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THE ROLE OF CHLORIDE ION IN PHOTOSYNTHESIS

III. THE EFFECT OF ARTIFICIAL ELECTRON DONORS UPON ELECTRON TRANSPORT*

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SUMMARY

4- to more than 10-fold acceleration of the Hill reaction (electron acceptor: indophenol dyes, ferricyanide, FMN, *etc.*) by Cl^- is consistently observed over a wide range of pH (5.7–8.3) when EDTA-uncoupled chloroplasts are used as experimental material. Using such highly Cl^--sensitive chloroplasts the effect of Cl^- was examined on the photooxidation of reducing agents which are capable of donating electrons to Photosystem II. No Cl^- effect is observed on the photooxidation of hydroxylamine (at substrate level with indophenol dye as electron acceptor) or of ascorbate (FMN as acceptor). These observations strongly suggest that Cl^- acts near the water-splitting end of Photosystem II.

High concentrations of ammonia and methylamine, like low concentrations of hydroxylamine, block water splitting on the oxidizing side of Photosystem II. Apparently the unprotonated forms of simple amines act as a specific inhibitor at a point near the site of Cl^- involvement.

Upon removal of Cl^- the steady-state fluorescence yield of chloroplasts is markedly and reversibly depressed. However, the addition of a substrate level of hydroxylamine obliterates the Cl^- effect on fluorescence and the yield becomes higher than in the presence of Cl^- alone. These observations are discussed in connection with the site of Cl^- involvement and with the current model of the chloroplast fluorescence quenching mechanism.

INTRODUCTION

The stimulatory effect of Cl^- on the Hill reaction was discovered by WARBURG AND LÜTTGENS[1] and confirmed by other investigators[2,3]. The site of Cl^- involvement

Abbreviations: BDHB, *n*-butyl-3,5-diiodo-4-hydroxybenzoate; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DCPI, phenolindo-2,6-dichlorophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TCPI, *o*-chlorophenolindo-2,6-dichlorophenol; MES, 2-(*N*-morpholino) ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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was later shown by BOVÉ *et al.*[4] to be the segment of the electron transport chain near Photosystem II[5] but no further attempts were made to pinpoint the site. Recently, HIND *et al.*[6] demonstrated a marked Cl^- effect in chloroplasts uncoupled by various agents and confirmed that the Cl^- is closely related to Photosystem II. Fluorescence studies[7] further indicated a location for the Cl^- action on the oxidizing (water-splitting) side of Photosystem II.

Evidence is accumulating that various reducing compounds, such as ascorbate[8,9], phenylenediamine[10], and hydroxylamine[11], can donate electrons to the oxidizing side of Photosystem II and thereby replace water as an electron donor. These artificial donor systems offer a way to ascertain whether the site of Cl^- action is nearer the step specifically involved in water splitting, or the photoact itself. The primary purpose of this paper is to describe the results of such experiments.

METHODS

Chloroplasts

In most of the experiments chloroplasts were isolated from field-grown spinach (*Spinacia oleracea* L.) and were then uncoupled by EDTA treatment[12]. Leaves were homogenized in a Waring blender with a medium containing 0.2 M Na_2SO_4 and 0.03 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)–NaOH buffer at pH 7.4. The homogenate, filtered through cheese-cloth, was centrifuged at 2500 × *g* for 5 min. The sedimented chloroplasts were resuspended and washed once with a medium containing 0.1 M sucrose and 5 mM TES–NaOH at pH 7.4, twice with 2 mM Tricine and 0.5 mM Na–EDTA at pH 7.8 and finally with 0.1 M sucrose, 5 mM TES–NaOH and 5 mM $MgSO_4$ at pH 7.4. In a few cases (fluorescence experiments) pea leaves were used as material, and the chloroplasts were isolated by the method described elsewhere[6].

Reagents

Tricine, TES and 2-(*N*-morpholino)ethane sulfonic acid (MES) buffers were purchased from Calbiochem (Los Angeles). Indophenol dyes were purified by transfer of the acid form of the dye into chloroform, extraction into dilute alkali and precipitation of the sodium salt with excess sodium acetate. Hydroxylamine sulfate was recrystallized from an aqueous solution by adding excess ethanol at 0°. The pH of hydroxylamine solutions was adjusted to the reaction pH with NaOH before use.

Assays

The reduction of ferricyanide and indophenol dyes was observed spectrophotometrically by recording the absorbance changes at 420 nm and 620 nm, respectively, during illumination. The actinic light used for ferricyanide reduction and ascorbate photooxidation was a broad-band red light (approx. 600–700 nm) of 570 kergs·cm^{-2}·sec^{-1} intensity, while for indophenol dye reduction a blue actinic light was used (370–500 nm) with an intensity of 100 kergs·cm^{-2}·sec^{-1}. Oxygen was assayed with a Clark-type oxygen electrode. The reaction temperature was 20°.

