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THE ROLE OF Cl^- IN PHOTOSYNTHESIS

II. THE EFFECT OF Cl^- UPON FLUORESCENCE

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SUMMARY

Cl^- is necessary for noncyclic electron transport in isolated chloroplasts. In order to locate its functional site, the fluorescence of chloroplasts at 684 nm was examined with respect to the reduction/oxidation poise of the quencher compound located on the reducing side of Photoact II. The fluorescence yield in the absence of Cl^- is lower due to a drop in the maximum fluorescence yield at saturating light intensity and a decrease in quantum efficiency. There is a good correlation between induction of the Cl^- effect by heating or by increasing the pH of the suspending medium, and decline of the fluorescence quantum efficiency. The fluorescence yield kinetics in the absence of Cl^- resemble more closely those obtained with added methyl viologen, which speeds electron transport, rather than those obtained with (dichlorophenyl)methylurea or *o*-phenanthroline which block the oxidation of the quencher by Photosystem I. It is therefore thought that the site of Cl^- involvement is on the oxidation or water-splitting side of Photosystem II and that the absence of Cl^- decreases the electron flow rate from water to the quencher.

INTRODUCTION

Cl^- is necessary for normal plant growth[1] and for the photosynthetic evolution of O_2 (refs. 2, 3). A Cl^- requirement can be demonstrated in isolated chloroplasts using electron acceptors, such as indophenol dyes and ferricyanide, which support noncyclic electron transfer involving Photosystem-II activity[3,4]. On the other hand, cyclic and certain noncyclic electron flows which depend entirely upon the activity of Photosystem I can operate with full efficiency in the virtual absence of Cl^- (refs. 3, 4). The site of Cl^- activity seems, therefore, to be in the area of Photosystem II, but whether on the reducing or oxidizing side of the photoact remains unknown.

DUYSENS[5] has shown that the yield of chlorophyll fluorescence from illuminated chloroplasts seems to be determined by the reduction/oxidation level of a compound located on the reducing side of Photoact II. It may be possible to locate the site of Cl^- action by observing the effect of Cl^- depletion on fluorescence and thus, indirectly, upon the redox state of this quencher compound.

Abbreviations: TCPI, *o*-chlorophenolindo-2,6-dichlorophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

METHODS

Chloroplasts were isolated as previously described[4] from either spinach or pea plants. The spinach was grown locally and the peas were grown in a greenhouse and harvested after 12 to 16 days of growth. Trichlorophenolindophenol (TCPI) reduction was carried out by standard procedures[4].

The fluorimeter was modified from that previously described[6], by incorporation of a chopped measuring beam (270 Hz) from a mercury lamp filtered by a Baird-Atomic filter (430 nm). The fluorescence was analyzed at a 40° angle to the impinging measuring beam. The output from the photomultiplier was amplified using a lock-in amplifier sensitive only to the modulated beam. The sample cuvette was illuminated from above with unmodulated blue actinic light (510 ± 40 nm) from a tungsten filament lamp.

A typical experiment consisted of suspending pea chloroplasts (40 μg chlorophyll) in 2.0 ml of Tricine–NaOH (0.015 M at pH 8.2) and $MgSO_4$ (0.005 M) at 16°. The fluorescence emission of this mixture at 684 nm was observed using a measuring beam intensity of about 20 ergs·cm^{-2}·sec^{-1} and an actinic light intensity of about $5 \cdot 10^3$ ergs·cm^{-2}·sec^{-1}.

