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THE ROLE OF Cl^- IN PHOTOSYNTHESIS

I. THE Cl^- REQUIREMENT OF ELECTRON TRANSPORT

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SUMMARY

1. Non-cyclic electron flow in isolated chloroplasts at high pH is dependent on Cl^- or on some other monovalent anion derived from an inorganic acid with ionization constant of approx. 0.01.

2. Whole chloroplasts generally do not show a Cl^- requirement until the internal Cl^- is released by some treatment to damage the outer membrane and promote swelling.

3. The basal component of non-cyclic electron flow is much less dependent on Cl^- than the uncoupled or coupled components. Cl^- effects have been observed after uncoupling by ammonia, chloroquine and carbonyl cyanide *m*-chlorophenylhydrazone.

4. The Cl^- effect is associated closely with a photochemical reaction (Photosystem II).

5. Photoinactivation in red light is potentiated by Cl^- deficiency, but is not in itself the cause of the Cl^- effect.

6. The role of Cl^- is discussed with reference to the chemi-osmotic coupling hypothesis.

INTRODUCTION

In 1946, WARBURG AND LÜTTGENS[1] showed that photosynthetic O_2 evolution by chloroplast fragments is dependent on the presence of Cl^-. Chloride was asserted to be essential to the primary photochemical process of the O_2-evolving system.

ARNON AND WHATLEY[2] questioned whether Cl^- could be an essential cofactor for photosynthesis and yet not be generally regarded as an essential element for the growth of higher plants, there being at that time only one very early report of Cl^- deficiency under field conditions. Failure to detect Cl^- dependence of plants grown hydroponically led them to propose that Cl^- might not be needed for photosynthesis *in vivo* but only for the protection of chloroplast components during isolation or assay. Evidence for such a protective role was obtained.

Abbreviations: CQP, 7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline diphosphate; TCPI, *o*-chlorophenolindo-2,6-dichlorophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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GORHAM AND CLENDENNING[3] prepared chloroplasts from plants grown in the absence of Cl^- and observed that they retained 30% of their control $Fe(CN)_6^{3-}$ reduction activity, even though the Cl^- concentration as measured by careful analysis would have indicated complete inhibition. They also showed that Cl^- protects chloroplasts against preillumination injury but that the principle effect of Cl^- is a direct stimulation of the Hill reaction. Later, however, in studies of chloroplast isolation techniques, PUNNETT[4,5] found that Cl^- stabilized the activity of chloroplast fragments and did not restore the photochemical reaction. The suggestion was made that earlier reports of Cl^- effects might be due to the protective rather than the cofactor role of the ion.

Investigations of pH dependence[3] led to the observation that the Cl^- effect is observed at alkaline pH values only. This seemed to provide a rational explanation for the supposed lack of Cl^- requirement for photosynthesis *in vivo*, since the pH of cell sap is close to neutral[6]. However, in 1954 BROYER *et al.*[7] demonstrated that with rigorous purification and assay techniques, symptoms of Cl^- deficiency could be demonstrated in cultured plants. Reports of Cl^- deficiency in soils also appeared[8,9].

BOVÉ *et al.*[10] later utilized a much improved understanding of the electron-transport pathway to localize the operative site of the Cl^- effect. Chloroplasts were prepared from normal leaves and rendered Cl^- free by washing. Cl^- was found to be necessary for coupled $NADP^+$ or $Fe(CN)_6^{3-}$ reduction, but not for cyclic photophosphorylation: necessary, that is, for any process dependent on water as a source of electrons.

At the biochemical level many questions remain unanswered, such as the possible protective role of Cl^- (ref. 2), the reason for the diminished Cl^- effect at acid pH values[3], and the location of the Cl^--dependent site relative to both the photochemical reaction and the step inhibited by substituted phenylurethanes[11].

This paper is addressed to the first two questions, while a subsequent article will report experiments in which we have attempted to locate the site of the Cl^- effect by means of fluorescence studies[12].

METHODS

Leaves from field-grown spinach plants or 20-day-old 'Alaska' pea seedlings were washed in distilled water, deveined (if spinach), and macerated in Cl^--free pH 7.4 buffer containing (in mM): sucrose 400, Tricine 30, $MgSO_4$ 5, and sodium ascorbate 2; using 15 ml of buffer per mg recovered chlorophyll. Chloroplasts were obtained from the filtered homogenate by centrifugation and then were washed once and finally resuspended in the same buffer *minus* ascorbate and with only 200 mM sucrose.

