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Inhibition of Electron Transport and Uncoupling of photophosphorylation in Spinach Chloroplasts by Lysolecithin

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Abstract Lysolecithin was added to spinach chloroplasts in suspension, and its effect as a detergent on activities of electron transport and photophosphorylation was examined. At low concentrations of lysolecithin, the detergent brought about the inhibition of cyclic photophosphorylation catalyzed by phenazine methosulfate, and also the stimulation of the activities of electron flow from water to ferricyanide and from reduced dichlorophenolindophenol to methyl viologen. The activity of electron flow from water to oxidized form of *p*-phenylenediamine was slightly stimulated by a small amount of the detergent. No lysolecithin-induced stimulation of the activity of electron transport was observed when the reaction was carried out in the presence of the uncoupler NH_4Cl. Lysolecithin-induced inhibition of dichlorophenolindophenol photoreduction was only slightly recovered by the addition of artificial electron donor to photosystem II, diphenyl carbazide, and the activity of electron transport from reduced dichlorophenolindophenol to methyl viologen was completely restored by the addition of plastocyanin. It seems that the inhibition of photosystem I causes the release of plastocyanin by the lysolecithin-induced disruption of thylakoid membranes, and the light reaction of photosystem I is not damaged. It was also shown that lysolecithin-induced inhibition of electron flow of photosystem II occurred mainly at the site(s) between water-splitting enzyme and the intermediate electron carrier of photosystem II which intercepted electrons by the lipophilic class III acceptor, the oxidized form of *p*-phenylenediamine.

Introduction

We previously reported that light-aged, isolated chloroplasts produced lysolecithin as a result of the hydrolysis of lecithin in thylakoid membranes (Hoshina *et al.*, 1975). Lysolecithin is known to be a powerful detergent which can disintegrate biological membranes (Neumann and Habermann, 1957; Rossiter and Strickland, 1960; Tanford and Reynolds, 1976). We also previously reported that the structural and functional changes in chloroplasts were induced by lysolecithin (Hoshina and Nishida, 1975). When

low concentrations of the detergent were added to chloroplast suspension, the activities of electron transport from water to ferricyanide and from reduced dichlorophenol-indophenol to methyl viologen were stimulated and partial disintegration of thylakoid membranes was observed by the measurement of the size distribution of chloroplasts using a Coulter counter (Hoshina and Nishida, 1975). At higher concentrations of lysolecithin, the activities of the electron transport decreased from maximum to 10-50% of the control activities and chloroplasts were further disintegrated into vesicles, particles or strands (Hoshina and Nishida, 1975).

The present study has been undertaken to elucidate the characteristic of the lysolecithin-induced stimulation of the activities of electron transport, and the sites of lysolecithin-induced inhibition of electron transport. Lysolecithin-induced inhibition of photosystem I was completely reactivated by the addition of plastocyanin, indicating that light reaction of photosystem I is not damaged by the detergent. It was also shown that lysolecithin-induced inhibition of photosystem II was observed at the site(s) between water-splitting enzyme and the intermediate electron carrier of photosystem II which intercepted electrons by lipophilic class III acceptor, the oxidized form of *p*-phenylene-diamine.

Materials and Methods

Chloroplasts were isolated from spinach leaves obtained from a local market. Leaves were homogenized in a Waring blender with a chilled medium containing 0.4M sucrose, 0.1M KCl, 5mM $MgCl_2$ and 20mM Tricine-NaOH (pH 7.5). The homogenate was filtered through two layers of Miracloth and the filtrate was centrifuged at 200 x g for 2min. Chloroplasts were collected by centrifuging the supernatant at 1,500 x g for 7min and were resuspended in a medium containing 0.1M KCl and 5mM $MgCl_2$. In this medium they were washed once by centrifugation. Chloroplasts were resuspended in the 0.1M KCl-5mM $MgCl_2$ medium to a concentration of 0.2-0.4mg chlorophyll per ml and kept on ice before use. In some experiments, a medium containing 0.3M NaCl and 50mM Tris-HCl (pH 7.5), or 0.1M NaCl, 3mM $MgCl_2$ and 50mM Tricine-NaOH (pH 8.0) was used for the grinding and resuspending medium instead of the medium above mentioned, as shown in the figure legend. Chlorophyll concentrations were determined in 80% acetone by the procedure of Arnon (1949).