RESULTS

Fig. 1a illustrates the relationship between fluorescence (F) at steady state and intensity of the modulated measuring light (I_m), for samples depleted in Cl^- and for samples to which Cl^- was added. Except at low intensities, there is a linear relationship between the fluorescence and the measuring light intensity[7]. The slope of the straight line plot is lower in the absence of Cl^-; however, these plots intercept the I_m axis at the same (non-zero) point. Thus, the fluorescence yield (F/I_m) is lower in Cl^--depleted chloroplasts. If the data from Fig. 1a are replotted as fluorescence yield *vs.* fluorescence yield divided by the intensity of light as in Fig. 1b, both the maximum fluorescence yield (intercept with the yield axis) and the quantum efficiency (reciprocal of the slope; *i.e.*, inverse light intensity required for half-saturation) are

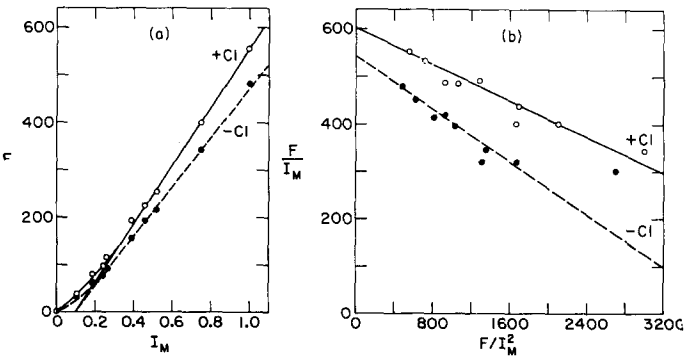

Fig. 1. Fluorescence of Cl^--depleted chloroplasts with and without restored Cl^-. Dependence of (a) fluorescence and (b) fluorescence yield on measuring light intensity. Spinach chloroplasts. Conditions as in METHODS. NaCl (10 mM). F = fluorescence (relative scale); I_m = light intensity (1.00 = 10^3 ergs·cm^{-2}·sec^{-1}) from 460 to 540 nm.

found to be higher in the presence of Cl^-. The lack of Cl^- apparently lowers the fluorescence yield by lowering the quantum efficiency.

The fluorescence yield[5], measured by modulated light of a constant mean intensity, is changed upon the addition of a continuous actinic light as shown in Fig. 2A. The actinic light causes a 3-fold increase in the fluorescence yield in 10–15 sec. If the actinic light is extinguished, the fluorescence yield decreases rapidly at first and then more slowly. A detailed analysis of this off-response will be presented in a later publication.

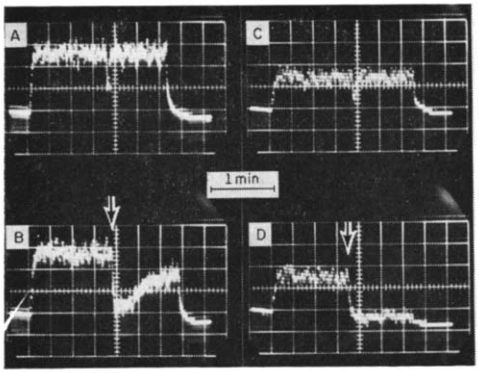

Fig. 2. Fluorescence kinetics of Cl^--depleted spinach chloroplasts. (A) +Cl; (B) +Cl, +TCPI; (C) −Cl; (D) −Cl, +TCPI. In A and B NaCl (10 mM) added initially; in B and D, TCPI (2 μM) added at arrow. Vertical scale: relative fluorescence yield. Actinic light on at 20 sec, off at 160 sec.

Illumination of rhodamine B dye, with the modulated measuring beam causes an instantaneous rise in the fluorescence of the dye, which is unaltered by the subsequent application of the actinic beam. This control shows that the changes in fluorescence recorded with the chloroplasts in response to the actinic beam reflect changes in the redox state of the quencher[5] and not, for example, leakage of a d.c. component through the amplification train.

If a small amount of TCPI is added to the chloroplast suspension during illumination by actinic light (at the arrow in Fig. 2B), the fluorescence yield decreases rapidly to almost the level obtained with no actinic light. The fluorescence yield then rises again as the chloroplasts reduce the dye; but the original level is not reached because the reduced dye undergoes a rapid reoxidation at pH 8.2. In the absence of Cl^-, the steady-state fluorescence yield is low (Fig. 2C). Addition of TCPI results in a prolonged suppression of fluorescence yield (Fig. 2D), since chloroplasts lacking Cl^- reduce indophenol dye very slowly.