The Cl^- content of the buffer is typically 14 μM, or 2 μM without sucrose (as used in some reaction mixtures). The Cl^- content of the wash supernatant is below 40 μM and additional washes are not helpful in improving the Cl^- effect. Specially purified chemicals, beyond the common analytical grades, are not required in preparing the buffers and assay media. Adenosine diphosphate, however, was purified by conversion to the barium salt[10], and *o*-chlorophenolindo-2,6-dichlorophenol (TCPI) by passage into chloroform and then into water, from the sodium salt was precipitated with sodium acetate. Chloroquine diphosphate (7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline diphosphate (CQP)), was obtained from Sigma.

Cl^- was estimated using an Aminco-Cotlove chloride titrator. Chloroplast volume was measured by means of a Coulter counter with plotting attachment and the packed volumes were obtained by centrifugation in hematocrit tubes. The number of chloroplasts containing 1 mg of total chlorophyll was determined by standard hemocytometer techniques. The osmotic volume was assumed to be 30% of the intact chloroplast[13].

The samples were illuminated with saturating intensities of red light ($Fe(CN)_6^{3-}$ reduction) or blue light (TCPI reduction) at 15° unless otherwise noted. Reduction rates were continuously monitored in a recording spectrophotometer by observing the decrease in absorbance at 420 nm ($Fe(CN)_6^{3-}$) or 620 nm (TCPI). Phosphorylation was assayed by the method of NIELSEN AND LEHNINGER[14].

RESULTS

Induction of the Cl^- effect

Chloroplasts obtained by the procedure given above initially show little or no Cl^- effect*. During the course of aging at 0° a Cl^- effect develops at a rate determined by the freshness and source of the leaf material. Spinach chloroplasts from fresh leaves develop a Cl^- effect of 2.0 with $Fe(CN)_6^{3-}$ as acceptor in 2–3 h, whereas the same result can be obtained from cold-stored leaves in less than an hour and often immediately upon isolation. Freshly cut pea seedlings give the most stable preparations, and a Cl^- effect of 2.0 may develop after 6 h.

TABLE I

INDUCTION OF THE Cl^- EFFECT BY HEATING

Cl^--free chloroplast suspension (pH 8.3); 400 μg chlorophyll/ml incubated as below then placed in an ice bath and assayed for uncoupled $Fe(CN)_6^{3-}$ reduction at 15° in the medium (mM): Tricine-NaOH 15, $MgSO_4$ 5, $Fe(CN)_6^{3-}$ 0.4, CQP 0.15. NaCl 10 mM where added.

Incubation time (min)	*Temperature*	*$Fe(CN)_6^{3-}$ reduction**		*Cl^- effect*
		−NaCl	*+NaCl*	
0	0°	662	630	1.0
30	0°	602	610	1.0
15	25°	336	535	1.6
20	25°	168	357	2.1
30	25°	94	189	2.0
15	30°	73	136	1.9
20	30°	52	63	1.2

* μmoles·h^{-1}·mg^{-1} chlorophyll.

The aging process can be accelerated by heating the chloroplasts or raising their pH, or by both in combination. The temperature effect was studied in order to select a treatment which would give the highest Cl^- effect and the highest control ($+Cl^-$) rate (Table I). The most useful temperature was 25°, giving Cl^- effects between 1.6 and 2.0 or up to 4.0 in other experiments, after 15–20 min incubation at pH 8.3.

* The Cl^- effect is here defined as the rate obtained by assay in the presence of added Cl^-, divided by the rate obtained without added Cl^-.

The corresponding control electron-transfer rates should be noted: they are 5- to 7-fold higher than those reported for chloroplasts prepared in Tris–acetate buffer[10].

Fig. 1 shows the pH dependence of the induction process for a representative chloroplast suspension incubated at 25° for 20 min. With older preparations or at higher temperatures and/or longer incubations the curves would typically be shifted along the pH axis to the acid side. Values of pH between 8.0 and 8.4 were used routinely in this investigation.

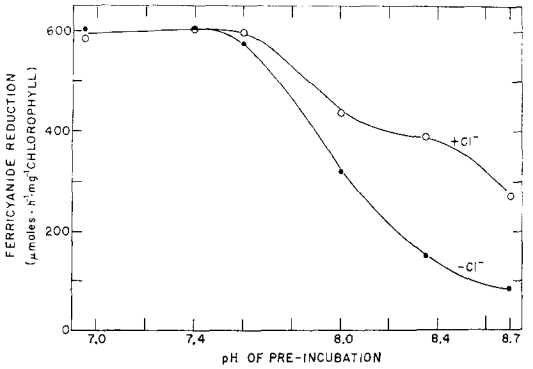


Fig. 1. Induction of the Cl^- effect by preincubation at different pH values. Samples were incubated at 25° for 20 min in (mM): sucrose 200, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) 20, Tricine–NaOH 15, $MgSO_4$ 5 (pH 7.4), then cooled rapidly. The assay medium consisted of (mM); sucrose 200, Tricine–NaOH 30, $MgSO_4$ 5, $Fe(CN)_6^{3-}$ 0.4, CQP 0.15 and spinach chloroplasts 20 μg/ml (pH 8.3).

