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MULTIPLE SITES OF DCIP REDUCTION BY SONICATED OAT CHLOROPLASTS: ROLE OF PLASTOCYANIN

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SUMMARY

1. Oat chloroplasts, in the presence of 0.02 M methylamine, reduce 2,6 dichlorophenolindophenol (DCIP) at a rate of 350–500 μmoles/mg chl per h, in saturating light. Brief sonication for approx. 1 min lowers the rate to approx. 50 μmoles/mg chl per h; longer sonication does not reduce activity further. During brief sonication, plastocyanin is lost from the chloroplasts. When plastocyanin is added back to sonicated fragments, DCIP reduction is approximately doubled to 100 μmoles/mg chl per h.

2. When oxidized plastocyanin is added, a transient is observed when light is first turned on: this is due to a reduction of the plastocyanin before DCIP reduction begins. When reduced plastocyanin is added, a different transient occurs: this is due to a fast photoreduction of DCIP by the plastocyanin and is followed by the slower steady state reduction of DCIP by water. When light is turned off before complete reduction of DCIP, a transient reduction of oxidized plastocyanin by reduced DCIP is seen. Insensitivity of these transients to 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and the greater effectiveness of 710 nm light, along with the known capacity of plastocyanin to mediate electron transfer to System I, prove that an intrinsically fast reduction of DCIP occurs at a site close to the primary photoreduced product of System I.

3. After brief sonication and washing, no residual plastocyanin was detected in chloroplast fragments, and the rate of the slow DCIP reduction (about 50 μmoles/mg chl per h) sustained by such fragments was essentially identical to that maintained by fragments of mutants lacking System I activity. Following KOK *et al.*[9], the simplest explanation for this slow DCIP reduction is that is occurs at a site close to System II and the system I is not involved.

4. A very slow transient reduction of DCIP occurs after extinguishing light; this presumably involves another reduction site close to System II, as suggested by KOK[9].

INTRODUCTION

Early comparisons of the Hill reaction activity of small, sonically prepared chloroplast fragments, with either large fragments[1,2] or osmotically disrupted chloro-

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCPIP, dichlorophenolphoshoindophenol; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; chl, chlorophylls.

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plasts[3], suggested that sonic disruption does not drastically reduce the electron transport activity of whole chloroplasts. However, more recent studies[4-8] show that sonication does reduce Hill reaction activity markedly. The decline accompanying brief sonication is especially dramatic in the case of $Fe(CN)_3$ and 2,6-dichlorophenol-indophenol (DCIP) Hill reactions measured in the presence of an uncoupler: typical rates with washed chloroplasts are 300–500 μmoles/mg chl per h and fall to around approx. 50 μmoles/mg chl per h after 2 min sonication[4,7,8]. Concerning the decline in activity, there are two important points. One is that, although 2 min sonication causes great loss of activity, the residual activity is comparatively resistant: further sonication for ten minutes or more causes little additional loss[5,6,15]. The second is that kinetic characteristics such as pH optimum, saturating oxidant concentration, and quantum yield in low light are different before and after sonication[8]. These observations show that DCIP and $Fe(CN)_3$ reduction occur by different mechanisms in whole chloroplasts and in sonically prepared fragments.

Evidence that DCIP can be reduced at different sites was presented by KOK *et al.*[9] and KE[10]. KOK *et al.*[9] showed that chloroplast fragments from wild type *Scenedesmus* reduce DCIP in fast and slow phases following a 2 μsec light flash. The slow phase but not the fast one is abolished by 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU); on the other hand, the slow phase but not the fast one is present in mutant No. 8 which lacks System I activity. KE[10] showed that lights I and II preferentially sensitize the fast and slow phases, respectively, of dichlorophenolphosphoindophenol (DCPIP) reduction by spinach chloroplasts. These observations point to two possible sites of DCIP reduction, one intrinsically fast and associated with System I, one intrinsically slow and associated with System II. KOK *et al.*[9] also demonstrated a third site close to System II at which DCIP can be very slowly reduced. Less direct evidence of two reduction sites are the different light-saturated rates of $Fe(CN)_3$ reduction by fragments from wild type and P_{700}-deficient Chlamydonomas strains[11] and the presence of enhancement in $Fe(CN)_3$ reduction in moderate but not low illumination[12].

This paper characterizes the kinetics of DCIP reduction in oat chloroplast fragments in the presence and absence of added plastocyanin. Our study confirms reported changes in activity brought about by sonication, and also confirms a rapid reduction at a System I site. Our results are furthermore consistent with intrinsically slower reductions at two sites close to System II; however, we cannot completely exclude the alternative of a slow, plastocyanin-independent path for electron transport to System I. In favoring the hypothesis of slow reduction at System II sites, we conclude tentatively that the marked fall in DCIP reduction activity, which attends brief sonication and is concomitant with a loss of plastocyanin, is due to a shift from fast reduction at a system I site to a slower reduction at a System II site. Addition of plastocyanin to fragments shifts reduction back to the System I site.

MATERIALS AND METHODS

Chloroplasts were isolated from 2–3-week-old oat seedings (*Avena sativa*, variety Gary) grown on vermiculite in the greenhouse. Routinely, 40–60 g of leaves with 240–300 ml of ice-cold medium containing 0.4 M sucrose, 0.01 M KH_2PO_4–

K_2HPO_4 (pH 7.2) and 0.01 M KCl, were homogenized in a Waring blendor operated at full power for 15 sec. After filtering through cheese cloth, the filtrate was centrifuged at 200 *g* for 2 min. The supernatant was centrifuged for 10 min at 1500 *g*. The sediment was resuspended and centrifuged again. The sediment of 'washed chloroplasts' was finally suspended at a chlorophyll concentration of 1–2 mg/ml and stored in an ice-bath.

To prepare sonicated fragments, washed chloroplasts (0.1–0.3 mg chl/ml) were suspended in 25 ml of buffer (pH 7.2, KH_2PO_4–K_2HPO_4, 0.01 M KCl 0.01 M) in 50 ml stainless teel centrifuge tubes. Sonication was performed with a Branson Model LS-75 (20 cycles, power setting 7, 5.8 A, standard 3/8" tip immersed 1 cm.). Sonication usually lasted 60 sec. Sonicated fragments were obtained by differential centrifugation (Sorvall RC-2 SS-34 angle rotor). Sediment fractions were designated '2K', after 10 min at 2000 × *g*, '2–12K', after an additional centrifugation at 12000 × *g* for 10 min, *etc.* Centrifigation lasted 20 min at 25000 × *g* and 30 min at 37000 × *g*. For forces higher than 37000 × *g*, a Spinco Model L ultracentrifuge with type 50 rotor was used and the time increased to 1 h. The extensively employed '3–25K' fraction was obtained by centrifuging 25 ml of a 90 sec sonicate at 25000 × *g* for 20 min, resuspending the sediment in 12 ml of buffer, and centrifuging again at 3000 × *g* for 10 min to remove large grana systems. This preparation contained about 40 % of the chloroplast chlorophyll.

