Local Anesthetics-induced Inhibition of Chloroplast Electron Transport

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Abstract. The effects of local anesthetics on photosynthetic activity of pea chloroplasts were investigated in order to elucidate the role of Ca^{2+} in photosynthetic electron transport. Dibucaine, benzocaine and tetracaine were found to inhibit the O_2-evolving activity. The inhibitory effect decreases in the order dibucaine > benzocaine > tetracaine > trimecaine similary as does the potency to inhibit propagation of exitation in nerve fibre. As demonstrated in experiments with artificial donors and acceptors, the site of inhibition is the water-splitting site of PSII. The inhibitory power of the anesthetics grows with increasing ionic strength of the incubating mixture (by adding NaCl or $MgCl_2$) and with pH; this is explained by occurrence of the neutral form of amine. At low concentrations the charged anesthetic acts as a protonofore; however, the inactivation of water splitting is not due to the protonophoric effect. The incubation is followed by the disappearance of ESR signal II_s. The role of Ca^{2+} and Ca^{2+}-binding protein in PSII electron transport and its localization are discussed.

Key words: Electron transport in chloroplasts — Local anesthetic — Photosystem II

Introduction

The structural and functional organisation of electron transport at the donor side of photosystem II (PSII) is one of the least studied aspects in the field of photosynthesis. Recently it has been reported that Ca^{2+} is necessary for normal operation of PSII both in cyanobacteria and chloroplasts of higher plants (Piccioni and Mauzerall 1978; Brand 1979; Barr et al. 1980). Calcium has been shown to play an important role in water splitting during reactivation of oxygen evolution (Ono and Inoue 1983; Pistorius and Schmid 1984). It was also found that the site of Ca^{2+} binding which occurs at a high rate, is one of the PSII proteins and that the binding is through proteins with molecular weights of 17 and 23 kDa (Ghanotakis et al. 1984). Probably, it is calmodulin-like protein.

The presence of such a protein in PSII was demonstrated not only in experiments using chlorpromazine and other calmodulin antagonists (Barr et al. 1984; Chamorovsky and Matorin 1984), but also by extracting a heat-resistant protein with a molecular weight of 13—15 kDa which had a capacity to complex with fluphenazine (Sparrow and England 1984). The Ca^{2+}-binding site of the latent water-splitting system shows properties which resemble those of the Ca^{2+}-binding center of calmodulin (Ono and Inoue 1983). The electron transport at the donor side of PSII was shown to be inhibited by agents that influence Ca^{2+}--dependent processes, such as chelators (Barr et al. 1980), calmodulin antagonists (Barr et al. 1982; Chamorovsky and Matorin 1984) and agents blocking the calcium channels (Carpentier and Nakatani 1985).

It is known that membrane processes involving Ca^{2+} and calmodulin are effected by local anesthetics (Low et al. 1979; Volpi et al. 1981). In the present work the effects of these substances on electron transport in chloroplasts were investigated.

Materials and Methods

Chloroplasts were isolated from 12—14-day-old pea seedlings. The incubation medium contained 300 mmol.l^{-1} NaCl, 30 mmol.l^{-1} Tris-HCl buffer (pH 7.8) 2 mmol.l^{-1} $MgCl_2$ and 0.5 mmol.l^{-1} EDTA. The homogenate of leaves was filtered through a caprone cloth and the obtained suspension was centrifuged at 300 × *g* for 1 min. The precipitate was discarded. Chloroplasts were sedimented by centrifugation at 2500 × *g* for 7 min and resuspended in the incubation medium. The chlorophyll content was determined by the method of Arnon (1949).

Electron transfer rates were measured by probing the reduction of DPIP* in illuminated chloroplasts. Rates of oxygen evolution were measured polarographically. The activity of PSI was determined from the oxygen uptake rate after the addition of MV (0.5 mmol.l^{-1}), sodium ascorbate (1 mmol.l^{-1}), DPIP (0.25 mmol.l^{-1}) and DCMU (0.005 mmol.l^{-1}). ESR spectra were recorded using an RE 1303 radiospectrometer. Data in the Figures and Tables are means of two-four experiments. The variance (σ) did not exceed 6 %.