The induction process is most readily accounted for as a loss of internal Cl^- by damaging treatments. To test this hypothesis the Cl^- content and physical parameters of chloroplasts in suspension were examined during induction of the Cl^- effect, with the results shown in Table II. Development of a Cl^- effect was accompanied by an increase in the proportion of non-refractive swollen chloroplasts lacking outer membranes, and a fall in the internal Cl^- of about 50% per 30 min. However, the data of WINOCUR, MACEY AND TOLBERG[15] would lead one to expect a Cl^- efflux half-time of

TABLE II

THE LOSS OF Cl^- FROM CHLOROPLASTS DURING INDUCTION

Samples containing 1.74 mg chlorophyll in 2 ml were incubated in (mM): sucrose 200, Tricine–NaOH 15, $MgSO_4$ 5 (pH 7.4) and centrifuged at 0° for packed volume and Cl^- determinations. TCPI reduction was assayed in this medium *plus* 0.05 mM TCPI, at pH 8.4. Coulter counter volumes were measured in (mM): sucrose 100, NaCl 100, Tricine–NaOH 15, $MgSO_4$ 5 (pH 7.4). 1 μg chlorophyll was equivalent to $1.09 \cdot 10^6$ chloroplasts having a chlorophyll *a*/*b* ratio of 4.0.

Incubation time at 30° (min)	*Modal volume* (μ^3)	*Internal* Cl^- (μM)	*TCPI reduction**		$+Cl^-/-Cl^-$ *rate*
			$-Cl^-$	$+Cl^-$	
0	26	245	88	193	2.2
30	33	125	27	167	6.2
60	48	57	9	99	11

* $\mu equiv \cdot h^{-1} \cdot mg^{-1}$ chlorophyll.

less than 4 min at 30°. This discrepancy can be explained if the chloroplast outer membrane is assumed to have much lower Cl^- permeability than the thylakoid membranes. The decline in internal Cl^- would thus correlate with the percentage loss of intact chloroplasts during heating and not the kinetics of the Cl^- efflux. Alternatively, one can avoid assigning unusual properties to the outer membrane by postulating that Cl^- is the counter-ion of an internal positively charged protein, which is released when the membrane breaks. We believe that chloroplasts isolated from sucrose media more commonly have membranes than those isolated from salt solutions. Outer membranes have been reported to be rare in chloroplasts isolated from 0.35 M NaCl[16]. Whether the spinach juice used by WINOCUR, MACEY AND TOLBERG[15] provides a high proportion of truly intact chloroplasts is not known and is difficult to estimate.

The interaction of uncouplers with the Cl^- effect

Cl^- deficiency in experiments reported thus far did not lead to total inhibition of electron flow. Even with extensive induction treatments a 4-fold Cl^- effect was roughly maximal, and it was not possible to account for the residual Cl^--free rate on the basis of contamination.

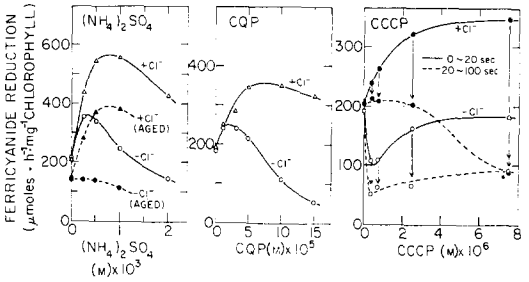


Fig. 2. Dependence of the Cl^- effect on uncoupled electron transport. Reaction mixture (mM) Tricine–NaOH 15, $MgSO_4$ 5, $Fe(CN)_6^{3-}$ 0.4 (pH 8.4), with uncoupler added as indicated. The time course with CCCP was biphasic. The initial rate is given in solid lines and the secondary rate in dashed lines.

Fig. 2 shows the effect of uncouplers on electron transfer, with or without Cl^-, in illuminated heat-induced spinach chloroplasts. In all three instances the Cl^- effect is very low or lacking in the absence of uncoupler, that is, the basal electron flow is independent of Cl^-. The persistence of the basal component as a Cl^--independent fraction of the total uncoupled electron flow could explain the apparent limit on the magnitude of the Cl^- effect with $Fe(CN)_6^{3-}$, as originally noted by GORHAM AND CLENDENNING[3]. A similar limit is observed if the electron flow is enhanced by coupled ATP synthesis.