Plastyocyanin was isolated by sonicating 50 ml of chloroplast suspension (obtained from 500 g of oat leaves) for 2 min in phosphate–KCl buffer. After centrifuging at 37000 × *g* for 1 h, the supernatant was fractionated with $(NH_4)_2SO_4$. The precipitate obtained between 55–100 % saturation was prufied by a gel-filtration with Sephadex G-75 and DEAE-cellulose chromatography[13]. The absorbance ratio ($A_{278\ nm}$: $A_{297\ nm}$) of the purified plastocyanin was 1.8. Plastocyanin was assayed using an extinction coefficient of $9.8 \cdot 10^3$ l·mole^{-1}·cm^{-1} or $4.9 \cdot 10^3$ l·equiv^{-1}·cm^{-1} at 597 nm[18].

The Hill reaction with DCIP was carried out in 2.4 ml reaction mixtures containing 0.01 M phosphate (pH 7.2), 0.01 M KCl, 30 μM DCIP, and about 24 μg of chlorophyll. Sucrose and methylamine–HCl (pH 7.2) when included were 0.4 M and 0.03 M respectively. The temperature was controlled at 20 ± 0.5°.

Strong actinic illumination and a weak monochromatic beam for spectrophotometric assay of DCIP were provided by a 'cross beam' optical system. The monochromatic (590 nm) beam, isolated from a battery-operated tungsten lamp by a Bausch and Lomb 250 mm f. l. monochromator, passed through the sample cuvette, a lucite light pipe, a 590 nm interference filter, a Corning No. 4308 blue filter, and fell on an RCA IP21 photomultiplier. Filters completely eliminated scattered actinic light. Actinic light (630–700 nm) was obtained from a 1000 W tungsten lamp *via* a Kohler projection system was freed of infrared by 1.5 cm of water, a piece of 'Solex' heat absorbing glass, and a XK-8 interference filter (Baird–Atomic), and of short waves by No. 2304 Corning and a No. 29 Wratten red filters. Unless otherwise stated, the actinic illumination was $6 \cdot 10^5$ erg cm^{-2}·sec^{-1}.

Reaction rate was measured with a calibrated log-derivative circuit which permitted direct recording of absorbance and its time derivative on a two-pen recorder. Details of the circuit are given elsewhere[13].

RESULTS

Steady state reaction: *Effects of storage, sonication and plastocyanin*

As in the ferricyanide Hill reaction, we found whole chloroplasts reduced DCIP at a moderate rate of about 50–70 μmoles/mg^{-1} chl per h. Greatly accelarated rates (350–500 μmoles/mg chl per h) occurred when an optimal concentration (20 mM) of the uncoupler methylamine was added. To avoid rate limitation due to a slow step coupled to phosphorylation, methylamine was routinely included. When stored in buffer containing 0.4 M sucrose, the uncoupled activity of chloroplasts declined less than 5 %/h. In buffer alone, activity increased about 15 % during the first 15 min, declined steadily at a rate about three times faster than that in the presence of sucrose. Subsequently, the original activity of unsonicated chloroplasts was taken as the average of five measurements performed between 20 and 45 min after resuspending chloroplasts in the sucrose-free buffer used for sonication

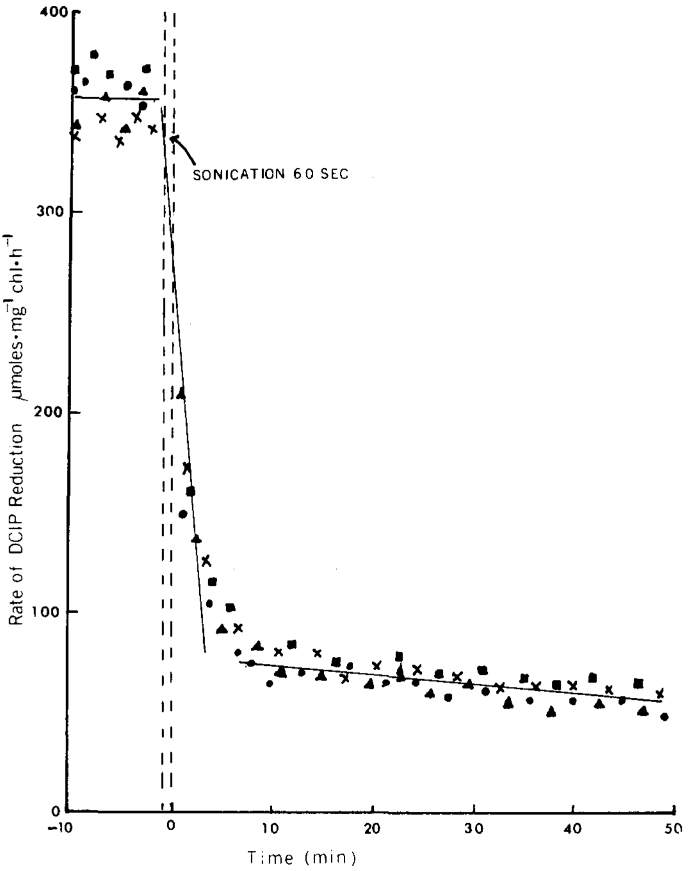

Fig. 1. Decay of DCIP reducing activity of sonicated chloroplasts. Sonication of freshly isolated chloroplasts was carried out as described under MATERIALS AND METHODS. DCIP was measured, before and at the indicated time after sonication, at 20° under red illumination (630 nm$<\lambda<$ 700 nm, $6 \cdot 10^5$ erg·cm^{-2}·sec^{-1}) in a final volume of 2.5 ml in a solution containing 0.01 M phosphate buffer, pH 7.2, 0.01 M KCl, 30 μM DCIP, 0.02 M methylamine (neutralized with HCl), and chloroplasts equivalent to 10 μg chlorophyll per ml.