Results

Local anesthetics used in the experiments were dibucaine, tetracaine, trimecaine and benzocaine. The addition of an anesthetic to the chloroplast suspension inhibited electron transport via the $H_2O \rightarrow$ DPIP path (Table 1). The effects of phenol and butanol, known to have weak anesthetic action are also shown. The

* Abbreviations: MV, methylviologen; DPIP, 2,6-dichlorphenolindophenol; DPC, diphenylcarbazide; CCCP, carbonylcyanide-*m*-chlorphenylhydrazone; DCMU, 3-(3,4-dichlorphenyl)-1,1-dimethylurea.

Table 1. Effect of anesthetics on electron transport activity in chloroplasts

Anesthetic	Anesthetic concentration of 50 % inibition of electron transport (mmol . l^{-1})		
	$H_2O \rightarrow DPIP$[a]	$H_2O \rightarrow$ ferricyanide[b]	$DPIP \rightarrow MV$[c]
Butanol	120	132	660
Phenol	15	13	190
Trimecaine	13	13.2	34.5
Tetracaine	5.3	5	11.5
Benzocaine	4	4.4	more than 10[d]
Dibucaine	1.5	1.7	9

The incubation medium contained sucrose 0.2 mol . l^{-1}, Tris-HCl 40 mmol . l^{-1} (pH 7.9), $MgCl_2$ 2 mmol . l^{-1}, KCl 10 mmol . l^{-1}. Chlorophyll concentration was 18 μg/ml. Incubation time: 1 min

[a] Chloroplasts in the incubating medium in the presence of 35 μmol . l^{-1} DPIP, the rate of DPIP reduction in control was 114 μmol DPIP/mg chl . h.

[b] Chloroplasts in the incubating medium in the presence of 0.25 mmol . l^{-1} ferricyanide, the rate of O_2 evolution in control was 63 μmol O_2/mg chl . h.

[c] Chloroplasts in the incubation medium in the presence of 0.5 mmol . l^{-1} MV, 1.0 mmol . l^{-1} Na ascorbate, 0.25 mmol . l^{-1} DPIP, 5 μmol . l^{-1} DCMU; the rate of O_2 consumption in controls was 206 μmol O_2/mg chl . h.

[d] Benzocaine concentrations below 10 mmol . l^{-1} did not inhibit electron transport, those exceeding 10 caused chloroplast precipitation.

inhibitory potency of the anesthetics studied increased in the order butanol < < phenol < trimecaine < tetracaine < benzocaine < dibucaine, when used in concentrations which block nerve excitation (Low 1979). It could be concluded that the site of inhibition is PSII. This was suggested by the fact the inhibition of the PSI electron transport required much higher anesthetic concentrations than suppression of oxygen evolution. In a chloroplast suspension inactivated by trimecaine, the addition of DPC, an artificial electron donor for PSII, caused recovery of PSII electron transport activity (Fig. 1). In chloroplasts with the water-splitting system inactivated by Tris the electron transport from DPC to DPIP could be inhibited by higher anesthetic concentrations (Fig. 2). The data suggest that local anesthetics interact predominantly with the water-splitting site of PSII.

The ionic strength of the solution had a significant effect on the potency of local anesthetics (Table 2). It is evident that the inhibitory effect of dibucaine depends not only on the ionic strength but also on pH. The results suggest that the interaction between chloroplast and anesthetic modified by the electric charge of the anesthetic and/or the membrane. The local anesthetics of the procaine series are either positive charged or carry no charge, depending on pH.