The excess fluorescence yield induced by the actinic illumination (ϕ) will be defined as the total fluorescence yield at a given intensity of actinic light (ϕ_F) *minus* that induced by only the measuring beam (ϕ_0). Values of ϕ for Cl^--depleted chloroplasts are shown for several intensities of actinic light in Fig. 3. The steady-state value of ϕ nearly reaches a maximum at high actinic light intensities. The plot of ϕ *vs.* ϕ/I results in a straight line with the reciprocal slope denoting the quantum efficiency (Φ_e). Cl^--depleted chloroplasts, again, have a lower quantum efficiency than those with added Cl^-.

In the preceding paper[4] it was reported that incubation of chloroplasts at 25°

induces or accentuates the Cl^- effect. Table I documents this phenomenon with TCPI serving as the terminal acceptor, and shows the variation of the maximum excess fluorescence yield (ϕ_{max}) and Φ_e as the Cl^- effect develops. The maximum fluorescence declines as a result of incubation and the values for *plus* and *minus* Cl^- converge.

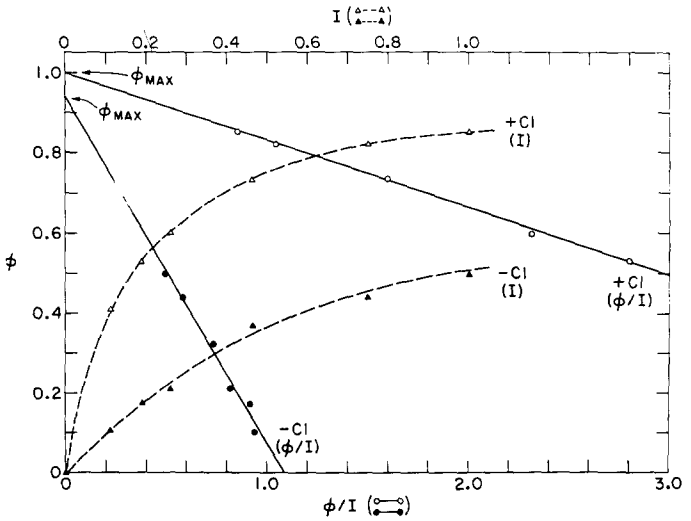

Fig. 3. Steady-state fluorescence yield induced by actinic light. Spinach chloroplasts. ϕ = fluorescence increase due to actinic light, as described in RESULTS. $\phi_{max} = 2.6\,\phi_0$ before normalization. I = actinic light intensity.

TABLE I

EFFECT OF HEAT TREATMENT OF Cl^--DEPLETED CHLOROPLASTS UPON ELECTRON TRANSPORT AND FLUORESCENCE

Heating was carried out on stock solution (400 μg/ml) of chloroplasts at 26°. Chloroplasts incubated as in METHODS for TCPI reduction. Maximum fluorescence (ϕ_{max}) and Φ_e defined by $\phi = (\phi_{max} \cdot I)/(I + 1/\Phi_e)$ as in Fig. 3 and RESULTS with ϕ_0 = yield of 1.0.

Heating time (min)	*Cl⁻*	*Dye reduction (μequiv·mg⁻¹·h⁻¹)*	*Maximum fluorescence (yield)*	*Φ_e ($cm^2 \cdot sec \cdot kerg^{-1}$)*
0	+	182	3.0	1.00
	−	79	2.3	0.71
5	+	200	2.5	0.53
	−	55	2.0	0.23
10	+	173	2.1	0.83
	−	19	1.9	0.13
15	+	170	1.7	0.67
	−	10	1.5	0.10

After 15 min of incubation, Φ_e is less than halved if Cl^- is added to the assay, whereas in the Cl^--free assay Φ_e decreases over 6-fold. The relative values of Φ_e are thus a sensitive reflection of the extent of the Cl^- effect.

