) and of FFA (total, saturated, unsaturated and linolenic acids) during aging in vitro at pH 7 (A) and pH 8 (B). The reaction mixture contained 50 mM Tricine (pH 8.4), 35 mM NaCl, 2 mM $NADP^+$, ferredoxin in excess and chloroplasts (60 µg chlorophyll per ml). Other conditions as in Materials and Methods.

DISCUSSION

Although electron flow activities through both systems were affected by aging, those through PS II were by far more sensitive toward aging than those through PS I (see Figs. 2A and C). Furthermore, the extent of inhibition of electron flow activity due to aging was pH-dependent. Aging caused a shift in the pH optimum toward acidity in all the electron flow systems studied (see Figs. 1A, 2A and C). This finding is similar to the observation of Punnett [24] who found that a high pH optimum was characteristic of high Hill activity preparations and that the pH optimum decreased as the chloroplasts lost their activity. Now, it is known that any uncoupling treatment (i.e. aging) causes a similar acid shift of electron flow [25].

During aging, a release of endogenous unsaturated FFA takes place due to hydrolysis of lipids [2,7,14,15,23]. These acids impair the integrity of the thylakoid membranes and their photochemical reactions. The effects of aging on the electron flow properties were strikingly similar to those of added $C_{18:3}$ as illustrated in Figs. 1—3.

This correlation was more directly verified by the determination of the evolution of endogenous FFA during aging (Fig. 3). When chloroplasts were aged at pH 7 and 8, a good inverse correlation existed (although with a time lag) between the diminution of NADP$^+$ photoreduction and the increase in the level of unsaturated fatty acids, especially linolenic acid. These observations are generally in good agreement with those of Constantopoulos and Kenyon [15] and Wintermans et al. [23].

Since galactolipids are the major lipids in the thylakoid membrane, the release of FFA during the aging process is mostly due to galactolipase activities. Galactolipases have been studied essentially in young leaves from *Phaseolus* [26—28] and only once from *Spinacia* [29]. All these enzymes were partially purified and their activities were measured with solubilized substrates [26—29] or with subchloroplast particles as substrates [28]. The pH optima of the enzyme from *Spinacia* [29] were 7.5 for monogalactosyldiglyceride (MGDG) and 5.9 for digalactosyldiglyceride. At both pH 7 and 8 the enzyme activities were equal and close to the maximum [29]. This explains why we found almost the same rate of FFA release at these two pH values (Figs. 3A and B). Since it is known that MGDG contains mainly $C_{18:3}$ and $C_{16:3}$ [30], one can infer from our results that MGDG is the preferential substrate hydrolyzed during aging in vitro. This observation is in agreement with the pH dependence and substrate specificity of the spinach galactolipase described by Helmsing [29]. However, our results disagree with those of McCarty and Jagendorf [14] who found that aging spinach chloroplast at pH 6 inactivated the electron flow measured at pH 8.5 whereas aging at pH 8 did not lead to these changes. This was related to the fact that $C_{18:3}$ was found only in extracts of chloroplasts aged at pH 6. The use of old spinach leaves by these authors may explain the discrepancy of their results with ours.

Although the levels of FFA were almost similar after 3 h of aging at both pH values, the inhibition of the electron flow activity was consistently greater at pH 8 than at pH 7 (see Figs. 1A and 3). This is an indication that in addition to the release of endogenous FFA in aged thylakoids other complex phenomena take place in the membrane.

For instance, it was shown that light- and, to a lesser extent, dark-aged chloroplasts produce lysolecithin as a result of the decomposition of endogenous lecithin in thylakoids [31]. Lysolecithin, a powerful detergent, was found to disintegrate chloroplasts into small particles and to impair the photochemical function in thylakoids [32]. Since no phospholipase A has been yet characterized in leaves, the enzyme involved in our system could be similar to the non-specific acyl-hydrolase described by Anderson et al. [28] which is membrane-bound, as in our case (unpublished results).

The inhibition of electron transport during aging could also be the result of the loss of lipids themselves which are essential for the functioning of the membrane. The release of lipids but not that of FFA, is enhanced under alkaline conditions [33] and may account for the greater inactivation of electron flow activity at pH 8 than at pH 7. However, Shaw et al. [34] have shown recently that when precautions are taken to prevent binding of fatty acids released by a galactolipid lipase to subchloroplast particles, large amounts of lipid may be removed without a marked effect on electron flow and thylakoid membrane structure.

It appears then that the factors which do regulate the aging process in the thylakoid membranes are quite complex. Another aspect, namely the influence of FFA on ΔpH, shall be reported soon (manuscript in preparation).

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RAPID ANALYSIS OF MEMBRANE LIPIDS USING A COMBINATION OF THIN-LAYER CHROMATOGRAPHY AND SCANNING OF PHOTOGRAPHIC NEGATIVES [1]

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A simple method is proposed for the analysis of the distribution and changes in membrane lipids subjected to different treatments (lipolytic, aging, etc.). This technique involves only one thin-layer chromatographic step followed by a scanning of the photographic negative of the charred thin layer. This method is time saving, inexpensive and does not require the technical skill usually demanded in lipidology. The precision of this method is compared with that obtained with the classical TLC—GC method: its variability is roughly twice that of the TLC—GC method.

Key words: lipid analysis; kinetics of lipolytic treatments; thin-layer chromatography; scanning of negatives; thylakoid membrane fractions.

INTRODUCTION

In studies designed to understand the role of lipids in photosynthetic [1–4] or in mitochondrial [5] membrane functions using the lipolytic treatment approach, one is always confronted with the difficult task of performing a large number of lipid distribution analyses. Although accurate, the classical thin-layer—gas chromatographic techniques [6,7] are often too laborious to allow for a large number of analyses. Indeed these techniques include several critical steps: lipid extraction, column chromatography followed by thin-layer chromatography, scraping, derivatization, extraction of methyl esters and injection in gas chromatograph. Under our working condi-

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[1] This work is part of a doctoral program which is being carried out by A.R. in The Laboratoire de Physiologie végétale et Biochimie, Université de Neuchâtel, Neuchâtel, Switzerland.

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Abbreviations: MG, monogalactosyldiglyceride; DG, digalactosyldiglyceride; PC, phosphatidylcholine; PG, phosphatidylglycerol; SL, sulfoquinovosyldiglyceride; LPG, lysophosphatidylglycerol; LPC, lysophosphatidylcholine; FFA, free fatty acids; TLC, thin-layer chromatography; GC, gas—liquid chromatography.

tions, we could analyse at best the lipid composition of two samples in one day.

In the present paper, we describe a technique for the analysis of acyl lipid distribution which involves only one chromatographic step and avoids the column chromatography, scraping, derivatization and gas chromatographic steps. The speed and convenience of this technique (up to 40 samples in one day) make it particularly well suited for lipid analyses in series.

MATERIALS AND METHODS

Preparation of membrane fractions

Spinach thylakoid membranes (CP) were prepared as described elsewhere [8]. Subchloroplast particles enriched in Photosystem II (SubCPII) or in Photosystem I (SubCPI) were prepared essentially as described in Ref. 9.

Extraction, separation and analysis of lipids

Classical method: combined thin-layer—gas chromatography (TLC—GC)
An aliquot (1—2 mg chlorophyll) of membrane suspension was subjected to lipid extraction according to Bligh and Dyer [10], separated into non-acidic and acidic lipids on a DEAE-cellulose column (acetate form) according to Roughan and Batt [11] and finally separated on silicagel plates, which were developed either with chloroform/methanol/H_2O (65 : 25 : 4) for non-acidic lipids or with chloroform/methanol/acetic acid/H_2O (85 : 15 : 10 : 3) for acidic lipids. After spraying the plates with alkaline bromothymol blue [12], zones of interest were scraped with a thin metal blade into glass tubes fitted with Teflon-lined screw caps and 1 ml methanol containing 5% (v/v) conc. H_2SO_4 or 3—5% (w/v) HCl was added. Derivatization was performed at 85—90°C under nitrogen. Extraction of the methyl esters was achieved with 3 × 2 ml pentane after addition of 2 ml water. The pentane extracts were pooled, evaporated to dryness with nitrogen, taken up in 50 μl hexane and injected into the chromatograph (Perkin Elmer Mod. 900). Gas chromatographic conditions were as follows: 180 cm glass column (i.d. 1.5 mm) packed with 3% DEGS on 80—100 mesh Chromosorb G AW-DMCS. The column was isothermally run at 160°C with nitrogen as carrier (30 ml/min). Injection port and detector (flame ionization detector) were held at 210—220°C. Identification and quantification of fatty acid methyl esters were made according to established procedures [6].

New method: combined thin-layer chromatography—negative scanning method
Step A: An aliquot (up to 0.5 ml) of membrane suspension (containing 0.5 mg chl/ml) was extracted as described elsewhere [10] in a 3 ml conical screw-cap vial. The resulting mixture was vigorously shaken and then centrifuged at 2000 × *g* until the upper phase (essentially aqueous methanol) was clear.

Step B: An aliquot of the lower phase (up to 100 μl) was removed with a Hamilton syringe (type 1710) and applied as a streak (6 × 1.5 mm) with the Linomat III sample applicator (Camag, Muttenz, Switzerland) on 10 × 20 cm silicagel 60 precoated aluminium sheets (Merck, Darmstadt, F.R.G.). Up to 20 samples can easily be applied in this way, each streak being separated from the next one by a 3 mm free space.

Step C: Development was achieved in a glass chamber (internally lined with fresh silicagel-coated plates) with chloroform/methanol/H_2O (65 : 25 : 4). When the front of the solvent had reached the top of the plate (about 8.0—8.5 cm), the thin-layer plate was removed, briefly dried in air, evenly sprayed with conc. H_2SO_4/ethanol/H_2O (34 : 33 : 33) and charred with a 'heat gun' (Ditzler, Dornach, Switzerland).

Step D: After the charring process, the plate was photographed with any convenient reflex camera (for instance, a Canon AT-1, fitted with a FD 50 mm, 1 : 1.8 S.C. objective plus a + 2 diopters Hoya close-up lens). The film was an Ilford FP-4 (medium speed black-and-white) 24 × 36 mm, with a recommended meter setting of 125 ASA (22 DIN). We found that daylight obtained from a correctly exposed window was convenient. The film was then processed according to the indications of the manufacturer in any available 'dark room' in the laboratory.

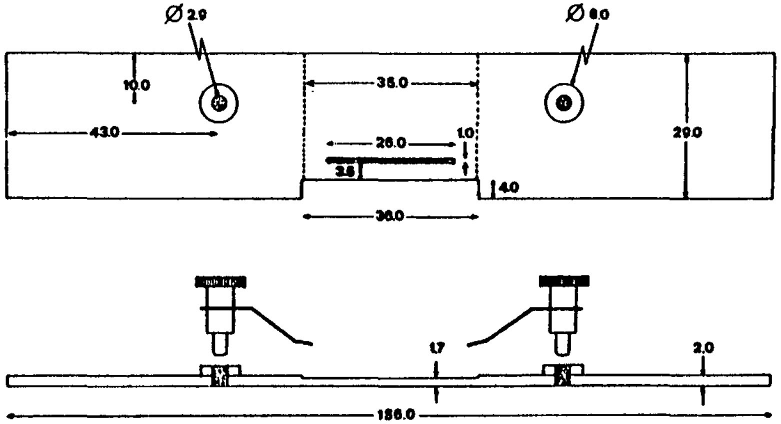


Fig. 1. Design of the holder used for the fixation of the negative for photometric analysis in the Zeiss Disk-ZK4 scanner. This device can be made out of any type of rigid plastic material such as polyvinylchloride. The negative (width 35 mm) was placed in the small groove between the two microscope clips which fixed the negative. Dimensions are in mm.

Step E: The film was cut into individual 24 X 36 mm negatives. Each negative was carefully wiped with a soft tissue and placed on a specially designed holder (Fig. 1), so that the slit (26 X 1 mm) intercepted a narrow band on a chromatographic lane. The holder was fixed with the appropriate clips onto the gel scanning device which is part of a Carl Zeiss (ZK 4) Scanner. The scanner itself was attached to a Carl Zeiss (PMQ 2) spectrophotometer. Scans were recorded with a Type 1100 W + W Recorder fitted with an integrator. Conditions for scanning were as follows: wavelength, 690 nm (this can vary somewhat with the type of film); scanning speed, 12 mm/min; recorder chart speed, 60 mm/min; scanner slit, completely opened; zero transmission, main slit closed; 100% transmission, on the clearest film zone. The negative should be placed with its shiny side facing the incident light beam. By moving slightly the negative along the groove, the next chromatographic lane can be placed in front of the slit.

RESULTS

Using the new method (see also Fig. 1), the linearity range of the technique was investigated for the three types of chloroplast fractions (CP, SubCPII and SubCPI). As an example, Fig. 2 shows the lipid chromatograms and their corresponding 'negative scannings' for a SubCPII lipid extract. Each band on the chromatogram (Fig. 2C) corresponded to a peak in the scanning profile (Fig. 2A), the peak area being determined by the integrator trace (Fig. 2B) and expressed as number of counts. When the amount of lipid extract applied on the chromatogram was increased, an excellent linearity was obtained for all three chloroplast fractions between the amount of lipid extract (expressed as µg chlorophyll) and the number of counts (Fig. 3). The slopes (*m*) were different for each separated compound, as illustrated in the graph and shown in the statistical analysis (see accompanying table). For minor lipids (PG + SL and PC), the linearity was maintained throughout the range of chlorophyll amounts used, whereas for major lipids (MG, DG) the curve showed two linear portions. Depending on the nature of the chloroplast extract (mainly on the pigment composition) and photographic conditions, the intercept of these portions occurred at various amounts of chlorophyll. The linear regression analysis showed that for the first linear portions the ordinates at the origin (*h*) were quite good and that the correlation coefficients (*r*) were excellent. In view of these results, one needs simply to work in a domain which is similar and linear for each lipid, i.e. from 0 to 30 µg chlorophyll for the CP fraction. It must be stressed that Fig. 3 presents only linearity domains and does not represent calibration curves.

In order to ascertain the precision of the new method the variability in the lipid content of thylakoid membrane fraction was determined and compared to that obtained with the classical TLC—GC method. This comparison was made with the same CP preparation. Table 1 shows the lipid composition of CP which is in good agreement with earlier results [13,14]. Using the TLC—

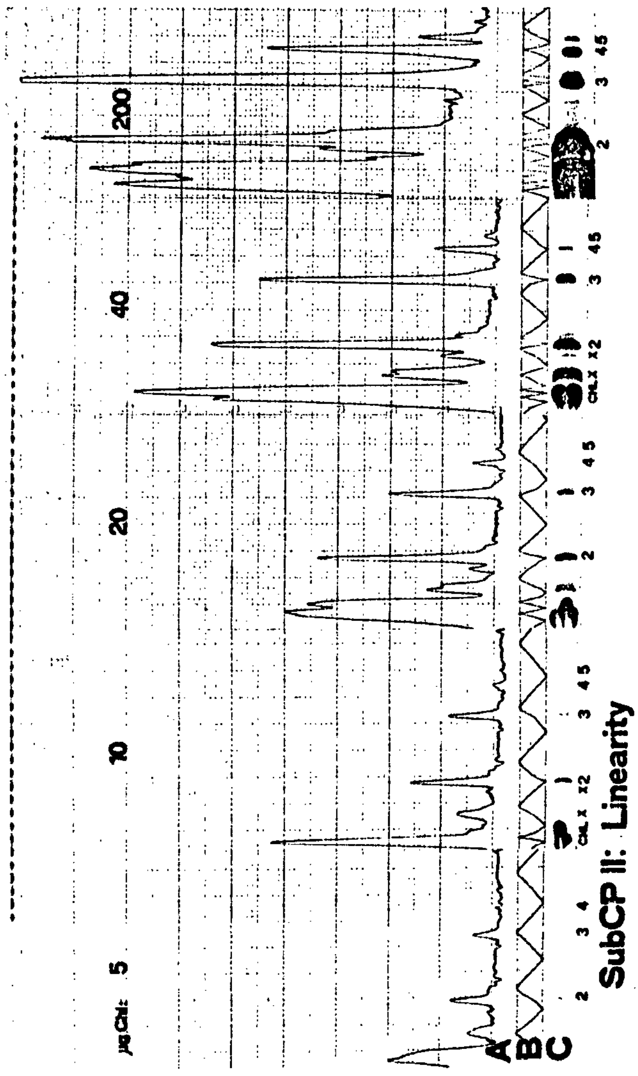
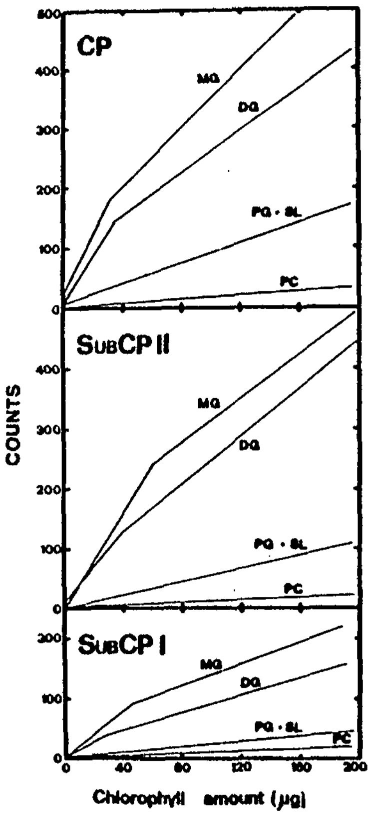


Fig. 2. Chromatograms of various amounts of SubCPII lipid extract and their corresponding 'negative scannings'. A, negative scannings; B, integrator traces; C, chromatograms. Chl, chlorophylls + β-carotene; x, xanthophylls; 2, monogalactosyldiglyceride; 3, digalactosyldiglyceride; 4, phosphatidylglycerol + sulfolipid; 5, phosphatidycholine.



		m	h	r
CP				
MG:	*	4.86	31.0	0.989
	**	2.46	104.0	0.998
DG:	*	3.87	12.6	0.943
	**	1.73	86.0	0.992
PG · SL:		0.80	11.6	0.989
PC:		0.18	3.7	0.994
SubCP II				
MG:	*	3.97	0.7	0.996
	**	1.81	134.0	0.993
DG:	*	3.07	9.6	0.996
	**	1.97	80.0	0.989
PG · SL:		0.66	-1.6	0.991
PC:		0.14	-1.0	0.995
SubCP I				
MG:	*	1.96	-0.4	0.995
	**	0.90	81.0	0.999
DG:	*	1.37	-0.8	0.983
	**	0.70	18.0	0.992
PG · SL:		0.23	1.6	0.993
PC:		0.10	0.6	0.991

Fig. 3. Study of the linear domains between the amount of material (μg chlorophyll from CP, SubCPII and SubCPI) applied on the chromatogram and the number of counts determined by the integrator trace. The linear regression analysis is expressed as m (slope), h (ordinate at the origin) and r (correlation coefficient). First (*) and second (**) linear portion of the curves.

GC technique the variability ranged from 1.8% for DG to 5.9% for PC. With the new technique, the variability was greater, i.e. from 2.2% for DG to 10.0% for PG + SL. Due to incomplete separation of PG and SL under the chromatographic conditions used in the new method, the variability for PG + SL (10%) must be compared with the sum of the variabilities (6.4%) of each compound determined separately with the classical TLC—GC technique.

This technique has been successfully applied to the study of the changes in lipid composition in thylakoid membrane fractions treated with lipolytic enzymes. Two extreme examples are presented. Firstly, thylakoid membranes were treated with phospholipase A_2 (Fig. 4, left). Due to the specificity of these enzyme, the hydrolysis of lipids was weak; in this case, the technique must detect very small variations in the phospholipid content besides high and constant levels of galactolipids. Indeed, Fig. 4 (left) shows that the amount of MG (band 2) and DG (band 3) did not change whereas PC (band 5) diminished strongly with a concomitant appearance of LPC (band 7). This change was much more obvious in the scanning than in the chromatogram. This was true also for band 4 (PG + SL) which decreased slightly due to a limited breakdown of PG into LPG (band 6) and free fatty acids (see arrow). Secondly, SubCPI were treated with lipolytic acyl hydrolase (Fig. 4, right side) which was isolated from potato according to [16]. In contrast to the first example, this less specific enzyme induced an

TABLE 1

COMPARISON OF THE LIPID COMPOSITION IN SPINACH THYLAKOID MEMBRANE FRACTION (CP) ANALYSED BY COMBINED THIN-LAYER AND GAS CHROMATOGRAPHY (TLC—GC) AND BY THE NEGATIVE SCANNING METHOD (NEG-SCAN)

From a unique fresh CP preparation five aliquots (2.83 mg chlorophyll/ml) were extracted as described in Materials and Methods. The variability of the extraction on the basis of chlorophyll concentration was ±0.85% and the average yield of the extraction was 99.3%. Each of these aliquots was submitted to TLC—GC as described in Materials and Methods. The initial fresh CP preparation was then diluted to 0.5 mg chlorophyll/ml and extracted by the same technique as above. Up to ten aliquots (40 μg chlorophyll) were spotted on a TLC plate and treated as described in Materials and Methods for negative scanning analyses.

Lipid	TLC—GC		NEG-SCAN	
	μmol lipid/ μmol chlorophyll ± S.D.	Variability (%)	Counts ± S.D.	Variability (%)
Monogalactosyldiglyceride (MG)	1.458 ± 0.032 (n = 5)	2.2	247 ± 11.0 (n = 9)	4.5
Digalactosyldiglyceride (DG)	0.654 ± 0.012 (n = 5)	1.8	167 ± 3.6 (n = 8)	2.2
Phosphatidylglycerol (PG)	0.192 ± 0.007 (n = 4)	3.4		
Sulfoquinovosyldiglyceride (SL)	0.115 ± 0.004 (n = 4)	3.0		
PG + SL	0.307 ± 0.011	6.4	12.9 ± 1.3 (n = 8)	10.0
Phosphatidylcholine (PC)	0.160 ± 0.000 (n = 4)	5.9	15.4 ± 1.0 (n = 10)	8.8
Phosphatidylinositol (PI)	0.012	n.d.		