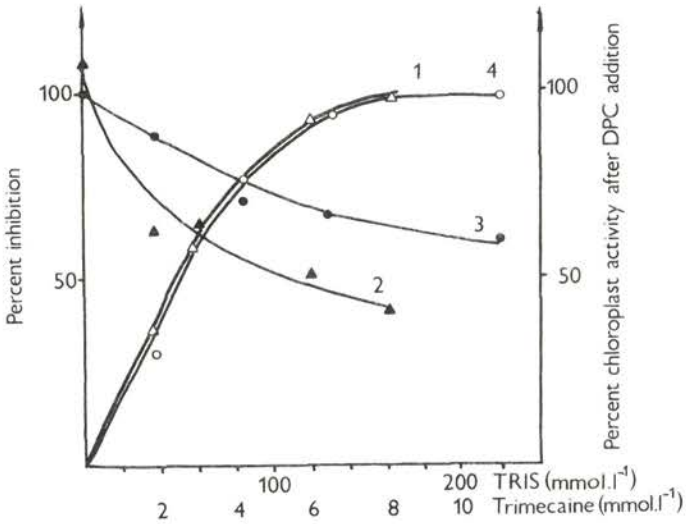

Fig. 1. Recovery of electron transport activity of chloroplasts inactivated by trimecaine or Tris after the addition of DPC. The incubation medium: sucrose 0.2 mol.l^{-1}, Tris-HCl 40 mmol.l^{-1} (pH 8.15), $MgCl_2$ 2 mmol.l^{-1}, KCl 10 mmol.l^{-1}. Chlorophyll concentration: 17 μg/ml; DPC 1.2 mmol.l^{-1}. Rate of DPIP reduction in controls: 90 μmol/mg chl.h. Percent inhibition by trimecaine (1), Tris (4). The activity of chloroplasts inhibited by trimecaine (2) or Tris (3) after the addition of DPC.

It was therefore useful to examine the pH dependence of their effects on chloroplast electron transport. Fig. 3 shows the pH dependence of the inhibitory effect of trimecaine on electron transport via the $H_2O \rightarrow$ DPIP path. The inactivation of O_2 evolution increased with increasing pH. The pattern of the inhibitory power indicates that the process in associated with deprotonation of either the anesthetic or some functional group of the membrane protein. Recently it was shown that the amino acid groups with pK around the 7.8 region are involved in the operation of the O_2 generating system (Laszlo et al. 1984). This groups, however, do not interact with local anesthetics. This is evidenced by the observation that benzocaine, a neutral anesthetic, exhibits no pH dependence. Obviously, the pH-dependent changes in trimecaine activity result from its protonation. This is suggested by the fact that pH of the 50 % inhibitory effect is dependent on the anesthetic concentration. This would not be the case if the activity of the anesthetic was determined by deprotonation of membrane groups (Fig. 4). pK for tetracaine is 8.5 (Ritchie and Greengard 1961).

In a study of the effects of local anesthetics on mitochondrial electron transport, the anesthetics were found to possess protonofore activity (Garlid

