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INHIBITION OF PHOTOSYSTEM II BY UNCOUPLERS AT ALKALINE pH AND ITS REVERSAL BY ARTIFICIAL ELECTRON DONORS

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SUMMARY

When chloroplasts are aged for 5 min at pH 9.6, or are exposed to uncouplers at pH 8.5–9.0, electron flow from water to Hill acceptors is inhibited. Both treatments induce rapid millisecond dark decay of delayed light emission. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea-sensitive electron transport through Photosystem II can be regenerated in both types of inhibited chloroplasts by the artificial electron donor, 1,5-diphenylcarbohydrazide. Neither treatment inhibits electron flow through Photosystem I. Uncouplers at alkaline pH, when added in the light, are less effective in producing the inhibition than when added in the dark. These results are interpreted as indicating inhibition of the oxygen-evolving apparatus by alkaline intrathylakoid pH.

INTRODUCTION

Uncouplers of photophosphorylation produce an acid shift of the pH optimum of the Hill reaction [1, 2] from about pH 9.0 to pH 8.0 or below. Above a pH of about 8.5, uncouplers actually inhibit the Hill reaction below the basal level and this inhibition becomes quite severe by pH 9.0.

In the present study, we have found that chloroplasts exposed to a wide variety of uncouplers at alkaline pH exhibit a very rapid decay of the delayed light emission in the absence of a Hill acceptor. This type of rapidly decaying emission has been associated with conditions that damage the water-splitting side of Photosystem II, such as Tris-ageing [3] or heating [4]. We have also observed that DCMU-

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CF_1, chloroplast photophosphorylation coupling factor; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbohydrazide; FMN, flavin mononucleotide; TES, (*N*-tris(hydroxymethyl))methyl-2-aminoethane sulfonic acid; Tricine, (*N*-tris(hydroxymethyl))-methyl glycine; Tris, (*N*-tris(hydroxymethyl))aminomethane.

sensitive photoreduction of Hill acceptors can be restored in the uncoupler-inhibited chloroplasts by the addition of artificial electron donors, such as DPC.

Our results suggest that it is the oxygen-evolving side of Photoreaction II that is damaged when chloroplasts are exposed to uncouplers at an alkaline external pH. This inhibition may be directly related to the elevated intrathylakoid pH associated with the decrease in the transmembrane pH gradient caused by uncouplers. This interpretation is in agreement with a similar suggestion recently made by Harth et al. [5].

MATERIALS AND METHODS

Chloroplasts were prepared from greenhouse-grown Good King Henry (*Chenopodium bonicus henricus*) or from market spinach by the method of Jagendorf and Avron [6]. The grinding medium contained 0.4 M sucrose, 0.05 M Tricine–NaOH (pH 7.8), and 0.01 M NaCl. Chloroplasts were resuspended in 100 mM sucrose, 20 mM NaCl and 5 mM TES (pH 7.4), and were stored on ice until use. Chlorophyll was determined according to Arnon [7].

EDTA-treated chloroplasts were prepared according to Cohen and Bertsch [8]. Chloroplasts were aged at alkaline pH by incubation in a medium containing 20 mM NaCl, 50 mM Tricine and 2 mM $MgCl_2$, adjusted to pH 9.6 at a concentration of 100 μg chlorophyll/ml for 5 min at 0 °C in the dark, followed by immediate titration to pH 7.5 and storage on ice until use.

Measurement of DCPIP reduction was carried out using an Aminco-Chance Dual-wavelength spectrophotometer following the absorbance change at 590–470 nm. The red actinic light, provided by a 650 W tungsten iodide lamp, was filtered through a red glass (Schott RG665) and a heat filter (Schott KG-1). The light intensity incident of the sample was $1.25 \cdot 10^6$ erg/cm^2/s. The photomultiplier tube was protected from the actinic light by two blue glass filters (Corning 4-76 and 4-96) and a green Kodak Wratten filter (No. 57). Ferricyanide reduction was monitored in the same instrument following the change at 420–480 nm. The exciting light was the same and the photomultiplier was protected by a blue glass filter (Corning 4-72) and 1 cm of a $CuSO_4$ solution (200 g/l). We report initial rates of reduction.