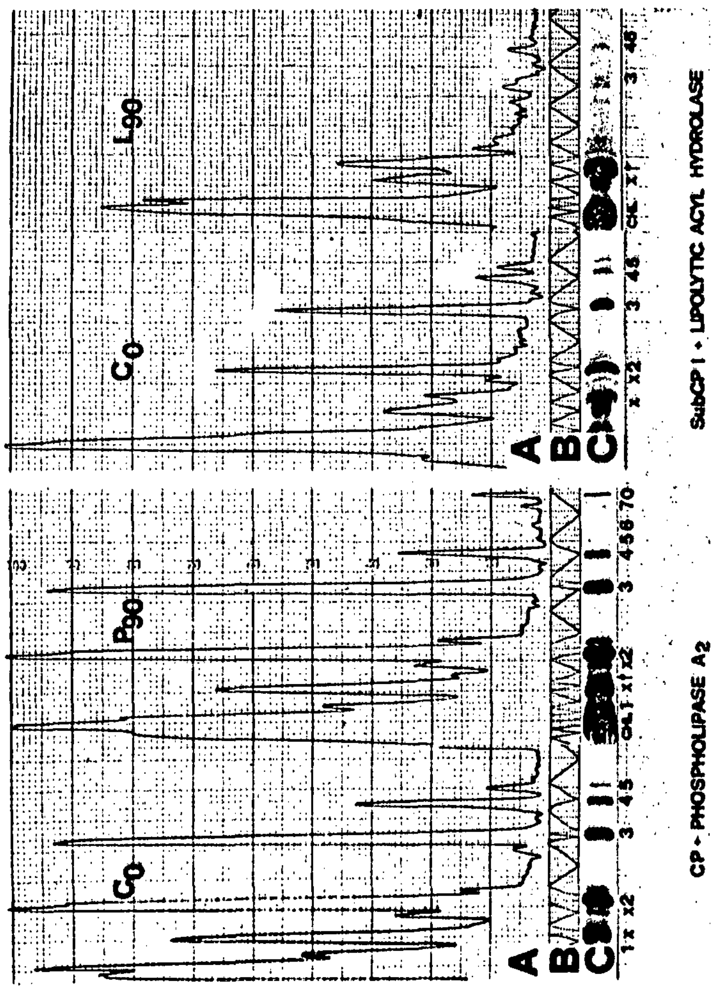


Fig. 4. Action of phospholipase A_2 (left trace) and lipolytic acyl hydrolase (right trace) on thylakoid membrane fractions (CP and SubCPI) followed by the changes in lipid composition. C_0, controls at 0 min; P_{90} and L_{90}, PLA_2 and LAH treatment at 90 min. A, B, C, Chl, X and 2—5 are as in Fig. 2. 1, acyl monogalactosyldiglyceride (see Ref. 15); 6, LPG; 7, LPC; O, origin; arrow, free fatty acids. The incubation mixture for PLA_2 treatment contained 20 mM Tricine (pH 8), 35 mM NaCl, 1 mM $CaCl_2$ and CP (0.5 mg chlorophyll/ml), 1500 mU PLA_2/μmol chlorophyll and for LAH treatment 50 mM 2-(N-morpholino)ethane sulfonic acid (pH 6), 35 mM NaCl, SubCPI (0.5 mg chlorophyll/ml), 40 μg LAH/mg chlorophyll. Incubation was in the dark at 20°C.

almost complete breakdown of lipids; in this case, the technique must detect great changes in the galactolipid content besides minor variations in the level of ionic lipids as shown in Fig. 4 (right). Thus, it appears that any rapid change in lipid composition, independent of the level of the lipid content, can be monitored easily with this new method.

DISCUSSION

Several techniques have been introduced to avoid the use of GC analysis for the quantification of lipid classes. These techniques were based on direct densitometric analyses of charred TLC plates [17] or of their photographic reproduction [18]. However these methods were applied essentially to neutral lipids and moreover needed calibration curves for each lipid. The necessity of analysing a great number of samples containing membrane lipids prompted us to reconsider the applicability of the above densitometric analyses to polar lipids. Since charred glycolipids and phospholipids gave different colored bands the rationale of our approach was to translate the color intensity and quality of each band into a single easy measurable signal. This signal must differentiate only the amount but not the chemical nature of each compound. This was achieved by the use of a black-and-white negative image of the chromatogram followed by the direct scanning of the negative in the transmission mode. Such an idea has been suggested already by Vioque and Vioque [19] and applied essentially to neutral lipids. This procedure must meet the following requirements: (a) each band represents a pure individual lipid. Although this assumption was generally verified, this was not the case for band 4 (PG + SL, Fig. 2C and 4C) and for the bands corresponding to FFA and xanthophylls (Fig. 4); (b) the shape (but not the area) of each band must be kept as constant as possible so that the scanning of the portion of the band defined by the slit is proportional to the whole band; this can be achieved by a narrow streak application of each sample and by a short distance of migration (8 cm in our case). Direct scanning of the photographic negative was prefered to direct photodensitometry because there was no risk of fading of the coloured bands and because the informative content of these negatives could be conveniently stored and reused, if necessary.

The linearity study of the method as a function of the amount of chloroplast extract added on the thin-layer plate showed two straight lines for MG and DG (Fig. 3). The slope of the first straight line was due to the increase of both surface and color intensity of the band. The slope of the second straight line was weaker and depended only on the area of the band as revealed by planimetric measurements (not shown).

Although the number of counts obtained from the integrator trace is a direct indication of the amount of each lipid it cannot be compared with the number of counts of other lipids. It is therefore not possible to give a relative lipid composition of the sample on the basis of number of counts. However, an absolute quantification can be achieved by relating the initial number of counts to the amount of each lipid determined by TLC—GC at zero time (Table 1). This relation is then easily extended to other samples by simple proportionality provided that (1) they contain exactly the same amount of chlorophyll; (2) the experiment is conducted in the same linear domain for all lipids; (3) each series of analysis can be referred to a control (zero time) on the same chromatographic plate. This latter point implies that photographic conditions have not to be strictly controlled between different series of analyses.

The variability of the results presented in Table 1 for the negative scanning method depends essentially on the quality of the chromatographic separation. As an example, DG which is well separated and appears as an ideal rectangular spot has a low variability (2.2%), which is close to that obtained with the TLC—GC method (1.8%). On the other hand, the higher variability of PG + SL (10%) is mainly due to an incomplete chromatographic separation and a low charring yield of the two lipids. If necessary, the precision of the method can be improved by using another solvent system which would resolve completely the two compounds.

The present technique has several advantages over the chromatographic procedures currently in use but is devised only for a lipid class analysis and not for a fatty acid distribution in the various lipids. This method can be highly recommended in all cases involving kinetic studies of lipolytic processes where numerous lipid determinations are desired.

SIMPLIFIED DESCRIPTION OF THE METHOD AND ITS ADVANTAGES

The method makes use of a single thin-layer chromatographic step to separate up to 20 lipid extracts in a short run. Quantitative and reproducible sample application as narrow bands by microsyringe is the only critical step. This is followed by charring and the thin layer is photographed in black-and-white. The negative is then scanned in the transmission mode and the recorder trace is converted into counts by an integrator. Peaks of identical chemical nature (same R_F) are directly comparable in terms of percent of control.

Avoiding the need of GC techniques, this method allows a rapid analysis of the changes in lipid composition of membranes subjected to lipolytic treatments, particularly during the early stages of the hydrolytic process. It can also be applied to the study of lipolytic enzymes with 'solubilized' lipid substrates.

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Role of Lipids in Functions of Photosynthetic Membranes Revealed by Treatment with Lipolytic Acyl Hydrolase

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1. Thylakoid membranes and subchloroplast particles I and II enriched in photosystem I and photosystem II, respectively, were treated with a potato lipolytic acyl hydrolase.

2. In the thylakoid membrane fraction, this treatment inhibited electron flows involving both photosystems and the associated photophosphorylations. However, electron flows involving either photosystem I of photosystem II were still preserved. The treatment of thylakoid membranes by lipolytic acyl hydrolase brought about a temporal convergence of different events such as maximal activity of reduced dichloroindophenol-supported electron flows, complete inhibition of photophosphorylations and electron transport activities through photosystem II + I, onset of the decay *N,N,N',N'*-tetramethyl-*p*-phenylenediamine-supported activity of photosystem activity and of the stimulation of photosystem activity (from reduced dichloroindophenol to $NADP^+$ by exogenous plastocyanin.

3. Lipolytic acyl hydrolase catalyzed a limited hydrolysis of each lipid but in a stepwise manner, the galactolipids being attacked before the ionic lipids. The extent of the hydrolysis was not more than 50% for each lipid. Most of the hydrolytic process occurred before any significant change in photochemical activities could be observed.

4. In subchloroplast particles I, a treatment by lipolytic acyl hydrolase did not greatly affect the electron transport whilst lipid hydrolysis was almost complete.

5. In subchloroplast particles II, neither the electron flow activities nor lipid content were significantly altered by lipolytic acyl hydrolase.

6. The sites of lipolytic acyl hydrolase action appeared to be localized between plastoquinones and P700. It is suggested that it is not possible to establish a quantitative and/or temporal correlation between the extent of lipid hydrolysis and the inhibition of photochemical activities.

7. The profile of the hydrolysis of lipids in thylakoid membranes suggests that ionic lipids are less accessible to lipolytic acyl hydrolase than galactolipids.

The thylakoid membrane is an example of a highly specialized, vectorially oriented membrane and its architecture has been extensively reviewed [1,2]. The lipid composition of spinach thylakoid membranes is well established [3–5]. These lipids all have a high linolenic acid content, but they differ strongly in their polar head groups; moreover, the main molecular species of phosphatidylglycerol offers the unique feature of being acylated in the 2-position by 3-*trans*-hexadecenoic acid [6]. Such a variety of lipid molecules allows the thylakoid membrane to sustain a wide range of activities [7].

We have previously investigated the effects of aging *in vitro* on the photochemical activities of the thylakoid membrane. Results showed that these effects [8–11] could be ascribed at least partly to a release of endogenous free fatty acids, mainly linolenic acid [8], which impaired the thylakoid membrane functions.

Since the level of free fatty acids seems to control the structure and the functions of the thylakoid membrane [8,12–15], it is primordial to know if lipids *per se* can play any specific role(s) in this respect. Toward this goal various approaches have been proposed, e.g. solvent extraction followed by reconstitution with lipids [16,17], immunological studies [18]

Abbreviations. Cl_2Ind, 2,6-dichlorophenol-indophenol; diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; $Ph(NMe_2)_2$, *N,N,N',N'*-tetramethyl-*p*-phenylene diamine; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; P700, reaction centre of photosystem 1.

and membrane lipid hydrogenation *in situ* [19]. Another technique involves lipolytic treatments of the thylakoid membrane [20–34]. Most of these studies failed to ascribe a specific role for lipids in sustaining a particular activity within the thylakoid membrane. Moreover, the reconstitution of lipase-treated thylakoid membranes with exogenous lipids may be complicated by the fact that, in most cases, the products of hydrolysis (fatty acids, lyso-compounds) remain in the membrane [35–37]. When such a reconstitution was attempted [17], it was shown to be non-specific.

In this investigation we have studied in detail the effect of a treatment with lipolytic acyl hydrolase from potato on the lipid composition and the photochemical activities of various thylakoid membrane fractions. We present evidence which tends to exclude a direct correlation between the amount of lipids destroyed, the kinetic aspects of this hydrolysis and the alteration of photochemical activities.

MATERIALS AND METHODS

Preparation of Chloroplast Membrane Fractions

Spinach (*Spinacia oleracea*, var. Nobel) was grown in a growth chamber under controlled conditions [15]. Leaves were harvested after 14 weeks of hydroponic culture.

Intact chlorplasts were first prepared according to Kalberer et al. [38] in a medium containing 25 mM Hepes (or Mops) pH 7.6 and 350 mM sucrose, spun down at 1935 × *g* for 30 s and then osmotically disrupted in the above medium diluted tenfold. Centrifugation at 17300 × *g* for 5 min yielded a pellet which was resuspended in a medium containing 25 mM Hepes (or Mops) pH 7.6 and 175 mM NaCl. The chlorophyll concentration was determined spectrophotometrically [39]. This suspension, which contained essentially type D chloroplasts according to Halliwell's nomenclature [40] will be referred to 'thylakoid membrane'.

Subchloroplast particles enriched either in photosystem I or in photosystem II were prepared by the Triton X-100 procedure of Vernon and Shaw [41], except that the above thylakoid membranes were submitted to the detergent treatment instead of the crude chloroplast fraction described by these authors. Subchloroplast particles I and II were checked for cross-contamination and were routinely found to be 90–95% pure as judged by measurements of electron transport activity.

Enzyme Preparation

Lipolytic acyl hydrolase was isolated from potato tubers by the method of Galliard [42] and partially purified through Sephacryl SP-200 and DEAE-cellulose column chromatography. The characteristics of the enzyme were similar to those described earlier [42]. Stored frozen in small aliquots, this enzyme preparation was stable over more than one year despite of numerous freeze-thawing cycles.

Enzyme Treatment of Membrane Fractions

The incubation of various chloroplast membrane fractions with lipolytic acyl hydrolase was carried out in the dark, at 20 °C. The mixture contained 50 mM Mes buffer pH 6.0, 35 mM NaCl, thylakoid membranes, subchloroplast particles I or subchloroplast particles II at a final chlorophyll concentration of 0.5 mg/ml, bovine serum albumin (when needed) at 0.5 mg/ml, and lipolytic acyl hydrolase at a final protein concentration of 20 µg/ml. In all treatments, the ratios lipolytic acyl hydrolase/chlorophyll and serum albumin/chlorophyll were thus kept constant (40 µg lipolytic acyl hydrolase/mg chlorophyll and 1 mg serum albumin/mg chlorophyll, respectively). Aliquots were taken at various times for lipid analysis and determination of photochemical activities.

Lipid Analysis

The method for lipid analysis is described elsewhere [4]. This technique is based on the combination of thin-layer chromatography and scanning of photographic negatives.

Determination of Electron Transport and Photophosphorylation Activities

These activities were assayed simultaneously to lipid hydrolysis. At any desired time, aliquots of the incubation mixture were pipetted into the appropriate reaction mixture.

Photosystem II activity in thylakoid membranes was measured by the photoreduction of Cl_2Ind at 590 nm (Balzers interference filter 589 nm) in a 0.2-cm glass cuvette (0.5 ml) containing 50 mM Hepes buffer pH 7.6, 35 mM NaCl, 0.2 mM Cl_2Ind, 0.01 mM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone and thylakoid membranes (40 µg chlorophyll/ml). Photosystem II activity in subchloroplast particles II was followed by the same method in 50 mM Mes buffer pH 6.6, 35 mM NaCl, 0.2 mM Cl_2Ind, 0.5 mM 1,5-diphenylcarbazide and subchloroplast particles II (30 µg chlorophyll/ml).

Photosystem I activity in thylakoid membranes was estimated by the autooxidation of photoreduced methylviologen with a Clark oxygen electrode in 5 ml containing 50 mM Tricine (pH 8.4), 35 mM NaCl, 2 mM NaN_3, 4 mM sodium ascorbate, 0.01 mM diuron, 0.15 mM methylviologen, 0.1 mM Cl_2Ind or

$Ph(NMe_2)_2$ and thylakoid membranes (20 µg chlorophyll/ml). Photosystem I activity in thylakoid membranes was also measured by the photoreduction of $NADP^+$ at 340 nm (Balzers interference filter 339 nm) in a 0.2-cm glass cuvette containing 50 mM Tricine pH 8.4, 35 mM NaCl, 4 mM sodium ascorbate, 0.01 mM diuron, 0.3 mM Cl_2Ind, 2 mM $NADP^+$, 6 µM ferredoxin and thylakoid membranes (100 µg chlorophyll/ml). Photosystem I activity in subchloroplast particles I was followed by the same method in 50 mM Tricine pH 8.4, 35 mM NaCl, 4 mM sodium ascorbate, 0.01 mM diuron, 0.3 mM Cl_2Ind, 2 mM $NADP^+$, 6 µM ferredoxin, 1 µM plastocyanin, 5 µM ferredoxin-$NADP^+$ reductase and subchloroplast particles I (50 µg chlorophyll/ml).

Photosystem II + I activity in thylakoid membranes was measured either by the photoreduction of $NADP^+$ at 340 nm in a 0.2-cm cuvette containing 50 mM Tricine pH 8.4, 35 mM NaCl, 2 mM $NADP^+$, 6 µM ferredoxin and thylakoid membranes (100 µg chlorophyll/ml), or by the autooxidation of photoreduced methylviologen in 50 mM Tricine pH 8.4, 35 mM NaCl, 2 mM NaN_3, 0.15 mM methylviologen and thylakoid membranes (20 µg chlorophyll/ml).

The intensity of actinic light at the level of the cuvette was around 250 mW · cm^{-2}. For optical measurements, the actinic light was passed through a Calflex and a Balzers DT-Red filter.

Cyclic photophosphorylation was allowed to proceed during 5 min of illumination under 30 mW · cm^{-2} in test tubes containing 50 mM Tricine pH 8.0, 35 mM NaCl, 5 mM $MgCl_2$, 1 mM Na_2HPO_4, 0.05 mM *N*-methylphenazonium methosulfate, 1 mM ADP, 0.01 mM diuron and thylakoid membranes (25 µg chlorophyll/ml) in a total volume of 1 ml. Non-cyclic photophosphorylation was studied in the same conditions as above. The reaction mixture contained 50 mM Tricine pH 8.4, 35 mM NaCl, 5 mM $MgCl_2$, 1 mM Na_2HPO_4, 1 mM ADP, 2 mM $NADP^+$, 6 µM ferredoxin and thylakoid membranes (25 µg chlorophyll/ml) in a total volume of 1 ml. The reactions were stopped by the addition of 0.1 ml trichloroacetic acid (30%, w/v). ATP formation was then estimated from the decrease in free inorganic phosphate, measured by the method of Chen et al. [43].

RESULTS

Fig. 1 shows the changes in the lipid composition of thylakoid membranes treated with lipolytic acyl hydrolase. Each band on the chromatogram (Fig. 1A, c) corresponded to a peak in the scanning profile (Fig. 1A, a), the peak surface being determined by the integrator trace (Fig. 1A, b). As seen in Fig. 1B (insert), the amount of free fatty acids increased strongly in the treated samples, whereas it was scarcely detectable in the controls. This was confirmed by the fact that in the controls, besides minor galactolipid transacylations [44], almost no change in the amount of the different lipids was observed on the chromatograms (not shown). Under the action of lipolytic acyl hydrolase, the changes in the membrane lipid content were characterized by three distinct phases. Firstly, the monogalactosyldiglyceride content diminished strongly down to 80%, and this was immediately followed by a similar decrease in digalactosyldiglyceride. During this short phase (4 min), the content of ionic lipids was not altered. Secondly, the amount of ionic lipids dropped abruptly (in 2 min) down to 60–70% whereas no further change occurred for the galactolipids. Eventually, during the third phase (6 to 90 min), the content of ionic lipids did not change whilst that of the galactolipids decreased slowly down to 50–60%.

The changes in the lipid composition arising from the action of lipolytic acyl hydrolase were accompanied by alterations in the photochemical activities of the thylakoid membranes. Fig. 2 shows the effect of lipolytic acyl hydrolase on different electron flow systems. The activities involving photosystem II + I (i.e. from H_2O to $NADP^+$, Fig. 2A; and from H_2O to methylviologen, Fig. 2B) decreased rapidly and were completely inhibited after 40 to 50 min of incubation with lipolytic acyl hydrolase. In contrast, lipolytic acyl hydrolase had no detectable effect on photosystem II electron flow (Fig. 2C) and a less pronounced action on $NADP^+$ photoreduction when reduced Cl_2Ind was the electron donor (Fig. 2D). The slight activation observed in the $H_2O/NADP^+$ and $Cl_2Ind/NADP^+$ systems in the presence of lipolytic acyl hydrolase was due to uncoupling since the enzyme did not display such an effect in the presence of uncoupled thylakoid membranes. Since albumin is generally known to protect the membrane structure and function by binding free fatty acids, the effect of bovine serum albumin was tested with the same enzyme-treated preparations. Indeed, albumin did protect the electron transport activities but only to a small extent whilst it had no effect on the controls (not shown).