The dependence of the Cl^- effect on $(NH_4)_2SO_4$ concentration given in Fig. 2 shows the characteristic inhibition of electron flow by high uncoupler concentrations[17,18]. Two states of induction are presented to display the lower control rate but greater Cl^- effect in the aged preparation. SO_4^{2-} is not inhibitory at the concentrations used[19].

The use of CQP as an uncoupler is reported here for the first time (Fig. 2). CQP is analogous in structure and activity to atebrin (quinacrine) but has the advantage of being colorless. It is particularly useful if $Fe(CN)_6^{3-}$ reduction is to be assayed

spectrophotometrically. CQP was superior to $(NH_4)_2SO_4$ in promoting Cl^--dependent electron flow (Fig. 2), possibly because it is less inhibitory at concentrations above the optimum (*cf.* 2 mM $(NH_4)_2SO_4$ with 150 μM CQP).

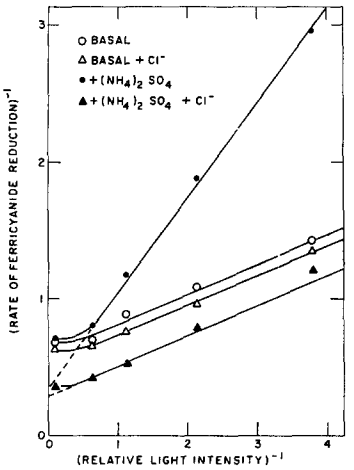
The uncoupling action of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) shown in Fig. 2 differs from that of ammonia and amines. Special properties of CCCP uncoupling have also been documented for other experimental conditions[20,21]. In the Cl^--free situation 0.4 μM CCCP inhibits electron flow by almost 50% whereas with Cl^- present the rate increases slightly. The rates improve with or without Cl^- as more CCCP is added.

The time course of CCCP-uncoupled $Fe(CN)_6^{3-}$ reduction shows a progressive inhibition by the uncoupler such that a distinct inflection occurs after 20 sec of illumination. If the rates observed after 20 sec are plotted, rather than the initial rates, a second set of concentration curves is obtained (Fig. 2, dashed lines). All CCCP levels now appear to be inhibitory, particularly in the Cl^--free samples, and at higher CCCP concentrations the Cl^- effect cannot be demonstrated. We are at present unable to explain these complex CCCP effects.

Other uncouplers with which a Cl^- effect has been obtained include arsenate, and EDTA treatment. Chloroplasts washed in EDTA can be made to show an exceptionally high Cl^- effect of 11.0 for $Fe(CN)_6^{3-}$ reduction at pH 7.6.

Light-intensity dependence

Fig. 3 shows the relationship between the reciprocals of $Fe(CN)_6^{3-}$ reduction rate and illumination intensity for four different conditions In the presence of Cl^-, the basal and ammonia-uncoupled systems give plots with the same slope but different intercepts. This demonstrates that ammonia affects a rate-limiting dark reaction[22].



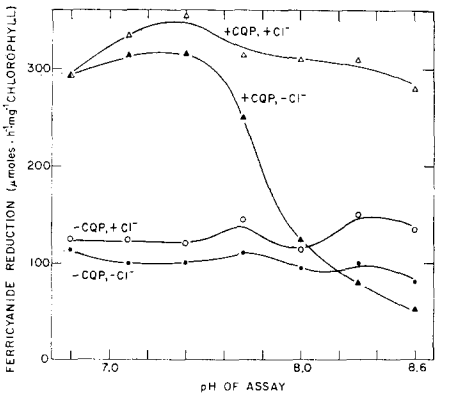


Fig. 3. Light-intensity dependence of the Cl^- effect under conditions of uncoupled and basal electron transfer. Reaction mixture as in Fig. 2, with 1 mM $(NH_4)_2SO_4$ where indicated. Maximum light intensity used (0.1 on abscissa) = 370 kerg.

Fig. 4. Dependence of the Cl^- effect on pH. A Cl^- effect (at pH 8.3) was induced by incubation of chloroplasts (400 μg chlorophyll/ml) in (mM): sucrose 200, Tricine–NaOH 15, $MgSO_4$ 5, for 30 min at 25° and pH 8.3. Assays were then run in (mM): sucrose 200, Tricine–NaOH 15, TES 20, $MgSO_4$ 5, $Fe(CN)_6^{3-}$ 0.4 (15°) with CQP omitted, or added so that the concentration was optimal at each pH. NaCl 5 mM where present. Chlorophyll, 20 μg/ml.