The incubation of chloroplasts with lysolecithin was performed by the method as previously described (Hoshina and Nishida, 1975).

Photoreduction of ferricyanide and dichlorophenolindophenol was measured by decrease in absorbance at 420 and 600nm, respectively, using a Shimadzu Multipurpose recording spectrophotometer model MPS-50L fitted with a cross-illumination attachment which supplied xenon light filtered with a Toshiba V-R66 (transmitted above 620nm). The energy incident on the sample was 5 x 10^5 ergs/cm^2/sec. Temperature during the reaction was approximately 25°C (room temperature). In some

experiments, the activity of ferricyanide reduction was measured by pH change of chloroplast suspensions as described by Gross *et al.* (1969). The apparatus employed was identical to the one described below for measurement of proton uptake.

The activity of methyl viologen photoreduction was measured with a Yello Spring Instrument model 53 oxygen monitor using a Clark-type electrode. Intensity of the actinic light (above 600nm, filtered with a Toshiba V-R62) was 5×10^5 ergs/cm^2/sec, and the temperature was maintained at 19°C by circulating water around a reaction vessel.

Light-induced proton uptake was measured with a combination glass electrode connected to a Beckman expandomatic pH meter model SS-2. The temperature was maintained at 19°C by circulating water around a reaction vessel. The sample was illuminated with red actinic light (above 600nm, intensity, 5×10^5 ergs/cm^2/sec). The extent of the pH changes was determined by titration with acid.

Cyclic photophosphorylation mediated by phenazine methosulfate was measured by the proton uptake method of Nishimura *et al.* (1962). The apparatus employed was identical to that described above for measurement of pH changes.

Lysolecithin (Type I, from egg yolk) was purchased from Sigma. Plastocyanin was purified from spinach chloroplasts by the method of Katoh (1971). *p*-phenylenediamine was recrystalized after adsorption of impurities on activated charcoal according to the procedure of Gould (1975).

Results and Discussion

The activity of ferricyanide photoreduction was enhanced by the addition of low concentrations of lysolecithin and showed a maximum at 0.5 LPC/chl (lysolecithin concentration given as lysolecithin per chlorophyll ratio, based on weight, in all the experiments described below), as previously described (Hoshina and Nishida, 1975). When the Hill reaction was performed in the presence of NH_4Cl, no stimulation of the activity caused by the detergent was observed and at concentrations higher than 0.5 LPC/chl, the activity decreased with an increase in concentration just as it did in the absence of the uncoupler (Fig. 1). Low concentrations of lysolecithin did not induce the stimulation of dichlorophenolindophenol photoreduction and at higher concentrations, the activity decreased with an increase in concentration as in the case of the effect of the detergent on ferricyanide reduction in the presence of the uncoupler. It seems reasonable that no stimulation of dichlorophenolindophenol photoreduction is observed, because the oxidized forms of indophenol dyes act as uncouplers of all types of photophosphorylation (Gromet-Elhanan and Avron, 1964 ; Keister, 1963).

As shown in Fig. 2, inhibition of phenazine methosulfate mediated photophosphorylation and proton uptake was observed at concentrations above 0.1 LPC/chl, which the activity of the Hill reaction began to stimulate. Approximately 50% inhibition of ATP synthesis occurred at a lysolecithin concentration of about 0.7 LPC/chl, which was in close agreement with maximum stimulation of ferricyanide photoreduction. With