Fig. 1 shows that the rate of DCIP reduction falls very rapidly during, and in the first 5–10 min following a 60-second sonication. After this period, the rate is about 60–70 μmoles/mg chl per h or about 16–20 % of the rate prior to sonication. From 10–50 min after sonication, a very slow further decline of activity occurs — the final rate being about 50 μmoles/mg chl per h. Apparently, the initial fast decline and the subsequent slow decline are due to different inactiviation mechanisms. In order to assure completion of the fast decline in activity, activity was measured at least 15 min after sonfication.

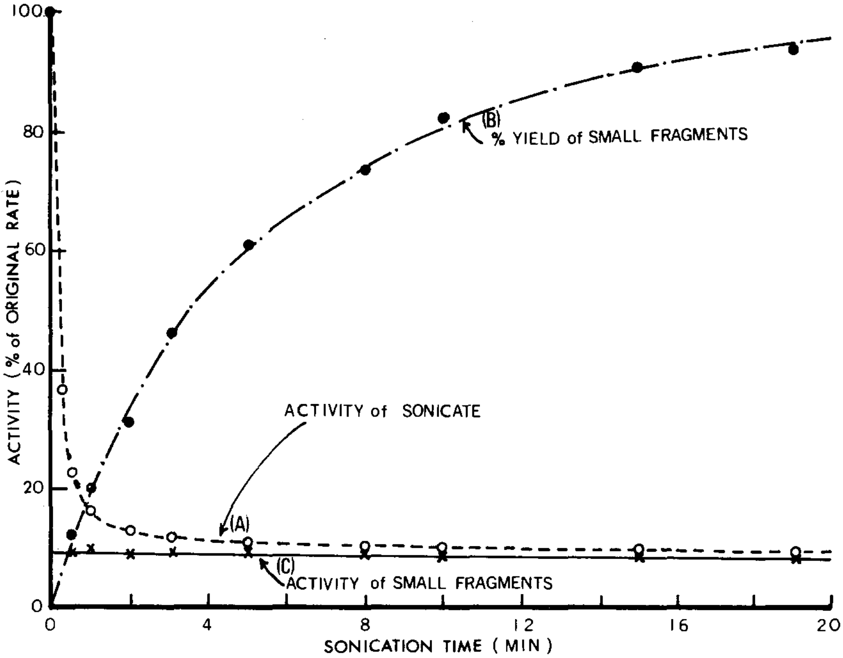

Fig. 2. DCIP reduction activity and yield of "small fragments" as a Function of the duration of sonication of chloroplasts. Freshly prepared chloroplasts were suspended in 0.01 M phosphate-buffer pH 7.2 0.01 M KCl; chl concentration, 126 μg/ml. From the suspension stored on ice, 25 ml aliquots were taken and sonicated for the times indicated. Sonication was performed in 30 sec intervals separated by 1 min intervals of cooling on ice. After the desired total sonication time, 5 ml of sonicate was used for assaying total chlorophyll and DCIP reducing activity. 20 ml of sonicate were centrifuged at 37000 × *g* for 60 min. The supernatant was assayed for chl concentration and DCIP reducing activity. DCIP reduction was measured under conditions as described in Fig. 1. Curve A, activity of sonicated suspensions prior to centrifugation; Curve B, yield of "small fragments", *i.e.* per cent of total chlorophyll in supernatant; Curve C, activity of "small fragments". Original activity of unsonicated chloroplasts (100% activity) was 341 ± 21 μmoles/mg chl per h.

Fig. 2 shows results of an experiment in which chloroplastst were sonicated for 0–20 min. Curve A shows the activity of the sonciated chloroplast suspension; Curve B, the yield of 'small fragments' (*i.e.* the fraction of total chlorophyll which remained in the supernatant after centrifuging at 37000 × *g* for 1 h); and Curve C, the activity of the 'small fragments'. It is seen that about 90 % of the original activity was lost in the first 2 min of sonication, but only about 30 % of the chloroplasts was converted to small fragments. As sonication lengthened to 20 min, essentially all of the chloroplasts were converted to small fragments, but little further loss of activity occured. Indeed, the activity of the small fragments was constant throughout the 20 min sonication period. Similar results have been reported previously[4-6,15].

The relationship between activity and fragment size was also examined in experiments in which fragments of different sizes were isolated by differential centrifugation from chloroplasts sonicated for 1 min. Typical data (Fig. 3) indicate that all fractions from Fraction 2K to 25-37K support essentially identical rates of 50-70 μmoles/mg chl per h. The smallest fragments (37-70K and 70-160K) give somewhat smaller rates, *i.e.* 25-40 μmoles/mg chl per h.

Evidently, the large loss of activity caused by brief sonication is primarily associated with an initial disruption of chloroplasts into relatively large fragments and not with the subsequent conversion of large fragments into small ones. We, therefore, considered the possibility that a necessary soluble factor(s) is lost. One may note that sonic disruption of suspensions containing 3-4 μl packed chloroplasts per ml (corresponding to about 10 μg chl per ml) would result in about a 1000-fold dilution of any soluble factor. A soluble factor supporting DCIP reduction was demonstrated as

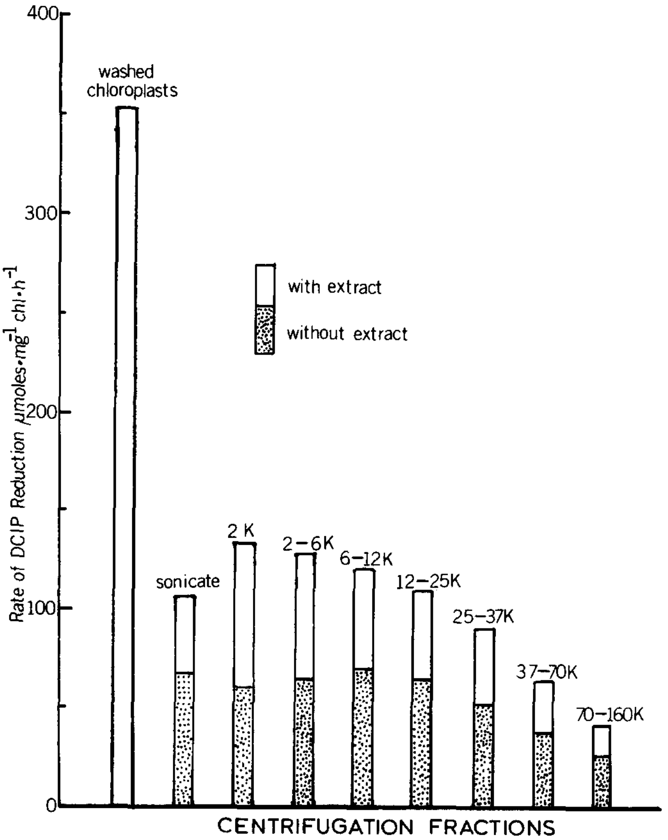

Fig. 3. Partial restoration of DCIP reducing activity of sonicated chloroplast fragments by a chloroplast extract. Chloroplast extract was prepared as described in the text. Chloroplast fragments of various sizes were obtained by differential centrifugation as described under MATERIALS AND METHODS. DCIP reduction was measured under conditions described in Fig. 1 except that 0.5 ml of the chloroplast extract was included in the 2.5 ml reaction mixtures when indicated.