The simultaneous analysis of the different lipids in the thylakoid membranes (Fig. 1B) has shown that the action of lipolytic acyl hydrolase was characterized by three hydrolytic steps. The question therefore was whether the different electron transport activities would be altered in the same way. In order to test this possibility six activities were determined simultaneously, including four electron donors [H_2O, reduced Cl_2Ind, reduced $Ph(NMe_2)_2$ and ascorbate] and two electron acceptors (methylviologen and $NADP^+$), as shown in Fig. 3. The interest of comparing Cl_2Ind and $Ph(NMe_2)_2$ as electron donors relies on the fact that the redox reaction of the former is linked to vectorial proton transport whereas the redox reaction of

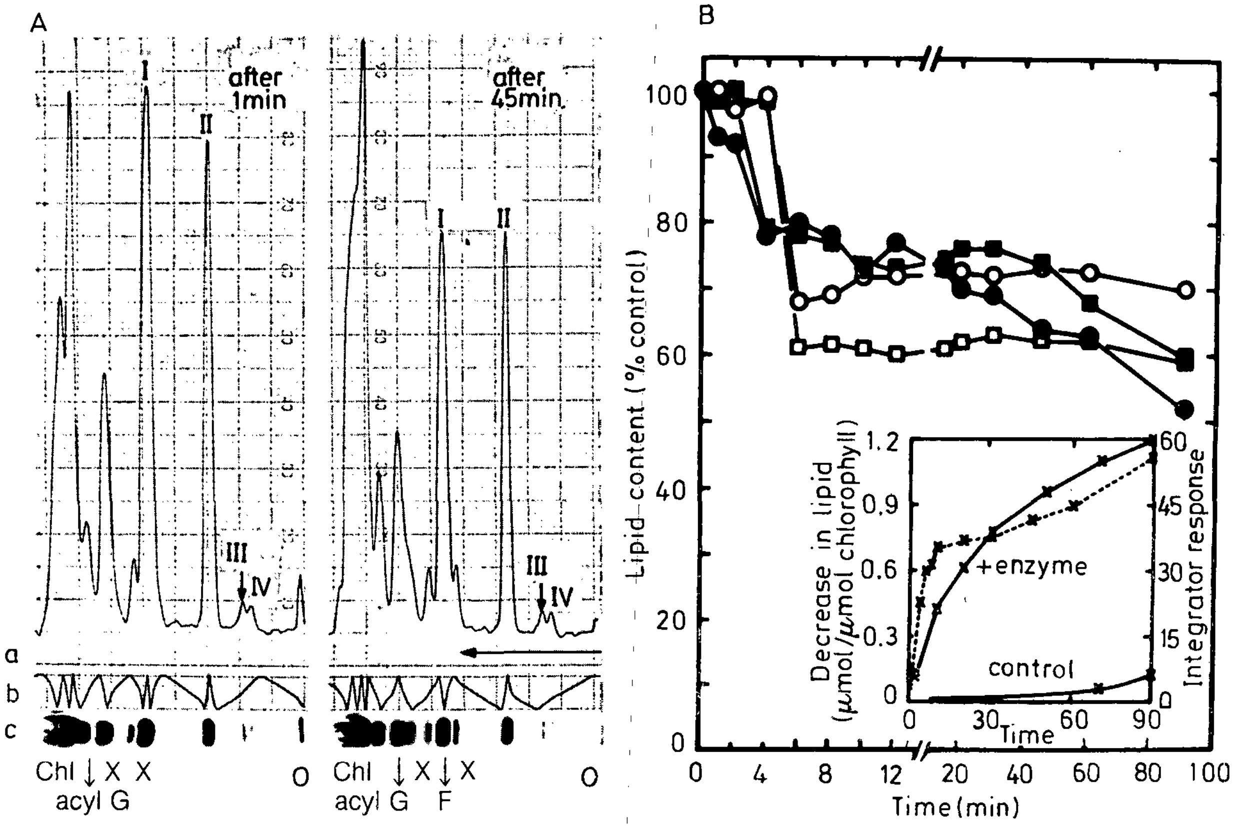

Fig. 1. *Action of lipolytic acyl hydrolase on thylakoid membrane followed by the evolution of lipid composition as a function of time.* (A) Chromatography; scanning of the photographic negatives after 1 min and 45 min (a); integrator traces (b); chromatograms (c); Chl, chlorophylls + β-carotene; acylG, acyl monogalactosyldiglyceride; X, xanthophylls; F, free fatty acids; O, origin of the chromatogram. Peak I corresponds to monogalactosyldiglyceride, peak II to digalactosyldiglyceride, peak III to phosphatidylglycerol + sulfoquinovosyldiglyceride, and peak IV to phosphatidylcholine. (B) Plot of lipid content against time. The 100% values corresponded to 1.43 µmol · µmol chlorophyll^{-1} for monogalactosyldiglyceride (●), to 0.70 for digalactosyldiglyceride (■), to 0.19 + 0.11 for phosphatidylglycerol and sulfoquinovosylglyceride, respectively, (○) and to 0.15 for phosphatidylcholine (□). Insert: amount of free fatty acids estimated by the integrator response (×——×) of from the decrease in the lipid content expressed as free fatty acids (×---×)

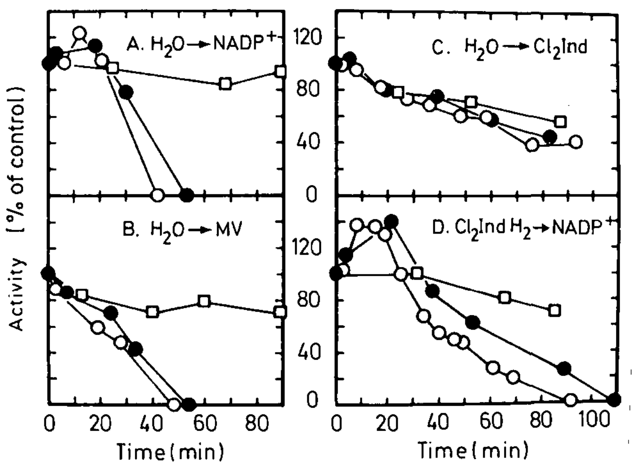

Fig. 2. *Action of lipolytic acyl hydrolase on thylakoid membrane fraction followed by the variations of electron transport activities as a function of time.* The 100% values corresponded to 128 µmol $NADPH_2$ · mg chlorophyll^{-1} · h^{-1} in the $H_2O/NADP^+$ system (A), to 56 µmol O_2 consumed · mg chlorophyll^{-1} · h^{-1} in the H_2O/methylviologen (MV) system (B), to 189 µmol Cl_2Ind reduced · mg chlorophyll^{-1} · h^{-1} in the H_2O/Cl_2Ind system (C) and 78 µmol $NADPH_2$ · mg chlorophyll^{-1} · h^{-1} in the reduced $Cl_2Ind/NADP^+$ system (D). Other conditions as described in Materials and Methods. (□) Control; (○) with enzyme; (●) with enzyme and bovine serum albumin

the latter involves no proton exchange and therefore no photophosphorylation [1]. Fig. 3 shows that complete inhibition of photosystem II + I electron flows (from H_2O to methylviologen and $NADP^+$) corresponded to maximum rates of photosystem I activity (from reduced Cl_2Ind to methylviologen and $NADP^+$) and to the beginning of the inactivation of photosystem I electron flow [from $Ph(NMe_2)_2$ to methylviologen]. This convergence of events which occurred after 1 h of incubation with lipolytic acyl hydrolase had to be monitored over a period of about 10 min because of technical limitations. Beyond this incubation time, activities supported by both reduced Cl_2Ind and $Ph(NMe_2)_2$ decreased, the first one being still higher than 100% whilst the second one was nil.

At low concentration (< 4 mM) ascorbate alone is not an electron donor for photosystem I electron flow because of its lack of accessibility to electron carriers embedded in the lipid phase [1]. Any treatment which would modify the lipid phase in the photosystem I region of the membrane and thus increase the accessibility to hydrophilic compounds could be monitored by an ascorbate-supported electron flow. As shown in Fig. 3 ascorbate did indeed become an

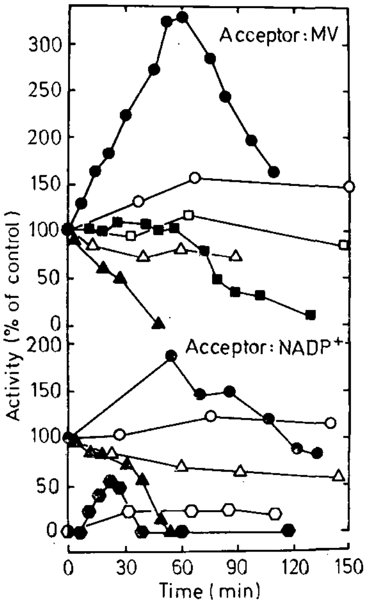

Fig. 3. *Simultaneous measurements of various electron transport activities in thylakoid membranes treated by lipolytic acyl hydrolase, as a function of time.* Filled symbols: treated thylakoid membranes; open symbols: controls; (○, ●) electron flow from Cl_2Ind to methylviologen (MV) (100% value = 190 μmol O_2 consumed · mg chlorophyll^{-1} · h^{-1}) or to $NADP^+$ (34 μmol $NADP^+$ reduced · mg chlorophyll^{-1} · h^{-1}); (□, ■) from reduced $Ph(NM_2)_2$ to methylviologen (629 μmol O_2 consumed · mg chlorophyll^{-1} · h^{-1}); (△, ▲) from H_2O to methylviologen (56 μmol O_2 consumed · mg chlorophyll^{-1} · h^{-1} or to $NADP^+$ (139 μmol $NADP^+$ reduced · mg chlorophyll^{-1} · h^{-1}); (○, ●) from ascorbate alone to $NADP^+$ (these activities were compared to the activity measured at 0 min in the presence of ascorbate + Cl_2Ind as the electron donor, i.e. 34 μmol ADP^+ reduced · mg chlorophyll^{-1} · h^{-1}). *Nota bene*: all photosystem-I-mediated reactions were performed in the presence of 10 μM diuron

Table 1. *Effect of lipolytic acyl hydrolase on electron transport activities and their associated photophosphorylations in the thylakoid membrane fraction*

Activity of Cl_2Ind and $Ph(NMe_2)_2$ to methylviologen is expressed as O_2 consumed, photophosphorylation is expressed as phosphate esterified and $H_2O/NADP^+$ reduction is expressed as $NADPH_2$ formed. Cyclic photophosphorylation is in the presence of *N*-methylphenazonium methosulfate, and non-cyclic with $H_2O/NADP^+$

Experiment 1

Time of incubation	Activity of	
	Cl_2Ind to methylviologen	cyclic photophosphorylation
min	μmol × mg chlorophyll^{-1} × h^{-1}	
0	142	181
10	236	79
40	408	0
60	270	0

Experiment 2

Time of incubation	Activity of			
	$H_2O/NADP^+$ reduction	Cl_2Ind to methylviologen	$Ph(NMe_2)_2$ to methylviologen	non-cyclic photophosphorylation
min	μmol × mg chlorophyll^{-1} × h^{-1}			
0	113	106	617	60
5	106	180	633	45
20	0	322	595	0
50	0	180	0	0

electron donor but only during the first period (from 10 to 40 min) of treatment by lipolytic acyl hydrolase. This phenomenon occurred also in the control, but the rate was slower and less pronounced.

Photophosphorylations were also measured in thylakoid membranes treated with lipolytic acyl hydrolase. Table 1 shows that cyclic photophosphorylation (experiment 1) was completely abolished (40 min) exactly when the basal electron transport activity (reduced Cl_2Ind/methylviologen) was maximal, corresponding to full uncoupling (see also Fig. 3). The same features occurred for non-cyclic photophosphorylation (experiment 2): maximal basal electron flow activity (Cl_2Ind/methylviologen) corresponded to still unchanged basal electron flow from $Ph(NMe_2)_2$ to methylviologen and to a complete inhibition of water-supported photoreduction of $NADP^+$ and its associated photophosphorylation.

The fact that lipolytic acyl hydrolase had an inhibitory action essentially between the two photosystems (see Fig. 2) and that the behaviour of photosystem I activity supported by reduced Cl_2Ind and $Ph(NMe_2)_2$ was quite different towards lipolytic acyl hydrolase treatment (see Fig. 3) strongly suggested that the lipid environment of at least one of the electron carriers between plastoquinone and P700 could be the site of lipolytic acyl hydrolase action. One way to test this hypothesis was to look for a restoration of the photosystem I electron flow activity in enzyme-treated membranes by one of the possible candidates, i.e. plastocyanin. Fig. 4A and B show the effect of plastocyanin on photoreduction of $NADP^+$ when reduced Cl_2Ind was the electron donor. The stimulation of the rate by plastocyanin, expressed by the ratio of the activity in presence and absence of added plastocyanin decreased as a function of incubation time (see also open symbols in Fig. 4D). In the presence of lipolytic acyl hydrolase, this ratio first decreased reaching a minimum value exactly when the activity of $H_2O/NADP^+$ was completely inhibited (50 to 60 min) then increased up to a value of two in the time limit of our experimental conditions (Fig. 4C and D).

Next, we have adopted the same enzymatic approach to study the effect of lipolytic acyl hydrolase on the electron transport activities and lipid com-

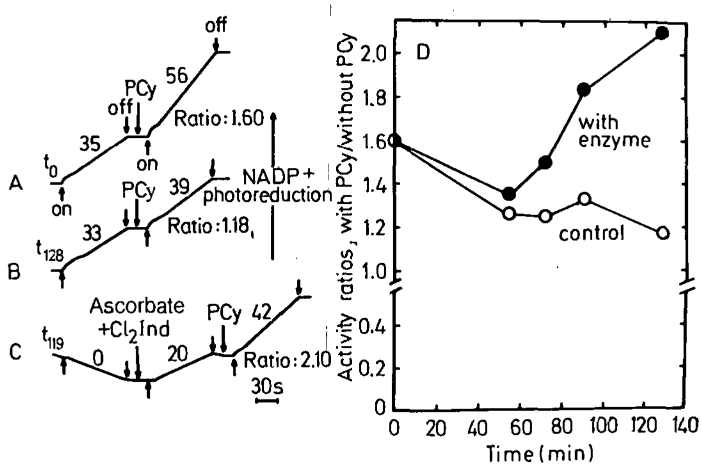

Fig. 4. *Recording traces showing how NADP$^+$ photoreduction was measured in the absence and presence of plastocyanin in thylakoid membrane fraction treated with lipolytic acyl hydrolase.* In A and B (untreated thylakoid membrane at 0 and 128 min) the reaction mixtures were as described in Materials and Methods for photosystem I activity in thylakoid membranes. In C thylakoid membranes treated by lipolytic acyl hydrolase for 119 min), the reaction mixture was as described in Materials and Methods for photosystem II + I in thylakoid membranes. After a first light-dark cycle to show complete inactivation of the electron flow from H_2O to NADP$^+$ by lipolytic acyl hydrolase, ascorbate (4 mM) and Cl_2Ind (0.3 mM) were added. Where indicated, plastocyanin (PCy) was at 1 μM. Numbers on the traces corresponded to μmol NADP$^+$ reduced · mg chlorophyll^{-1} · h^{-1}. The ratios of the reaction rates with and without plastocyanin are indicated separately in A, B and C and plotted as a function of incubation time in D

position in more simple membrane structures such as subchloroplast fractions. Fig. 5A shows the effect of lipolytic acyl hydrolase on the lipid composition of subchloroplast particles I. The lipolytic process was again characterized by three steps. Firstly, the phosphatidylglycerol + sulfoquinovosyldiglyceride content diminished strongly down to 80%, and this was immediately followed by a similar decrease in monogalactosyldiglyceride. During this short step (6 min), the content of digalactosyldiglyceride and phosphatidylcholine remained constant. The second step (6 to 10 min) was characterized by no change in the content of phosphatidylglycerol + sulfoquinovosyldiglyceride, but by a strong decrease in all the other lipids. In the third step (10 to 90 min), 50% phosphatidylglycerol + sulfoquinovosyldiglyceride were hydrolysed whereas most monogalactosyldiglyceride, digalactosyldiglyceride and phosphatidylcholine were destroyed. A first-order kinetic was found for the hydrolysis of monogalactosyldiglyceride and digalactosyldiglyceride throughout the experiment but not for the hydrolysis of ionic lipids (not shown). The decrease in acyl lipid content during the three steps was accompanied by a parallel increase in free fatty acids which accounted for the hydrolytic process. Since the initial amount of galactolipids was much higher than that of ionic lipids (see legend to Fig. 5A), the increase in the content of free fatty acids was mainly a contribution of the hydrolysis of galactolipids. In control subchloroplast particles I (not treated by lipolytic acyl hydrolase), no trace of free fatty acids and transacylation reactions between galactolipids were observed (not shown). The drastic changes in galactolipids and phosphatidylcholine content of subchloroplast particles I treated by lipolytic acyl hydrolase could not be related to similar decrease in electron transport activity (Fig. 5B). Although the decrease in phosphatidylglycerol + sulfoquinovosyldiglyceride content appeared to be of the same extent as that of the electron transport activity, this was not true for longer incubation period with lipolytic acyl hydrolase since subchloroplast particles I lipids were completely hydrolyzed whilst the electron transport activity was still high (not shown). If there was a correlation between the extent of lipid hydrolysis and decrease in activity after 90 min (see Fig. 5A and B), it would be therefore fortuitous. In this system, serum albumin had no protective action, neither in the control activity (not shown) nor in the activity of subchloroplast particles I.

In subchloroplast particles II, lipolytic acyl hydrolase had almost no effect on the lipid content, except a slight hydrolysis of galactolipids during the last 30 min of incubation (not shown). The effect of lipolytic acyl hydrolase on the electron transport activity in subchloroplast particles II is illustrated in Fig. 6. In both 1,5-diphenylcarbazide/Cl_2Ind and H_2O/Cl_2Ind systems the activities were inhibited down to around 50 to 60% in the treated subchloroplast particles II; in the controls, the 1,5-diphenylcarbazide/Cl_2Ind activity was more resistant than the H_2O/Cl_2Ind activity.

DISCUSSION

The relationship between lipids and photochemical activities in the thylakoid membrane is an intriguing question. Using lipolytic enzymes several authors

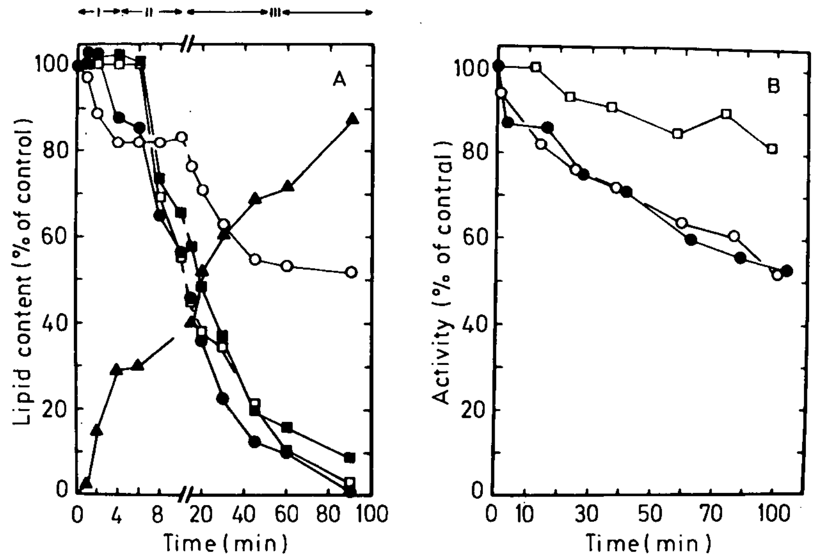

Fig. 5. *Action of lipolytic acyl hydrolase on subchloroplast particles I (SubCPI) followed by the changes in lipid composition (A) and by the electron transport activity from Cl_2Ind to $NADP^+$ (B), as a function of time.* In A, the 100% values were 0.333 μmol · mg chlorophyll^{-1} for monogalactosyldiglyceride (●), 0.282 for digalactosyldiglyceride (■), 0.062 and 0.010 for phosphatidylglycerol and sulfoquinovosyldiglyceride respectively, (○), and 0.052 for phosphatidylcholine (□). I, II and III correspond to three hydrolytic steps. (▲) Free fatty acids. In B, the 100% value was 656 μmol $NADPH_2$ · mg chlorophyll^{-1} · h^{-1}. (□) Control; (○) with enzyme; (●) with enzyme and bovine serum albumin

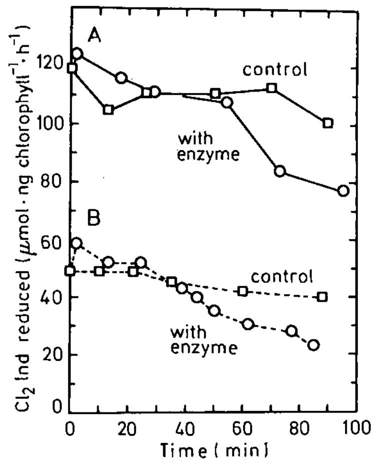

Fig. 6. *Action of lipolytic acyl hydrolase on subchloroplast particles II followed by the electron transport activity as a function of time.* Cl_2Ind reduced · mg chlorophyll^{-1} · h^{-1} when H_2O (B) and 1,5-diphenylcarbazide (A) were the electron donors, respectively

have tried to find a quantitative correlation between the amount of lipids destroyed and the decay in photochemical activities [20–34]. So far, such a correlation has not been described unequivocally. In order to gain the best from this enzymatic approach it seems necessary to emphasize the following points. Firstly, the specificity of the lipolytic enzyme must be known. Secondly, the ratio enzyme/chlorophyll should be low enough to allow the detection of the earliest events in the membrane functions which could be linked to hydrolysis of lipids. Thirdly, the action of lipolytic enzymes is governed by the nature of the membrane considered (thylakoid membranes, subchloroplast particles I, subchloroplast particles II), the distribution of lipids between the two membrane surfaces, the compactness of the membranous lipid layer and the degree of shielding of the lipids by membrane proteins or other lipids [40]. Fourthly, it is most important to monitor, simultaneously to the destruction of lipids, the greatest number of functional parameters and to measure both changes at short intervals of time. Fifthly, it may be helpful to make a distinction between structural and functional lipids. Lipolytic enzymes destroy only the lipids they have access to but cannot distinguish between these two types. Moreover, there is no chemical difference between structural and functional lipids. Therefore, the lipids destroyed arise from both types which makes questionable any strict correlation between the extent of lipid hydrolysis and the changes in function. Finally, it is noteworthy that the lipidic products of the hydrolytic process remain in the membrane [35–37] where they may alter its structure and/or function, as suggested by a previous report [45].

The lipolytic acyl hydrolase from potato used in this investigation acts on a wide variety of substrates and does not form lyso-compounds as final hydrolytic products [42]. When added exogenously, these lyso-compounds are known to be deleterious to the thylakoid membrane structure and function [46,47]. Contrary to most authors [22,24,25,27–30] we have chosen a rather low concentration of enzyme (40 μg per mg chlorophyll) in order to analyse in more details the different phases of the hydrolytic process. This is obvious in Fig. 1B and 5A where a small amount of lipolytic acyl hydrolase made it possible to show that lipid hydrolysis occurred stepwise.

These different steps can be interpreted as due to the heterogeneity of the distribution of the lipid as well as to the accessibility of the enzyme at the surface of the membrane. In addition to surface charges, this accessibility depends not only on the size of the enzyme molecule but also on the relief of the membrane surface. From this one can infer that in thylakoid membranes monogalactosyldiglyceride then digalactosyldiglyceride are the first lipids to be attacked by lipolytic acyl hydrolase and then, due to reorganization of the membrane components, this enzyme has access to ionic lipids. The fact that anionic lipids are hydrolysed before galactolipids and phosphatidylcholine in subchloroplast particles I (Fig. 5A) indicates that the molecular organization of the components of this particle is quite different from that of thylakoid membranes (Fig. 1B). This compartmentation concept has already been suggested by Costes et al. [48, 49] who used mild extraction of lipids from freeze-dried thylakoid membranes with organic solvents.

Our results show quite clearly that in essence there is no quantitative correlation between the extent of lipid hydrolysis and decrease in photochemical activities. In thylakoid membranes, complete inactivation of non-cyclic electron flow was observed (Fig. 2A and B) whilst only 25–40% of each lipid were destroyed (Fig. 1D). Similar conclusions can be drawn from the activity of each photosystem measured separately (Fig. 2C and D). In subchloroplast particles I the situation is inverted: complete hydrolysis of lipids still corresponded to good rates of electron transport (Fig. 5). In subchloroplast particles II, the photochemical activities diminished although few lipids were hydrolysed. The observed effects of lipolytic acyl hydrolase on the electron transport activities in thylakoid membranes are in good agreement with those reported by Hirayama and Matsui [30]. However, our results do not support the conclusions of these authors that there is a correlation between degradation of galactolipids, particularly monogalactosyldiglyceride and decay in photosystem I activity. This discrepancy may be due to the fact that they have measured the level of lipids at only one time. This would imply that the rate of lipid hydrolysis is linear during the whole process which is obviously not the case (Fig. 1B and 5A). Shaw et al. [31], using a bean-leaf galactolipid lipase, reported that this enzyme caused a marked inhibition (50% to 60%) of electron flow through both photosystems, a less pronounced inhibition (30% to 40%) of O_2 evolution and no alteration in photosystem I activities. These changes were accompanied by a 50% to 60% drop in the lipid content of the subchloroplast particles. These results are not in agreement with ours. This may be due to differences in the specificity of the bean-leaf galactolipid lipase and of lipolytic acyl hydrolase, as well as in the type of membranes used as substrates. Furthermore, the claim that 50% to 60% of the acyl lipid of chloroplast membranes may be removed without affecting the rate of electron flow through photosystems I or II seems, in our opinion, questionable on the basis of the results presented by these authors [31]. However, such a conclusion is unequivocally shown for the electron flow activity through photosystem II in thylakoid membranes (Fig. 1 and 2) and for photosystem I electron flow in subchloroplast particles I (Fig. 5).