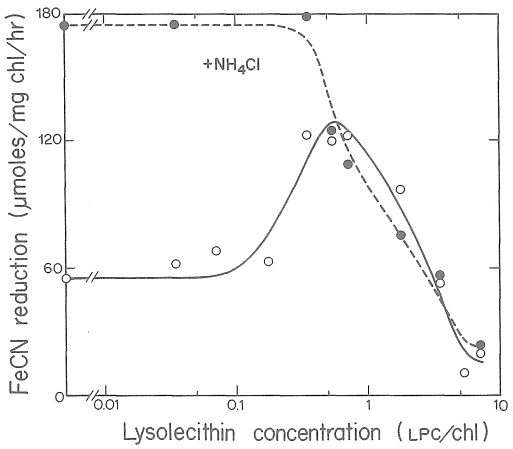

Fig. 1. Effect of NH_4Cl on lysolecithin-induced stimulation of ferricyanide photoreduction. Chloroplasts were isolated with a medium containing 0.3M NaCl and 50mM Tris-HCl (pH 7.5). Ferricyanide photoreduction was colorimetrically determined. The reaction mixture contained 50mM Tris-HCl (pH 7.5), 0.3M NaCl, 300μM potassium ferricyanide, and chloroplasts equivalent to 18.9μg chl/ml. The final concentration of ethanol in the reaction mixture was kept lower than 1% in all the experiments described below. For uncoupled rates of electron transport, NH_4Cl (final concentration, 10mM) was added to the reaction mixture. LPC/chl is given on the basis of weight and FeCN stands for ferricyanide in all experiments described below.

increasing concentration of the detergent, proton uptake was inhibited more than phosphorylation, and at concentrations above 1.5 LPC/chl, both activities were completely inhibited. This result suggests that disorganization of membrane by lysolecithin may induce more preferential inhibition of proton translocation through membranes than membrane potential. The preferential inhibition of proton uptake of chloroplast membranes treated with detergents was previously reported by Hauska and Sane (1972) and Nelson *et al.* (1970). In the experiment presented above, proton uptake was measured under the condition of coupling of photophosphorylation. A similar result was obtained when the reaction was carried out in the absence of ADP. These results show that low concentrations of lysolecithin acts as an uncoupler of cyclic and noncyclic photophosphorylation.

Scince plastocyanin is known to be released from thylakoid membranes by sonication, incubation with detergents or French press treatment (Sane and Hauska, 1972), the effect of plastocyanin on lysolecithin-induced inhibition of methyl viologen photoreduction was investigated (Table 1). Plastocyanin recovered photosystem I activity in lysolecithin-incubated chloroplasts but did not affect it in non-treated ones. The inhibition of activity caused by incubation with the detergent was completely overcome by the addition of 170nM plastocyanin to the reaction mixture. However, the electron transport from water to ferricyanide in the presence of lysolecithin was only

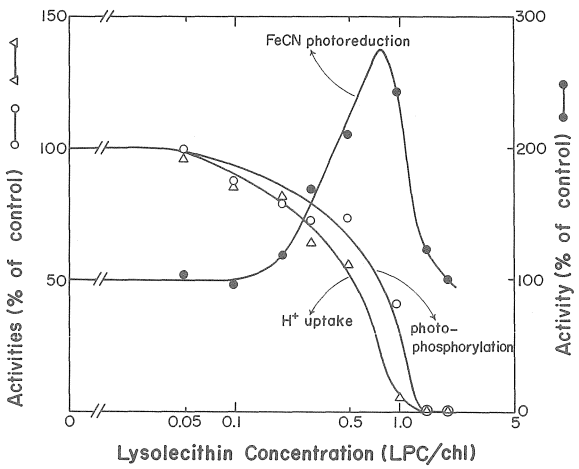

Fig. 2. Effect of various concentrations of lysolecithin on phenazine methosulfate mediated photophosphorylation and light-induced proton uptake. Chloroplasts were isolated with a medium containing 0.4M sucrose, 0.1M KCl, 5mM $MgCl_2$, and 20mM Tricine-NaOH (pH 7.5). The reaction mixture for ATP formation and proton uptake contained 0.1M KCl, 5mM $MgCl_2$, 2mM potassium phosphate, 2mM ADP, 30μM phenazine methosulfate, and chloroplast equivalent to 29.9μg chl/ml. Initial pH of the reaction mixture was ajusted to pH 7.5 before measurement of the activity. The activity of ferricyanide photoreduction was determined by the measurement of proton release. The reaction mixture of ferricyanide reduction was the same as described above, except that ADP and phenazine methosulfate were omitted and 500μM potassium ferricyanide was added. Control activities were 104.1 μmoles ferricyanide reduced, 146.4 μmoles ATP formed and 386.0 μmoles H^+ taken up/mg chl/hr.

partially restored by the addition of plastocyanin (Table 1). This suggests that lysolecithin-induced inhibition of photosystem I activity may be due to releasing plastocyanin from chloroplast membranes without interferring with the primary photochemistry of photosystem I.