The fluorescence of the chloroplasts was measured as previously described[7] using a modulated exciting beam (270 Hz) of blue light (436 nm) and, where indicated, a continuous blue actinic light (480–540 nm). In addition, in some experiments the

exciting beam was used unmodulated and the resulting signal from the photomultiplier was amplified with a DC-amplifier. The reaction temperature was 14°.

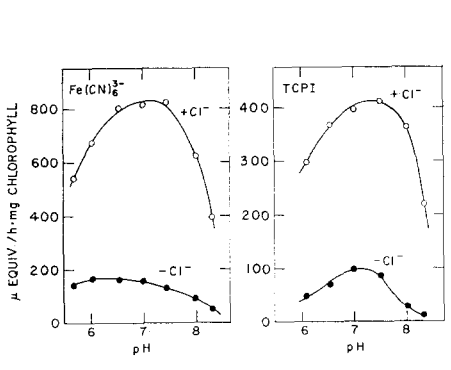
RESULTS

Effect of Cl^- on the Hill reaction of EDTA-uncoupled chloroplasts

Previously a marked and consistent Cl^- effect ($Rate_{+Cl^-}/Rate_{-Cl^-}$) on the Hill reaction was observed only at alkaline pH (>7.5) even with chloroplasts intensively washed in Cl^--free media[2,4,6]. Below this pH the Cl^- effect was, in most cases, near enough to unity to cast some doubt on the interpretation that Cl^- is an essential cofactor of photosynthetic electron transport. However, by using EDTA-uncoupled chloroplasts a Cl^- effect of greater than unity is demonstrable over a wide range of pH (Fig. 1). With either indophenol dye or ferricyanide as electron acceptor, the electron flow is increased 4- to more than 10-fold by Cl^- over a pH range of from 5.7 to 8.3. Only rarely did samples of EDTA-washed chloroplasts show a poor Cl^- effect (less than 2) at lower pH and these were readily rendered highly Cl^- sensitive upon exposing the chloroplast preparation to pH 8.1 for several minutes at room temperature. Immediately after such treatment the pH was brought back to the optimal pH for chloroplast storage, pH 7.2–7.4.

In normal (EDTA-untreated) chloroplasts a similar and prolonged treatment is often crucial for obtaining a significant Cl^- effect even at pH 8 (ref. 6).

It appears that the EDTA treatment facilitates the release of internal Cl^- (as does alkaline pH (ref. 6)) presumably by making the thylakoid membrane more permeable. The use of EDTA-uncoupled chloroplasts also eliminates the need for addition of uncouplers (or phosphorylating agents) to the reaction mixture in order to observe the Cl^- effect[6]. For these reasons the EDTA-washed spinach chloroplasts are employed as material throughout the present work, except for a few instances where the chloro-



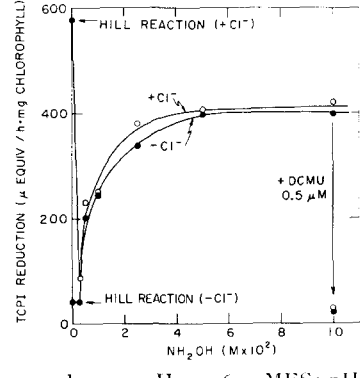


Fig. 1. The pH dependence of the Cl^- effect. The buffers used were: pH 5.7–6.5, MES; pH 7–7.5, TES; >pH 8, tricine. The reaction mixtures (2 ml) contained: sucrose (0.1 M); buffer (25 mM); $MgSO_4$ (3 mM); TCPI (25 μM) or potassium ferricyanide (400 μM); NaCl (if added, 5 mM); and chloroplasts (30 μg chlorophyll). For other conditions, see METHODS.

Fig. 2. Effect of NH_2OH concentration on TCPI photoreduction. Reaction conditions were essentially the same as in Fig. 1 except for the addition of NH_2OH (sulfate). Reaction pH 8.0. The lack of O_2 production (or consumption by Mehler-type reaction) in the presence of excess (> 1 mM) NH_2OH was confirmed with a Clark O_2 electrode.

plast type is specified. The effect of Cl^- is always examined by restoring NaCl (>5 Mm) to Cl^--depleted chloroplasts.

The lack of Cl^- effect on photooxidation of hydroxylamine

Hydroxylamine, a classical inhibitor of the Hill reaction[13], has recently been found capable of donating electrons to Photosystem II and, apparently, of by-passing its own inhibition site at high concentration[11]. In the present study *o*-chlorophenol-indo-2,6-dichlorophenol (TCPI) (or phenolindo-2,6-dichlorophenol (DCPI)) was successfully used as an electron acceptor to follow the reaction quickly by photometry. Ferricyanide cannot be used because its rate of reduction by hydroxylamine is too rapid. The reaction rates were computed from the initial slopes of the recorder tracings. Corrections were made for the rate of the dark (chemical) reduction of dye by hydroxylamine, which was slow under the experimental conditions used (10–20 % of the net photoreaction).