follows: A grana-free chloroplast extract was prepared by sonicating a dense chloroplast suspension (about 3 mg chl per ml) for 2 min followed by centrifugation at 190000 × *g* for 2 h. Fig. 3 shows the additions of the extract almost doubled the activity of all sizes of chloroplast fragments. Fractionation of the extract by a combination of methods (see MATERIALS AND METHODS) led to identification of the active factor — plastocyanin. Subsequently, when highly purified plastocyanin from oat chloroplasts was used, identical results were obtained, and it was found that the maximum stimulation (about two-fould) was obtained with $\geqslant 3\ \mu M$ plastocyanin (see Fig. 4).

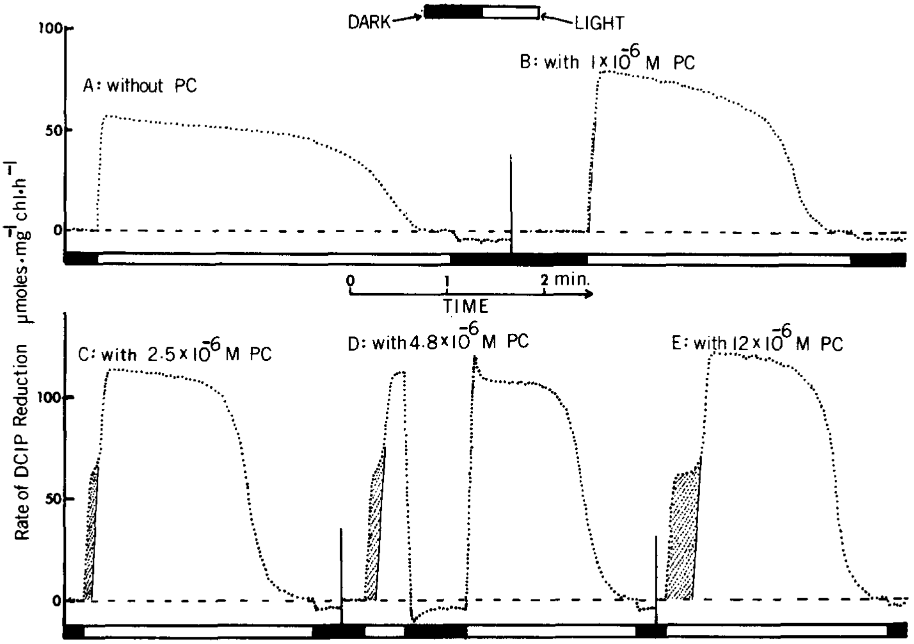

Fig. 4. Time courses of the rate of DCIP reduction with 3–25 K Fragments in the presence and absence of plastocyanin(PC). Conditions as described in Fig. 1, except that oxidized plastocyanin was added as indicated. The decline in rate after 1–3 min is due to exhaustion of DCIP and the small negative rate in darkness indicates a slow autooxidation of reduced dye.

Induction dependent on plastocyanin reduction

Addition of plastocyanin not only accelerated steady state DCIP reduction, but also led to the appearance of an initial induction transient (see Fig. 4). In the absence of plastocyanin, a steady-state rate developed immediately upon turning on the light. In contrast, with plastocyanin there was an initial period of slower bleaching prior to the attainment of maximum rate. The initial slower bleaching is ascribable to reduction of plastocyanin, the later higher rate of bleaching to reduction of DCIP. This assigment is supported by the following: (a) the initial lag period is absent when reaction mixtures were prepared with reduced plastocyanin, (b) the duration of the induction period is proportional to the amount of oxidized plastocyanin present in the mixture, (c) the rate of change of absorbance early in the induction period is about half that of the steady state rate — this correlates with the extinction coefficient of plastocyanin being half that of DCIP[18,23].

Evidently, the rate of plastocyanin reduction during the induction period and the steady state rate of DCIP reduction (maximally stimulated by plastocyanin) are equal in terms of electrons transported per unit time. KATOH *et al.*[16] observed a similar plastocyanin-dependent lag period in DCIP reduction with a washed grana preparation from spinach, and attributed it to competitve inhibition of the reduction of DCIP by oxidized plastocyanin.

Curve D of Fig. 4 shows an additional property. When the illumination is interrupted in the middle of the reaction, there is no lag period when a second illumination begins. This means there is no appreciable dark oxidation of plastocyanin by DCIP. This in accord with the known redox potentials of plastocyanin (E_0 = 0.37 V)[18] and DCIP (E_0 = 0.21 V)[24] and with the fact that reduced DCIP spontaneously donates electrons to System I via plastocyanin.