Induction of the Cl^- effect depends upon the pH as well as temperature, and the Cl^- effect itself is much more pronounced at higher pH values[4]. A study of the

steady-state fluorescence yield indicates that although ϕ_{max} decreases with increasing pH, it declines more rapidly in the absence of Cl^- (Fig. 4a). Furthermore, only in the absence of Cl^- does Φ_e decrease with increasing pH (Fig. 4b). These observations confirm the utility of Φ_e as a measure of the Cl^- effect.

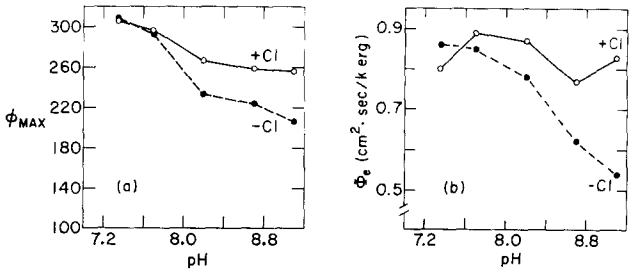

Fig. 4. pH dependency of maximum fluorescence yield and quantum efficiency of Cl^--depleted chloroplasts. Pea chloroplasts. ϕ_{max} = maximum fluorescence. Φ_e = quantum efficiency as determined by: $\phi = [\phi_{max} I]/[I + 1/\Phi_e]$ (see RESULTS), where ϕ_0 = yield of 1.0.

Table II shows that the addition of Cl^- to chloroplasts before illumination gives a greater increase in Φ_e than does the same addition made after 1 min of illumination. This incomplete restoration of Φ_e correlates well with the observed incomplete restoration of electron flow by Cl^-, following pre-illumination in its absence[4].

The initial kinetics of the fluorescence rise at low light intensity (from only the modulated measuring beam) demonstrate the basic difference between inhibition of O_2 evolution by *o*-phenanthroline or 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), and by lack of Cl^- (Fig. 5). The fluorescence rise of chloroplasts (Fig. 5A) exhibits multi-phasic kinetics with three distinct phases[8,9]. The initial sharp rise of fluorescence yield (denoted Phase I) is distorted due to the limiting time response of the instrumentation. Following this there is a short period of a rapid increase in yield (Phase II) which nearly levels off. Changing the time scale of the recording at this point (shown by the dashed line) makes apparent the slowest increase in yield (Phase III) to the steady-state level. In Fig. 5B the absence of Cl^- changes the rate constants (lower

TABLE II

RESTORATION OF FLUORESCENCE BY NaCl IN Cl^--DEPLETED CHLOROPLASTS

ϕ_{max} and Φ_e defined as for Table I. Chloroplasts from two preparations (1 and 2) incubated as in METHODS.

NaCl (mM)	ϕ_{max} *(yield)*	Φ_e *($cm^2 \cdot sec \cdot kerg^{-1}$)*
1. 10	2.6	0.46
10*	2.3	0.40
0	2.2	0.23
2. 10	3.3	0.35
10*	2.9	0.11
0	2.5	0.03

* NaCl added after 1 min illumination.

steady state and slower Phase III rise) but does not alter the basic character of this curve.

Addition of methyl viologen, which is known to promote electron transfer through Photosystem I, mimics the effect of Cl^- deficiency (Fig. 5C). Both in the presence and absence of Cl^-, the steady-state level and the Phase-III rate are lowered by methyl viologen (Fig. 5D), but it is difficult to see changes in earlier kinetic phases. However, *o*-phenanthroline (Fig. 5E) or DCMU (Fig. 5F) alter the kinetics markedly. The rise of Phase II is much faster and the higher steady-state yield is reached rapidly. Phase III is apparently absent.