Saha *et al.* (1971) demonstrated that lipophilic class III acceptores, such as the oxidized form of *p*-phenylenediamine intercepted electrons by reacting with some intermediate carrier which normally transfered electrons from photosystem II to photosystem I, and finally donated electrons to ferricyanide. Fig. 3 shows the effect of lysolecithin on the activity of the electron transport from water to oxidized form of *p*-phenylenediamine. In the presence of a small amount of lysolecithin (0.5-1.0 LPC/chl), the rate of electron flow from water to *p*-phenylenediamine was slightly increased by 15-30% of that of control chloroplasts, while the electron transport from water to ferricyanide was markedly stimulated by it. This indicates that in the reaction of electron transport from water to *p*-phenylenediamine, ferricyanide is reduced mainly by the class III acceptor, *p*-phenylenediamine, and accepts electrons only partially as a class I acceptor through the coupling site I (the site between plastoquinone and cytochrome *f*), because the coupling site I has control while the coupling site II (the site

Table 1 Reactivation of lysolecithin-induced inhibition of photosystem I activity by plastocyanin

		$DCPIPH_2 \rightarrow MV \rightarrow O_2$	$H_2O \rightarrow FeCN$
		μequiv/mg chl/hr	
control		223.2	79.9
+ LPC		122.8	18.8
+ LPC + plastocyanin	17 nM	188.4	—
+ LPC + plastocyanin	85 nM	207.6	—
+ LPC + plastocyanin	170 nM	230.8	—
+ LPC + plastocyanin	510 nM	230.8	—
+ LPC + plastocyanin	760 nM	—	27.4

The isolation medium was 0.3M NaCl and 50mM Tris-HCl (pH 7.5). $DCPIPH_2 \rightarrow MV \rightarrow O_2$ was assayed in the reaction mixture which contained 0.3M NaCl, 50mM Tris-HCl (pH 7.5), 0.2mM methyl viologen, 0.5mM NaN_3, 10μM dichlorophenolindophenol, 5mM sodium ascorbate, 10μM dichlorophenyl dimethylurea, and chloroplasts equivalent to 16.5μg chl/ml. The concentration of lysolecithin was 4.7 LPC/chl. $H_2O \rightarrow FeCN$ was assayed in the reaction mixture which contained 0.3M NaCl, 50mM Tris-HCl (pH 7.5), 0.3mM potassium ferricyanide, and chloroplasts equivalent to 13.3μg chl/ml. The concentration of lysolecithin was 4.7LPC/chl. DCPIP and MV stand for dichlorophenolindophenol and methyl viologen, respectively.

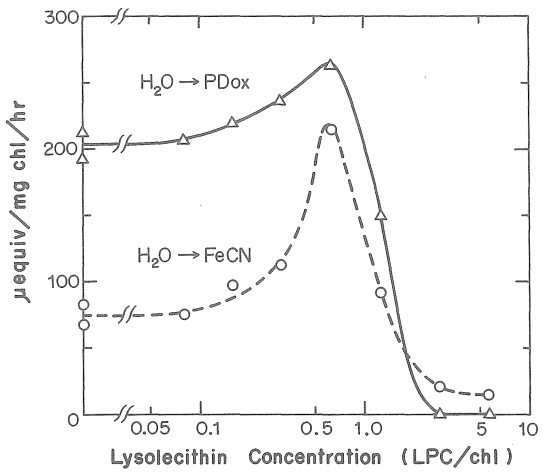

Fig. 3. Effect of lysolecithin on the activity of the electron transport from water to oxidized *p*-phenylenediamine. Chloroplasts were isolated with medium containing 0.4M sucrose, 10mM $MgCl_2$, and 50mM Tris-HCl (pH 7.5). $H_2O \rightarrow FeCN$ was assayed by following ferricyanide reduction in a reaction mixture which contained 100mM NaCl, 3mM $MgCl_2$, 500μM potassium ferricyanide, 50mM Tricine-NaOH (pH 8.0), and chloroplasts equivalent to 16.5μg chl/ml. $H_2O \rightarrow PD_{ox}$ was assayed in the reaction mixture described above for $H_2O \rightarrow FeCN$, except that 0.5mM *p*-phenylenediamine plus an equivalent amount of ferricyanide to oxidize the phenylenediamine was added. PD_{ox} stands for oxidized form of *p*-phenylenediamine.