Fast transients; evidence of DCIP reduction by Photosystem I

The presence of plastocyanin also leads to the appearance of fast reversible

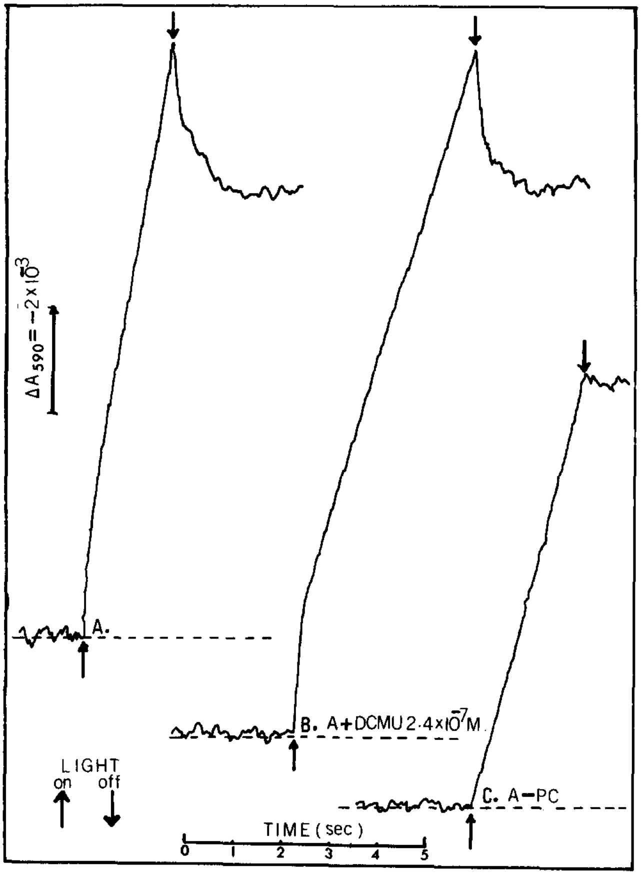

Fig. 5. Biphasic kinetics of absorbance decrease at 590 nm during illumination and the initial transient increase upon darkening. The 3–25K fragments were prepared as described under MATERIAL AND METHODS. Measurement of DCIP was carried out as described in Fig. 1, except that 2.5 μM of plastocyanin (PC) (reduced form) was included in the reaction mixtures.

transients. An example is seen in Curve D of Fig. 4. Upon ending the first short illumination, during which plastocyanin and some DCIP were reduced, there was an initial minimum in the rate of absorbance change, followed by a small, fast transient increase. Later, at the start of the second light period, there was a transient maximum in the rate of absorbance change. The rate curves in Fig. 4 were obtained using the slow differentiator circuit (time constant, 0.6 sec). For further characterization of the fast transients, we recorded absorbance directly; in this case, instrumental response was limited by the recorder pen speed (10 inch/sec).

To observe the fast transients, oxidized DCIP and at least partly reduced plastocyanin must both be present. Proof of this is found in Figs. 4, 5, 6 and 7. In Fig. 4, the transient was not seen at the first illumination when plastocyanin and DCIP were both fully oxidized, nor was it seen when light was extinguished after complete reduction of both components. In Fig. 5 the requirement for reduced plastocyanin is

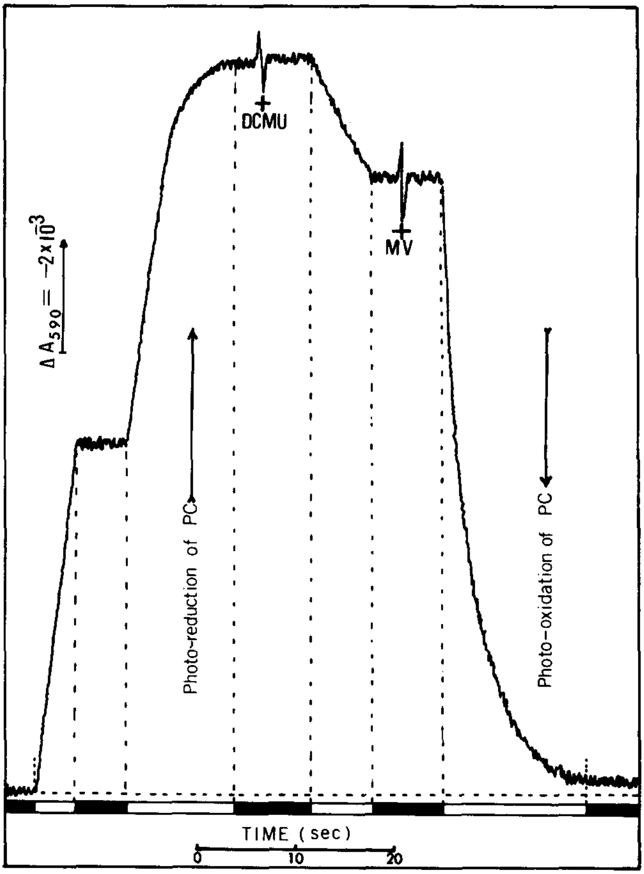

Fig. 6. Photoreduction (without DCMU) and photooxidation (in the presence of DCMU and methylviologen) of plastocyanin (PC) by 3–25K fragments. Original reaction mixture (2.5 ml) contained 0.01 M phosphate-buffer (pH 7.2), 0.01 M KCl, 30 μM DCIP, 0.02 M methylamine, fragments (5.1 μg chlorophyll per ml) and 1.2 μM of plastocyanin added in oxidized form. Reaction was carried out at 20° under $6\cdot10^5$ erg$\cdot$cm$^{-2}\cdot$sec^{-1} of red light which was turned on and off as indicated by the white black bars at the bottom of the graph. DCMU (10 μM) and methylviologen (MV) (0.1 mM) were added at the time indicated in the figure.

seen. In Curve C, no plastocyanin was added and there was no transient; rather the steady state reduction of DCIP began immediately. In Curve A, with reduced plastocyanin present, the initial fast transient occurred and was followed by steady state reaction which was faster than in the absence of plastocyanin. Curve B shows DCMU at low concentration ($2.4 \cdot 10^{-7}$ M) does not affect the fast transient, but does slow the steady state reaction. Fig. 5 also shows that, where fast transients are seen at the start of illumination, they also appear in similar magnitude, but in reverse sense, at the end of illumination, provided the illumination is too short for complete reduction of DCIP. Finally, in Fig. 6, no fast transients occurred during the photoreduction of plastocyanin in the absence of DCIP — either initially when the plastocyanin was fully oxidized, or later when the plastocyanin was partially or fully reduced.