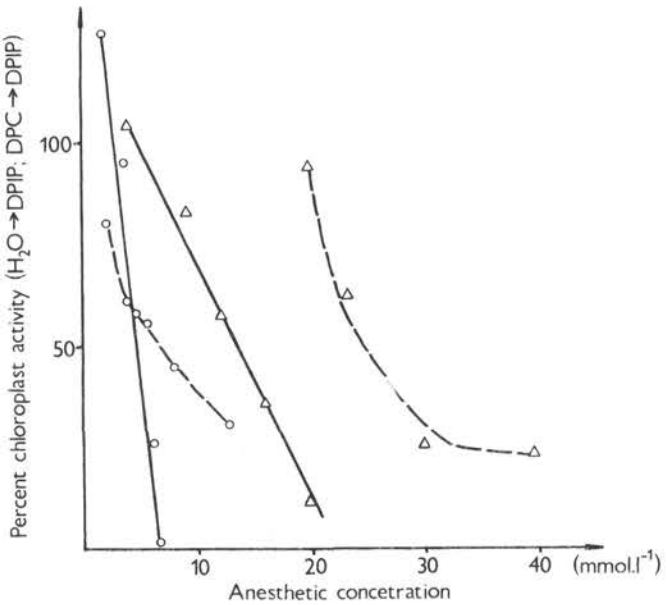

Fig. 2. The effect of local anesthetics tetracaine (○) and trimecaine (△) on the electron transport chain: $H_2 \rightarrow$ DPIP (———), DPC → DPIP (– – –). The incubation medium: Sucrose 0.2 mol . l^{-1}, Tris-HCl 40 mmol . l^{-1} (pH 7.9), $MgCl_2$ 2 mmol . l^{-1}, KCl 10 mmol . l^{-1}. Chlorophyll concentration: 9.5 μg/ml, DPC 0.6 mmol . l^{-1}. The water-splitting system was inactivated by incubation of chloroplasts in 0.6 mol . l^{-1} Tris-HCl buffer (pH 8.2) for 5 min. The rate of DPIP reduction in controls: 75.8 μmol/mg chl . h ($H_2O \rightarrow$ DPIP) and 32 μmol/mg chl . h (DPC → DPIP).

and Nakashima 1983). The protonophores are known to be able to inactivate O_2-production by chloroplasts at alcaline pH (Cohen et al. 1975). It was however unknown whether or not the local anesthetics produce the protonophoric effect on chloroplasts. We investigated the effects of small concentrations of an anesthetic (tetracaine) on the electron transfer rate at neutral pH. Fig. 5 shows that low concentrations of tetracaine similary as the known protonophores CCCP and NH_4Cl, enchance DPIP reduction, indicating that tetracaine can act as a protonophore. The inhibition of water splitting by the anesthetics observed in our experiments does not result from their protonophore (data not shown), effectively inactivates water splitting.

It is evident that the pH-dependent activation of the inhibitory effect of anesthetics is associated with the presence of the neutral form of the anesthetic. Deprotonation of anesthetics is shown to be accompanied by a significant redistribution of their contents between lipids and water. For tetracaine the lipid/water distribution factor is 22 at pH 5.5 and 660 at pH 9.5 (Boulanger et al. 1980). The activation of the inhibitory activity of the anesthetic during its

Table 2. Effect of ionic strength of the medium on the inhibitory action of dibucaine during DPIP reduction

Medium	Dibucaine concentration, mmol . l^{-1}	Percent inhibition of chloroplasts activity ($H_2O \rightarrow$ DPIP)
pH 7.3		
Sucrose 0.2 mol . l^{-1}, NaP_i		
50 mmol . l^{-1}		17
+ 150 mmol . l^{-1} NaCl	0.66	39
+ 300 mmol . l^{-1} NaCl		44
pH 7.75		
Sucrose 0.2 mol . l^{-1}, NaP_i		
50 mmol . l^{-1}		28
+ 100 mmol . l^{-1}		67
+ 150 mmol . l^{-1}	0.33	75
+ 200 mmol . l^{-1}		100
pH 7.67		
Sucrose 0.2 mol . l^{-1},		
Tris 40 mmol . l^{-1}		13
+ 15 mmol . l^{-1} $MgCl_2$	0.33	20
+ 30 mmol . l^{-1} $MgCl_2$		31
pH 7.94		
Sucrose 0.2 mol . l^{-1},		
Tris 40 mmol . l^{-1}		45
+ 15 mmol . l^{-1} $MgCl_2$	0.33	67
+ 30 mmol . l^{-1} $MgCl_2$		100

deprotonation may be a consequence of an increase of its concentration in the membrane. Nevertheless, it cannot be ruled out that the observed pH dependence may be a result of different chemical activities of the protonated and deprotonated form. For example, Tris, a hydrophilic amine exhibits a stronger activity in interactions with the water-splitting complex when in neutral form (Yamashita and Butler 1969).