Conversely, the plots for the effect of Cl^- on uncoupled electron flow have almost the same intercept but very different slopes, indicative of an effect of Cl^- close to the rate-limiting light reaction. Deficiency of Cl^- results in a marked drop in the quantum efficiency of electron transport. This observation supports the accepted view (see INTRODUCTION) that Cl^- is necessary for some component reaction of Photosystem II.

Relationship to pH

The Cl^- effect has a marked dependence on pH, as shown in Fig. 4. The general form of the curves resembles that seen in Fig. 1 for the induction phenomenon, but it should be remarked that in Fig. 4, the chloroplasts were already induced and the electron-flow rates were linear. Thus the curves in Fig. 4 do not indicate an induction effect occurring during assay. It is clearly preferable to operate above pH 8.0 in studies of the Cl^- effect. The basal electron transfer is relatively insensitive to pH or Cl^-.

Replacement of Cl^- by other ions

WARBURG, LÜTTGENS[1] and BOVÉ *et al.*[10] observed that Br^- and NO_3^- could substitute for Cl^- in depleted chloroplasts. Fig. 5 confirms this for CQP-uncoupled $Fe(CN)_6^{3-}$ reduction. The half-maximal concentrations of Cl^- and Br^- were approx. 200 μM and of NO_3^- 330 μM. In Table II it was reported that a sample of chloroplasts having an average internal Cl^- content of 245 μM had an electron flow rate 46% of the maximum, which agrees well with the value predicted by Fig. 5. It should be borne in mind, however, that the chloroplast populations used in Table II may be much more heterogeneous with regard to Cl^- content than those obtained by restoration of Cl^- to depleted chloroplasts of the type used in Fig. 5.

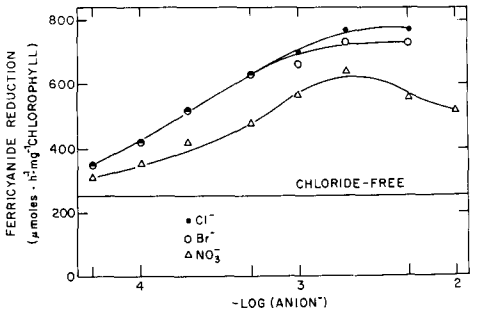


Fig. 5. Dependence of the Cl^- effect on the external anion concentration. Assays were conducted as for Fig. 1, with the indicated sodium salts added to the suspending medium.

Besides NO_3^-, weak enhancements of uncoupled electron flow rates were given by I^- and ClO_4^-. The other monovalent anions tested (Table III) were essentially without effect. Table III lists the dissociation constant of the acid corresponding with each anion, from which it is evident that the activity of an ion in this system is a function of the K_{diss} of the acid, weak acid anions being ineffective. There must be other determinants of activity, however, since not all strong acid anions are effective. Mono-, di- and tri-chloroacetate are inactive even though the corresponding K_{diss} values are $1.4 \cdot 10^{-3}$, $3.3 \cdot 10^{-2}$, and $2 \cdot 10^{-1}$. It is reasonable to suppose that ion volume is a significant factor; therefore we have entered values of relative ion mobility (u_{rel})

TABLE III

ENHANCEMENT BY MONOVALENT ANIONS OF ELECTRON FLOW IN Cl^--DEFICIENT CHLOROPLASTS

Reaction mixture as described for Fig. 1. All additions as 5 mM sodium salts.

Addition	*Rate**	*K***	u_{rel}***
None	230		
PO_4^{3-}	225		
N_3^-	210	$2 \cdot 10^{-5}$	
CNO^-	250	$7 \cdot 10^{-10}$	
HCO_3^-	255	$4 \cdot 10^{-7}$	
Acetate	265	$1.7 \cdot 10^{-5}$	
F^-	280	$3.5 \cdot 10^{-4}$	
ClO_4^-	465	§	0.803
NO_3^-	455	§	0.810
I^-	560	§	0.795
Br^-	700	§	0.807
Cl^-	735	§	0.815

* μmoles $Fe(CN)_6^{3-}$ reduced $\cdot h^{-1} \cdot mg^{-1}$ chlorophyll.
** Dissociation constant of equivalent acid in 0.1 M aqueous solution.
*** Mobility relative to OH^- in 10% sucrose at 25° (ref. 41).
§ Strong acids with $K > 10^{-2}$.

for the active ions in Table III. Although the mobility (inversely correlated with size) does not relate well to the observed order of effectiveness in Table III, the u_{rel} of acetate and presumably the Cl^--substituted acetates is 0.4 or less. A significant difference in activity could hence be expected.