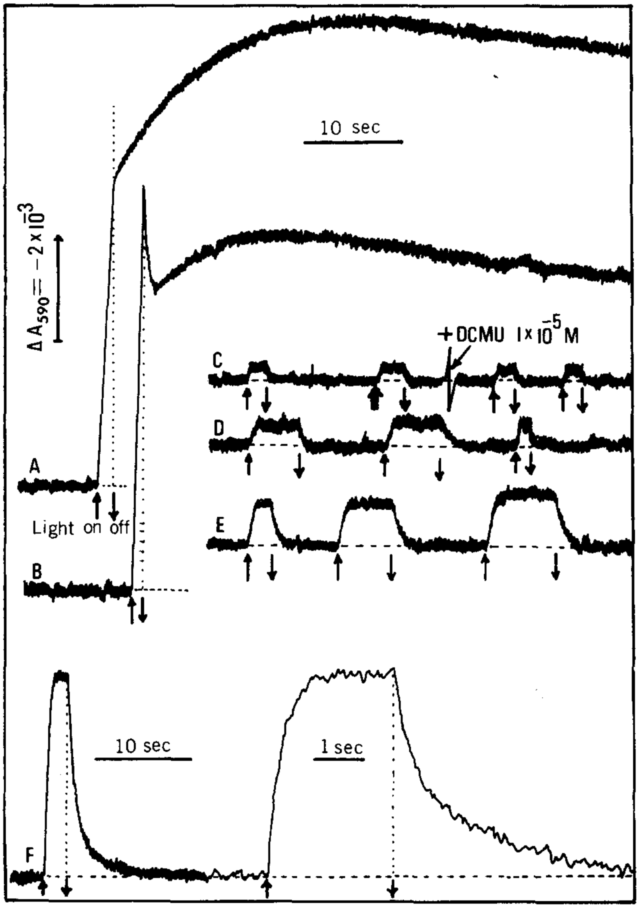

Fig. 7. Absorbance changes at 590 nm caused by brief illumination of 3–25K fragments. Basic reaction mixture (2.5 ml) contained 0.01 M phosphate-buffer, 0.01 M KCl, 0.02 M methylamine. Plastocyanin when used was 2.5 μM. DCIP when added was 30 μM and unless stated otherwise fully oxidized DCIP was used. Reaction were carried out at 20° under $6 \cdot 10^5$ erg · $cm^{-2} \cdot sec^{-1}$ of red light. Curve A, Fragments (10.8 μg chlorophyll per ml) + DCIP; Curve B, Fragments + DCIP + plastocyanin (fully reduced); Curve C, Fragments only (first part without DCMU, second part with 10 μM DCMU); Curve D, Fragments + DCMU + DCIP (initially 10% in reduced form); Curve E, Fragments + DCMU + DCIP + plastocyanin (about 90% in oxidized form); Curve F, Fragments + DCMU + DCIP + plastocyanin (fully reduced).

In Fig. 7, Curves C, D, E, and F show how the magnitude of the fast transients increase with consecutive additions of DCIP, oxidized plastocyanin, and reduced plastocyanin. Chloroplast fragments alone (Curve C) give a very small absorbance change the nature of which is unknown. Addition of DCIP (Curve D) gives a slightly larger reversible absorbance change. (The increment increase in magnitude due to DCIP corresponds to about 1 equivalent of DCIP reduced per 500 moles chlorophyll; KE[10] reported a similar observation). Addition of plastocyanin (about 90 % oxidized increased the transient amplitude somewhat more (Curve E). Finally, addition of reduced plastocyanin (Curve F) magnifies the transient markedly. In similar experiments, we found that the amplitude of the fast transients is directly proportional to chlorophyll content, and that the maximum absorbance change ($4 \cdot 10^{-4}$ per μg chl per ml) is developed in the presence of approx. 1 reduced plastocyanin molecule per 5 chlorophylls.

Several observations show the fast transients are sensitized by Photosytem I: (a) the transients are insensitive to DCMU (Figs. 5 and 7), (b) far-red (710 nm) is more effective than red (648 nm). In Fig. 8, although the 648 nm light provided 1.6 times more incident energy and was more strongly absorbed, the 710 nm light produced an equal or larger transient both in the presence and absence of DCMU. On the other hand, the 648 nm light supported a 10-fold faster steady state reaction, (c) in experiments with Chlamydomonas mutants, sonically prepared fragments from mutant ac-206 (lacking cytochrome f_{553} and System I activity), exhibited no detectable fast transient

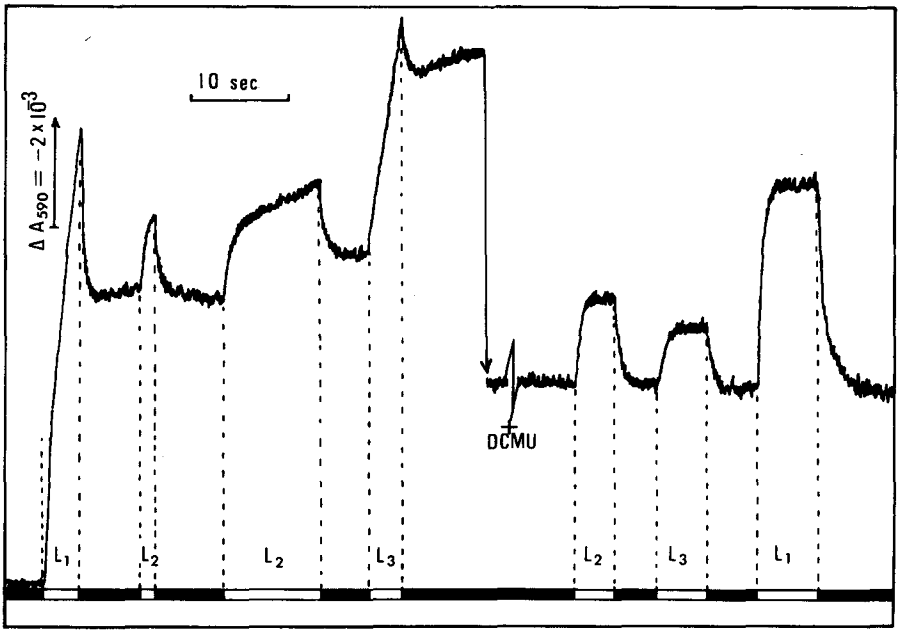

Fig. 8. Effect of red and far-red lights on the time course of absorbance changes at 590 nm with 3–25K fragments. Reaction mixture (2.5 ml) contained 0.01 M phosphate-buffer (pH 7.2), 0.01 M KCl, 30 μM DCIP, 2.5 μM plastocyanin and 0.02 M methylamine–HCl, and fragments (9.7 μg chlorophyll per ml). DCMU (20 μM) was added as indicated in the graph. Reaction was carried out at 20°. The same sample was used throughout different illumination conditions. L_1: 630–700 nm $6 \cdot 10^5$ erg·cm^{-2}·sec^{-1}; L_2: 710 nm; $3 \cdot 10^4$ erg·cm^{-2}·sec^{-1}; L_3: 648 nm, $5 \cdot 10^4$ erg·cm^{-2}·sec^{-1}.

in the presence of DCIP and reduced plastocyanin, but did catalyze a DCMU-sensitive steady state reduction of DCPIP (Fig. 9, A–D). With mutant ac-115 (lacking cytochrome *b* and System II activity), there was no steady state reaction, but there were typical fast transient which were insensitive to DCMU and dependent on added plastocyanin (Fig. 9, E–H), with fragments prepared from the wildtype cells, both the steady state reaction and the transients were oserved (Fig. 10). (The small transients observed in Fig. 9, F and Fig. 10, B were probably due to imcomplete extraction of endogenous plastocyanin.)