In addition to the absence of quantitative correlation between lipid hydrolysis and activity decay, there is no temporal correlation between the different phases of these two processes (compare Fig. 1–3). However, the treatment of thylakoid membranes by lipolytic acyl hydrolase brings about a temporal convergence of different events (Fig. 3 and Table 1). This convergence suggests that the lipolytic acyl hydrolase treatment eventually alters a critical locus, i.e. the lipidic environment of an electron carrier (plastocyanin?) and/or of the reaction center of photosystem I, which sustains all the above activities. However, by no means can this convergence be correlated with a significant change in the lipid composition of thylakoid membranes.

Two action sites of lipolytic acyl hydrolase on the photosynthetic electron transport (between plastoquinones and P700) are revealed by the behaviour of the activities of the different portions of the chain during the incubation of thylakoid membranes (Fig. 2 and 3). The first, most sensitive site, seems to be localized before the entry of electrons from Cl_2Ind, whereas the second, after the entry of Cl_2Ind (see Fig. 2). This is in general agreement with the results reported by Hirayama and Matsui [30]. It is noteworthy that the extreme portions of the photosynthetic chain, i.e. from H_2O to plastoquinone and from the primary acceptor of photosystem I to $NADP^+$, are not significantly affected by the lipolytic acyl hydrolase. In contrast, this enzyme is likely to bring about a change in the lipidic environment of plastocyanin (site II) which might induce a delocalization of this electron carrier in the thylakoid membrane resulting in a loss of activity. These membranes become more and more dependent on exogenous plastocyanin for full activity (Fig. 4). When photosystem I activity was inhibited by other treatment, plastocyanin was also found to be an efficient restoration agent [17]. This interpretation is further supported by the fact that ascorbate alone, which is generally not an electron donor for photosystem I in thylakoid membranes, mediates $NADP^+$ photoreduction for a short period of time (Fig. 3).

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FUNCTION AND DISTRIBUTION OF PHOSPHOLIPIDS IN SPINACH THYLAKOID MEMBRANES AS REVEALED BY PHOSPHOLIPASE A_2 TREATMENT

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INTRODUCTION

Lipolytic treatments have been widely used to ascribe a possible role to lipids in sustaining a particular activity within the thylakoid membrane. Using a non specific potato acyl hydrolase, we have shown that there was no direct correlation between the extent and rates of lipid hydrolysis and the alteration of photochemical activities[1]. In this investigation we study the effects of pancreatic PLA_2 on the extent of phospholipid hydrolysis and various electron transport activities. Furthermore, from the hydrolytic patterns at different temperatures, an asymmetric transmembrane distribution of PG and PC is proposed.

MATERIAL AND METHODS

Isolation of CP and measurements of electron transport activities are described elsewhere[2]. The incubation of CP with PLA_2 was carried out in darkness in 50 mM Tricine (pH 8), 35 mM NaCl, 1 mM $CaCl_2$, CP (0.5 mg chlorophyll/ml) and PLA_2 (1,6 sigma unit/mg chlorophyll). Aliquots were taken at various times for the determination of photochemical activities[2] and for lipid analysis[3].

RESULTS AND DISCUSSION

Fig. 1 shows the effect of PLA_2 at 20°C on different electron flow activities in CP. The activities involving PSII+I (Fig. 1A and B) decreased rapidly and were completely inhibited after 60 to 80 min. In contrast, PLA_2 brought about a stimulation of PSI activity which remained higher than the control (Fig. 1C). A greater activation was found when MV was used as the electron acceptor

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Abbreviations : CP, thylakoid membrane; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl p-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DCPIP, 2,6-dichlorophenol-indophenol; DPC, 1,5-diphenylcarbazide; MV, methylviologen; PC, phosphatidylcholine; PG, phosphatidylglycerol; PLA_2, pancreatic phospholipase A_2; PS, photosystem.

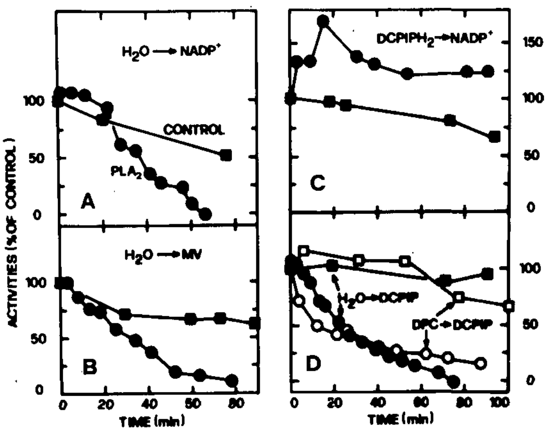

Fig. 1. Action of PLA_2 on CP followed by the variations of electron transport activities as a function of time: electron flows from H_2O to $NADP^+$ and from H_2O to MV (PSII + PSI), from $DCPIPH_2$ to $NADP^+$ in the presence of DCMU (PSI) and from H_2O or DPC to DCPIP in the presence of DBMIB (PSII). Incubation with PLA_2 was at 20°C.

(not shown). This stimulation is only partially due to uncoupling since the long-term activation was also observed in uncoupled CP. The inhibition of electron flow through both photosystems (Fig. 1A, B) could be ascribed to the decrease in PSII activity as shown in Fig. 1D. Comparing the DPC- and H_2O-supported PSII activities it seems unlikely that the water-splitting system is inactivated by the PLA_2 treatment. Thus, these results suggest the existence of at least two action sites of PLA_2. The first, leading to an inhibitory effect, is located between the electron entry point from DPC and the plastoquinones. The second site, leading to a stimulatory effect, is located between the electron entry point from $DCPIPH_2$ and the primary acceptor of PSI.

At 20°C, the hydrolysis of PG and PC displayed two phases (Fig. 2). The extent and the rate of the first, fast hydrolytic step were greater for PG than for PC. The difference in rates reflects, at least in part, the preference of PLA_2 for anionic lipids whilst the difference in the extent of the first hydrolytic step suggests that a greater amount of PG than PC is easily accessible to the enzyme. The rates of the second phase were much slower and the hydrolysis was eventually complete for both lipids. The shapes of the hydrolysis curve of PC at 20°C (Fig. 2) and of the decrease of PSII activity (Fig. 1D) together with other evidence (unpublished data) seem to indicate that most of PC is involved in PSII activity. Surprisingly, a huge increase in PSI activity (even in uncoupled CP) corresponded to a great decrease in PG level.

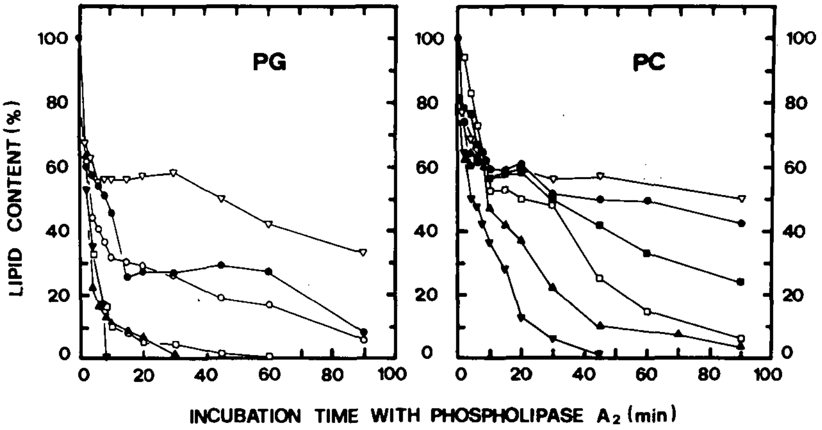

Fig. 2. Time course of phospholipid hydrolysis at various temperatures in PLA_2-treated CP. ▽(0°C), ●(5°C), ○(10°C), ■(15°C), □(20°C), ▲(25°C), ▼(35°C).

The difference between the extents of PG and PC hydrolysis at 20°C as well as the biphasic nature of the hydrolytic process may indicate an asymmetric distribution of these lipids in the thylakoid membrane. Such a possibility has been substantiated by investigating the effect of various temperatures on the hydrolysis. Fig. 2 shows that the hydrolytic patterns of PG and PC were different and that the second phase was strongly temperature-dependent. An increase in temperature tended to suppress the second phase in PG hydrolysis whereas both phases were still present in PC.

In order to draw conclusions on the asymmetric distribution of the phospholipids in the membrane we have checked in a series of control experiments (according to[4]) that lysis of the membrane did not occur during the treatment by PLA_2 (morphological and functional evidences, not shown) and that when the two sides of the membrane are exposed to the enzyme all the phospholipids were hydrolyzed (membranes sonicated in the presence of PLA_2 and subsequent addition of Ca^{++} to initiate the reaction, not shown). However, the hydrolytic process did modify the distribution of the two lipids which experienced an important "flip-flop", as suggested by the temperature dependency of the second phase (Fig. 2). As a consequence, an unequivocal conclusion cannot be drawn from this enzymatic approach. However, a sensible estimation of the actual phospholipid distribution in the membrane is given by computing the fast reacting pool of

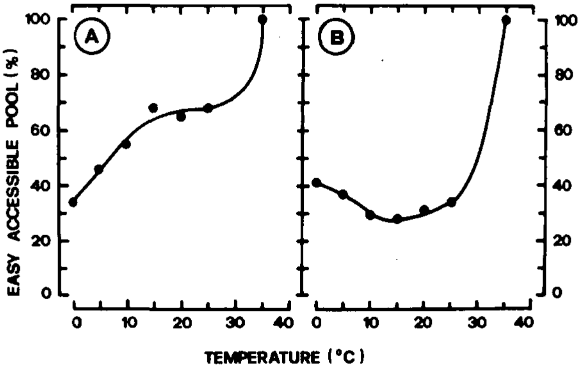

Fig. 3. Effect of temperature on the easy accessible pools of PG (A) and of PC (B) in PLA_2-treated CP. The estimation of the easy accessible pools was obtained as described previously[5].

lipids as shown in Fig. 3. The easy accessible pool of PG, which was 35% at 0°C, increased with temperature to reach a plateau between 15 and 25°C. On the other hand, the corresponding pool of PC remained essentially constant within 30 to 40% of the total PC between 0 and 25°C. Above 25°C, the accessibility of both lipids became total.

From these results, we conclude that around 70% of PG and 35% of PC could be localized in the outer layer of the thylakoid membrane. The above results favor the idea that the well-known functional asymmetry of the thylakoid membrane could be underlined at least partly by a phospholipid asymmetric distribution.

ACKNOWLEDGEMENTS

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Photosynthesis II. Electron Transport and Photophosphorylation
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A NEW TYPE OF CORRELATION BETWEEN CHANGES IN LIPID COMPOSITION AND LOSS OF ELECTRON TRANSPORT ACTIVITIES DURING AGING IN VITRO

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ABSTRACT

Purified and non-purified thylakoids from spinach and lettuce were aged in vitro (darkness, 20°C, pH 7.6) and the variations in their lipid composition and photochemical activities were followed. The purification of both thylakoid species affected greatly the changes in their lipid patterns. In contrast, it did not alter significantly the degree of inhibition of their electron transport activities during aging. The results suggest that most of the lipid modifications were not the actual cause of loss of electron transport activities. The occurrence of a weak endogenous lipolytic activity in thylakoids is responsible for the release of a small amount of free fatty acids arising from "essential" lipids; this appears to be one of the factors which lead to the impairment of the membrane functions in the aging process. The results are discussed in terms of the localization of this lipolytic activity in the membrane and of its time-dependent effects on the photochemical activities.

INTRODUCTION

It has been shown that aging in vitro of non-purified spinach thylakoids is accompanied by an increase in free fatty acids and lyso-derivatives (1,2). This accumulation coincides with a decrease in their photochemical activities (1,3,4). Since external additions of free

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Abbreviations : AMG, acylmonogalactosyldiglyceride; DG, digalactosyldiglyceride; MG, monogalactosyldiglyceride; LAMG, lyso-acylmonogalactosyldiglyceride; LDG, lyso-digalactosyldiglyceride; LMG, lyso-monogalactosyldiglyceride; LPC, lyso-phosphatidylcholine; PC, phosphatidylcholine; PG, phosphatidylglycerol; SL, sulfoquinovosyldiglyceride; any type of fatty acid is described by number of carbon atoms : number of double bonds.

fatty acids (e.g. $C_{18:3}$) to freshly isolated thylakoids mimic the effects of aging in vitro (1,4,5) it was postulated that the endogenous levels of free fatty acids control the structural and physiological state of the thylakoids (6,7). However, it remains to be established if loss of electron transport activities is a direct consequence of lipid hydrolysis and interconversions, as recently challenged (8,9). For instance, it was pointed out that the relative increases in endogenous free fatty acids reported to occur during aging (1-9) are often disproportionately small with respect to the observed functional changes (9,10). A similar situation was found in thylakoids treated by various lipolytic enzymes in that no correlation, neither quantitative nor temporal, was established between lipid hydrolysis and the alteration of photochemical activities (11). It can be asked if all free fatty acid molecules released during aging are effectively involved in the loss of activities. It should be emphasized that in the above studies (1-9), the thylakoids used were non-purified and therefore contained several contaminants (12) which could affect the aging of the membrane itself, e.g. enzymes involved in lipid hydrolysis or interconversions. The question was therefore whether a correlation between the presence of free fatty acids in the membrane and the loss of electron flow activities still holds in absence of extraneous contaminants.

To reduce contaminating lipolytic enzymes, experiments were performed with highly purified thylakoids from spinach and lettuce and the results were compared to those obtained with non-purified thylakoids. Lettuce is often used during the summer time to replace spinach. Moreover, the lipid composition of lettuce and spinach thylakoid membranes present interesting differences.

MATERIALS AND METHODS

Spinacia oleracea L. and Lactuca sativa var. "Cos" were obtained from the local market. Chloroplast isolation and preparation of non-purified and highly purified thylakoids were carried out as described in (12). Electron flow activities were measured as in (11). Extraction of chloroplast lipids, thin layer chromatographic analyses as well as quantitative analyses of the individual lipids were carried out as in (13).

TABLE 1 : ACYL LIPID COMPOSITION OF LETTUCE AND SPINACH THYLAKOIDS

Lipid class	umoles/µmole chlorophyll lettuce	 spinach	% Lettuce	 Spinach
MG	1.139	1.431	49.5	55.0
DG	0.930	0.696	40.4	26.8
PG	0.151	0.195	6.5	7.5
PC	0.061	0.150	2.6	5.7
SL	0.021	0.116	0.9	4.5
Total	2.302	2.588	100	100

Thylakoids from both species were incubated in the dark at 20°C (pH 7.6); at intervals aliquots were used for electron transport measurements and lipid analyses.

RESULTS AND DISCUSSION

As shown previously (12), the contaminations of the mitochondria (estimated by the cytochrome c oxidase), stroma ($NADP^+$ glyceraldehyde 3-phosphate dehydrogenase) and peroxisomes (glycollate oxidase) were reduced by a factor of 10, 26, >100, respectively, by the purification procedure. It was also confirmed that the lipid composition of purified thylakoids was similar to that of freshly isolated non-purified thylakoids (12).

Table 1 shows the major lipid classes of both spinach and lettuce thylakoids. Significant differences occurred between the two species. In lettuce thylakoids there was a higher amount of DG together with lower amounts of PC and SL than in spinach thylakoids. The content of MG was roughly similar in both species. The ratio MG/DG was 2.0 for spinach and 1.2 for lettuce. Table II shows the fatty acid composition of the different lipid classes of both species. Surprisingly, in lettuce there was an absence of $C_{16:3}$ which is the second most abundant fatty acid after $C_{18:3}$ in spinach. Moreover there was a higher total content of $C_{18:3}$ and lower total content of $C_{16:0}$.

In attempting to relate changes in lipid composition with the impairment of electron transport activities we explored how the purity

TABLE 2 : FATTY ACID COMPOSITION OF LETTUCE (L) AND SPINACH (S) THYLAKOIDS

LIPID TYPE		µMOLES % OF FATTY ACIDS								
		16:0	16:1(3T)	16:1	16:2	16:3	18:0	18:1	18:2	18:3
MG	L	0.8	-	0.2	TR	-	TR	TR	1.0	96.0
	S	0.7	-	0.4	0.9	21.5	0.1	1.3	1.8	73.3
DG	L	5.2	-	0.3	-	-	0.8	0.5	1.3	91.8
	S	7.3	-	0.4	0.1	3.0	0.9	3.0	3.9	81.4
PG	L	17.3	35.9	-	-	-	0.6	1.3	4.3	40.5
	S	18.7	32.4	-	-	-	1.5	1.5	5.9	40.0
PC	L	22.1	-	2.5	1.6	-	6.6	3.3	24.6	39.3
	S	16.1	-	0.7	1.0	1.8	2.8	7.6	15.0	55.0
SL	L	30.9	-	1.6	1.1	-	7.7	6.6	5.0	47.0
	S	37.5	-	0.4	-	2.2	1.7	1.7	7.8	48.7
TOTAL	L	4.5	2.3	0.3	TR	-	0.6	0.4	2.0	89.7
	S	6.1	2.5	0.4	0.6	13.3	0.8	1.9	3.1	71.3

of the membrane could influence the lipolytic activities. Fig. 1 shows thin-layer chromatographic separations of the various lipid classes of both species as a function of aging <u>in vitro</u>. As expected, in non-purified spinach thylakoids there was a strong increase in the level of endogenous free fatty acids. In non-purified lettuce thylakoids, the same phenomenon occurred but to a lesser extent. Moreover, significant galactolipid interconversions were observed in spinach as evidenced by increasing levels of AMG, LAMG and LDG. In contrast these interconversions were not observed in lettuce.

The purification of thylakoids had important consequences on the evolution of lipid pattern during aging in vitro. Fig. 1 shows that in contrast to non-purified thylakoids, the release of free fatty acids was considerably reduced in both spinach and lettuce purified thylakoids; nevertheless, the free fatty acid band was always above the level detectable by thin-layer chromatography. In addition, there was a significant decrease in the extent of lipid interconversions in spinach. Again no AMG and lyso-galactolipids were encountered in lettuce. However, a weak band corresponding to LPC appeared in the chromatograms of purified lettuce thylakoids.

These data suggest that most of the lipolytic activities encountered in non-purified thylakoids is attributable to extraneous enzymes which

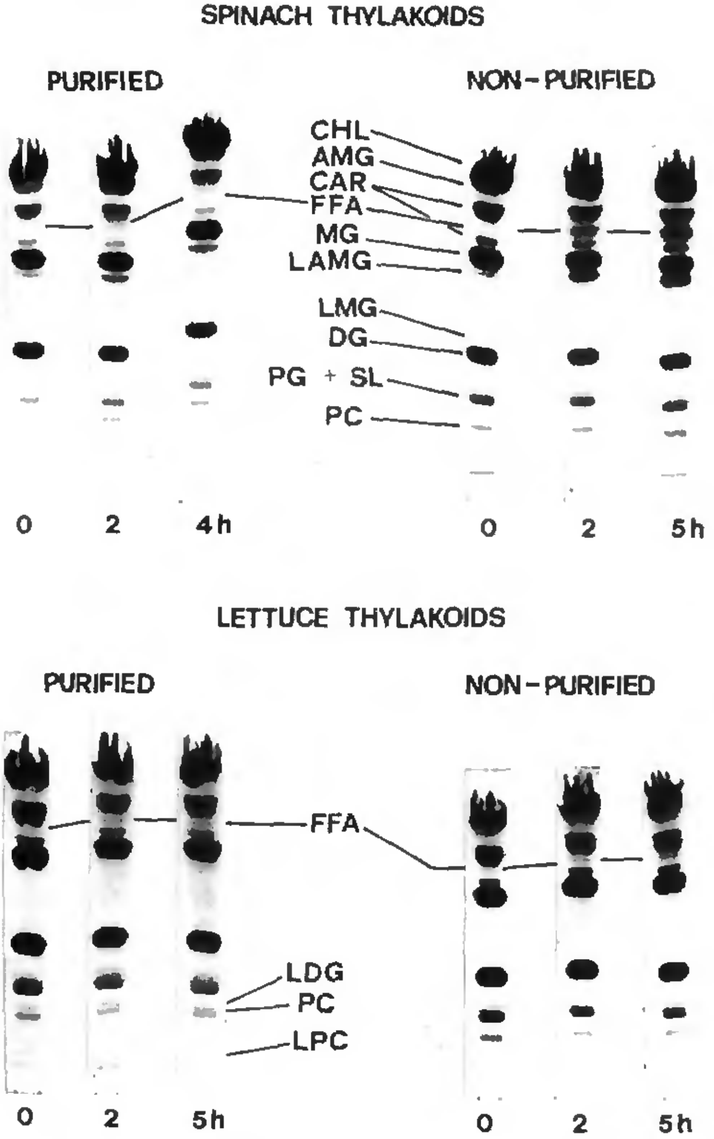

Fig. 1. Lipid composition of different thylakoid preparations as a function of incubation time. CHL, chlorophylls; CAR, carotenoids; FFA, free fatty acids.

are easily removable by the purification procedure. In contrast, only part of the galactolipid interconversion activities in spinach thylakoids are removed by the purification technique. This suggests that some of these enzymes responsible for these transformations are integral membrane components or remain firmly adsorbed to the thylakoid membrane despite the purification procedure.

TABLE 3 : EFFECT OF THE PURIFICATION OF SPINACH AND LETTUCE THYLAKOIDS ON $H_2O/NADP^+$ (PHOTOSYSTEM II + I) AND H_2O/DCPIP (PHOTOSYSTEM II) ELECTRON TRANSPORT ACTIVITIES

The results are expressed as % of the zero-time controls. Due to variations in the aged thylakoid preparations, the range of the activities are given. For measurements of the activities thylakoids were uncoupled with 2 mM NH_4Cl and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) was added in photosystem II reaction mixture. The 100% values varied between 220-350 µmoles $NADP^+$ reduced · mg chlorophyll^{-1} · h^{-1} and between 110-220 µmoles 2,6-dichlorophenolindophenol reduced (DCPIP) · mg chlorophyll^{-1} · h^{-1} for both spinach and lettuce thylakoids.

Species	Thylakoids	Aging time (h)	$H_2O/NADP^+$	H_2O/DCPIP
Spinach	Non-purified	0	100	100
		4-5	0-13	0-15
	Purified	0	100	100
		4-5	23-43	30-50
Lettuce	Non-purified	0	100	100
		4-5	13-33	10-30
	Purified	0	100	100
		4-5	27-47	20-40

Table 3 shows the effect of the purification of spinach and lettuce thylakoids on the electron transport activities as a function of aging *in vitro*. Upon aging, all activities diminished in all four thylakoid preparations. As shown previously (1,4), the decrease in the $H_2O/NADP^+$ system is mainly attributable to the inhibition of photosystem II activity, since photosystem I activity was much more resistant (not shown). The purification of spinach thylakoids resulted in a greater resistance of the photosystem II-mediated reactions. Nevertheless, this purification could not prevent the occurrence of a marked inhibition of the activities. In contrast, the efficiency of the purification procedure was rather small in lettuce thylakoids.