Fig. 2 represents the effect of increasing concentrations of hydroxylamine on TCPI reduction. The Hill reaction in the presence of Cl^- is severely inhibited by low concentrations of hydroxylamine (< 1 mM). However, as the hydroxylamine concentration increases, the rate of TCPI reduction sharply increases again, indicating the commencement of electron flow from hydroxylamine to Photosystem II. The involvement of Photosystem II in this hydroxylamine-supported TCPI reduction is indicated by the high sensitivity of the reaction to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). In the absence of Cl^- the low rates of the Hill reaction can be increased by hydroxylamine (>5 mM) to the $+Cl^-$ level. Hence, the hydroxylamine-supported TCPI reduction is completely Cl^- independent.

Fig. 3 illustrates the pH–activity profile for the hydroxylamine reaction, which

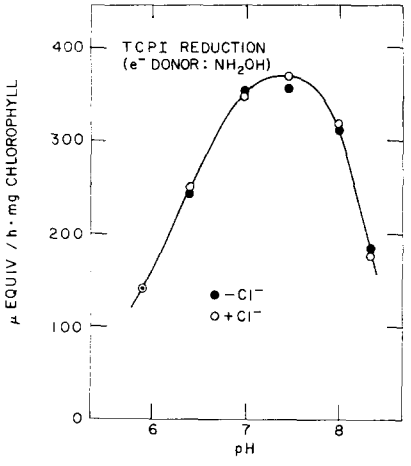


Fig. 3. pH/activity profile of TCPI photoreduction with NH_2OH as electron donor. Experimental conditions were the same as in Fig. 1 except for the addition of NH_2OH (25 mM).

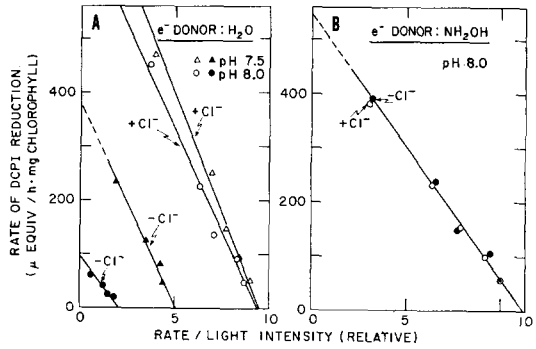


Fig. 4. Effect of light intensity on DCPI reduction with water and NH_2OH as electron donors. Reaction conditions were the same as in Fig. 1, with NaCl (5 mM) and NH_2OH (25 mM, sulfate salt) where indicated. Light intensity was altered by means of calibrated screen filters. The highest and lowest light intensities used in these experiments were 67 and 3.1 kergs·cm^{-2}·sec^{-1}, respectively. For explanations, see text.

is rather similar to that for the normal Hill reaction (see Fig. 1). Cl^- has no effect at any pH.

Previously it was shown that Cl^- has a striking effect upon the dependence of the Hill reaction on light intensity[6]. Fig. 4A shows that the quantum efficiency of the Hill reaction of EDTA-washed chloroplasts also depends critically on the presence or absence of Cl^-. Removal of Cl^- causes a very marked, reversible drop of quantum efficiency of electron flow (intercepts on the abscissa) and also suppresses the rate-limiting dark reaction or maximum rate (intercepts on the ordinate). In short, the two lines in Fig. 4A are nearly parallel and the Cl^- effect is practically independent of light intensity (if the rates are expressed in relative units with both maxima equal to 1). This situation was already suggested by the data of GORHAM AND CLENDENNING[2]. The effect on the limiting dark reaction was not readily observed in experiments reported earlier[6], performed with preparations less completely deficient in Cl^-.

If water is replaced by the artificial electron donor hydroxylamine, the quantum efficiency of electron flow with or without added Cl^- remains at the same level as (or at a level slightly higher than) that of the normal Hill reaction with Cl^- added (Fig. 4B). However, the maximum rate declines somewhat. These observations will be discussed in the final section.