The above consideration of ion volume or mobility need not presuppose a role for Cl^- in ion transport as the cause of the Cl^- effect. Size could also be important to the possible functioning of Cl^- as an enzyme activator or electron-transfer catalyst, as will be discussed later.

MACROBBIE[23] and RAVEN[24] pointed out that although Cl^- movement in the cell is dependent on Photosystem-II activity, K^+ uptake in the light can be mediated by Photosystem I alone, and is sensitive to uncouplers. We were interested to see if, in the isolated chloroplast, this apparent independence of cation and anion fluxes is maintained. The uncoupler nigericin was employed to promote a rapid H^+/K^+ exchange across the thylakoid membranes[25].

TABLE IV

THE INABILITY OF K^+ FLUX TO SUBSTITUTE FOR Cl^-

All samples contained nigericin, 1 μM, and where indicated Cl^- (as NaCl, 5 mM) and K^+ (as K_2SO_4, 2 mM). Reaction mixture (mM): sucrose 200, Tricine–NaOH 30, $MgSO_4$ 5, $Fe(CN)_6^{3-}$ 0.4, spinach chloroplasts 20 μg/ml (pH 8.3).

Addition	*Rate**
None	75
K^+	95
Cl^-	110
K^+, Cl^-	360

* μmoles $Fe(CN)_6^{3-}$ reduced $\cdot h^{-1} \cdot mg^{-1}$ chlorophyll.

The results shown in Table IV indicate that the combination of nigericin and K^+ has no effect on the Cl^- requirement of an O_2-evolving system. Furthermore, since only the uncoupled electron-flow component is dependent on Cl^- (Fig. 2), K^+ is necessary to demonstrate the Cl^- effect.

Absence of Cl^- requirement in Photosystem I

Table V shows the results of an experiment designed to check the observation[10] that only non-cyclic photophosphorylation is Cl^- dependent. Good agreement with the data of BOVÉ *et al.*[10] was obtained. A minor difference was our observation that Cl^- slightly but consistently inhibited pyocyanine-mediated cyclic phosphorylation. Photosystem-II activity must affect the redox poise of electron-transfer components common to cyclic and non-cyclic pathways in such a way as to diminish the rate of electron flow through the cyclic coupling site. A comparable effect has been observed[26] upon addition of ascorbate to cyclic phosphorylation assays.

Studies of the electron flow between ascorbate and methyl viologen provided a more critical test for the non-involvement of Cl^- in Photosystem I. In this system,

TABLE V

THE EFFECT OF Cl^- ON PHOTOPHOSPHORYLATION

Reaction mixture (μmoles): Tricine 40, $MgCl_2$ 6.5, sodium [^{32}P]orthophosphate 25, ADP 1.5, $Fe(CN)_6^{3-}$ 3 or pyocyanine 0.1, NaCl 0 or 10, and chlorophyll 40 μg. Volume 2 ml, pH 8.3. Illuminated 3 min at 25°. Cl^- effect induced by incubating chloroplasts at 25° for 5 min.

Electron flow	*Electron acceptor (cofactor)*	*NaCl*	*Phosphorylation**	*Reduction***	*P/e_2*
Non-cyclic	$Fe(CN)_6^{3-}$	−	101	186	1.1
		+	170	315	1.1
Cyclic	Pyocyanine	−	410		
		+	358		

* μmoles ATP formed·h^{-1}·mg^{-1} chlorophyll.
** μmoles $Fe(CN)_6^{3-}$·h^{-1}·mg^{-1} chlorophyll.

TABLE VI

THE EFFECT OF Cl^- ON PHOTOSYSTEM I ELECTRON TRANSFER

A peroxide-trapping system was not necessary under the conditions employed with water as electron donor. The modified Mehler reaction used below to isolate Photosystem-I activity has been described elsewhere[27]. Reaction mixture (mM): sucrose 200, Tricine–NaOH 30, $MgSO_4$ 5, $(NH_4)_2SO_4$ 1, methyl viologen 0.05 and spinach chloroplasts 20 μg/ml; pH 8.4. Also present where indicated were NaCl 5 mM, TCPI 50 μM, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) 1 μM and ascorbate 3 mM.

Electron donor system	*NaCl*	*O_2 uptake**
H_2O	−	45
H_2O	+	430
Ascorbate, TCPI, DCMU	−	400
Ascorbate, TCPI, DCMU	+	415

* μequiv·h^{-1}·mg^{-1} chlorophyll.

detailed elsewhere[27], electrons flow from ascorbate to the acceptor through a catalytic amount of TCPI. The reduced viologen is oxidized to form H_2O_2 which is in turn reduced by ascorbate, providing a net uptake of 1 O_2 molecule for every electron pair transferred by Photosystem I.