Using the fact that neutral and charged forms of anesthetics have different inhibitory power, we estimated the rate of inhibition of electron transport and the effect of light on the process. pH of the incubation medium containing suspended chloroplasts was adjusted to neutral after a specified interval following the addition of trimecaine (Table 3). The inactivation was slow: after 2 min of incubation in the presence of the anesthetic, the chloroplast retained 40 percent of its activity; complete inactivation occurred only after 5 min. In contrast, with no change in pH, a 100 percent inhibition was obtained as early as after 10 s. This indicates that illumination of chloroplasts with the anesthetic

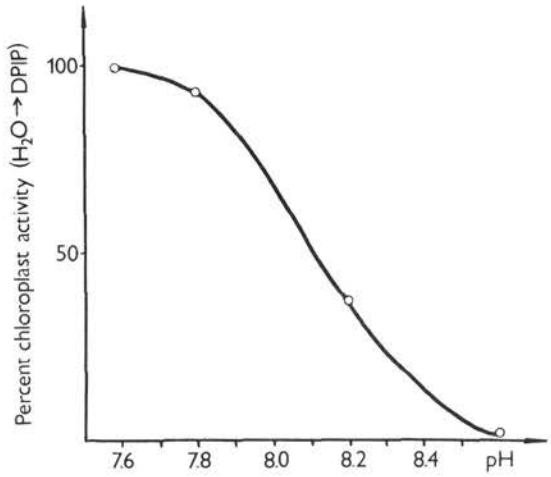

Fig. 3. pH dependence of inhibitory activity of trimecaine (4.12 mmol . l^{-1}). For the composition of the incubation medium see legend to Fig. 2. Chlorophyll concentration 17 μg/ml.

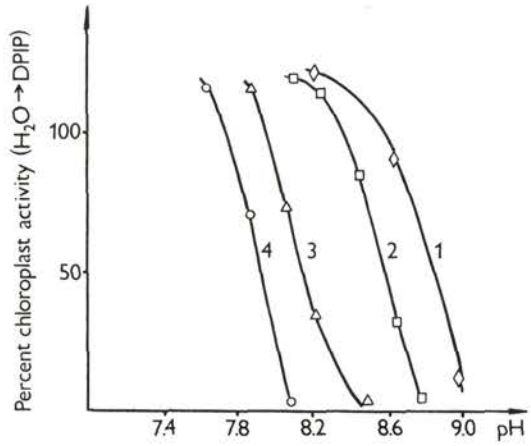

Fig. 4. The inhibitory power of tetracaine as a function of pH for different tetracaine concentrations: 0.28 mmol . l^{-1} (1), 0.57 mmol . l^{-1} (2), 1.73 mmol . l^{-1} (3), 3.75 mmol . l^{-1} (4). For the composition of the incubation medium see legend to Fig. 2. Chlorophyll concentration 19 μg/ml; incubation time 15 s.

during acting measurement produced an immediate inactivation. Hence, in the dark inactivation of electron transport by the anesthetic is quite a slow process. It is probably due to a detergent-like effect, accompanied by a structural alteration of the membrane. The effect is largerly accelarated in light, probably

Table 3. Changes of the inhibitory activity of trimecaine on decreasing pH of the medium. Chloroplasts incubation medium (sucrose 0.2 mol . l^{-1}, Tris-HCl 40 mmol . l^{-1}, $MgCl_2$ 2 mmol . l^{-1}, KCl 10 mmol . l^{-1}), chlorophyll concentration 23.5 μg/ml. pH of the medium was decreased to 7.2 after incubating the samples in the dark.