The lack of Cl^- effect on ascorbate photooxidation

It is known that ascorbate added alone to chloroplasts also undergoes a DCMU-sensitive photooxidation[8,9]. In our experiments FMN was used as the electron acceptor and the electron flow measured as the reoxidation of reduced FMN (O_2 uptake). In Table I the results are compared for EDTA-treated chloroplasts and for chloroplasts in which the mechanism of water oxidation was destroyed. Selective destruction of the O_2 evolution system was achieved by ageing the chloroplasts in an alkaline

TABLE I

THE LACK OF Cl^- EFFECT ON PHOTOSYSTEM II-DEPENDENT OXIDATION OF ASCORBATE (FMN AS ELECTRON ACCEPTOR)

Reactions were assayed with a Clark oxygen electrode as O_2 consumption resulting from the reoxidation of reduced FMN. The H_2O_2 trap was found to be unnecessary because of the insignificant catalase activity of the chloroplast preparations used. The reaction kinetics were linear for at least 90 sec. The reaction mixtures (4 ml) contained: sucrose (0.1 M); TES buffer (30 mM at pH 7.0); $MgSO_4$ (3 mM); FMN (100 μM); chloroplasts (100 μg chlorophyll) and where added: sodium ascorbate (10 mM); NaCl (5 mM); DCMU (0.5 μM)

Electron donor	*Rate of O_2 uptake (μequiv/h·mg chlorophyll)*					
	Normal chloroplasts			*Aged chloroplasts**		
	$-Cl^-$	$+Cl^-$	$(+Cl^-/Cl^-)$	$-Cl^-$	$+Cl^-$	$(+Cl^-/-Cl^-)$
A. H_2O (Hill reaction)	33	370	(11.2)	5	44	(8.8)
B. H_2O + ascorbate	215	530	(2.5)	203	265	(1.3)
C. Ascorbate (net)**, (B − A)	182	160	(0.9)	198	221	(1.1)
D. H_2O + ascorbate + DCMU	23	45	—	25	37	—

* For ageing treatment, see text.

** The computation is based on the assumption that water oxidation (Hill reaction) continues unchanged in the presence of ascorbate.

medium for 30 min at room temperature[14,*]. The lack of a Cl^- effect on the ascorbate photooxidation is quite evident in EDTA-treated chloroplasts and even more striking in the aged chloroplasts. The last line of Table I confirms that this ascorbate photooxidation is highly DCMU sensitive, thus ruling out the possibility that the ascorbate may donate electrons to some component between Photosystems I and II.

Inhibitors of water oxidation

The ability of hydroxylamine to donate electrons around the Cl^--deficient block suggested the use of this donor system in screening for other possible inhibitors of the water-splitting mechanism. Table II shows that most of the compounds tested appeared to act in a DCMU-like fashion, inhibiting regardless of the electron source.

TABLE II

COMPARISON OF THE EFFECT OF VARIOUS COMPOUNDS ON TCPI PHOTOREDUCTION WITH WATER (HILL REACTION) AND WITH NH_2OH AS ELECTRON DONORS

The results shown in this table are a summary of several sets of experiments with different chloroplast preparations. In the runs for ammonium chloride and methylamine the same batch of chloroplasts was used. The reaction mixtures (pH 7.5, unless otherwise noted) contained 5 mM NaCl. The concentration of hydroxylamine (sulfate) as electron donor was 25 mM. For experimental conditions, see Fig. 1.

Compound	*Concn. (M)*	*Inhibition of TCPI reduction (%)*	
		H_2O as donor	*NH_2OH as donor*
DCMU	$2.5 \cdot 10^{-7}$	93	82
o-Phenanthroline	$4 \cdot 10^{-5}$	88	80
n-Butyl-3,5-diiodo-4-hydroxybenzoate (BDBH)	$5 \cdot 10^{-5}$	92	90
Usnic acid	$2.5 \cdot 10^{-5}$	89	81
CCCP	$1 \cdot 10^{-4}$	88	87
Pentachlorophenol	$1 \cdot 10^{-4}$	90	97
Dicumarol	$5 \cdot 10^{-4}$	90	93
$NH_2OH \cdot \frac{1}{2}H_2SO_4$	$1 \cdot 10^{-3}$	76	(0)
NH_4Cl	$3.4 \cdot 10^{-2}$	41	3
NH_4Cl (pH 8.3)	$3.4 \cdot 10^{-2}$	87	2
CH_3NH_3Cl	$1.5 \cdot 10^{-1}$	0	9
CH_3NH_3Cl (pH 8.3)	$1.5 \cdot 10^{-1}$	70	0

Ammonia and methylamine at high concentrations, however, inhibited the oxidation of water but not of hydroxylamine. Marked pH dependence of the amine inhibition suggested that the unprotonated base was the form responsible for inhibition (as for phosphorylation uncoupling), in confirmation of an earlier report[15]. In Table III it is shown that the DCMU-sensitive ascorbate photooxidation is nearly completely resistant to 1 mM hydroxylamine which is sufficient to block water splitting by 80 %. Thus, ascorbate and hydroxylamine must donate electrons at very nearly the same site on the oxidizing side of Photosystem II, since both reaction systems are DCMU

* The medium contained 0.03 M tricine and 3 mM $MgSO_4$ at pH 8.3. After the ageing treatment the chloroplasts were transferred to a pH 7.2 medium (see METHODS) for storage.

sensitive, hydroxylamine insensitive, resistant to mild ageing treatment, and Cl^- independent.