Oxygen evolution in the presence of a Hill acceptor was monitored using a Beckman 39065 electrode mounted in a specially built, thermostatically controlled lucite chamber. The exciting light. provided by a 650 W tungsten iodide lamp, was passed through a red glass filter (Corning 2-62) and 8 cm of H_2O. The same apparatus was also used to monitor O_2 consumption associated with the reduction of methyl viologen.

Delayed light emission from 0.8 to 3.0 ms after the centers of repeating flashes of white light was measured with a modified Becquerel phosphoroscope which has been described previously [9]. The sample received 250 flashes/s and each flash had a duration of 0.4 ms. To increase the sensitivity of the measurement a RCA No. 8852 "Quantacon" photomultiplier was used to detect the delayed light. All samples were exposed to 30 s of flashes at 10 % intensity and then 30 s at full intensity to reach a steady-state before data were taken by photographing the oscilloscope screen. Then a Hill acceptor ($K_3Fe(CN)_6$ or DCPIP) was injected into the sample and a steady-state was again reached before data were photographed in the presence of the acceptor.

RESULTS

Table I shows the effects of gramicidin at pH 7.5 and 8.8 on electron-transport rates through both photosystems, and through Photosystem I alone. Electron flow through both photosystems was stimulated several fold by the uncoupler at pH 7.5, but was severely inhibited at pH 8.8. In contrast, the Photosystem I reaction was stimulated by the uncoupler both at pH 7.5 and at pH 8.8.

Similar effects were observed with the following uncouplers: NH_4Cl (10 mM), nigericin (0.5 μM), chloroquinphosphate (0.1 mM), CCCP (5 μM), methylamine (40 mM), hexylamine (5 mM) and in EDTA-treated chloroplasts. In each case the indicated concentration of the uncoupler-stimulated electron transport at pH 7.5,

TABLE I

THE EFFECT OF GRAMICIDIN ON ELECTRON TRANSPORT AT pH 7.5 AND AT pH 8.8

The 5 ml reaction mixture for $H_2O \rightarrow$ methyl viologen contained 20 mM NaCl, 50 mM Tricine (pH 7.5 or 8.8), 2 mM $MgCl_2$, 0.1 mM methyl viologen, 0.5 mM NaN_3, and Good King Henry chloroplasts equivalent to 100 μg chlorophyll. For DCPIP $\rightarrow$ methyl viologen, the following components were added: 0.5 mM neutralized ascorbate, 0.04 mM DCPIP and 0.002 mM DCMU. Gramicidin, when added, was 1 μM final concentration. Rates are expressed as μequiv. of electrons/mg chlorophyll/h.

Condition	Rate of electron transport	
	$H_2O \rightarrow$ methyl viologen	DCPIP $\rightarrow$ methyl viologen
pH 7.5	103	111
+gramicidin	803	480
pH 8.8	332	148
+gramicidin	69	554

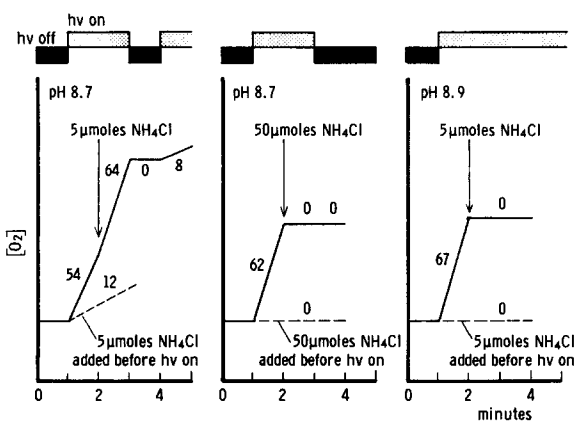
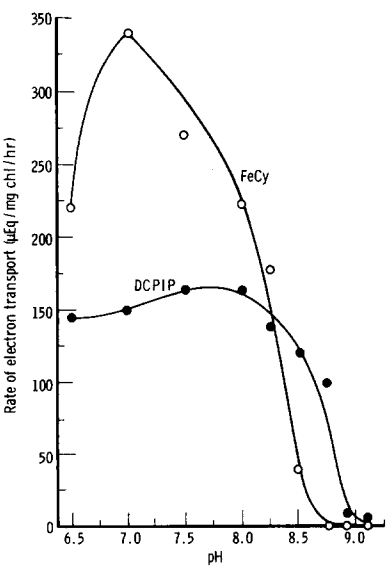