The effects of Cl^- on the kinetic parameters are tabulated in Table III as yields, with the Phase I yield, ϕ_0 (which was constant under these conditions) set at unity. In all cases, the rate as well as the steady-state level declines (20–30%) in the absence

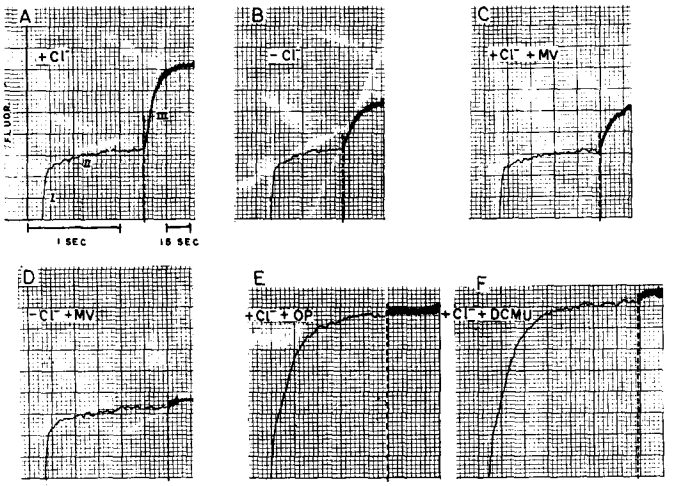

Fig. 5. Fluorescence-rise kinetics in modulated light. Pea chloroplast suspension containing, where indicated: NaCl (10 mM), DCMU (10 μM), *o*-phenanthroline (OP) (100 μM), methyl viologen (MV) (0.2 μM). Measuring beam intensity $\approx$ 250 ergs·cm^{-2}·sec^{-1}. Speed change of recorder is indicated by dashed line. Kinetic phases denoted by Roman numerals.

TABLE III

EFFECT OF INHIBITORS UPON FLUORESCENCE RISE KINETICS OF Cl^--DEPLETED CHLOROPLASTS

NaCl (10 mM), DCMU (5 μM) or *o*-phenanthroline (100 μM) in less than 0.1 % ethanol. Modulated light intensity approx. 170 ergs·cm^{-2}·sec^{-1}.

Conditions	*+Cl⁻*		*−Cl⁻*	
	*Initial rate (yield/sec)**	*Steady-state yield**	*Initial rate (yield/sec)**	*Steady-state yield**
Control				
Phase II**	0.7	—	0.6	—
Phase III**	0.094	3.3	0.066	2.8
+DCMU, Phase II	4.6	5.5	3.3	5.6
+*o*-phenanthroline, Phase II	3.5	4.7	2.8	4.1

* Yield with $\phi_0 = 1.0$.
** See Fig. 5A.

of Cl^-. Only in the presence of DCMU is the steady-state value nearly the same. The action of *o*-phenanthroline and DCMU are qualitatively the same.

When the pathways into Photosystem I are blocked by DCMU, the relationship between the rise rate of Phase II of the fluorescence yield and the intensity of exciting light is linear (Fig. 6). Although the absence of Cl^- decreases the rate of fluorescence rise about 15%, this decrease is not as large as might be expected.

Phase-II kinetics in the absence of DCMU are difficult to measure. The relation of Phase-III kinetics to light intensity in the absence of DCMU is shown in Fig. 7. The basic form of the rate/intensity curve remains the same with or without Cl^- or methyl viologen, only the absolute rates are effected. The similarity between the effect of Cl^- depletion and methyl viologen addition, first noted in Fig. 5, is seen to apply over a range of light intensities.