Table VI displays the results from which we conclude that only when oxidation of water occurs, as in the electron transfer between water and viologen, is there a significant requirement for Cl^-.

The photoinactivation effect

The time course of $Fe(CN)_6^{3-}$ reduction in the presence of rate-limiting Cl^- concentrations is always less linear than in the control Cl^--saturated system. Reduction often stops after 4 min of illumination. In view of early suggestions[2,3] that the role of Cl^- might be to protect against photoinactivation, we studied the ability of Cl^- to restore the control rate of electron flow to preilluminated Cl^--depleted chloroplasts.

Fig. 6 demonstrates that the decline in reduction rate resulting from illumination of Cl^--depleted chloroplasts is not fully reversed upon adding Cl^-. The reversibility is nevertheless sufficient to show that the entire Cl^- effect cannot be ascribed to photoinactivation. An interesting feature of the restoration curves is the lag of up to 30 sec after addition of Cl^- before the maximum rate is achieved.

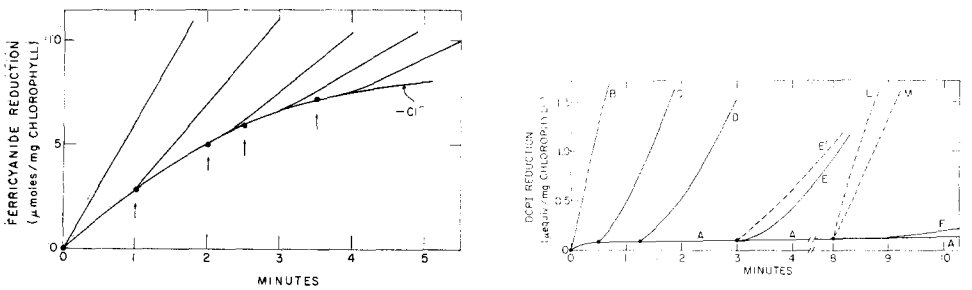


Fig. 6. The photoinactivation of Cl^--deficient chloroplasts. Samples were assayed as in Fig. 1 with 5 mM NaCl added initially, or during illumination at the arrows. Decline in rate, and lag after adding Cl^- are indicative of photoinactivation.

Fig. 7. Restoration of DCPI reduction by Cl^-. Cl^--free chloroplasts were illuminated in the absence of Cl^- (curve A) or with 10 mM NaCl added at various times (solid dots on Curve A), as explained in the text. Reaction mixture (mM): Tricine–NaOH 15, $MgSO_4$ 5, TCPI 0.05 (pH 8.4). Chlorophyll 20 μg/ml.

Further information on the photoinactivation process was sought using TCPI as the electron acceptor. This system has the advantage that the dye serves both as uncoupler and as electron acceptor[28]; but more importantly, the basal electron transfer rate (defined as the component resistant to Cl^- depletion) is absent. With a given chloroplast preparation the Cl^- effect is therefore much greater than for $Fe(CN)_6^{3-}$ reduction.

The experiment shown in Fig. 7 used the same protocol as Fig. 6. The reduction rate in the absence of Cl^- rapidly declined almost to zero (Curve A). Addition of Cl^- at different times during the illumination (Curves C–F) restored the reduction rate to an extent which decreased as the addition of Cl^- was delayed. Here again the maximum

restoration of electron-flow rate was attained only after a considerable lag period. Curve E' (Fig. 7) shows that the lag in reactivation can be overcome by incubation with Cl^- in the dark. Thus the lag is most probably a measure of the penetration rate of added Cl^- into a region where it can exert its effect on electron flow.

Curve L of Fig. 7 is addressed to the question whether the deleterious effect of light might also be observed if electron flow were curtailed by elimination of electron acceptor rather than of Cl^-. The data indicate that little inhibition results from preillumination in the absence of dye, and we conclude that the photoinactivation observed in Curves C–F is a special attribute of Cl^- deficiency.

Curve M of Fig. 7 is simply a control to curve F, showing that a sample incubated in darkness for 8 min is readily reactivated by addition of Cl^-.

In summary, Cl^--deficient chloroplasts can show both a Cl^- effect and a photoactivation effect, which are easily distinguishable in the dye reduction system. There seems to be no possibility that the Cl^- effect is an artifact resulting from photoinactivation under Cl^--free conditions.