Time of incubation	Sample	Percent chloroplast activity (H_2O — DPIP)	
		pH 8.5	pH 8.5 → 7.2
10 s	control	100	89
	+ trimecaine 5.5 mmol . l^{-1}	6	109
2 min	control	100	100
	+ trimecaine 5.5 mmol . l^{-1}	0	41
5 min	control	100	87
	+ trimecaine 5.5 mmol . l^{-1}	0	6

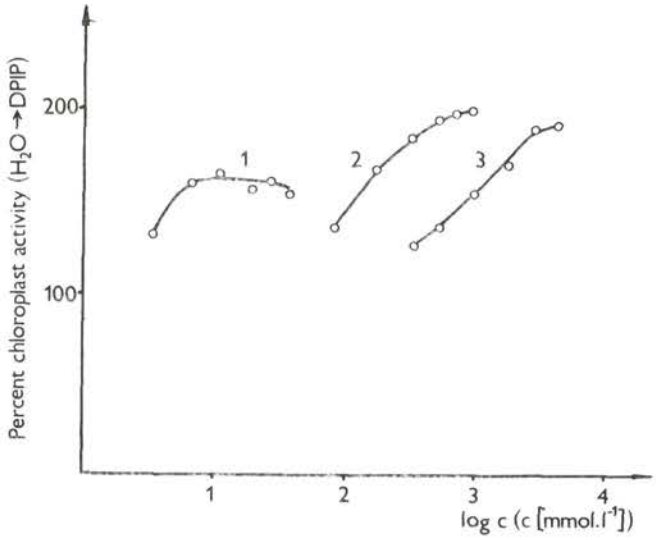

Fig. 5. Effect of protonophores and tetracaine on the rate of DPIP reduction. The incubation medium contained: Sucrose 0.2 mol . l^{-1}, Tris-HCl 40 mmol . l^{-1} (pH 7.4), $MgCl_2$ 2 mmol . l^{-1}, KCl 10 mmol . l^{-1}. Chlorophyll concentration: 12 μg/ml. The rate of DPIP reduction in controls (with no addition): 80 μmol/mg chl . h. CCCP — 1; tetracaine — 2; NH_4Cl — 3.

due to the higher S-states of the water-splitting system, induced by light which interacts efficiently with the inhibitor. A similar mechanism is operative with Tris (Cheniae and Martin 1978).

ESR spectra of chloroplasts treated with tetracaine showed a disappearance of ESR signal II_s. The reduction of signal II_s after tetracaine was concentration

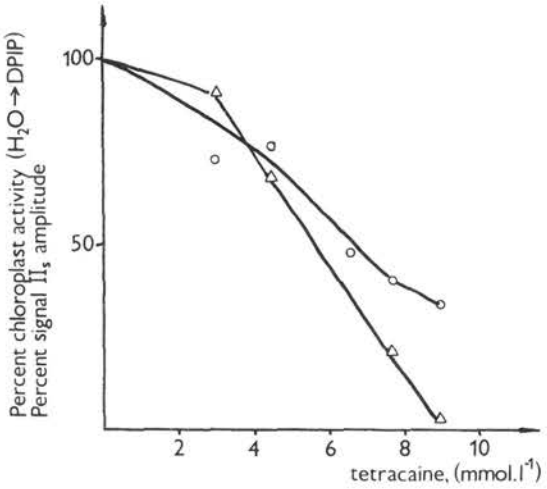

Fig. 6. Effect of tetracaine on the rate of DPIP reduction (△) and signal II_s amplitude (○). The incubation medium contained: sucrose 0.2 mol.l^{-1}, Tris-HCl 40 mmol.l^{-1}, $MgCl_2$ 2 mmol l^{-1}, KCl 10 mmol.l^{-1}. Chloroplasts (180 μg chl/ml) in incubation medium, pH 8.4, were treated by tetracaine during 1 min; then one part of the mixture was diluted to obtain chlorophyll concentration of 18 μg/ and the reduction activity of chloroplasts was measured; the other portion was centrifuged at 5100 × g for 4 min, the resulting pellet was resuspended in incubation medium (pH 7.0) to a final chlorophyll concentration of 4.5 mg/ml and signal II_s was measured. ESR conditions: modulation amplitude, 4G; scan rate, 50 G/min; time constant, 0.1 s.

and pH dependent and correlated with the inhibition power of the anesthetic (Fig. 6).