Fig. 1. Effect on O_2 evolution of NH_4Cl concentration, pH, and time of uncoupler addition. The reaction mixture contained, in 5 ml: 20 mM NaCl, 50 mM Tricine (pH 8.7 or 8.9), 2 mM $MgCl_2$, 0.4 mM $K_3Fe(CN)_6$ and spinach chloroplasts equivalent to 100 μg of chlorophyll. The numbers above the traces indicate the rates of O_2 evolution in μmoles/mg chlorophyll/h. The dashed lines indicate experiments in which the uncoupler was added just before turning the light (hv) on.

but inhibited electron flow from Photosystem II at higher pH values (8.5–9.0). The pH at which significant inhibition of Photosystem II activity occurred varied slightly from day to day, but always fell into the range of pH 8.5–9.0 with the above uncoupler concentrations.

Uncoupler-induced inhibition depended on the uncoupler concentration, and uncouplers were observed to inhibit more effectively when added in the dark. These effects are illustrated for NH_4Cl in Fig. 1. As shown in this figure, with a low concentration of NH_4Cl (1mM) the inhibition occurred only if the uncoupler was present with chloroplasts in the dark. When the pH was raised, this uncoupler concentration inhibited even when added in the light. At a higher concentration of the uncoupler (10 mM) the inhibition was independent of when the uncoupler was introduced. A similar pattern was observed with gramicidin and other uncouplers.

Fig. 2 compares the pH dependence, in the presence of gramicidin, of the ferricyanide and DCPIP Hill reactions. The pH dependencies of the coupled reactions were very similar to one another and were comparable to those observed previously [10]. Both uncoupled Hill reactions were inhibited at alkaline pH, but the pH at which this inhibition occurred was reproducibly slightly lower for ferricyanide than for DCPIP. Hill reactions using methyl viologen or FMN as acceptors showed a pH dependence similar to that of the DCPIP Hill reaction.



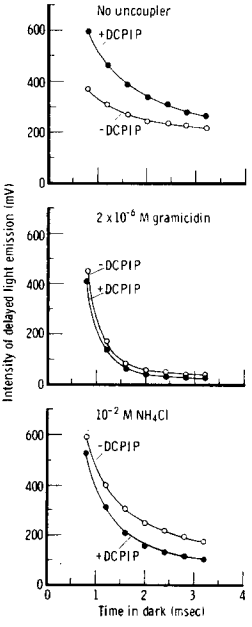


Fig. 2. pH dependence of electron transport in the presence of 1 μM gramicidin. Reaction mixtures contained, in 5 ml: 20 mM NaCl, 2mM $MgCl_2$ spinach chloroplasts equivalent to 50 μg chlorophyll, either 50 mM TES (pH 6.5–7.5) or 50 mM Tricine (pH 8.0–9.5), and either 0.3 mM potassium ferricyanide (FeCy) or 0.012 mM DCPIP. Rates were determined spectrophotometrically.

Fig. 3. Effects of gramicidin and NH_4Cl at pH 8.8 on ms delayed light emission. Reaction mixtures contained, in 2 ml: 20 mM NaCl, 50 mM Tricine (pH 8.8), 2 mM $MgCl_2$, spinach chloroplasts equivalent to 20 μg chlorophyll, and, when added, 0.001 mM DCPIP.

Fig. 3 shows the effects of gramicidin and of NH_4Cl on ms-delayed light emission and electron transport from chloroplasts at pH 8.8. The effects of uncouplers at this pH were quite different from those observed at pH 7.5 [11, 12]. At pH 7.5, uncouplers lower the intensity of the emission without causing a large change in the kinetics of the dark decay regardless of presence or absence of acceptors. At pH 8.8, on the contrary (in the absence of an electron acceptor), uncouplers caused a marked increase in the decay kinetics, and this rapid dark decay was accompanied by an increase in the intensity of the emission at 0.8 ms after illumination (Fig. 3). Addition of a Hill acceptor, in this case, had little or no effect.