DISCUSSION

A precondition for development of the Cl^- effect seems to be some treatment which will increase the permeability of the chloroplast outer membrane. Heating[29] and aging[30] are known to promote hydrolysis of non-chlorophyll lipid. The resultant free fatty acid swells the chloroplasts and partially inhibits O_2 evolution[19,30]. In our experience with pea and spinach chloroplasts this process, as reflected in the Cl^- effect, is most rapid at high[31] rather than low pH[30]. Swelling is accompanied by loss of internal Cl^- (Table V) and a corresponding decline in the Cl^--free reduction rate. The retention of Cl^- against a concentration gradient during chloroplast isolation is most probably associated with the presence of internal positively charged proteins. These would leak out during swelling. The thylakoids must be quite permeable to Cl^- as illustrated by Curves E and E' of Fig. 7, in which a 75-sec-dark incubation with a saturating Cl^- concentration removed about 65% of the extrapolated lag in electron flow.

The specificity of the Cl^- effect for monovalent anions of strong acids may be evidence that anion transfer across a positively charged matrix in the membrane is a necessary accompaniment to electron flow in Photosystem II. Possibly Cl^- and the other active ions move as counter-ions to a H^+ flux—a rapid H^+ uptake closely associated with the photochemical step of Photosystem II has indeed been described[32–34]. The same reasoning applies if Photosystem-II activity deposits H^+ inside the thylakoid by oxidation of water and Cl^- exchanges with electrons across the membrane.

Since H^+ uptake can occur as an accompaniment to cyclic electron flow[35] it would seem that two sites of ion translocation occur, one associated with Photosystem II and the other (cyclic) with Photosystem I[23,36]. It follows from the nigericin data (Table VI) that these two sites are not equivalent, for under conditions of free H^+ and K^+ interchange a Cl^- requirement is still demonstrable for H^+ uptake at the non-cyclic site.

The low sensitivity of the basal component of $Fe(CN)_6^{3-}$ reduction to Cl^- depletion cannot easily be explained on the basis of alternative electron-transport pathways since TCPI reduction appears to have no such resistant component. Thus the branch point of an alternative pathway and the site of Cl^- action would need to be

closer to Photosystem I than the site of TCPI reduction. Fluorescence studies do not support this location for Cl^- involvement[12].

A more probable explanation for the lack of Cl^- effect under basal conditions is as follows. In the absence of inhibitors, the coupling site between the photosystems is the rate-limiting step in electron flow to $Fe(CN)_6^{3-}$ (ref. 37). Omission of coupling reagents increases this limitation to the point where a Cl^- effect is no longer recognizable owing to our inability to make the chloroplasts sufficiently free of Cl^- to shift the limiting reaction from the coupling site to Photosystem II. In the presence of coupling or uncoupling reagents (including TCPI) the turnover rate improves at the coupling site and it becomes possible to demonstrate the effect of Cl^- deficiency. The greatest Cl^- effects are observed with TCPI as electron acceptor because it most effectively uncouples or bypasses the coupling site between the photosystems.

However, according to the above hypothesis, the uncoupled electron flow rate inhibited by lack of Cl^- should not be less than the basal rate. This does not always hold true, as can be seen in Fig. 2 where the increase in Cl^- effect upon adding uncoupler results, in part, from a suppression of the *minus*-Cl^- rate below the basal rate. This contradiction can be explained on the basis of evidence, to be presented in a later publication, that the inhibitions by Cl^- deficiency and by excess uncoupler occur in the same segment of the electron-transfer chain, between water and Photosystem II, and are in fact synergistic.

At present we prefer the above interpretation of the Cl^- effect. It is noteworthy, however, that many peptidases are activated in free solution by the same anions in the same concentration ranges as are operative in the Cl^- effect[38]. Anion activation is also reported for glutaminase, α-amylase and arylsulfatase. ZELITCH[39] observed that glyoxylate reductase from leaves is activated by several anions, though in this case the order of effectiveness is $I^- > Cl^- > SO_4^{2-}$.

Perhaps more relevant to this discussion is the well-documented catalysis by Cl^- and similar ions of electron transfer between metal chelates in solution[40]. Weak acid anions such as azide are very effective in such systems, so that a direct analogy to the chloroplast situation is ruled out. Nevertheless it is reasonable to propose that the chloroplast manganese, also necessary for O_2 evolution, undergoes changes in valency and that Cl^- is involved in this reaction step. Evidence from fluorescence studies supports the view that the Cl^--dependent and Mn^{2+}-dependent sites are in the same segment of the photosynthetic electron-transfer chain, namely, between water and Photosystem II (ref. 12).

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