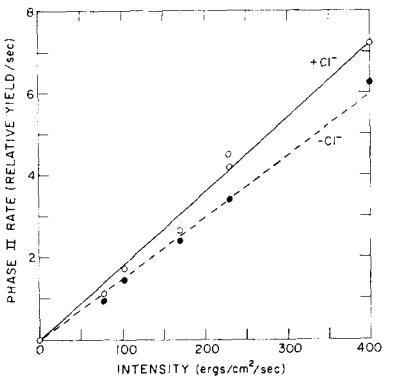

Fig. 6. Light-intensity dependence of the rate of Phase-II fluorescence rises in the presence of DCMU. Cl^--depleted pea chloroplasts with added NaCl (10 mM) where indicated and DCMU (5 μM). Measuring beam intensity approx. 170 ergs·cm^{-2}·sec^{-1}. Relative yield defined as ϕ/ϕ_0.

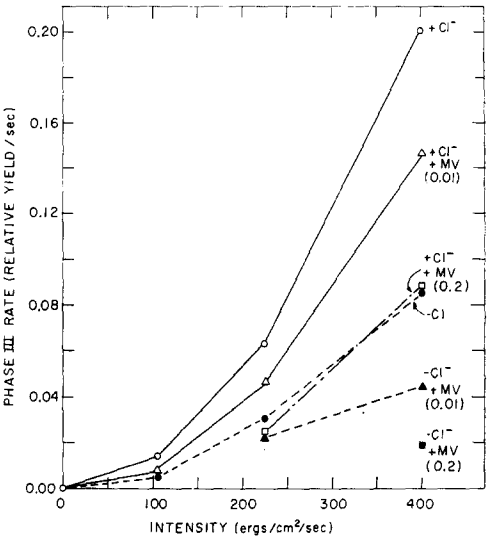

Fig. 7. Light-intensity dependence of the rate of Phase-III fluorescence rise as affected by methyl viologen (MV) and Cl^-. Pea chloroplasts with NaCl (10 mM) and methyl viologen (μM) where indicated, as in Fig. 6.

DISCUSSION

The level of fluorescence, as DUYSENS[5] has observed, seems to be an excellent indicator of the reduction/oxidation poise of the quencher compound which, presumably, is accepting electrons from the photoact itself. We assume that the Phase I or ϕ_0 yield of fluorescence attained with extremely low light intensities and in less than 5 μsec at higher light intensities[9] indicates the lower limit of fluorescence and thus the totally oxidized state of the quencher. Although the weak measuring beam causes some reduction of the quencher, this is very small even in the absence of an electron acceptor. The addition of actinic light causes a relatively rapid reduction of the quencher, measurable as the increase of fluorescence yield.

At the fluorescence steady state the rates of oxidation and reduction of Q are equal. At steady state we can assume that: the flow of electrons into Q is proportional (K) to the intensity of light (I) and the flow of electrons out of Q is dependent upon the amount of reduced Q, the intensity of light and a combination of both. Thus,

$$KI = j_0 I + j_c[Q]_{red} + j_c' I[Q]_{red} \tag{1}$$

where j_0, j_c and j_c' are constants and $[Q]_{red}$ = concentration of reduced Q. This will give the simplest formulation which is thus far consistent with the observations. The j_0 term most probably represents Photosystem-I reactions and the j_c and j_c' terms account for possible back reactions involving oxidants on the water-splitting side of Photoact II. Solving for the value of $[Q]_{red}$ at steady state we obtained:

$$[Q]_{red} = \frac{KI - j_0 I}{j_c + j_c' I} \tag{1'}$$

The maximum amount of reduced Q expected at very high light intensity when j_0 is blocked is:

$$[Q]_{red}^{max} = \frac{K}{j_c'} = Q_0 \tag{2}$$

where Q_0 is the total amount of Q available. Eqn. 2 becomes the definition of j_c' and Eqn. 1' is now:

$$[Q]_{red} = \frac{(K - j_0)Q_0 I}{j_c Q_0 + KI} \tag{1''}$$

If the fluorescence is expressed as the Stein–Volmer equation[10] with the quencher being active only in the oxidized form[5], then:

$$\phi_F = \frac{k_F}{k' + Q_{oxid}} = \frac{k_F}{k' + Q_0 - Q_{red}} \tag{3}$$

where k' and k_F are constants. Substituting Eqn. 1' into Eqn. 3 we obtain:

$$\phi_F = \frac{k_F j_c + k_F KI}{(k' + Q_0) j_c Q_0 + (k' + Q_0) KI - (K - j_0) Q_0 I} \tag{4}$$

which fits the data of Fig. 1b if j_c is less than KI; however, at lower light intensities a plot of ϕ_F *vs.* ϕ_F/I would deviate from linearity.