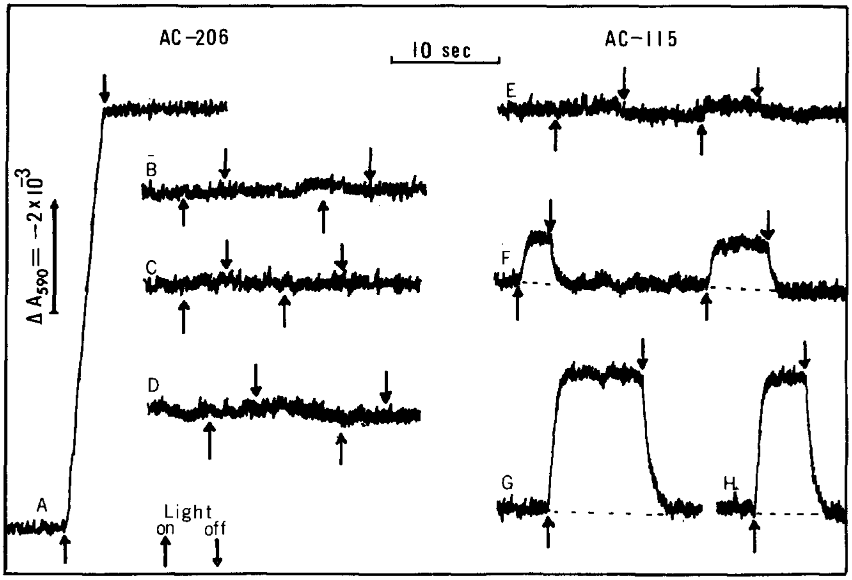

Fig. 9. Absorbance changes at 590 nm with 3–25K fragments from mutant strains of *Chlamdomonas reinhardi*. Fragments from the cells of mutants (ac-206 and ac-115) were prepared by washing and resuspending the freshly harvested cells with 0.01 M phosphate-buffer, pH 7.2, containing 0.01 M KCl. Upon sonicating 25 ml of the cell suspension (containing 0.1 mg chlorophyll per ml) for 60 sec, the 3–25 Fragments were isolated by differential centrifugation as described under MATERIALS AND METHODS. The basic reaction mixtures (2.5 ml) contained 0.01 M phosphate-buffer pH 7.2, 0.01 M KCl, 30 μM DCIP, 0.02 M methylamine–HCl and fragments (equivalent to 9.5 μg chlorophyll per ml for traces A – D and 12.3 μg chlorophyll per ml for E – H).

Curve A, fragments (ac-206) in basic reaction mixture; Curve B, A *minus* DCIP; Curve C, A + DCMU (10 μM); Curve D, C + plastocyanin (2.5 μM reduced form); Curve E, Fragments (ac-115) in basic reaction mixtures *minus* DCIP; Curve F, E + DCIP (30 μM total, 10% added in reduced form); Curve G, F + plastocyanin (2.5 μM reduced form); Curve H, G + DCMU (10 μM).

The properties of fast transients described thus far show that the transient is associated with System I and requires the presence of reduced plastocyanin and oxidized DCIP. The maximum absorbance change in the transient is 0.004 in a sample made up to 10 nmoles·ml^{-1} in chl, 30 nmoles·ml^{-1} in oxidized DCIP and 2.5 nmoles·ml^{-1} of reduced plastocyanin. Three alternative mechanisms of the fast transient reaction can be conceived.

(1) Photoreduction of oxidized plastocyanin, which is present in small amounts in the dark equilibrium with DCIP, by endogenous donors. This mechanism appears impossible for two reasons. First, the absorbance change would indicate about 0.5

nmole/ml (or 1.0 nequiv/ml) of plastocyanin reduced, corresponding to more than 40 equivalents per photosynthetic unit of 500 chl. This is much too large to account for by endogenous electron carriers which lie between Systems I and II and are not oxidized in the dark. Secondly, from the oxidation potentials of plastocyanin (+ 0.37) and DCIP (+0.21), and the concentrations of reduced plastocyanin and DCIP in the reaction mixture, one can calculate that, at equilibrium, the concentration of oxidized plastocyanin would be about 0.015 nmoles/ml which is too small to supoort the observed extent of reaction.

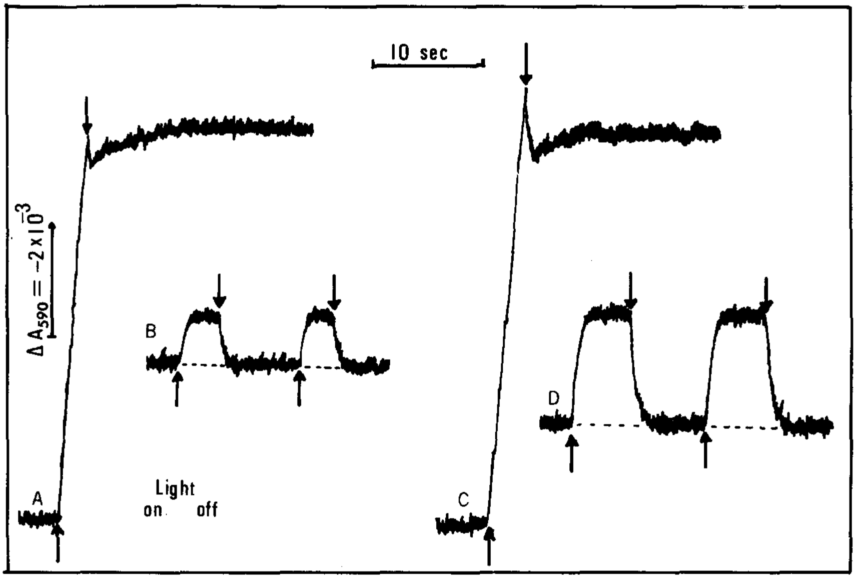

Fig. 10. Absorbance changes at 590 nm with 3–25 fragments from wild-type Cells of *Chlamydomonas reinhardi*. Fragments from the wild-type cells were prepared as described in Fig. 9. Basic reaction mixtures and measuring conditions were the same as in Fig. 9 except that the amount of fragments used in this experiment was 11.7 μg chlorophyll per ml. Curve A, Fragments in basic reaction mixtures; Curve B, A + DCMU (10 μM); Curve C, A + plastocyanin (2.5 μM reduced form); Curve D, C + DCMU (10 μM).