near photosystem II) does not (Gould and Izawa, 1973). The activity of the electron flow from water to oxidized *p*-phenylenediamine was decreased with an increase in concentration of lysolecithin and was totally inhibited at the concentration of the detergent above 3-4 LPC/chl. The activity of water to ferricyanide remained at a level of 10-50% of the control rate even if high concentrations of the detergent were present. This may suggest that high concentrations of lysolecithin cause not only the inhibition of the electron transport near photosystem II but also cause the lowering of the affinity of *p*-phenylenediamine with ferricyanide or with the intermediate electron carrier of photosystem II.

We know that the donor side of photosystem II is quite labile to various treatments such as mild heating (Katoh and San Pietro, 1967), incubation with high concentration of alkaline Tris buffer (Yamashita and Butler, 1969), hydroxylamine extraction (Cheniae and Martin, 1971) and treatment with chaotropic reagents (Lozier *et al.*, 1971). A number of compounds have now been described as electron donors for photosystem II (Trebst, 1974). Vernon and Shaw (1969) recommended dichlorophenolindophenol as electron acceptor and diphenyl carbazide as electron donor for a more satisfactory measurement of the activity of photosystem II in Tris-washed chloroplasts and subchloroplast fragments. As shown in Table 2, lysolecithin-induced inhibition of dichlorophenolindophenol photoreduction is only partially reversed by the addition of diphenyl carbazide. The recovered rate of dichlorophenolindophenol reduction was only 18% of the rate of untreated chloroplasts using water or diphenyl carbazide as the electron donor. Thus, lysolecithin only slightly inhibited electron flow before reaching the site of electron donation by diphenyl carbazide, that is, between water and water-splitting enzyme.

Table 2 Effect of diphenyl carbazide on lysolecithin-induced inhibition of dichlorophenolindophenol photoreduction

	electron donors		
	H_2O	DPC (0.1mM)	DPC (1mM)
	μequiv/mg chl/hr		
control	116.6	121.3	—
+ LPC	11.2	16.7	20.7

The isolation medium was 0.3M NaCl and 50mM Tris-HCl (pH 7.5). The reaction mixture contained 0.3M NaCl, 50mM Tris-HCl (pH 7.5), 30μM dichlorophenolindophenol, 0.9% methanol, and chloroplasts equivalent to 16.5μg chl/ml. The concentration of lysolecithin was 4.7 LPC/chl. DPC stands for 1, 5-diphenyl carbazide.

Renger (1976) suggested that the proteinaceous shield was an essential structural element probable by its orientation effects for the functional connection between the plastoquinone pool and X 320 (the primary electron acceptor of photosystem II), and was leaving X 320 highly inaccessible to external redox agents. Katoh (1977) also reported that C 550 (the primary acceptor of photosystem II) was covered by a lipophilic layer. It will be described elsewhere (Hoshina, 1979) that lysolecithin molecules bind to thylakoid membranes and chloroplast lipids are released into the medium when chloroplasts are incubated with the detergent. The present result suggests that one of the factors inhibiting electron transport of photosystem II by lysolecithin may be due to the modification of lipophilic acceptor site as a result of replacing of lipid in chloroplasts by the detergent.

It is clear from these results that lysolecithin acts as an uncoupler of photophosphorylation and also as inhibitor of electron transport of both photosystem I and II. The lysolecithin-induced inhibition of the electron flow of photosystem I is due to the release of plastocyanine from its site in thylakoid membranes. The lysolecithin-induced inhibition of photosystem II may take place mainly at the site(s) between water-splitting enzyme and the intermediate electron carrier which intercepts electrons by the oxidized form of *p*-phenylenediamine.

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