These results show that in all types of thylakoids the effect of aging on the electron transport activities did not differ significantly, However, if the correlation invoked previously (1-9) would have been correct, one would have expected, on the basis of the lipid patterns during aging, important differences in the activities between the four thylakoid preparations. It appears that the loss of photochemical activities does not depend on the presence of galactolipid transformation products in the membrane since similar inhibitions were observed despite large differences in the levels of these products (Fig. 1 and Table 3). On the other hand, the free fatty acids released from the membrane lipids are the unique compounds formed during aging which appear in all thylakoid preparations, the only difference being their relative amount. On the basis of our results, there is obviously no quantitative correlation between the total amount of free fatty acids present in a membrane and the degree of inhibition of its activities. However, our results can be explained if one assumes that in non-purified thylakoids most of the free fatty acids released on the external side of the membrane by contaminating lipolytic enzymes are not involved in the inhibitory mechanism of the activities. It is thus suggested that only that small amount of free fatty acids released in the purified membranes by an intrinsic lipase(s) is actually responsible for the observed inhibitions.

Concerning the nature of the correlation between lipid modifications and alterations of photochemical activities, we believe that the dose-response concept which is applicable only when membranes are treated by exogenous fatty acids, cannot explain the effects of aging _in vitro_, and therefore should be considered with caution. We suggest that the searched correlation should be considered in terms of the identity and localization of the lipid(s) hydrolyzed during aging and in terms of the (amount of free fatty acids) x (aging time) parameter.

In conclusion, the main part of the free fatty acids released during aging _in vitro_ of non-purified thylakoids is due to the activity of contaminating lipases which attack in a non-specific manner the lipids localized in the outer layer of the membrane. These fatty acids are relatively inefficient inhibitors since they cause only 30-33% decrease in the photochemical activities. In contrast, the small amount of free fatty acids released in purified thylakoids can be related to the major part of the inhibition. This is so because these fatty acids probably arise from lipids which are essential in sustaining photosystem II electron flow activity. These lipids would be localized in the inner layer of the thylakoid membrane as suggested previously (4) and evidenced in (14). However, it should be emphasized that the aging process is very complex and involves not only the lipid moiety of the membrane but also the protein portion, namely the chlorophyll-protein complexes (12).

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Photosynthesis III. Structure and Molecular Organisation of the Photosynthetic Apparatus
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TRANSMEMBRANE DISTRIBUTION OF PHOSPHOLIPIDS AND THEIR INVOLVMENT IN PHOTOSYNTHETIC ELECTRON TRANSPORT ACTIVITIES

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ABSTRACT

Thylakoid membranes were treated with pancreatic or snake venom phospholipase A_2 (PLA_2), and the residual phospholipid content of these membranes was determined in relation to photosystem II and/or photosystem I electron transport activities.

The hydrolysis patterns of both phosphatidylglycerol (PG) and phosphatidylcholine (PC) under different conditions and at various temperatures suggest that these lipids are asymmetrically distributed across the thylakoid membrane. In addition, a quantitative estimation of the external and internal pools of phosphatidylglycerol and phosphatidylcholine is given.

When acting on uncoupled thylakoid membranes, PLA_2 exerts an inhibitory effect on photosystem II activity and a stimulatory effect on photosystem I activity. The involvment of phosphatidylglycerol and phosphatidylcholine as well as the role of their respective external and internal pools in electron transport activities of photosystem II and photosystem I is discussed.

INTRODUCTION

The vectorial function of the thylakoid membrane lies so far essentially on the sidedness of the proteins involved in the electron transport (1). The question is whether the photosynthetic membrane is also asymmetric with regard to lipids. One of the approaches to answer this question is to treat membranes with lipolytic enzymes and determine the hydrolytic kinetics of lipids under different conditions (temperature, etc.). Moreover, this lipolytic approach can give information on the involvment of lipids in the electron transport activities.

Using a non specific potato acyl hydrolase, we have previously found that there was no direct correlation between the extent and rates of lipid hydrolysis and the alteration of photochemical activities (2). With such a non specific enzyme it is inevitable to destroy lipids which do not directly support the measured activity. It is therefore desirable to use more specific enzymes such as phospholipases (e.g. phospholipase A_2) which permit to diminish the degree of uncertainty on the lipid class related to a given function. Phospholipases have already been used by others (3,4) but in these studies no attempt was made to correlate alterations of photochemical activities with the structural organization of the thylakoid membrane lipids. The aim of this investigation is to determine the role of external and internal phospholipids in sustaining electron transport activities in thylakoid membranes.

MATERIAL AND METHODS

Spinacia oleracea (var. Nobel) was grown in a growth chamber and the leaves were harvested after 14 weeks of hydroponic culture (5). The isolation of thylakoid membrane was carried out as described in (2). The incubation of thylakoid membranes (0.5 mg Chl/ml) with PLA_2* was carried out in darkness in 50 mM N-tris-(hydroxymethyl)-methylglycine (pH 8), 80 mM NaCl, 1 mM $CaCl_2$ and PLA_2 either from pig pancreas (1.6 sigma unit/mg Chl) or from Vipera russelli venom (0.11 sigma unit/mg Chl). Aliquots of the above incubation media were taken at various times for the determination of electron transport activities (2) and lipid analysis (6). All the results were expressed in % of the initial values. a) The amount of PG and PC were 0.195 and 0.150 µmoles lipid/µmole Chl; b) The electron flow activities were around 140 µmoles $NADPH_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$ ($H_2O/NADP^+$), 75 µmoles O_2 consumed $\cdot$ mg $Chl^{-1} \cdot h^{-1}$ (H_2O/methylviologen); 57 µmoles $NADPH_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$ (ascorbate-DCPIP/$NADP^+$); 128 and 159 µmoles $DCPIPH_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$ when H_2O and 1,5-diphenylcarbazide were the electron donors, respectively.

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*Abbreviations : PLA_2, phospholipase A_2; PS, photosystem; PC, phosphatidylcholine; PG, phosphatidylglycerol; DCPIP, 2,6-dichlorophenol indophenol; Chl, chlorophyll.

RESULTS AND DISCUSSION

When thylakoid membranes were treated with PLA_2, the hydrolysis of PG and PC displayed two phases (Fig. 1). The first was almost temperature-independent whilst the second was strongly dependent on this factor. The extent and rate of the first, fast hydrolytic step were greater for PG than for PC. The difference in rates reflects, at least in part, the preference of the pancreatic PLA_2 for anionic lipids whilst the difference in the extents of the first hydrolytic step suggests that a greater amount of PG than of PC is easily accessible to the enzyme. The rates of the second phase were much slower and above 15°C the hydrolysis was eventually complete for both lipids. These results may indicate an asymmetric distribution of the phospholipids in the thylakoid membrane.

In order this to be true, at least three criteria should be satisfied in control experiments (8). Firstly, no lysis of the thylakoids should occur when the enzyme attacks the membrane from the outside. This is indirectly established by the fact that a proton gradient was still maintained across the membrane when the first hydrolytic step was achieved (not shown). Secondly, all lipids should be hydrolysed when the enzyme attacks the membrane from both sides. Fig. 2 shows that when PLA_2 attacks the membrane from the outside only, e.g. in normal (incubated

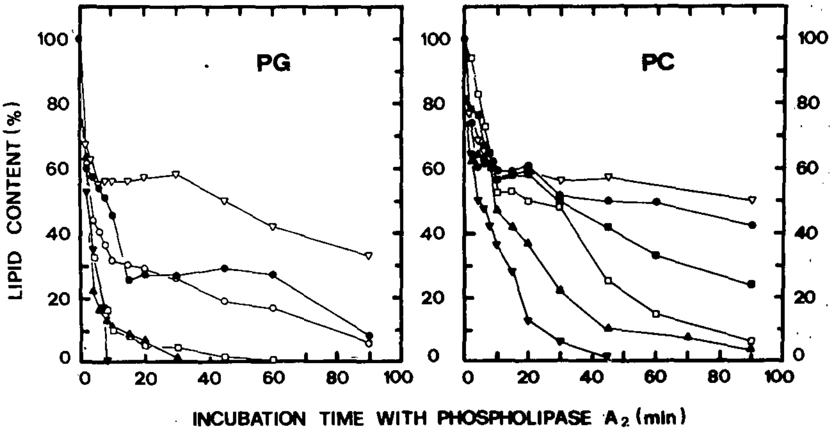

Fig. 1. Time course of phospholipid hydrolysis at various temperatures in thylakoid membranes treated with pancreatic PLA_2. ▽ (0°C), ● (5°C), ○ (10°C), ■ (15°C), □ (20°C), ▲ (25°C), ▼ (35°C).

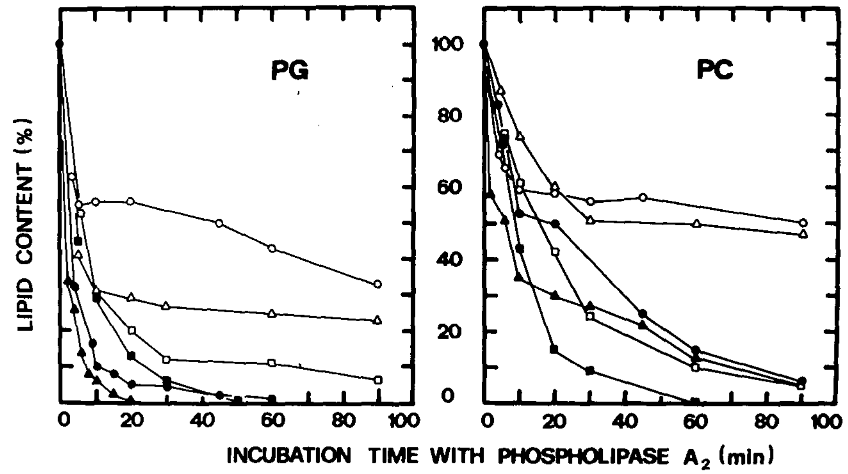

Fig. 2. Time course of phospholipid hydrolysis at two temperatures in normal, swollen and disrupted thylakoid membranes treated with pancreatic PLA_2. Open symbols : 0°C; closed symbols, 20°C. Normal (○,●) and swollen (△,▲) thylakoid membranes. Disrupted thylakoid membranes (□,■) were obtained by sonication for 3 min in an incubation mixture containing PLA_2, but no NaCl and $CaCl_2$; Ca^{2+} was added after the sonication process to initiate the hydrolytic reaction.

with NaCl) and swollen thylakoids (incubated without NaCl) the hydrolysis of both phospholipids at 0°C was partial. At this temperature the flip-flop mechanism is negligible. On the other hand, when PLA_2 had access to both sides, e.g. in disrupted thylakoids (Fig. 2), the hydrolysis of PG and PC was almost complete. This indicates that some of the lipids are located in the inner layer of the membranes. Thirdly, an unequivocal distribution of lipids in the membrane can be obtained only if the lipolytic treatment does not modify the actual distribution of the lipids. This implies that no flip-flop should occur during the hydrolytic process. Although this was the case at low temperatures, the flip-flop mechanism increased when the temperature was raised (see second phase in Figs 1 and 2). Consequently, the best estimation of the actual phospholipid distribution in the membrane is given by computing the fast-reacting pool of lipids as shown in Fig. 3. The easy accessible pool of PG, which was 35% at 0°C, increased with temperature to reach a plateau between 15 and 25°C. On the other hand, the corresponding pool of PC remained essentially constant within 30 to 40% of the total PC between 0 and 25°C. Above 25°C, the accessibility of both lipids

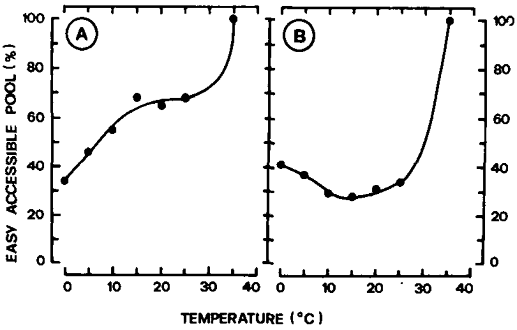

Fig. 3. Effect of temperature on the easy accessible pools of PG and PC in thylakoid membranes treated by pancreatic PLA_2. The estimation of these pools was obtained as described in (7).

became total. We conclude that around 70% of PG and 35% of PC could be localized in the outer layer of the thylakoid membrane.

The effect of pancreatic PLA_2 at 20°C on different electron flow activities of thylakoid membranes is shown in Fig. 4. The activities involving PSII + I (Fig. 4A and B) decreased rapidly and were completely inhibited after 60 to 80 min. In contrast, PLA_2 brought about a stimulation of PSI activity which remained higher than the control (Fig. 4C). A greater activation was found when methylviologen was used as the electron acceptor (not shown). This stimulation was only partially due to uncoupling since the long-term activation was also observed in uncoupled thylakoid membranes. The inhibition of electron flow through both photosystems (Fig. 4A,B) could be ascribed to the decrease in PSII activity as shown in Fig. 4D. Comparing the 1,5-diphenylcarbazide (DPC)- and H_2O-supported PSII activities it seems unlikely that the water-splitting system is inactivated by the PLA_2 treatment. Thus, these results suggest the existence of at least two action sites of PLA_2. The first, leading to an inhibitory effect, is located between the electron entry point from DPC and the plastoquinones. The second site, leading to a stimulatory effect, is located between the electron entry point from $DCPIPH_2$ and the primary acceptor of PSI. The shapes of the hydrolysis curve of PC at 20°C (Fig. 1) and of the decrease of PSII activity (Fig. 4D) seem to indicate a possible involvment of PC in PSII activity. Surprisingly, the huge increase in PSI activity (even in uncoupled thylakoid membranes) corresponded to a great decrease in PG level (see also Fig. 6).

The comparison between the action of PLA_2 from pancreas and snake venom (which have inverse preference toward PC and PG) supports the

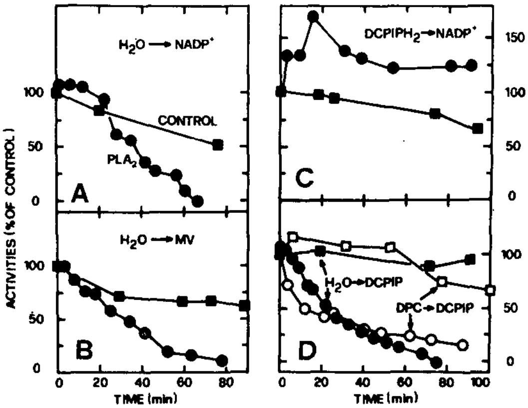

Fig. 4. Action of pancreatic PLA2 on thylakoid membranes followed by the variations of electron transport activities as a function of incubation time. Electron flows were : from H_2O to $NADP^+$ and from H_2O to methylviologen (MV) (PSII + PSI), from $DCPIPH_2$ to $NADP^+$ in the presence of DCMU (PSI) and from H_2O or 1,5-diphenylcarbazide (DPC) to DCPIP in the presence of 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (PSII).

hypothesis that PC is the main phospholipid involved in PSII electron flow activity (Fig. 5). When the concentrations of both enzymes were chosen so as to obtain the same hydrolysis rate of PG, the extent and rate of the hydrolysis of PC by the pancreatic PLA_2 were smaller than that by the snake venom enzyme (Fig. 5B). This difference in the hydrolytic patterns of PC can be related to the inhibition of PSII electron flow activity which was smaller with pancreatic PLA_2 than with the snake venom enzyme (Fig. 5A).

The involvment of PC in PSII-mediated activity is further substantiated by the comparison, at different temperatures, of the action of pancreatic PLA_2 on the phospholipid content and on PSII electron transport activities in uncoupled thylakoid membranes. When the external pool of PC was hydrolysed (for instance up to 10 min of PLA_2 treatment at 5°C and 20°C, Fig. 6a and b) no change in PSII activity was observed. However, the hydrolysis of the internal pool of PC under the conditions where the flip-flop mechanism occurs (for instance at 35°C and after 10 min of PLA_2 treatment at 20°C, Fig. 6 b and c) was directly related to the inhibition of the PSII activity. On the other hand, when no flip-flop occurred (for instance after 10 min at 5°C) there was no inhibition of PSII activity. In addition, Fig. 6 shows that the hydrolysis of the external pool of PG was associated to an increase in PSI activity, which was markedly stimulated when the internal pool of PG was further hydrolysed, due to the flip-flop mechanism. It is noteworthy that the great stimulation of PSI activity is not due to uncoupling. This unexpected observation suggests that PG plays a

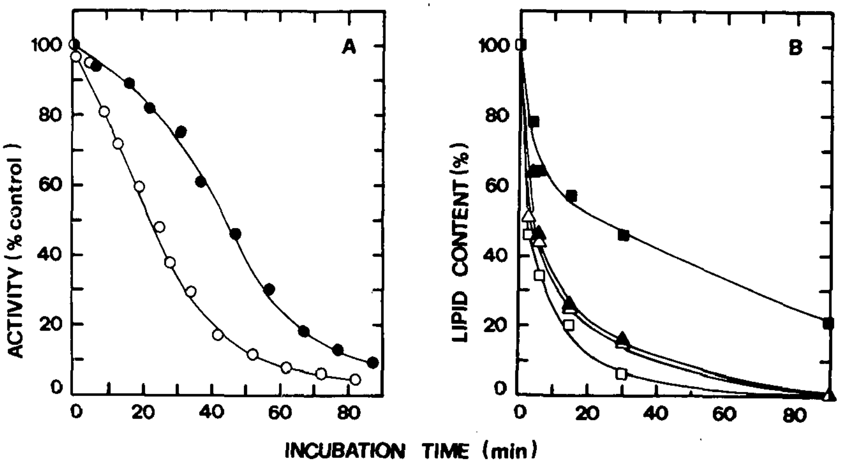

Fig. 5. Comparison of the action of pancreatic and *Vipera russelli* venom PLA_2 on PSII electron flow activity (A) and on the hydrolysis of phospholipids (B) as a function of incubation time. Closed symbols : pancreatic PLA_2; open symbols : snake venom PLA_2. A, PSII electron flow H_2O/DCPIP (+ DBMIB); B, phospholipid content; ▲, △ : PG; ■, □ : PC.

privileged role in the function of PSI. These results point out the great importance of the internal pool of PC to sustain the PSII electron flow activity and a complex relationship between both external and

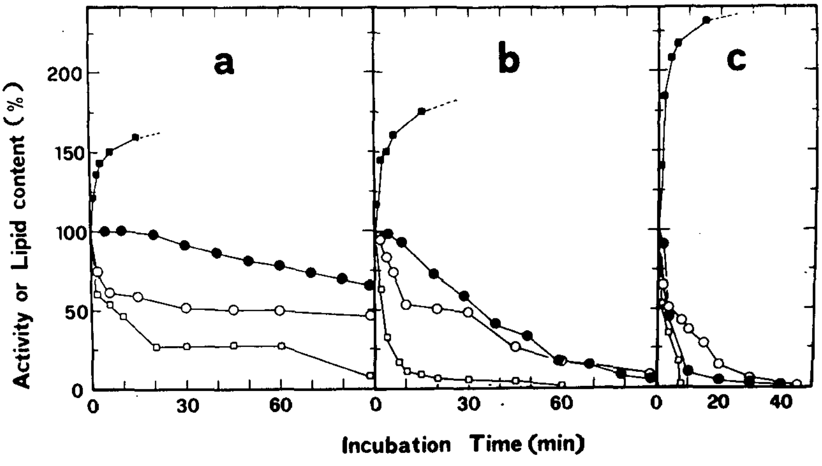

Fig. 6. Temperature dependence of the action of pancreatic PLA_2 on the phospholipid content and on PSII and PSI electron transport activities of uncoupled thylakoid membranes. a, 5°C; b, 20°C; c, 35°C. ●, PSII electron flow, H_2O/DCPIP; ■, PSI electron flow, $DCPIPH_2/NADP^+$; ○, PC; □, PG. Uncoupling and blocking of hydrolysis were achieved by addition of 2 mM EDTA in the reaction media.

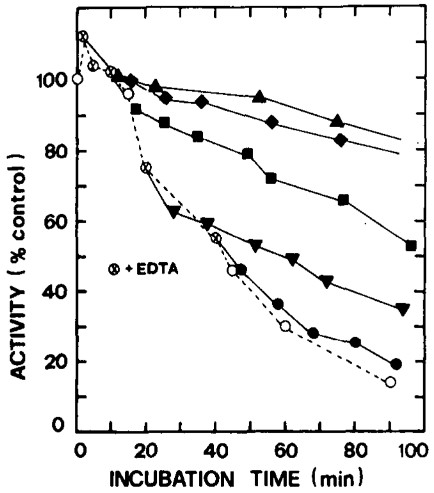

Fig. 7. Effect of various incubation times on the PSII-catalysed photoreduction of DCPIP by water in thylakoid membranes treated with pancreatic PLA_2. Hydrolysis is blocked by addition of 2 mM EDTA (⊗) after 2 (▲), 5 (◆), 10 (■), 20 (▼) and 40 minutes (●) of treatment. Non-interrupted treatment with PLA_2 (○ , ⊗). Incubations were made at 20°C.

internal pools of PG and the PSI electron flow activity.

Another approach to show the importance of the internal phospholipids was to control the degree of phospholipid depletion of thylakoid membranes and to observe the subsequent behaviour of PSII electron flow activity. Such an experiment is shown in Fig. 7 where EDTA was added in the incubation mixture to block PLA_2 action by Ca^{2+} chelation. Short treatments (2 and 5 min) with PLA_2, which destroy only partially the external pool of PC (Figs 1 and 6 a,b) and which do not induce sizable flip-flop, did not inhibit significantly PSII activity (Fig. 7). In contrast, longer treatments with PLA_2 which cause increasing flip-flop and progressive destruction of internal lipids, did inactivate PSII electron flow activity. However, the flip-flop of internal PC, but not its destruction, is in itself necessary and sufficient to inactivate PSII electron flow (Figs 6b and 7). It is noteworthy that the presence of lyso-compounds produced _in situ_ did not appear to contribute significantly to the inactivation of electron flow, as indicated by the fact that small amount of lyso-compounds (e.g. 5 min of treatment with PLA_2 and 75 min incubation) had the same inhibitory effect than larger amount (10 min of treatment and 35 min incubation). We conclude that pancreatic and snake venom PLA_2 inhibit the electron flow at the PSII level. The presence of an intact pool of phosphatidylcholine in the inner layer of the thylakoid membrane is required for PSII electron flow activity. PSI activity is associated in a complex manner with phosphatidylglycerol.

The above results favor the idea that the well-known functional asymmetry of the thylakoid membrane could be supported at least in part by an asymmetric distribution of its phospholipids.