TABLE III

THE LACK OF HYDROXYLAMINE INHIBITION OF ASCORBATE PHOTOOXIDATION (FMN AS ELECTRON ACCEPTOR)

The method of assay and experimental conditions were the same as in Table I. NaCl (5 mM) was added to the normal (EDTA-treated) spinach chloroplasts to restore Hill reaction activity but not to the aged chloroplasts (to minimize water oxidation). The concentration of NH_2OH (sulfate) was 1 mM.

Electron donor	*Rate of O_2 uptake (μequiv/h·mg chlorophyll)*					
	Normal chloroplasts			*Aged chloroplasts*		
	Control	*+NH_2OH*	*Inhibition (%)*	*Control*	*+NH_2OH*	*Inhibition (%)*
A. H_2O (Hill reaction)	372	75	(80)	12	22*	-
B. H_2O + ascorbate	615	284	(54)	176	195	(−11)
C. Ascorbate (net)**, (B − A)	243	204	(16)	164	173	(−5)

* The apparent lack of hydroxylamine inhibition of the residual Hill reaction is probably due to a masking of inhibition by a small contribution of electrons from the hydroxylamine added.

** The computations are based on the assumption that water oxidation continues unchanged in the presence of ascorbate.

Effect of hydroxylamine (as an electron donor) on chloroplast fluorescence

It is clear that replacement of water with an artificial electron donor (hydroxylamine or ascorbate) obliterates the Cl^- requirement of electron flow. This section describes the effect of hydroxylamine on the fluorescence of chloroplasts. It should be emphasized that hydroxylamine is here used at a high concentration as an electron donor to Photosystem II, the effect of low concentrations of hydroxylamine on fluorescence being already known[16].

The most striking of adding hydroxylamine to a chloroplast suspension is an increase in fluorescence[19]. Fig. 5 shows the dependence of the chloroplast fluorescence at 684 nm (± 5 nm) upon the exciting light intensity. The control curve has a non-zero intercept with the intensity axis[7,17]. This non-zero intercept is shifted towards zero in the presence of hydroxylamine. The fluorescence yield (F/I) at the higher intensities of light is increased by hydroxylamine nearly 13 % over that of the control. The emission spectrum of the fluorescence of chloroplasts is almost unchanged by the addition of hydroxylamine, although there is apparently a small shift of the 684-nm peak to a lower wavelength (1–1.5 nm).

The excess fluorescence yield (ϕ), defined as in HEATH AND HIND[7], is related to the actinic light intensity, as shown in Fig. 6. The plot shows that the maximum fluorescence yield (intercept on the ϕ axis) is increased by Cl^- as previously noted[7]. When hydroxylamine is added to the chloroplasts, the maximum yield increases dramatically. Furthermore, the presence or absence of Cl^- does not alter this high yield. The quantum efficiency (Φ_e = inverse slope of the curves in Fig. 6) of the fluorescence yield is higher (4 $cm^2 \cdot sec \cdot kerg^{-1}$) with hydroxylamine than with either $-Cl^-$

(0.56 $cm^2 \cdot sec \cdot kerg^{-1}$) or $+Cl^-$ alone (1.0 $cm^2 \cdot sec \cdot kerg^{-1}$). In the presence of hydroxylamine, the absence of Cl^- does not affect the quantum efficiency.

Fluorescence kinetics, such as shown in Fig. 7, can be arbitrarily divided into three phases (I, II, III)[7]. The kinetics of the Phase III fluorescence rise seems to indicate the difference between electron transport rates through Photosystem I and II[7]. Fig. 7 demonstrates the lower rate of the Phase III increase and lower steady-state level of fluorescence in the absence of Cl^- compared with chloroplasts with added Cl^-, as previously noted[7]. The addition of hydroxylamine substantially overcomes the