Discussion

Our results show that local anesthetics block electron transport at the donor side of PSII before of the site of DPC involvement. An important observation is that the inhibitory effect correlates with the anesthetic activity. This, together with the vast amount of data on interaction of anesthetics with membranes, may be useful in studies of the structural and functional organization of the water-splitting complex.

One possible mechanism that has been advanced in attempt to explain the anesthetic activity is that the anesthetic effect is due to some modification of the lipid phase. Numerous reports have shown that anesthetics can significantly influence the structural organisation lipid bilayers. The effect include widening

of the lipid membrane without changing ist thickness (Kita et al. 1981), reduction of temperature of phase transition of the lipids (Hill 1974); lowering of the viscosity of the lipid bilayer (Gordon et al. 1980). The ability to change the permeability and phase properties of lipid bilayers decreases in the order dibucaine > tetracaine > procaine, i.e., in parallel to the anesthetic activity (Singer and Jain 1980). There are also data showing that modification of the lipid phase affects PSII electron transport activity. Gounaris and coworkes (1983a) showed that thermal inactivation of water splitting is accompanied by a redistribution of lipids that form the bilayer structure. They also observed inhibition of oxygen evolution in PSII particles after the addition of negatively charged lipids (Gounaris et al. 1983b). Incubation of lipids with weakly active PSII particles containing a small amount of high-potential cytochrome b-559 caused the low potential cytochrome to convert to high-potential form, and also resulted in a significant increase in the rate of oxygen evolution (Matsuda and Butler 1983). An interesting fact is that the viscosity of the lipid phase in PSII particles is greater than that of stromal membrane (Ford et al. 1982; Aronson et al. 1983).

The above facts, together with the correlation observed between inhibitory power and anesthetic activity, suggest that the effect is either by a modification of the lipid surrounding of the water-splitting complex or by an internaction of the anesthetic with the hydrophobic regions at the donor side of PSII. Buchet and coworkes (1985) reported that the interaction of anesthetics with the hydrophobic region is accompanied by breaks of hydrogen bonds.

Another possible mechanism of the action of anesthetics is an effect on Ca^{2+}-binding components which correlates with the anesthetic activity (Low et al. 1979). The local anesthetics are also known to be antagonists of calmodulin (Volpi et al. 1981). On the other hand, data are available which show the involvement of Ca^{2+} and/or calmodulin in the operation of electron transport at the donor side of PSII (Brand 1979; Barr et al. 1982; Ghanotakis et al. 1984). Fluorescence data showed that the site of inhibition of electron transport by depletion of Ca^{2+} is located close to the reaction center (Brand et al. 1983). Hales and Gupta (1981) suggested that Ca^{2+} binds to carrier Z. They observed that signal II is reduced in amplitude in cells of blue-green algae grown in Ca^{2+} poor medium and recovers after the addition of Ca^{2+}. The metal/quinone complex has been proposed as a possible candidate for the role of carrier Z. In this complex the metallic component is an ion of a divalent alkaline metal (Hales and Gupta 1981). All these results taken together point to the possible participation of Ca^{2+} in the formation of signal II. The disappearence of signal II_s in the presence of anesthetics may be considered inderect evidence for Ca^{2+}-linked action of anesthetic on the photosynthetic electron transport.

Our results provide evidence that local anesthetics inhibit electron transport at the donor side of PSII. To date it is difficult to conclude which of the two

mechanisms — modification of the membrane hydrophobic region or influence on Ca^{2+}/calmodulin-dependent processes, is responsible for this effect. The analysis of reports in the literature, supported by the similarity with the chlorpromazine effect (as shown by fluorescence data, not shown), speak in favour of the second mechanism.

Acknowledgement. The authors wish to thank Dr. K. N. Timofeev for technical assistance in ESR-spectroscopy and L. Spektrova for translating the manuscript.

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Final version accepted December 12, 1988