ACKNOWLEDGEMENTS

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TRANSMEMBRANE DISTRIBUTION OF PHOSPHOLIPIDS AND THEIR INVOLVEMENT IN ELECTRON TRANSPORT, AS REVEALED BY PHOSPHOLIPASE A_2 TREATMENT OF SPINACH THYLAKOIDS

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Key words: Phospholipid distribution; Electron transport; Phospholipase A_2; Chloroplast; (Thylakoid membranes)

Summary

Thylakoid membranes were treated with either pancreatic or snake venom phospholipase A_2, and the residual phospholipid content of these membranes was determined and compared to the rates of Photosystem II and/or Photosystem I electron transports. The hydrolysis curves of both phosphatidylglycerol and phosphatidylcholine displayed a first, rapid phase which was almost temperature-insensitive, followed by a second, slower phase which depended strongly on the temperature. When pancreatic phospholipase A_2 had access either to the outer face or to both faces of the thylakoid membrane, either only part of or all the phospholipids, respectively, could be hydrolysed. These results were interpreted as indicating an asymmetric distribution of phospholipids across the thylakoid membrane, phosphatidylglycerol and phosphatidylcholine being preferentially located in the outer and the inner layer, respectively. When acting on uncoupled thylakoid membranes, phospholipase A_2 exerted an inhibitory effect on Photosystem II activity and a stimulatory effect on Photosystem I activity. The involvement of phosphatidylcholine and of phosphatidylglycerol in electron transport activities of Photosystem II and of Photosystem I are discussed with special reference to the role of the external and internal pools of these phospholipids.

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* This work is part of a doctoral program which is being carried out by A.R. in the Laboratoire de Physiologie Végétale et Biochimie, Université de Neuchâtel, Neuchâtel, Switzerland.

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

The relationship between the functional asymmetry of the thylakoid membrane and its structural sidedness, thoroughly discussed in 1974 by Trebst [1], has gained continuous support during the past six years. So far, this relationship is mainly concerned with the proteins involved in electron transport and proton movements. However, our knowledge of the distribution of lipids in thylakoid membranes is rather scarce [2—4]. The only major contribution to the problem of the localization of lipids in chloroplast membrane fractions was made by the group of Radunz (Ref. 5 and references therein), who used immunological techniques. However, this approach did not provide a quantitative estimation of the transmembrane distribution of lipids, although an asymmetric picture of the thylakoid membrane emerged from these studies.

Lipolytic treatments of biological membranes were designed for a double aim. Firstly, they have been widely used with bacterial and animal membranes in an attempt to establish the quantitative distribution of lipids with respect to a possible asymmetry of these components across the membrane. Advantages and limitations of this approach have been critically reviewed recently by Op den Kamp [6]. Secondly, lipolytic treatments were often used to assess the direct involvement of lipids in the functionality of various membranes. This latter approach was also applied to thylakoid membranes [7—20]; however, the results were often ambiguous [8—20]. In our view, there are at least two reasons for this lack of clear-cut results. Most authors did not recognize clearly that a lipolytic enzyme destroys almost inevitably lipids which do not support directly the measured function. In addition, the fact that biological membranes are generally described in terms of a lipid bilayer, the outer and inner monolayers of which being mutually independent up to a certain degree [21], was neglected in these studies [8—20]. We believe that the knowledge of the distribution of lipids across the thylakoid membrane is a prerequisite to any attempt to understand whether and, if so, how the lipids are involved in the functions of this membrane.

In this investigation, we present the thylakoid transmembrane distribution of phosphatidylcholine and of phosphatidylglycerol as revealed by pancreatic phospholipase A_2 treatment. In addition, we report on the role of external and internal pools of these phospholipids in sustaining electron transport activities in spinach thylakoid membranes. A preliminary report of these findings has been published [22].

Materials and Methods

Enzymes

Phospholipases A_2 (EC 3.1.1.4) from pig pancreas and from *Vipera russelli* venom, purchased from Sigma Chemical Co., were used without further purification.

Preparation of thylakoid membranes

Spinacia oleracea (var. Nobel) was grown in a growth chamber and the leaves were harvested after 14 weeks of hydroponic culture [23]. Intact chloroplasts were first prepared according to Kalberer et al. [24] in a medium containing 25 mM Mops (pH 7.6) and 350 mM sucrose, spun down at 1935 × *g* for 30 s and then osmotically disrupted in the above medium diluted 10-fold. Centrifugation at 17 300 × *g* yielded a pellet which was resuspended in a medium containing 25 mM Mops (pH 7.6) and 175 mM NaCl. The chlorophyll concentration was determined spectrophotometrically [25] and adjusted to 2 mg/ml; this suspension was used without delay for the enzymatic treatments.

Enzymatic treatments

The incubation of thylakoid membranes (0.5 mg chlorophyll/ml) with enzymes was carried out in darkness in 50 mM Tricine (pH 8), 80 mM NaCl, 1 mM $CaCl_2$ and phospholipase A_2 either from pig pancreas (not less than 1.6 Sigma unit/mg chlorophyll) or from *Vipera russelli* venom (not less than 0.11 Sigma unit/mg chlorophyll). Except where indicated, the incubations were performed at 20°C. Aliquots of the above incubation media were taken at various times (including a zero time control) for the determination of electron transport activities and for lipid analysis.

Determination of electron flow activities

Photosystem II activity was measured by the photoreduction of DCIP at 590 nm in a glass cuvette (0.2 cm optical path); the reaction mixture contained 50 mM Hepes (pH 7.6), 35 mM NaCl, 0.2 mM DCIP, 0.005 mM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone and thylakoid membranes (40 μg chlorophyll/ml). When used instead of water as an electron donor, 1,5-diphenylcarbazide was at 0.5 mM in the reaction mixture.

Photosystem I activity was estimated by the photoreduction of $NADP^+$ at 340 nm; the reaction mixture contained 50 mM Tricine (pH 8.4), 35 mM NaCl, 4 mM sodium ascorbate, 0.3 mM DCIP, 0.01 mM DCMU, 2 mM $NADP^+$, 6 μM ferredoxin and thylakoid membranes (100 μg chlorophyll/ml).

Photosystem II + I activity was measured as a $H_2O/NADP^+$ electron flow. The reaction mixture was the same as for Photosystem I, except that ascorbate, DCIP and DCMU were omitted. When the H_2O/methylviologen electron flow activity was followed with a Clark oxygen electrode, the reaction mixture contained 50 mM Tricine (pH 8.4), 35 mM NaCl, 2 mM NaN_3, 0.15 mM methylviologen and thylakoid membranes (20 μg chlorophyll/ml).

The intensity of actinic light after filtration through a Calflex and a DT-Red filter (both from Balzers) was 250 mW · cm^{-2} at the level of the cuvettes.

Lipid analysis

The changes in the phospholipid content of thylakoid membranes treated with phospholipases A_2 were followed by the method of Rawyler and Siegenthaler [26]. This method is based on the combination of thin-layer chromatography and scanning of photographic negatives of the charred plates. The results are expressed as percentages of the initial (control) values. The amounts of phosphatidylglycerol and phosphatidylcholine in freshly isolated thylakoid membranes were 0.195 and 0.150 μmol/μmol chlorophyll, respectively [26].

Results and Discussion

When thylakoid membranes were treated with pancreatic phospholipase A_2, the hydrolysis of phosphatidylglycerol and phosphatidylcholine displayed two phases (Fig. 1). The first was almost temperature-independent whilst the second was strongly dependent on this factor. The extent and rate of the first, fast hydrolytic step were greater for phosphatidylglycerol than for phosphatidylcholine. The difference in rates reflects, at least in part, the preference of the pancreatic phospholipase A_2 for anionic phospholipids whilst the difference in the extents of the first hydrolytic step suggests that a greater amount of phosphatidylglycerol than of phosphatidylcholine is easily accessible to the enzyme. The rates of the second phase were much slower; however, above 15°C the hydrolysis of both lipids was eventually complete. These results may indicate an asymmetric distribution of the phospholipids in the thylakoid membrane.

In order for this to be true, at least three criteria should be satisfied in control experiments [27]. Firstly, no lysis of the thylakoids should occur when the enzyme attacks the membrane from the outside. This was indirectly established by the fact that a proton gradient was still maintained across the membrane when the first hydrolytic step was achieved (data not shown). Secondly, all lipids should be hydrolyzed when the enzyme attacks the membrane from both sides. Fig. 2 shows that when phospholipase A_2 attacks the membrane from the outside only (e.g. in normal thylakoids), the hydrolysis of both phospholipids at 0°C was only partial. At this temperature the transbilayer movement mechanism is negligible. On the other hand, when phospholipase A_2 had access to both sides, e.g. in disrupted thylakoids (Fig. 2), the hydrolysis of phosphatidylglycerol and phosphatidylcholine was almost complete. This indicates that some of the phospholipids are located in the inner layer of the thyla-

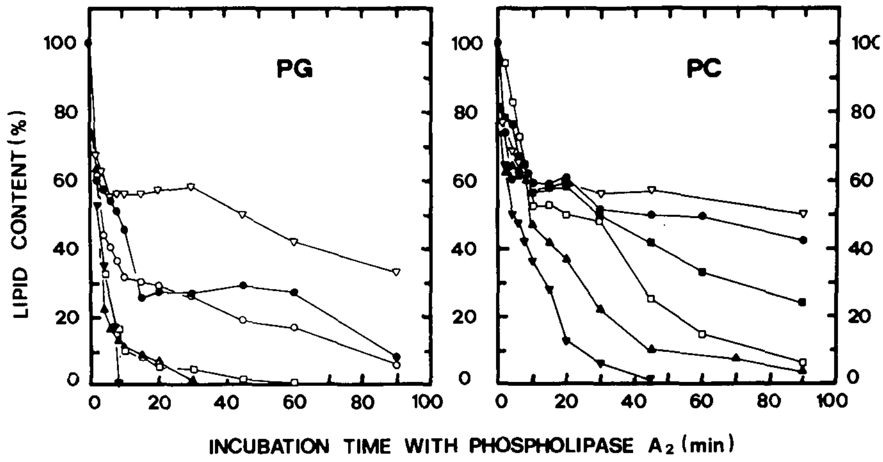

Fig. 1. Time course of phospholipid hydrolysis at various temperatures in thylakoid membranes treated with pancreatic phospholipase A_2. ▽——▽, 0°C; ●——●, 5°C; ○——○, 10°C; ■——■, 15°C; □——□, 20°C; ▲——▲, 25°C; ▼——▼, 35°C. PG, phosphatidylglycerol; PC, phosphatidylcholine.

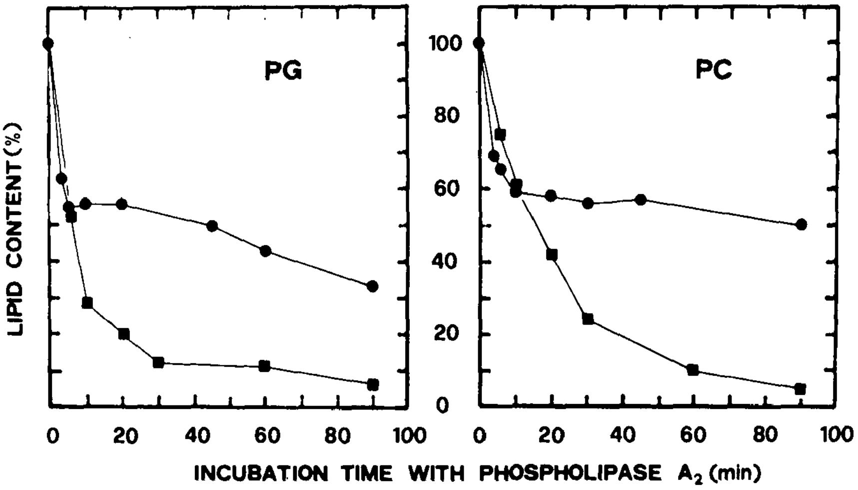

Fig. 2. Time course of phospholipid hydrolysis at 0°C in normal (●——●) and disrupted (■——■) thylakoid membranes treated with pancreatic phospholipase A_2. Disrupted thylakoid membranes were obtained by sonication of normal membranes for 3 min in an incubation mixture containing phospholipase A_2, but no NaCl and no $CaCl_2$; vesicles containing the trapped enzyme were thus formed and Ca^{2+} was added to initiate the hydrolytic reaction. PG, phosphatidylglycerol; PC, phosphatidylcholine.

koid membrane. Thirdly, an unequivocal localization of the lipids in the membrane can only be obtained if the lipolytic treatment does not modify the actual distribution of these lipids. This implies that no transbilayer movement should occur during the hydrolytic process. Although this was the case at low temperatures, the intensity of this phenomenon increased when the temperature was raised (see second phases in Figs. 1 and 2). Consequently, the best estimation of the actual distribution of phospholipids in the membrane is given by computing the fast-reacting pools of phosphatidylglycerol and phosphatidyl-

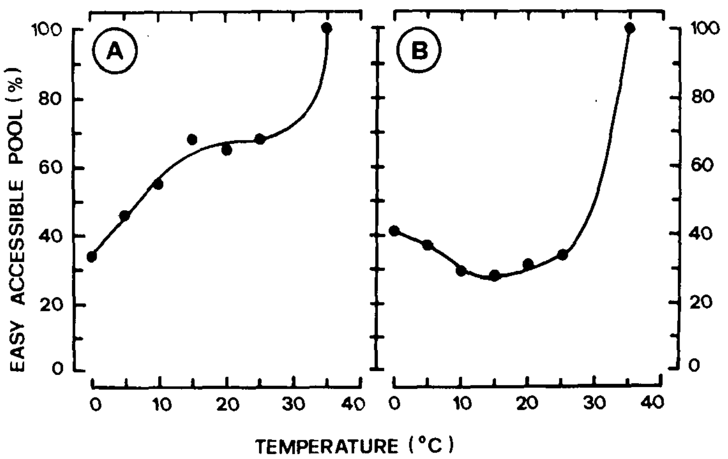

Fig. 3. Effect of temperature on the easy accessible pools of phosphatidylglycerol (A) and phosphatidylcholine (B) in thylakoid membranes treated with pancreatic phospholipase A_2. The estimation of these pools was made from the data of Fig. 1 by the logarithmic treatment of Demant et al. [28].

choline as shown in Fig. 3. The easily accessible pool of phosphatidylglycerol, which was 35% at 0°C, increased with temperature to reach a plateau between 15 and 25°C. On the other hand, the corresponding pool of phosphatidylcholine remained essentially constant within 30—40% of the total phosphatidylcholine between 0 and 25°C. Above 25°C, the accessibility of both lipids became total. We conclude that around 70% of phosphatidylglycerol and 35% of phosphatidylcholine could be localized in the outer layer of the thylakoid membrane.

Previous reports have shown that phospholipase A_2 treatment of spinach [19] and pea [20] thylakoid membranes resulted in a rapid hydrolysis of about 80% of the total phosphatidylglycerol. Our data on phosphatidylglycerol are in good agreement with these results, although these earlier works were not aimed at the study of the phospholipid transmembrane distribution. Moreover, our results give a quantitative support to the qualitative estimation of the distribution of phosphatidylglycerol in *Antirrhinum* thylakoid membranes [5]. In contrast, the distribution of phosphatidylcholine presented here is just the opposite of that suggested by Radunz [5].

The effects of pancreatic phospholipase A_2 at 20°C on different electron flow activities of thylakoid membranes are shown in Fig. 4. The activities involving Photosystem II + I (Fig. 4 A and B) decreased rapidly and were completely

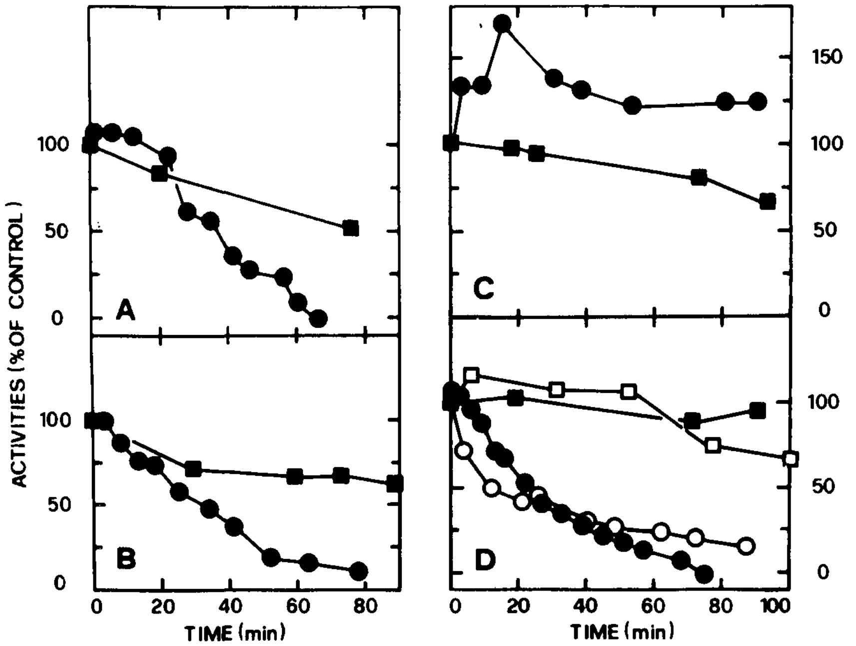

Fig. 4. Action of pancreatic phospholipase A_2 on thylakoid membranes followed by the time-dependent variations of electron transport activities measured from H_2O to $NADP^+$ (A) or to methylviologen (B) (Photosystem II + I), from reduced DCIP to $NADP^+$ (C) (Photosystem I) and from H_2O (closed symbols) or 1,5-diphenylcarbazide (open symbols) to DCIP (D) (Photosystem II). □ and ■, control membranes; ○ and ●, treated membranes. The 100% values were 140 and 70 μmol $NADPH_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ in A and C, respectively; 80 μmol O_2 consumed $\cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ in B and 130 μmol $DCIPH_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ in D.

inhibited after 60 to 80 min. In contrast, phospholipase A_2 brought about a stimulation of Photosystem I activity which remained higher than the control (Fig. 4C). A greater activation was found when methylviologen was used as the electron acceptor (data not shown). This stimulation was only partially due to uncoupling [9] since the long-term activation was also observed in uncoupled thylakoid membranes. The inhibition of electron flow through both photosystems (Fig. 4 A and B) could be ascribed to the decrease in Photosystem II activity as shown in Fig. 4D. Comparing the 1,5-diphenylcarbazide- and H_2O-supported Photosystem II activities, it seems unlikely that the water-splitting system is inactivated by the phospholipase A_2 treatment. Thus, these results suggest the existence of at least two action sites of phospholipase A_2. The first, leading to an inhibitory effect, is located between the electron entry point from 1,5-diphenylcarbazide and the plastoquinone pool. The second site, leading to a stimulatory effect, is located between the electron entry point from $DCIPH_2$ and the primary acceptor of Photosystem I.

These results are in partial agreement with those of Hirayama and Matsui [17] but disagree strongly with more recent works by Hirayama and Nomotobori [19] and Duval et al. [20]. Indeed, our phospholipase A_2 treatments always led to an inhibition of Photosystem II electron transport activity which was not found by these authors [19,20]. This discrepancy can be explained, at least in part, by the fact that these two groups did not add 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone to their H_2O/DCIP reaction mixture and thus did not measure a true Photosystem II-mediated electron flow, but a preferential DCIP reduction through Photosystem I [29,30]. Moreover, it should be emphasized that the hydrolysis of the lipid moiety of membranes, treated either with the lipolytic acyl hydrolase from potato [7] or with the pancreatic and snake venom phospholipase A_2 (see Figs. 1, 4 and 5), begins before any

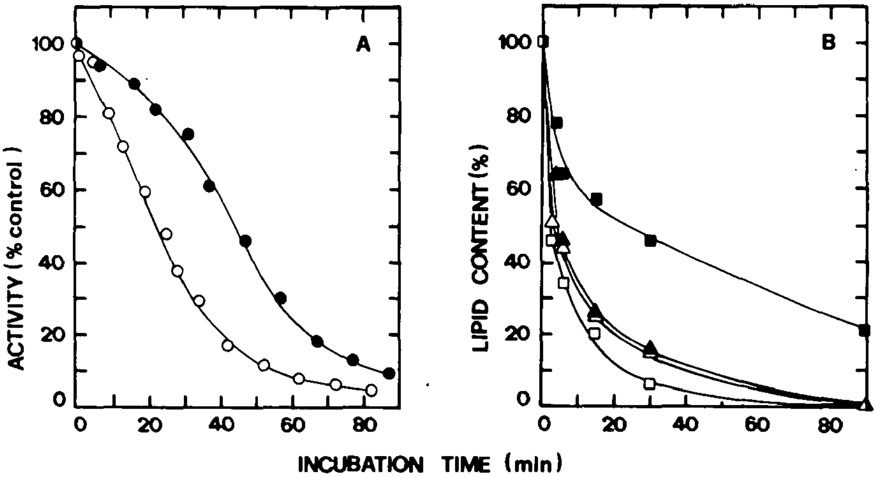

Fig. 5. Comparison of the action of pancreatic and *Vipera russelli* venom phospholipase A_2 on Photosystem II electron flow activity (A) and on the hydrolysis of phospholipids (B) as a function of incubation time. Closed symbols: pancreatic phospholipase A_2; open symbols: snake venom phospholipase A_2. A, Photosystem II electron flow H_2O/DCIP; B, phospholipid content; ▲ and △, phosphatidylglycerol; ■ and □, phosphatidylcholine.

appreciable change in electron flow activities can be detected. Hirayama and Nomotobori [19] and Duval et al. [20] did not present the time-dependent changes in electron flow activities of their phospholipase A_2-treated thylakoid membranes. Thus, the weak inhibition of Photosystem II electron flow activity observed by these authors could be explained by the limited time of incubation of their membranes in the presence of phospholipase A_2.

The shapes of the hydrolysis curve of phosphatidylcholine at 20°C (Fig. 1) and of the decay in Photosystem II activity (Fig. 4D) seem to indicate a possible participation of phosphatidylcholine in Photosystem II structures. The comparison between the action of phospholipase A_2 from pancreas and snake venom (which have inverse preference toward phosphatidylcholine and phosphatidylglycerol) supports the hypothesis that phosphatidylcholine is the main phospholipid involved in Photosystem II electron flow activities (Fig. 5). When the concentrations of both enzymes were chosen so as to obtain the same hydrolysis rate of phosphatidylglycerol, the extent and rate of the hydrolysis of phosphatidylcholine by the pancreatic phospholipase A_2 were smaller than that by the snake venom enzyme (Fig. 5B). This difference in the hydrolytic patterns of phosphatidylcholine can be related to the inhibition of Photosystem II electron flow activity which was smaller with pancreatic phospholipase A_2 than with the snake venom enzyme (Fig. 5A).