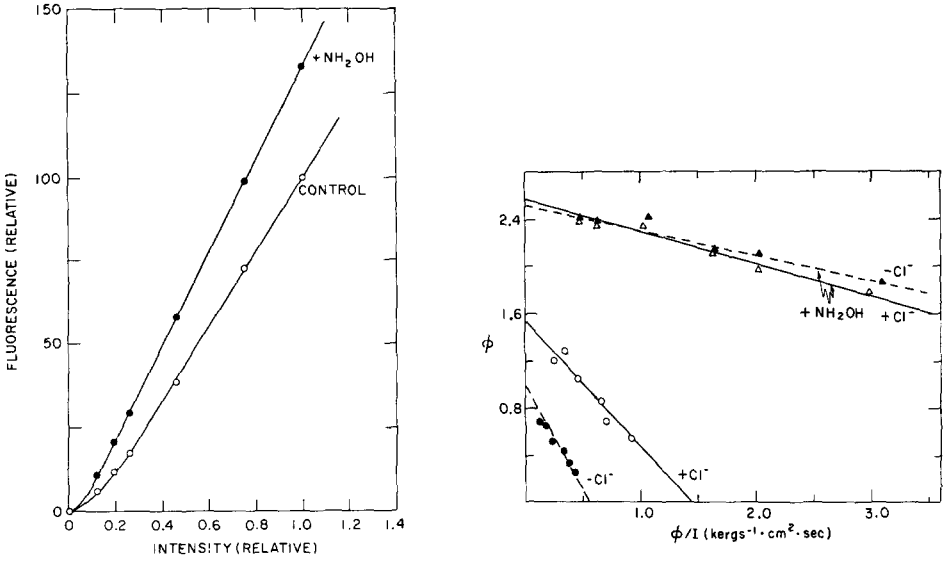


Fig. 5. Fluorescence of chloroplasts in the presence of excess NH_2OH. Pea chloroplasts (Cl^- deficient, EDTA untreated; chlorophyll, 40 μg) were incubated in a medium (2 ml) containing: sucrose (0.2 M); tricine buffer (30 mM at pH 8.4); Na_2SO_4 (30 mM); $MgSO_4$ (5 mM) at 12°; and where indicated, NH_2OH (as sulfate), 60 mM. Intensity of light (435 $\pm$ 10 nm), 1 $kerg \cdot cm^{-2} \cdot sec^{-1}$ = 1.00 unit (modulated at 270 Hz).

Fig. 6. Dependence of fluorescence yield upon light intensity in the presence of excess NH_2OH. For experimental conditions, see Fig. 5. Intensity of actinic light (510 $\pm$ 40 nm), 5 $kergs \cdot cm^{-2} \cdot sec^{-1}$ at $\phi/I = 1.00$. Intensity of measuring light (435 nm), 40 $ergs \cdot cm^{-2} \cdot sec^{-1}$. $\phi = \phi_F$ (total fluorescenceyield) $- \phi_0$ (yield from measuring light) with $\phi_F = 1.00$.

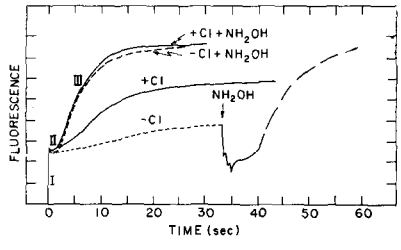


Fig. 7. Initial kinetics of fluorescence in the presence of Cl^- and NH_2OH. EDTA-treated spinach chloroplasts (40 μg chlorophyll) were suspended in a medium (2 ml) containing: sucrose (0.1 M), TES buffer (15 mM at pH 7.4); Na_2SO_4 (25 mM); $MgSO_4$ (5 mM); and where indicated, NaCl (10 mM); NH_2OH (50 mM). Light intensity (435 $\pm$ 10 nm), 200 $ergs \cdot cm^{-2} \cdot sec^{-1}$ (nonmodulated). The fluorescence rise curve is divided into phases (I, II and III) as previously defined[7].

TABLE IV

EFFECT OF Cl^- AND NH_2OH ON THE KINETICS OF FLUORESCENCE

EDTA-treated spinach chloroplasts (40 μg chlorophyll) were suspended in a medium (2 ml) containing: sucrose (0.1 M); TES buffer (15 mM at pH 7.4); Na_2SO_4 (25 mM); $MgSO_4$ (5 mM). Where indicated NH_2OH (sulfate) (50 mM); NaCl (10 mM); DCMU (10 mM); $Na_2S_2O_4$ (several crystals). Light intensity (436 ± 10 nm), 260 ergs·cm^{-2}·sec^{-1} (non modulated). Phases (I, II, III) defined in Fig. 7 (see also ref. 7).

Condition	*Fluorescence level (relative)*				*Initial rate of fluorescence rise (relative units/sec)*		
	Phase I	*Steady state*					
		Normal	*+DCMU*	*+$Na_2S_2O_4$*	*Phase II*	*Phase III*	*Phase II +DCMU*
$+Cl^-$	21.0	53	87	102	0.90	2.45	26.0
$+Cl^-$, $+NH_2OH$	21.0	67	99	105	0.85	6.30	30.5
$-Cl^-$	21.0	35	82	104	0.55	0.40	10.4
$-Cl^-$, $+NH_2OH$	20.5	77	97	115	0.30	7.20	29.0

Cl^- deficiency and gives a higher rate of Phase III increase and a higher steady-state fluorescence. The same effect is seen if hydroxylamine is added after 30 sec of illumination.