Fig. 3 also shows that at pH 8.8, gramicidin and NH_4Cl had somewhat different effects on delayed light emission. Gramicidin at high pH caused the emission to decay extremely rapidly. This was also the case when nigericin was used as the uncoupler and following EDTA treatment. With NH_4Cl the dark decay was not quite so fast, the major effect of the uncoupler being an overall rise in the intensity of the delayed emission. This effect is duplicated at pH 7.5 if the concentration of NH_4Cl is raised to 10^{-1} M. Under these conditions inhibition of Photosystem II was also observed. Again it was found that electron transport to ferricyanide was more sensitive to this type of inhibition than electron transport to DCPIP; that is, at pH 7.5, lower concentrations of NH_4Cl were needed to inhibit ferricyanide reduction than DCPIP reduction. In the presence of 10^{-6} M valinomycin, inhibition of electron transport was observed at lower NH_4Cl concentrations than were otherwise necessary

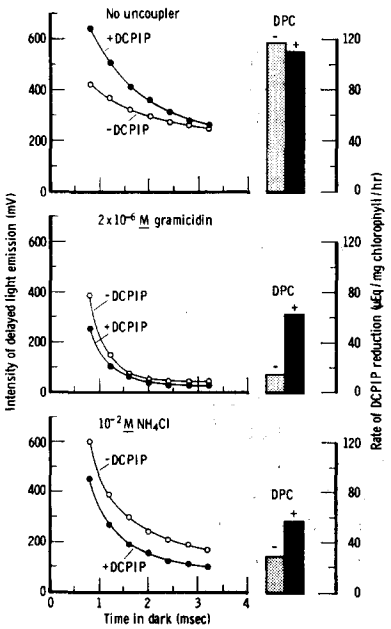


Fig. 4. Effect of DPC on ms-delayed light emission in the presence of gramicidin or NH_4Cl at pH 8.8. Reaction conditions for delayed light emission were the same as those in Fig. 3, except that all reaction mixtures contained 0.5 mM DPC. Rates of DCPIP reduction were determined spectrophotometrically as in Fig. 2.

(data not shown). Delayed light emission in the valinomycin plus NH_4Cl case resembled that observed with gramicidin at alkaline pH.

We also examined the effect of alkaline pH in the absence of uncouplers. Ageing chloroplasts at pH 9.6 severely inhibited electron transport involving Photosystem II, but did not inhibit electron flow through Photosystem I. ms-Delayed light emission from chloroplasts aged at pH 9.6 had characteristics similar to those shown in Fig. 3 for gramicidin at pH 8.8.

The extremely rapid decay of the delayed light emission observed in the absence of a Hill acceptor at pH 8.8 with gramicidin, or observed following alkaline incubation, is similar to that observed in chloroplasts which have been Tris-aged [3] or heated [4]. In all these systems electron transport from Photosystem II can be restored by the addition of exogenous electron donors. Fig. 4 shows the effect of the electron donor DPC on the photoreduction of DCPIP, and on the delayed emission, from chloroplasts at pH 8.8 in the presence of NH_4Cl and gramicidin. All the incouplers mentioned above were similarly tested, and in all cases DCMU-sensitive electron transport was restored in the presence of DPC. This was also true of chloroplasts which had been inhibited by a 5 min incubation at pH 9.6.

In preliminary experiments (data not shown) we observed that variable fluorescence was severely attenuated when chloroplasts at pH 8.8 were exposed to either gramicidin or NH_4Cl. Addition of the electron donor DPC had little or no effect on the level of variable fluorescence in the uncoupler-treated chloroplasts.

DISCUSSION

Exposure of chloroplasts to uncouplers of photophosphorylation at alkaline pH (8.5–9.0), or incubation of chloroplasts at pH 9.6, results in inhibition of electron transport reactions involving Photosystem II, but not those which involve only Photosystem I. Associated with the inhibition of electron transport is a rapid decay of ms-delayed light emission in the absence of an electron acceptor. In addition, variable fluorescence is considerably lowered when chloroplasts are exposed to NH_4Cl or gramicidin at pH 8.8. These characteristics of delayed light emission [3] and fluorescence [13] have been associated with treatments that damage the O_2-evolving apparatus of Photosystem II. This interpretation is supported by restoration of DCMU-sensitive electron transport upon the addition of the artificial electron donor DPC.

However, the addition of DPC to chloroplasts inhibited by uncouplers at high pH does not restore the normal decay of the delayed light (Fig. 4), nor does it increase the level of the variable fluorescence as is the case when electron donors are added to Tris-aged chloroplasts [3, 13]. The failure of donors to affect prompt or delayed fluorescence has also been noted in chloroplasts in which the O_2-evolving apparatus has been inactivated by ultraviolet irradiation or NH_2OH treatment [14–16]. This may suggest that different sites in the O_2-evolving apparatus are affected by these treatments than are affected by Tris-ageing.