Again, this result is not in agreement with that of Duval et al. [20] who suggested that phosphatidylglycerol was mainly associated with Photosystem II structures. These authors have apparently neglected the fact that the phospholipase A_2 from *Vipera russelli* venom shows a preference for zwitterionic phospholipids such as phosphatidylcholine as compared to anionic phospholipids such as phosphatidylglycerol [31]. Consequently, they did not measure the changes in the phosphatidylcholine content of their phospholipase A_2-treated thylakoids, considering that the phosphatidylcholine level was constant. This was obviously not the case (Fig. 5B).

The involvement of phosphatidylcholine in Photosystem II-mediated activity is further substantiated by the comparison, at three different temperatures, of the action of pancreatic phospholipase A_2 on the phospholipid content and on Photosystem II electron transport activities in uncoupled thylakoid membranes. When the external pool of phosphatidylcholine was hydrolyzed (for instance up to 10 min of phospholipase A_2 treatment at 5°C and at 20°C, Fig. 6a and b) no change in Photosystem II activity was observed. However, the hydrolysis of the internal pool of phosphatidylcholine under the conditions where transbilayer movement occurs [28] (for instance at 35°C and after 10 min of phospholipase A_2 treatment at 20°C, Fig. 6 b and c) was directly related to the inhibition of Photosystem II activity. On the other hand, when no transbilayer movement occurred (for instance after 10 min of phospholipase A_2 treatment at 5°C) there was no appreciable inhibition of the Photosystem II activity. In addition, Fig. 6 shows that the hydrolysis of the external pool of phosphatidylglycerol was associated with an increase in Photosystem I activity, which was markedly stimulated when the internal pool of phosphatidylglycerol was further hydrolyzed, due to the transbilayer movement. It is noteworthy that the great stimulation of Photosystem I activity is not due to uncoupling. This unexpected observation suggests that phosphatidylglycerol plays

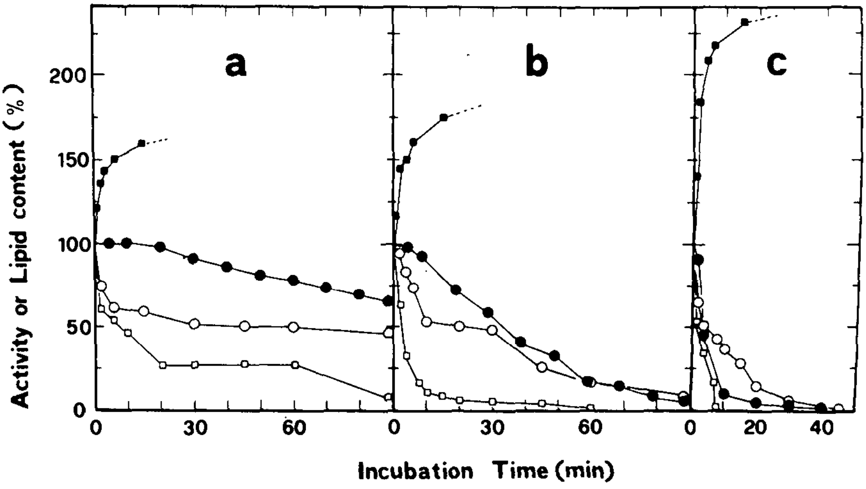

Fig. 6. Temperature dependence of the action of pancreatic phospholipase A_2 on the phospholipid content and on Photosystem II and Photosystem I electron transport activities of uncoupled thylakoid membranes. a, 5°C; b, 20°C; c, 35°C. •——•, Photosystem II electron flow, H_2O/DCIP; ▪——▪, Photosystem I electron flow, $DCIPH_2/NADP^+$; ○——○, phosphatidylcholine; □——□, phosphatidylglycerol. Uncoupling and blocking of hydrolysis were achieved by addition of 2 mM EDTA in the reaction media.

a privileged role in the function of Photosystem I. These results point out the great importance of the internal pool of phosphatidylcholine to sustain the Photosystem II electron transport activity and the complex relationship between both external and internal pools of phosphatidylglycerol and the

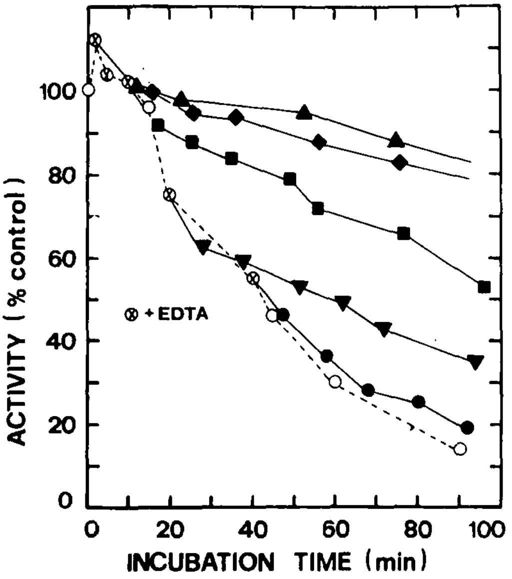

Fig. 7. Effect of various incubation times on the Photosystem II-catalysed photoreduction of DCIP by water in thylakoid membranes treated with pancreatic phospholipase A_2. Hydrolysis is blocked by addition of 2 mM EDTA (⊗) after 2 (▲), 5 (◆), 10 (■), 20 (▼) and 40 min (●) of treatment. Non-interrupted treatment with phospholipase A_2 (○,⊗). Incubations were made at 20°C.

Photosystem I electron flow activity.

Another approach to show the importance of the internal phosphatidylcholine was to control the degree of phospholipid depletion of thylakoid membranes and to observe the subsequent behavior of Photosystem II electron flow activity. Such an experiment is shown in Fig. 7 where EDTA was added in the incubation mixture to block phospholipase A_2 action by Ca^{2+} chelation. Short treatments (2 and 5 min) with phospholipase A_2, which destroyed only partially the external pool of phosphatidylcholine (Figs. 1 and 6 a and b) and which do not induce a sizable transbilayer movement, did not inhibit significantly Photosystem II activity (Fig. 7). In contrast, longer treatments with phospholipase A_2, which causes an increasing transbilayer movement and a progressive delocalization of internal phospholipids, did inactivate Photosystem II electron flow. However, the delocalization of internal phosphatidylcholine, but not its further destruction by phospholipase A_2, was in itself necessary and sufficient to cause the inhibition of Photosystem II activity (compare Figs. 6b and 7). It is noteworthy that the presence of lyso-compounds produced in situ did not appear to contribute significantly to the inactivation of electron flow. Indeed, even if all phosphatidylcholine is converted into its lyso-derivative in phospholipase A_2-treated thylakoid membranes, the molar ratio of lysophosphatidylcholine to chlorophyll (0.15) is far below that (0.70) required to obtain a detectable inhibition on the Photosystem II electron flow activity [32,33]. It must be emphasized that this threshold value of 0.70 was obtained with various amounts of lysophosphatidylcholine added to thylakoid membranes [32,33]; moreover, a given amount of lysophosphatidylcholine [34] or free fatty acids [35] was much more inhibitory when added exogenously than when the same amount was released in situ by lipolytic enzymes.

General conclusions

Treatments of thylakoid membranes by phospholipase A_2 permit us to draw the following conclusions:

1. Phospholipids are asymmetrically distributed in the thylakoid membrane. The outer layer is enriched in phosphatidylglycerol whereas the inner layer is enriched in phosphatidylcholine.
2. Phosphatidylcholine is the main phospholipid associated with the thylakoid structures responsible for Photosystem II electron flow. The presence of an intact pool of phosphatidylcholine in the inner layer of the membrane is absolutely required for maximal Photosystem II activity.
3. Phosphatidylglycerol appears to be preferentially associated with the thylakoid structures responsible for Photosystem I electron flow. Both outer and inner pools of phosphatidylglycerol seem to be involved in the modulation of this activity.
4. The role of a given lipid in a thylakoid membrane function is determined by its transmembrane distribution, its localization in the plane of the two leaflets of the membrane and by its chemical identity, but not necessarily by its relative abundance in the membrane.

The above results favor the idea that the well-known sidedness of the thylakoid membrane is supported in part by an asymmetric distribution of its phospholipids.

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REGULATION OF PHOTOSYSTEM I ELECTRON FLOW ACTIVITY BY PHOSPHATIDYLGLYCEROL IN THYLAKOID MEMBRANES AS REVEALED BY PHOSPHOLIPASE TREATMENT

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Thylakoid membranes were treated by potato lipolytic acyl hydrolase, phospholipases A_2 from pancreas and snake venom, and by phospholipase C from *Bacillus cereus* under various conditions. The changes in the uncoupled rates of electron transport through Photosystem I (PS I) and in lipid composition were followed during these treatments. Pancreatic phospholipase A_2 which destroyed all phospholipids in thylakoid membranes stimulated the $NADP^+$ reduction supported by reduced 2,6-dichlorophenolindophenol. This stimulation concerned only the dark but not the light reactions of this pathway. The main site of action of pancreatic phospholipase A_2 may be located on the donor side of PS I; the hydrolysis of phospholipids at this site caused an increased ability of reduced 2,6-dichlorophenolindophenol and ascorbate alone to feed electrons into PS I. A second site may be located on the acceptor side of PS I, probably between the primary acceptor and the ferredoxin system. When thylakoid membranes were first preincubated with or without lipolytic acyl hydrolase at 30°C (pH 8), the $NADP^+$ photoreduction was inhibited whilst the methyl viologen-mediated O_2 uptake was stimulated. A subsequent addition of pancreatic phospholipase A_2 (which had the same hydrolysis rates for phosphatidylglycerol but not for phosphatidylcholine) further stimulated the O_2 uptake and restored $NADP^+$ photoreduction. The extent of this stimulation, which depended on the presence of lipolytic acyl hydrolase, was ascribed partly to the hydrolysis of the phospholipids and partly to the generation of their lyso derivatives but not to the release of free fatty acids. On the contrary, phospholipase C which destroyed only phosphatidylcholine failed to restore this activity. It is suggested that phosphatidylglycerol is the only phospholipid associated with thylakoid membrane structures supporting PS I activities and that this lipid may play a physiological role in the regulation of these activities.

Introduction

In the thylakoid membrane, the vectorial electron transport is achieved through an adequate organization of chlorophyll-protein complexes along with proteic and nonproteic redox components [1,2]. All these constituents are distributed within a lipid environment, of which 85% are glycolipids (mono- and digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol) and 15% phospholipids (mainly phosphatidylglycerol and phosphatidylcholine).

We have previously shown that when thylakoid membranes were incubated in the presence of a nonspecific lipolytic acyl hydrolase, one could observe an inhibition of various electron-transport activities which was best explained by the hydrolysis of certain strategic lipids; the importance of these lipid molecules has been suggested to depend more upon their localization and their chemical identity than upon their relative abundance within the membrane [3]. More recently, we have studied the transmembrane

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; Tricine, *N*-tris(hydroxymethyl)methylglycine; PS, photosystem; Chl, chlorophyll.

distribution of both phosphatidylglycerol and phosphatidylcholine in spinach thylakoid membranes as revealed by pancreatic phospholipase A_2. It was concluded that phospholipids are asymmetrically distributed across the membrane, 70% of phosphatidylglycerol and 35% of phosphatidylcholine being located in the outer leaflet and the remainder present in the inner leaflet of the membrane [4,5]. In addition, we have shown that phosphatidylcholine was the main phospholipid associated with the structures supporting PS II activity and that the phosphatidylcholine pool of the inner leaflet was essential for this activity [5]. Previous observations have indicated that pancreatic phospholipase A_2 was able to stimulate PS I electron flow activity, even under uncoupled conditions [5]. It is rather uncommon to observe an enhancement in a membrane function when part of the structures supporting this function is destroyed. Therefore, we have investigated the stimulatory effect of pancreatic phospholipase A_2 on PS I activity to determine the identity of the phospholipid(s) involved and to characterize the action site(s) of this enzyme in the thylakoid membrane.

Materials and Methods

Enzymes. Phospholipase A_2 (EC 3.1.1.4) from pig pancreas and from *Vipera russelli* venom and phospholipase C (EC 3.1.4.3) from *Bacillus cereus* were purchased from Sigma Chemical Co. and used without further purification. Lipolytic acyl hydrolase (EC 3.1.1.26) was isolated from potato tubers as described previously [3].

Biological material. Spinach (*Spinacia oleracea* var. Nobel) was grown as hydroponic culture [6]. After 14 weeks, the leaves were harvested; the thylakoid membranes were prepared as described earlier [3], suspended at a concentration of 2 mg Chl/ml and used without delay. Chlorophyll was determined as described previously [7].

Enzymatic treatments. The incubation medium for all experiments involving the lipolytic acyl hydrolase and both phospholipases A_2 was 50 mM Tricine (pH 8.0), 80 mM NaCl, 1 mM $CaCl_2$ and thylakoid membranes (0.5 mg Chl/ml). To this basic medium, lipolytic acyl hydrolase (40 μg protein/mg Chl), pancreatic phospholipase A_2 (1.6 Sigma U/mg Chl) or snake venom phospholipase A_2 (0.12 Sigma U/mg Chl) was added as indicated in the figures. For phospholipase C experiments, the incubation medium was the same as above except that 50 mM Tricine (pH 7.5) was used as buffer; phospholipase C was present at 1.4 Sigma U/mg Chl. Incubations were generally carried out in the dark at 30°C. Other conditions are specified in the legends to the figures.

Lipid analysis. The changes in the lipid content of thylakoid membranes were measured by a combination of thin-layer chromatography and scanning of the photographic negatives of the charred plates [8].

Photochemical measurements. The electron-transport activity of PS I was measured by the $NADP^+$ photoreduction (followed at 340 nm) or by the oxygen uptake in the presence of methyl viologen. The reaction mixture for $NADP^+$ photoreduction contained 50 mM Tricine (pH 8.4), 35 mM NaCl, 2 mM NH_4Cl, 4 mM sodium ascorbate, 0.3 mM DCIP, 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 2 mM $NADP^+$, 6 μM spinach ferredoxin and thylakoid membranes (100 μg Chl/ml). The oxygen uptake was measured with a Clark oxygen electrode in the same reaction mixture as above except that 2 mM NaN_3 and 0.15 mM methyl viologen were used instead of $NADP^+$ and ferredoxin; thylakoid membranes were at 20 μg Chl/ml. Other conditions are specified in the legends. The actinic light was passed through a Calflex and a wide-band DT-Red filter, both from Balzers. The irradiance was usually 250 mW/cm^2.

Results

Fig. 1 shows that when thylakoid membranes were incubated at 30°C (pH 8) in the presence of 1 mM Ca^{2+}, two uncoupled PS I electron flows (e.g., reduced DCIP to either methyl viologen/O_2 or ferredoxin/$NADP^+$) had a completely different behavior. The photoreduction of $NADP^+$ was first inhibited (approx. 50%) then progressively recovered up to the initial level. On the contrary, the methyl viologen-mediated oxygen uptake was stimulated and reached a plateau at 180%. An analysis of the lipid composition during this incubation revealed that the only change was the appearance of a small amount of free fatty acids (Table I); this could be correlated with the formation of 6-*O*-acylmonogalactosylmonoacylglycerol from 6-*O*-acylmonogalactosyldiacylglycerol (not shown in Table I but see Ref. 9). When the incubation was carried out in the presence of lipolytic acyl hydrolase, essentially the same changes in activities (Fig. 1) and in lipid composition (Table I) were observed. However, the release of free fatty acids was somewhat higher than in the control, due to an additional hydrolysis of monogalactosyldiacylglycerol.

When pancreatic phospholipase A_2 was added (after 42 min of incubation), the photoreduction of $NADP^+$ was restored rapidly and reached 110–120% of the initial activity in the controls and 80–90% in the membranes pretreated with lipolytic acyl hydrolase (Fig. 1). On the other hand, pancreatic phospholipase A_2 caused a rapid additional stimulation of the methyl viologen-mediated reaction which was also higher in the controls than in the pretreated membranes. This stimulation was followed by a decrease in these activities. In the presence of pancreatic phospholipase A_2, both phosphatidylglycerol and phosphatidylcholine were rapidly and completely hydrolyzed. In the thylakoid membranes which had not been treated with the lipolytic acyl hydrolase, the hydrolysis of phospholipids by pancreatic phospholipase A_2 was accompanied by the appearance of the corresponding lyso compounds whilst in the pre-

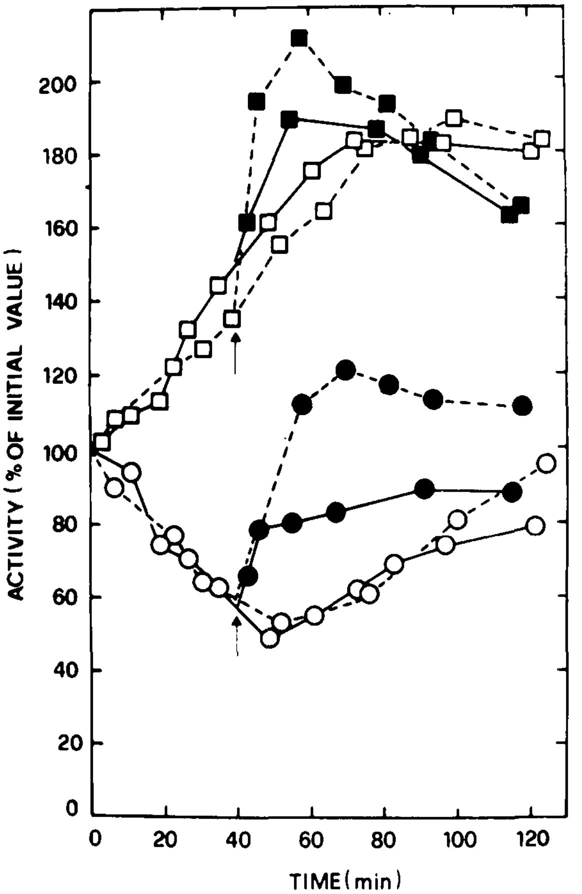

Fig. 1. Time course of the changes in the photoreduction of $NADP^+$ (○,●) and in the methyl viologen-mediated oxygen uptake (□,■) catalyzed by PS I in thylakoid membranes. The membranes were preincubated with (open symbols, full lines) and without (open symbols, dashed lines) lipolytic acyl hydrolase. After 40 min of preincubation (arrows) pancreatic phospholipase A_2 was added (closed symbols) to aliquots of both samples. The initial rates were 124 μmol O_2 consumed/ mg Chl per h and 103 μmol $NADPH_2$/mg Chl per h.

TABLE I

CHANGES IN LIPID COMPOSITION UNDER THE VARIOUS CONDITIONS DESCRIBED IN FIG. 1

Pancreatic phospholipase A_2 was added after 40 min of preincubation with or without lipolytic acyl hydrolase. Values expressed as nmol lipid/μmol Chl. tr, trace.

Lipid	Control				Lipolytic acyl hydrolase		
	0 min	40 min	120 min	120 min (+ phospholipase A_2)	40 min	120 min	120 min (+ phospholipase A_2)
Monogalactosyldiacylglycerol	1 431	1 316	1 260	1 260	1 280	1 121	1 068
Digalactosyldiacylglycerol	696	675	661	661	643	576	589
Phosphatidylglycerol	195	191	182	0 [a]	190	174	0
Phosphatidylcholine	150	142	141	0 [a]	134	130	0
Sulfoquinovosyldiacylglycerol	116	116	112	112	112	106	101
Free fatty acids	tr	40	98	443	87	140	830

[a] Stoichiometric amounts of lysophosphatidylcholine and lysophosphatidylglycerol present.

treated membranes no lysophospholipid could be detected due to the high activity of lipolytic acyl hydrolase towards these compounds [10]. In order to exclude the possibility of the contribution of lysophospholipids in this restoration phenomenon, we have deliberately chosen to investigate further this phenomenon under conditions where no lyso compounds can accumulate in the membrane, although the restoration was less pronounced than in the presence of lyso compounds. Since the free fatty acids released by the addition of phospholipase A_2 (see Table I) could also be part of the mechanism involved in the observed reactivation of PS I electron flow, their effect was tested under our experimental conditions. After a preincubation of thylakoid membranes in the presence of lipolytic acyl hydrolase for 40 min (see Fig. 1), a subsequent addition of linolenic acid (the most abundant free fatty acid released by the action of phospholipase A_2 and that of lipolytic acyl hydrolase on both galactolipids and 2-lysophospholipids generated by the phospholipase A_2) to these membranes (with molar ratio linolenic acid/Chl ranging from 0.1 to 20.0) failed completely to reactivate the $NADP^+$ photoreduction (data not shown). Altogether, these results indicated that the restoration of $NADP^+$ photoreduction was correlated with the hydrolysis of phospholipids but apparently not with free fatty acids per se. Furthermore, the extent of this restoration induced by phospholipase A_2 depended upon the presence of an enzyme (e.g., lipolytic acyl hydrolase) which is able to destroy the lysophospholipids generated by the phospholipase A_2.

Next, the pancreatic phospholipase A_2-induced restoration was estimated after various times of incubation with the lipolytic acyl hydrolase. Fig. 2 shows that when both enzymes were added simultaneously, the activity was first inhibited (about 40%) then decreased very slowly. In contrast, when thylakoid membranes were first incubated in the presence of lipolytic acyl hydrolase, a subsequent addition of phospholipase A_2 at different times resulted in a reactivation of the $NADP^+$ photoreduction which eventually reached the same level (about 80% of the initial rate). In addition, the longer the first incubation with the lipolytic acyl hydrolase was, the stronger was the reactivation rate (see Fig. 2). Since the reactivation was essentially due to the hydrolysis of phospholipids by the pancreatic phospholipase A_2 (see Fig. 1 and Table I), an experiment was designed to attempt to identify which phospholipids were involved in the restoration effect. As previously described [5], two phospholipases A_2 (from pancreas and snake venom) having opposite specificities toward phosphatidylcholine and phosphatidylglycerol were used. Fig. 3 shows that when the concentration of both enzymes was chosen so as to obtain the same hydrolysis rate of phosphatidylglycerol (and consequently different rates of phosphatidylcholine, see Fig. 5B in Ref. 5), the rate and extent of reactivation were identical. This result suggested that the rapid destruction of phosphatidylglycerol was responsible for the restoration.