Table IV tabulates both the steady-state fluoresescence and the rates of the various phases of the fluorescence rise. It is important to note that both the initial level of fluorescence (Phase I, Fig. 7) and the maximum fluorescence level obtained with added hydrosulfite[19] are the same for all conditions employed. With added hydroxylamine the steady-state fluorescence level is raised for both the $+Cl^-$ and $-Cl^-$ cases, and the rates of the Phase III fluorescence rise are increased. Only the Phase II rate is practically unaffected by hydroxylamine; however, the Phase II rate is difficult to determine accurately. Hydroxylamine also increases the steady-state level and the rate of rise of fluorescence of chloroplasts in which electron flow into Photosystem I is blocked by DCMU.

DISCUSSION

The present status of our knowledge concerning the effect of Cl^- on the photosynthetic electron transport chain may be summarized as follows: (1) The Hill reaction, regardless of the electron acceptors used (quinone, $NADP^+$, FMN, ferricyanide, indophenol dye, *etc.*) requires Cl^- (refs. 1–4, 6). (2) Electron transport mediated by Photosystem I (as observed for DCMU-insensitive cyclic photophosphorylation and $TCPIH_2$ photooxidation) does not require Cl^- (refs. 4, 6). (3) The modified Hill reaction in which artificial electron donors (hydroxylamine and ascorbate) are substituted for water does not require Cl^- (*cf.* RESULTS).

The site of Cl^- involvement seems to be on the oxidizing side of Photosystem II as suggested by previous fluorescence studies which showed that Cl^- removal results in a reversible decrease in steady-state fluorescence yield in illuminated chloroplasts[7]. Tris-washing of chloroplasts[10], and removal of manganese from chloroplasts[19] also result in a decreased fluorescence yield. Hydroxylamine inhibition, however, does not change the fluorescence yield[16]. The fluorescence decline caused by the blocking of

electron transfer on the water side of Photosystem II or a speeding of electron transfer to Photosystem I (*e.g.*, by methyl viologen) is to be contrasted with the fluorescence rise resulting from a blocking of electron transfer (*e.g.*, by DCMU) on the reducing side of Photosystem II[20]. These changes in fluorescence can be best explained in terms of the reduction/oxidation poise of a quenching compound (Q), which is the primary electron acceptor from Photoact II[18,20]. A suppressed supply of electrons from water to the quencher will allow Q to become oxidized by Photosystem I and the fluorescence to fall. A block of the pathway into Photosystem I will allow Q to become reduced and the fluorescence to rise.

HEATH AND HIND[7] have interpreted the dependence on light intensity of the effect of Cl^- on fluorescence as indicating that there may be a cycle through which the reduced Q is reoxidized by an oxidant (Y) produced on the water side of Photosystem II. Removal of Cl^- from chloroplasts is assumed to activate this cycle (*e.g.*, by causing the oxidant to accumulate). The observed decrease of quantum efficiency of electron flow (Hill reaction) upon Cl^- removal could also be explained by the same mechanism (Fig. 4A and ref. 6). That is, at low light intensities the cycle, stimulated by Cl^- removal, may operate fast enough, even in the presence of an electron acceptor, to carry electrons around the photoact, resulting in a net loss of electrons (per absorbed quantum) available for the acceptor. This would mean a drop in the quantum efficiency of (measurable) electron flow. If, however, substrate level hydroxylamine is added to Cl^--depleted chloroplasts, the cycle should collapse since the key oxidant is now reduced. Enough electrons would be provided by Photosystem II to keep Q largely reduced and hence the fluorescence yield would increase. Chloroplasts under such conditions would be expected to photoreduce electron acceptors quite efficiently—possibly even with a slightly better quantum efficiency than in normal Hill reactions (*cf.* Fig. 4B). A similar recovery of fluorescence yield was observed by YAMASHITA AND BUTLER[10] when they added an ascorbate-phenylenediamine couple to "Tris-washed" chloroplasts and by HOMANN[19] when he added hydroxylamine or Mn^{2+} to Mn-deficient chloroplasts. The large increase observed in the fluorescence quantum efficiency (Φ_e) with added hydroxylamine may also be related to a decline in the back (or cyclic) reaction (Fig. 6 and ref. 7).

KATOH AND SAN PIETRO[21] reported that ascorbate photooxidation in Euglena chloroplasts with NADP as the Hill acceptor is sensitive to DCMU, triazines, *o*-phenanthroline, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), and significantly, to hydroxylamine. Their last observation implies that either ascorbate donates electrons to the chain between hydroxylamine inhibition and water splitting, or hydroxylamine causes an inhibition at another site in their system (NADP reductase?) and, therefore, lowers the rate. In our system utilizing dye, it is quite clear that neither hydroxylamine nor ascorbate electron donations are sensitive to hydroxylamine (Table III).