The measurement of fluorescence yield is not as Eqn. 4 but rather a difference of fluorescence between that obtained in actinic light and low measuring light (in which Q is largely oxidized)

$$\phi = \phi_F - \phi_0 = \phi_F - \frac{k_F}{k' + Q_0} \tag{5}$$

Substituting Eqn. 4 into Eqn. 5 and rearranging we obtain:

$$\phi = \frac{\phi_{max} \cdot I}{I + 1/\Phi_e} \tag{6}$$

where

$$\phi_{max} = \frac{k_F(k - j_0)Q_0}{(k' + Q_0)(j_0 Q_0 + k'K)} \tag{7}$$

$$\Phi_e = \frac{j_0 Q_0 + k'K}{j_c Q_0 (k' + Q_0)} \tag{7'}$$

Eqn. 6 matches the experimental data of Fig. 3.

The absence of Cl^- seems, in the main, to give low values of Φ_e (Fig. 3) while restoring Cl^- to the chloroplasts gives a high value of Φ_e (Table II). A decrease in Φ_e from Eqn. 7' indicates an increase in j_c or in the amount of possible back reaction. The possibility of k_F, k' or Q_0 changing must be ruled out by the constancy of ϕ_0 ($= k_F/[k'+Q_0]$) and ϕ ($=k_F/k'$) in the presence of DCMU with or without Cl^- (see Table III and text). Back reactions of Photosystem II have been postulated previously[7]. A decrease in ϕ_{max}, due to high pH or heating as in Fig. 4 and Table I, indicates a decrease in the ratio of K/j_0, with either K decreasing or j_0 increasing. The j_0 term most probably represents electron flow through Photosystem I and should be fairly constant under the above experimental conditions; on the other hand, heating, high pH and the absence of Cl^- are known to decrease the electron flow from Photosystem II[4]. Thus the rate constant (K) for electron flow from Photosystem II into the quencher most probably declines during the above treatments.

A lower steady-state level (and Phase-III rate) is also observed in the presence of methyl viologen (Fig. 5). The similarities between the effects of methyl viologen and the lack of Cl^- (Fig. 7) cannot be ascribed to the same process since Cl^- deficiency decreases and methyl viologen increases the noncyclic electron flow rate[11]. Therefore, the lack of Cl^- must lower fluorescence by decreasing the electron flow rate into the quencher.

The Phase-II rate of fluorescence increase is thought to reflect the initial electron flow into the quencher, whereas the Phase-III rate most probably indicates electron flow from Q into subsequent pools[8]. Table III shows that although the absence of Cl^- slows the Phase-II rate, the Phase-III rate is much more pronouncedly decreased. When Phase II is enhanced (*e.g.*, by the addition of DCMU) the rise rate is decreased by the lack of Cl^-, but not greatly. The low sensitivity to the absence of Cl^- under these conditions may be due to a pool of electrons located between the photoact and water-splitting, with the site of Cl^- function lying on the water-splitting side of the pool. Initially there would be little limitation to electron flow since electrons would be obtained from the pool. The pool could represent the bound manganese which is present at a concentration of 1 manganese per 50 chlorophylls[12].

The best location for the functional site of Cl^- from the data presented here is near the water-splitting step. The absence of Cl^- would limit the flow of electrons and thereby generate a high concentration of oxidizing equivalents near the reaction center. These intermediates might speed back reactions (j_c) from the reduced quencher and promote deleterious oxidations within the photochemical apparatus[13].

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