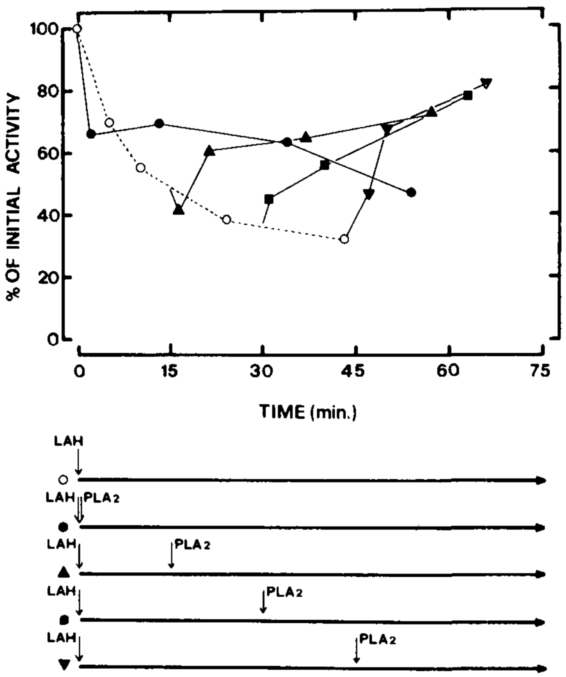

Fig. 2. Effect of pancreatic phospholipase A_2 (PLA_2) on the $NADP^+$ photoreduction catalyzed by PS I in thylakoid membranes preincubated for various times in the presence of lipolytic acyl hydrolase (LAH). (○) Treatment with lipolytic acyl hydrolase alone. The pancreatic phospholipase A_2 was added either immediately (●) or after 15 (▲), 30 (■) or 45 min (▼) of treatment with lipolytic acyl hydrolase. The initial rate was 93 μmol $NADPH_2$/mg Chl per h.

A second argument which supports this suggestion is given in Fig. 4 where the abilities of pancreatic phospholipase A_2 and phospholipase C from *B. cereus* were compared. In contrast to the rapid restoration

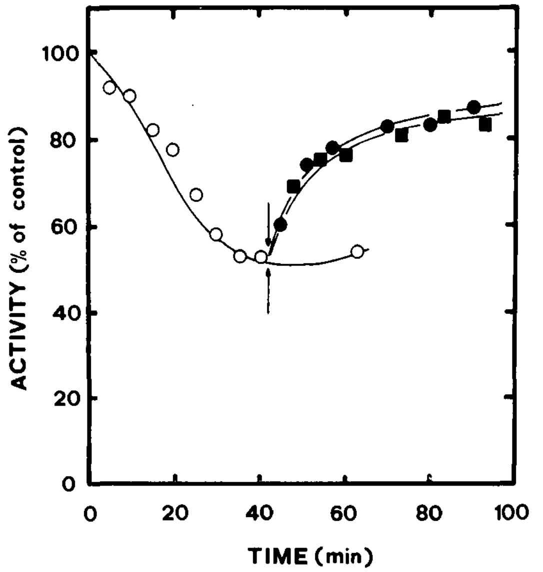

Fig. 3. Comparison between the effect of pancreatic phospholipase A_2 and that of *Vipera russelli* venom phospholipase A_2 on the $NADP^+$ photoreduction catalyzed by PS I in thylakoid membranes preincubated in the presence of lipolytic acyl hydrolase. After 42 min of treatment with lipolytic acyl hydrolase alone (○), pancreatic phospholipase A_2 (●) or snake venom phospholipase A_2 (■) was added (arrows). The initial rate was 70 μmol $NADPH_2$/mg Chl per h.

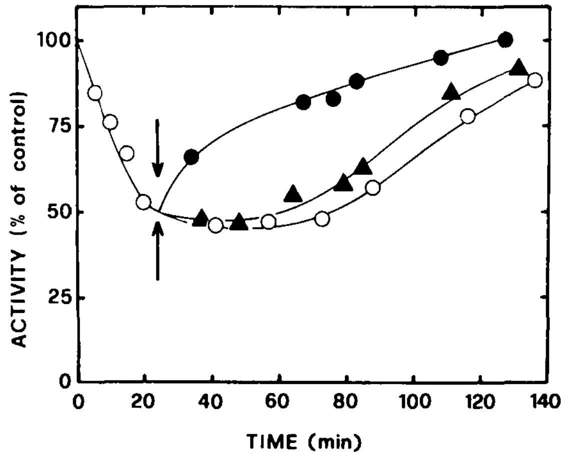

Fig. 4. Comparison between the effect of pancreatic phospholipase A_2 and that of phospholipase C from *Bacillus cereus* on the $NADP^+$ photoreduction catalyzed by PS I in thylakoid membranes preincubated in the presence of lipolytic acyl hydrolase. After 24 min of treatment with lipolytic acyl hydrolase alone (○), pancreatic phospholipase A_2 (●) or phospholipase C (▲) was added (arrows). The initial rate was 76 μmol $NADPH_2$/mg Chl per h.

induced by phospholipase A_2, the reactivation due to phospholipase C was slow and similar to that of lipolytic acyl hydrolase alone. The changes in the lipid composition occurring during this particular experiment are depicted in Fig. 5. During the treatment of the lipolytic acyl hydrolase a weak decrease in all classes of lipids (with the exception of sulfoquinovosyldiacylglycerol) was observed (see also Table I). After the addition of pancreatic phospholipase A_2, both phosphatidylcholine and phosphatidylglycerol were completely hydrolyzed. In contrast, phospholipase C destroyed completely the phosphatidylcholine (see Fig. 5, sample E_1) with a parallel production of diacylglycerol (sample E_2); this enzyme, however, attacked only slightly phosphatidylglycerol (compare samples C_2, D_2 and E_2). Again, these results suggested that the restoration of PS I electron flow was associated with the hydrolysis of phosphatidylglycerol.

The question was whether the destruction of this lipid would be related to the dark or light reactions of PS I. By investigating the relation of light intensity to the rate of the PS I electron flow reaction, one can differentiate between effects related to the dark reaction and those related to the light reaction, using the following linear equation:

$$1/V = 1/K_L \cdot 1/I + 1/K_D$$

where I is the light intensity, K_D a constant related to the dark reaction, K_L a constant related to the light reaction and V the rate of the reaction [11]. If phospholipase A_2 affects the dark reaction a change in $1/K_D$ should occur, whilst if it affects the light reaction a change in $1/K_L$ should take place. Table II shows clearly that the phospholipase A_2 treatment affected the dark reaction(s) of the $DCIPH_2$ to $NADP^+$ electron flow.

Next, we investigated whether the hydrolysis of phosphatidylglycerol would be related to the donor or acceptor side of PS I. To this end, we have measured the photoreduction of $NADP^+$ under various conditions (i.e., light intensity, concentration of electron donor or acceptor). Table III shows that 10 min incubation had essentially no effect in the controls, whatever the conditions used. On the other hand, a treatment of thylakoid membranes by phospholipase A_2 always stimulated the electron-transport rates,

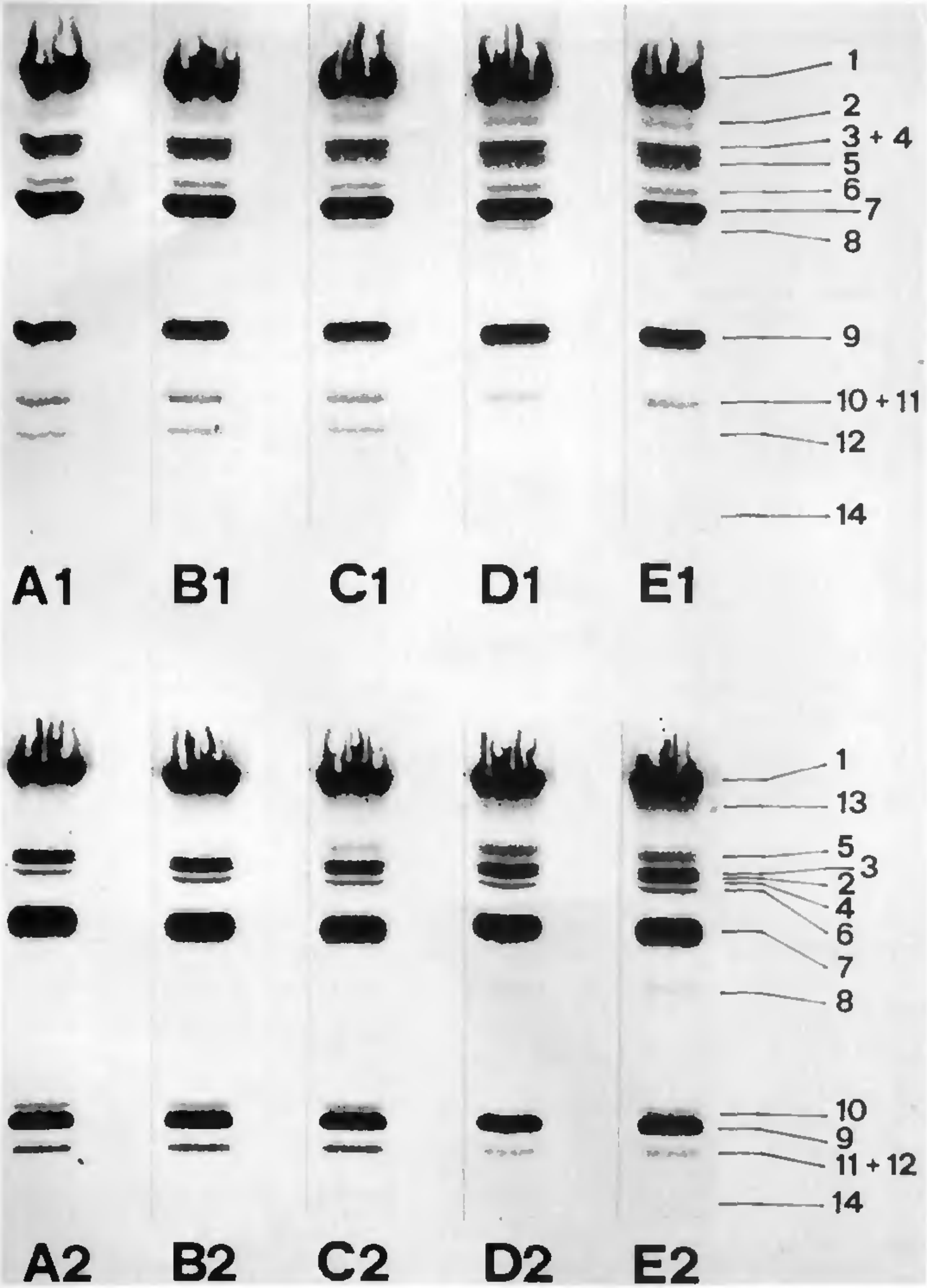

Fig. 5. Thin-layer chromatographic analysis of the lipid changes occurring during the treatment of thylakoid membranes with lipolytic acyl hydrolase alone or in combination with pancreatic phospholipase A_2 or phospholipase C from *Bacillus cereus*, as described in Fig. 4. Samples were spotted on silica gel plates and developed in $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v/v) (first row) and in $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (85 : 15 : 10 : 3, v/v/v/v) (second row). Lipids were then visualized by sulfuric acid charring [8]. Initial control (A); 24 min (B) and 130 min (C) of treatment with lipolytic acyl hydrolase alone; 130 min of lipolytic acyl hydrolase treatment with added pancreatic phospholipase A_2 (D) or with added phospholipase C (E). 1, chlorophylls + β-carotene; 2, 6-*O*-acylmonogalactosyldiacylglycerol; 3, lutein; 4, violaxanthin; 5, free fatty acids; 6, neoxanthin; 7, monogalactosyldiacylglycerol; 8, 6-*O*-acylmonogalactosylmonoacylglycerol; 9, digalactosyldiacylglycerol; 10, phosphatidylglycerol; 11, sulfoquinovosyldiacylglycerol; 12, phosphatidylcholine; 13, diacylglycerol; 14, origin.

TABLE II

CHARACTERISTICS OF THE LINES EXPRESSING THE DOUBLE-RECIPROCAL PLOTS OF RATES OF PS I ELECTRON FLOW VS. IRRADIANCE, $1/V = 1/K_L \cdot 1/I + 1/K_D$

The rates (V) were measured up to an irradiance of 290 mW/cm^2; $1/K_D$ constant related to the dark reactions) and $1/K_L$ (constant related to the light reactions) were derived from the regression analysis of the experimental data; r, correlation coefficient. After 10 min incubation at 20°C, EDTA (2 mM) was added to stop the action of pancreatic phospholipase A_2 and the rates were measured.

	$1/K_D$ ($\times 10^3$)	$1/K_L$ ($\times 10^3$)	r
Control	8.51	6.7	0.987
+ phospholipase	4.67	6.7	0.981

TABLE IV

EXTENT OF THE STIMULATION OF PS I NADP$^+$ PHOTOREDUCTION BY PANCREATIC PHOSPHOLIPASE A_2 UNDER THE SAME CONDITIONS AS DESCRIBED IN TABLE III

The extent of the stimulations was expressed as:

$$\frac{\text{Rate (phospholipase, 10 min)} - \text{rate (control, 10 min)}}{\text{Rate (control, 10 min)}} \times 100$$

Irradiance (mW/cm^2)	Electron donor		Ferredoxin	
	Type	Concentration (µM)	1 µM	10 µM
64	$DCIPH_2$	30	27	27
		300	6	16
290	$DCIPH_2$	30	58	88
		300	15	39

maximum stimulation being obtained at high irradiance and concentration of ferredoxin but at low reduced DCIP concentration. Ascorbate alone which does not support PS I electron transport in intact thylakoid membranes [12] became functional in phospholipase A_2-treated membranes under saturating conditions. The extents of the phospholipase A_2 stimulation of NADP$^+$ photoreduction are presented in Table IV. Whatever the concentrations of ferredoxin, the highest extents of stimulation were observed with limiting amounts of reduced DCIP, suggesting that the donor side of PS I is affected by the hydrolysis of phosphatidylglycerol. In addition, the extents of the stimulation were 4-times higher at low

TABLE III

DEPENDENCE OF PS I NADP$^+$ PHOTOREDUCTION ON THE IRRADIANCE, AMOUNTS OF FERREDOXIN AND ELECTRON DONORS IN THE ABSENCE AND PRESENCE OF PANCREATIC PHOSPHOLIPASE A_2

NADP$^+$ photoreduction given as uncoupled rates (2 mM NH_4Cl). At the end of each incubation period, 2 mM EDTA was added to block phospholipase A_2 activity. Incubations were at 20°C.

Irradiance (mW/cm^2)	Electron donor		NADP$^+$ photoreduction (µmol/mg Chl per h)					
	Type	Concentration	1 µM ferredoxin			10 µM ferredoxin		
			Controls		+ phospholipase (10 min)	Controls		+ phospholipase (10 min)
			0 min	10 min		0 min	10 min	
64	$DCIPH_2$	30 µM	15	15	19	40	41	52
		300 µM	14	18	19	48	49	57
290	$DCIPH_2$	30 µM	20	19	30	67	59	111
		300 µM	37	34	39	87	93	129
64	Ascorbate	1 mM	–	–	–	0	0	0
		4 mM	–	–	–	0	0	2
290	Ascorbate	1 mM	–	–	–	0	0	0
		4 mM	–	–	–	3	1	22

as compared to high concentrations of reduced DCIP with a rate-limiting amount of ferredoxin whilst they were only doubled with a saturating amount of ferredoxin. This suggests that the reducing side of PS I is also affected by the phospholipase A_2 treatment.

Discussion

It is generally believed that the destruction of certain lipids results in the impairment of the membrane functions sustained by these lipids. Adopting the enzymatic approach to make conspicuous the role of lipids in the functionality of the thylakoid membrane, several authors have shown that this was indeed the case (see Ref. 3 and references therein). In this investigation, we give evidence that under certain conditions the hydrolysis of certain membrane phospholipids gives rise to an enhancement of PS I-mediated reactions. This is rather unusual but the type and extent of the response to phospholipase A_2 were found to depend on the physiological state of the membrane at the time of the treatment.

Four cases have been considered under identical conditions of temperature (30°C) and pH (pH 8): (a) Freshly isolated thylakoid membranes were treated at zero time with phospholipase A_2; in this case phospholipids were destroyed with the formation of the corresponding lyso derivatives resulting in a stimulation of PS I activity (see Table III and Ref. 5). (b) Freshly isolated membranes were treated at zero time with a combination of phospholipase A_2 and lipolytic acyl hydrolase (Fig. 2). (c) Freshly isolated membranes were first preincubated for 40 min in the absence of lipolytic acyl hydrolase then treated with phospholipase A_2 (Fig. 1). (d) Freshly isolated membranes were first preincubated for 40 min in the presence of lipolytic acyl hydrolase then treated with phospholipase A_2 (Fig. 1).

Comparing cases a and b suggests that the stimulation of PS I activity by phospholipase A_2 can be essentially attributable to the formation of lysophospholipids. This is due to the fact that when the accumulation of lyso compounds was prevented by the presence of lipolytic acyl hydrolase, the destruction of the parent phospholipids led to an inhibition of PS I activity. Furthermore, it can be recalled that the degradation of phospholipids into their corresponding lyso derivatives and free fatty acids does not affect the bilayer structure in erythrocyte membranes [13–15]; however, it induces an increase in permeability to ions [16]. A similar situation in thylakoid membranes could explain the stimulation of the activity by an increased permeability of the membrane to electron donors. It is noteworthy that our interpretation concerning the stimulatory effect of lyso derivatives (e.g., at least in cases a and c) is in disagreement with the classical conception of the deleterious effect of lyso compounds on thylakoid membranes [17–20]. In the first phase of cases c and d, the mirror behavior of PS I activities (e.g., the stimulation of the methyl viologen-mediated reaction and the inhibition of $NADP^+$ photoreduction, see Fig. 1) was really unexpected as well as the subsequent recovery of $NADP^+$ photoreduction activity beyond 50 min of incubation at 30°C. This behavior accounts for a reversible modification of the membrane at the level of the acceptor side (i.e., from X to ferredoxin). The fact that this phenomenon did not occur at 20°C (not shown) could indicate that between 20 and 30°C a phase transition had occurred, namely in the molecular species of phosphatidylglycerol containing palmitic and 3-*trans*-hexadecenoic acids which might have changed from the gel to liquid-crystalline state. Indeed, dimyristoylphosphatidylglycerol has been shown by differential scanning calorimetry to undergo a sharp transition at about 25°C [21].

In the second phase of case d, the restoration of the activities can be explained by the hydrolysis of phospholipids. The additional restoration of the activity in case c seems to be due mainly to the presence of lysophospholipids and possibly to some galactolipids which have not been destroyed by lipolytic acyl hydrolase.

It is noteworthy that at the beginning of the preincubation in the presence of lipolytic acyl hydrolase, certain phospholipids are required (case b) whilst during the preincubation they must be destroyed (case d) for full photochemical activity (see Fig. 2). This switch in the role of certain phospholipids may be an expression of a lateral redistribution of the membrane components.

One can also ask whether the free fatty acids per se released by these enzymes could be involved in the mechanism(s) of the reactivation. The effects of added free fatty acids on photosynthetic electron transports depend on their concentration and on the pH of the reaction medium [19,22–24]. At pH values higher than 7.5, these effects are stimulatory at low fatty acid/Chl ratios and inhibitory at higher ratios. Under our experimental conditions, an addition of linolenic acid instead of phospholipase A_2 (see Fig. 1) did not reactivate the PS I NADP$^+$ photoreduction. However, it is not correct, sensu stricto, to compare the effects of an addition of lipid on an intact membrane with those of an equal amount of lipid released endogenously (see, for instance, Refs. 9 and 13). In these two cases, a threshold fatty acid/Chl ratio might exist above which an inhibition of electron transport occurs. The extent of the reactivation observed in the presence of a relatively small amount of free fatty acids (443 nmol/μmol Chl, case c) was much greater than that obtained in the presence of twice this amount (830 nmol/μmol Chl, case d; cf. Fig. 1 and Table I). If these endogenously released free fatty acids had been actively involved in the reactivation, then the opposite should have been observed. Finally, one may suggest that the type of free fatty acid released but not its concentration could be responsible for the reactivation. In this case, such a free fatty acid could have been released only from the 2 position of certain phospholipids by the action of the phospholipases A_2. Obviously, the levels of this hypothetical, particular free fatty acid would be equal in cases c and d, since the pancreatic phospholipase A_2 was able, in both cases, to destroy all phospholipids of the thylakoid membrane (Table I). As a consequence, the extent of reactivations should be the same in cases c and d, which is obviously not the case (Fig. 1).

Previous studies presented indirect evidence that phospholipids were associated with PS I structures in the thylakoid membrane [25–28]. Here, phosphatidylglycerol has been identified as the only phospholipid the destruction of which was responsible for both stimulation (case a) and restoration (cases c and d) of PS I activity. This has been indirectly verified by the experiments comparing the effect of phospholipases A_2 from snake venom and pancreas (Fig. 3) and demonstrated by comparing the effect of phospholipases A_2 and C (Figs. 4 and 5). These results exclude the possibility of the involvement of phosphatidylcholine in the structures which participate in the restoration of PS I activity. This conclusion supports our previous results [5] but disagrees with other findings [29–31].

Concerning the type of reactions which are affected by the hydrolysis of phosphatidylglycerol, the results showing the stimulatory action of phospholipase A_2 alone on PS I electron flow (case a) permit us to exclude the possibility of the effect of this enzyme on the light reactions (Table II). Two types of dark reactions can be concerned, e.g., those on the oxidizing and those on the reducing sides of PS I. To discriminate between these two possibilities, the following rationale has been adopted. Under conditions where the irradiance and ferredoxin were not rate limiting, an effect of phospholipase A_2 on the donor side should be stronger at low than at high concentration of the electron donor. This was indeed the case, suggesting that the hydrolysis of phosphatidylglycerol resulted in increased permeability of the membrane to reduced DCIP. This was further substantiated by the appearance of a PS I activity supported by ascorbate alone (Table III). Such a phenomenon was already observed when thylakoid membranes were treated with lipolytic acyl hydrolase [3] although in this latter case the ascorbate-supported activity was suppressed by a longer incubation period. On the other hand, this activity was permanent with phospholipase A_2 treatment, suggesting the involvement of lysophospholipids in this process.

The donor side thus appears to be the main target of pancreatic phospholipase A_2 in the PS I region. However, the extent of the stimulation can be modulated by the action of the enzyme on a second target located at the acceptor side. This modulation is expressed by the fact that the stimulation at a low as compared to a high concentration of reduced DCIP was 2-times higher at limiting than at saturating ferredoxin concentration (Table IV).

It has been suggested that the phosphatidylglycerol molecules, namely those containing 3-*trans*-hexadecenoic acid, could exert a cementing effect in the building of appressed regions in thylakoid membranes [32]. However, this hypothesis has been challenged [33]. Although the aim of this study was not to solve this problem, we can now suggest another possible physiological role of phosphatidylglycerol as an element which may regulate the rate of PS I electron transport.

In conclusion, from these results and those recently published [5], it may be suggested that in addition to their asymmetric transmembrane distribution [5], the phospholipids are located in two main, spatially distinct pools within the plane of the membrane, phosphatidylcholine being associated with PS II [5] and phosphatidylglycerol with PS I structures.

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