The mechanism of the cofactor action of Cl^- in water oxidation is still open to question, as is the mechanism of water splitting itself. However, an interesting fact has emerged from the present study; namely, that the reaction step involving Cl^- falls close to the site of hydroxylamine inhibition. Like Cl^- removal, hydroxylamine affects only the reaction steps involving water oxidation. It does not inhibit either ascorbate or hydroxylamine photooxidation. Further, ammonia and methylamine act as inhibitors much like hydroxylamine. The experimental results, thus far, seem to be most

consistent with the scheme of electron transport in Photosystem II as presented in Fig. 8. There are two major sites of electron transport inhibition (DCMU-like and $-Cl^-$-like) which can be easily differentiated by fluorescence kinetics[7]. The two donors (ascorbate and hydroxylamine) add electrons to the chain between the $-Cl^-$ block and the photoact. The need of a by-pass, a cycle, seems to be required from fluorescence and from light-intensity studies to explain changes in quantum efficiency (Fig. 4).

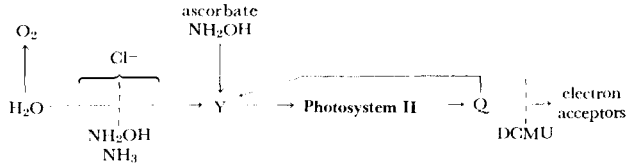


Fig. 8. Diagram showing the relative positions of sites of Cl^- involvement, hydroxylamine inhibition, DCMU inhibition, and of intervention of artificial electron donors.

Further work is in progress on the identification of Y and the elucidation of the components of the by-pass. In addition, the question of whether the sites of $-Cl^-$ inhibition and ammonia inhibition are the same needs to be answered.

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REFERENCES

1 O. WARBURG AND W. LÜTTGENS, *Biokhimiya*, 11 (1946) 303.
2 P. R. GORHAM AND K. A. CLENDENNING, *Arch. Biochem. Biophys.*, 37 (1952) 199.
3 T. PUNNETT, *Plant Physiol.*, 34 (1959) 283.
4 J. M. BOVÉ, C. BOVÉ, F. R. WHATLEY AND D. I. ARNON, *Z. Naturforsch.*, 18b (1963) 683.
5 L. N. M. DUYSENS AND J. AMESZ, *Biochim. Biophys. Acta*, 64 (1962) 243.
6 G. HIND, H. Y. NAKATANI AND S. IZAWA, *Biochim. Biophys. Acta*, 172 (1969) 277.
7 R. L. HEATH AND G. HIND, *Biochim. Biophys. Acta*, 172 (1969) 290.
8 S. IKEDA, *Mem. Res. Inst. Food Sci. Kyoto Univ.*, 18 (1959) 57.
9 A. TREBST, H. ECK AND S. WAGNER, in *Photosynthetic Mechanisms in Green Plants*, Publ. No. 1145, Natl. Acad. Sci-Natl. Res. Council, Washington, D.C., 1963, p. 174.
10 T. YAMASHITA AND W. L. BUTLER, in K. SHIBATA, A. TAKAMIYA, A. T. JAGENDORF AND R. C. FULLER, *Comparative Biochemistry and Biophysics of Photosynthesis*, University of Tokyo Press, Tokyo, 1968, p. 179.
11 S. VAKLINOVA, *Compt. Rend. Acad. Bulg. Sci.*, 17 (1964) 283.
12 A. T. JAGENDORF AND M. SMITH, *Plant Physiol.*, 37 (1962) 135.
13 C. S. FRENCH, A. S. HOLT, R. D. POWELL AND M. L. ANSON, *Science*, 103 (1946) 462.
14 J. W. HINKSON AND L. P. VERNON, *Plant Physiol.*, 34 (1959) 268.
15 G. HIND AND C. P. WHITTINGHAM, *Biochim. Biophys. Acta*, 75 (1963) 194.
16 A. JOLIOT, *Biochim. Biophys. Acta*, 126 (1966) 587.
17 N. MURATA, M. NISHIMURA AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 112 (1966) 213.
18 L. N. M. DUYSENS, *Proc. Roy. Soc. London, Ser. B.*, 157 (1963) 301.
19 P. HOMANN, *Biochim. Biophys. Acta*, 162 (1968) 545.
20 L. N. M. DUYSENS AND H. E. SWEERS, in *Studies on Microalgae and Photosynthetic Bacteria*, Japan. Soc. Plant physiol., University of Tokyo Press, Tokyo, 1963, p. 353.
21 S. KATOH AND A. SAN PIETRO, *Arch. Biochem. Biophys.*, 122 (1967) 144.