It has previously been suggested that the rate of electron transport is dependent on the internal pH (designated pH_i) of the thylakoid [10, 17]. Our results support this idea and indicate that the O_2-evolving apparatus is a site which is sensitive to changes in the pH_i, in agreement with the recent suggestion of Harth et al. [5].

Uncouplers reduce ΔpH established in the light [10, 18] and lead to an elevation of the internal pH. Incubation of chloroplasts at pH 9.6 for 5 min in the absence of uncouplers could also lead to an elevation of the internal pH to a level sufficient to inactivate the O_2-evolving apparatus.

It has been suggested by a number of workers that the O_2-evolving apparatus is located on the inner side of the thylakoid membrane [19–21]. This suggestion is consistent with our hypothesis that the O_2-evolving apparatus is sensitive to the pH of the intrathylakoid space.

The inhibition of electron transport caused by NH_4Cl at alkaline pH has previously been interpreted as being due to the free amine [22]. This is consistent with the fact that the inhibition can be achieved at lower pH (7.5) by a higher concentration of NH_4Cl. However, these data are also consistent with the idea that the internal pH of the thylakoid is the critical factor. The ΔpH across the thylakoid membrane is decreased, and the pH_i is raised, as the NH_4Cl concentration is increased.

The observation that the delayed light emission from NH_4Cl-inhibited chloroplasts differs from that observed from chloroplasts inhibited by gramicidin, nigericin, etc. may reflect some action of the amine in addition to its effect on ΔpH. In the presence of valinomycin, which increases the permeability of the thylakoid membrane to NH_4^+ [23], the delayed emission from NH_4Cl-inhibited chloroplasts is converted to a pattern like that from the other inhibited systems. This suggests that the unusual delayed light decay in the NH_4Cl-inhibited case may be related to the accumulation of NH_4^+ by the chloroplast or to the large amount of swelling that accompanies this accumulation. A similar interpretation of the effect of NH_4Cl on delayed light emission has recently been made by Felker et al. [12].

The observation that the effect of an uncoupler at alkaline pH may depend on whether it is introduced in the light or in the dark (Fig. 1) reflects the interaction of electron transport, ΔpH and pH_i. When chloroplasts are illuminated in the presence of an electron acceptor, the pH_i would fall as protons are pumped into the thylakoid. Addition of an uncoupler in the light would increase the rate of dissipation of the accumulated protons, thus raising pH_i. However, if this rate of dissipation were not too high compared to the rate of proton accumulation, the pH_i would not rise enough to inhibit oxygen evolution. This could result in an increase in the rate of electron transport by reducing the ΔpH against which protons must be pumped and/or by bringing the new pH_i closer to the optimal pH_i for electron transport. In contrast, addition of the uncoupler in the dark to chloroplasts at alkaline pH would alkalinize the interior to a much greater extent, causing inhibition of the water-oxidizing apparatus. If the concentration of uncoupler were raised, or if the external pH were too alkaline, addition of the uncoupler even in the light might result in the pH_i being raised to a level which inhibits the oxygen-evolving side of Photoreaction II.

The rate of electron transport in the presence of an uncoupler exhibits a rather abrupt decrease as the external pH is raised (Fig. 2). This would result from the fact that although the rate of electron transport is influenced by pH_i and ΔpH, it is electron flow that is responsible for the injection of protons into the thylakoid space. Thus, if the pH_i is initially only somewhat above that optimal for electron transport, then, upon illumination, the resulting electron transport will act to lower

the pH_i. This would result in an increased rate of electron transport which would further lower the pH_i, etc. Only at sufficiently high external pH is a point reached where inhibition of the oxygen-evolving apparatus by high pH_i causes such low initial rates of electron flow that the light-induced proton pump can no longer significantly compete with the rate of dissipation of protons caused by the uncoupler. The observation (Fig. 2) that electron transport to ferricyanide and to DCPIP in the presence of gramicidin are inhibited at slightly different pH values may be due to a difference between the two Hill reactions in the rate of proton accumulation into the thylakoid.

In conclusion, the inhibition of electron transport by uncouplers at alkaline pH is seen to be a direct result of the presumed mode of action of uncouplers in dissipating the proton gradient across the thylakoid membrane, together with the effect of the intrathylakoid pH on the oxygen-evolving apparatus of Photosystem II.

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