(2) Photoreduction of DCIP by endogenous donors. In this case, the observed absorbance change would indicate about 0.25 nmole/ml of DCIP reduced. This again corresponds to about 20 equivalents per unit and would require more reduced entogenous donors than is present in darkness.

(3) Photoreduction of DCIP by reduced plastocyanin. This mechanism, which we presume is correct, is not subject to restriction by endogenous donors, and it accounts simply for the conditions which give the maximum transient. In this case, the extent of reactions is calculated as:

$$n\ (\text{equiv/l}) = \frac{2\Delta A_{590\,\text{nm}}}{\varepsilon_{\text{M(DCIP)}} - \varepsilon_{\text{M(PC)}}}$$

where $\varepsilon_{\text{M(DCIP)}}$ and $\varepsilon_{\text{M(PC)}}$ are molar extinction coefficients. From this, one estimates that, during the fast transients, about 32 electrons are transferred per unit of

500 chlorophylls and that the initial rate of DCIP reduction is about 800 μmoles DCIP/mg chl per h.

Slow reduction of DCIP in the absence of plastocyanin

A number of observations indicate that the slow reduction of DCIP by sonicated fragments, in the absence of added plastocyanin, does not depend upon a small residue of plastocyanin retained by the fragments nor upon the presence of a functional System I. These observations thus point to a mechanism involving reduction at a site close to System II.

First, we tried to show that no plastocyanin remains in sonicated oat chloroplast fragments. For this, reaction mixtures containing chloroplasts or chloroplast fragments (30 μg chl per ml), DCMU (20 μM), methylamine (0.02 M), sodium ascorbate (50 μM), methyl viologen (1 mM) and phosphate-buffer (pH 7.2) were illuminated with red light ($6 \cdot 10^5$ erg·cm^{-2}·sec.$^{-1}$). Under these conditions plastocyanin is reduced in darkness and oxidized in the light. With whole chloroplasts, the absorbance increase in the light corresponded to about 1 plastocyanin per 300 chlorophyll. With sonicated fragments, no absorbance change was detectable and the plastocyanin content must be less than 1 molecule per 3000 chlorophylls. When the test was carried out with sonicated fragments to which plastocyanin had been added, over 80 % of the plastocyanin was oxidized in the light. One concludes that sonication removes $>$90 % of the plastocyanin of the chloroplast. It is thus improbable that the slow reduction of DCIP involves a residual, plastocyanin-dependent electron transport to System I, and reduction at a System I site.

Secondly, as stated earlier, essentially all of the decrease in activity brought about by sonication occurs in the first 1–2 min, and longer sonication (up to 10–20 min) does not further reduce the activity of small fragments. This indicates, again, that the residual activity does not depend on a small residue of plastocyanin remaining in the fragments: were this true, activity should continue to decline as prolonged sonication further solubilizes the plastocyanin.

Thirdly, as KOK *et al.*[9] found with *Scenedesmus* mutant No. 8, so we found (Table I) that chloroplast fragments of *Chlamydomonas reinhardii* mutant ac-206 (deficient in cyt *f* and Photosystem I activity) reduced DCIP at a rate of about 35 μmoles/mg per h. This rate, which cannot be raised by methylamine and plastocyanin, is essentially indentical to that maintained by oat chloroplast fragments in the absence of added plastocyanin. With fragments from wild type Chlamydomonas, again the same rate is obtained, but it is raised by methylamine and plastocyanin. It thus appears that the residual activity is independent of System I and can function with either cytochrome or plastocyanin deficiency.

Evidence of a very slow DCIP reduction of System II

With particles of *Scenedesmus* mutant No. 8, KOK *et al.*[9] found a very slow reduction of DCIP continuing for 15 sec after the end of illumination. The size of the pool of the light-generated reductant was about 1 equiv per 75 chl. Evidence of a similar reaction was presented by FUJITA AND MYERS[17].

We also observed this very slow reduction of DCIP. In Fig. 7, Curve A shows that sonically prepared chloroplast fragments reduce DCIP at a moderate rate in the light. Upon darkening, the rate falls instantaneously to a much lower value, and the

TABLE I

RATE OF DCIP REDUCTION BY CHLOROPLAST FRAGMENTS PREPARED FROM *Chlamydomonas reinhardi* MUTANT ac-206 AND WILD-TYPE CELLS

The 3–25K fragments were isolated by sonication of whole cells, as described in Fig. 10. Reaction mixture (2.5 ml) included 0.01 phosphate-buffer (pH 7.2), 0.01 M KCl, fragments 9.4 μg chlorophyll per ml, 30 μM DCIP and as indicated, 0.02 M methylamine (MA) and 2.5 μM plastocyanin. The reaction was carried out at 20° under $6 \cdot 10^5$ erg·cm^{-2}·sec^{-1} of red light.

Fragments (3–25K)	*Contents of a reaction mixture*	*Rate of DCPIP reduction* (μmoles/mg chl per h)*
ac-206 (30 sec sonication)	Fragments + DCIP	35 ± 4
	Fragments + DCIP + MA-HCl	38 ± 4
	Fragments + DCIP + MA-HCl + plastocyanin	36 ± 3
ac-206 (120 sec sonication)	Fragments + DCIP	34 ± 4
	Fragments + DCIP + MA-HCl	33 ± 3
	Fragments + DCIP + MA-HCl + plastocyanin	33 ± 3
Wild type (30 sec sonication)	Fragments + DCIP	68 ± 5
	Fragments + DCIP + MA-HCl	168 ± 11
	Fragments + DCIP + MA-HCl + plastocyanin	166 ± 10
Wild type (120 sec sonication)	Fragments + DCIP	48 ± 5
	Fragments + DCIP + MA-HCl	61 ± 7
	Fragments + DCIP + MA-HCl + plastocyanin	74 ± 6

* Average of 4 measurements

reaction continues at a decaying rate — the half-time being approx. 8 sec. The instantaneous change in rate at the moment of darkening clearly indicates that the steady reaction in the light proceeds mainly by a mechanism different from that of the decaying reaction in darkness. The magnitude of the very slow reduction was limited to 1 equiv per 60 ± 15 chl.

Several observations support the conclusion of KOK *et al.* that the site of the very slow reduction lies between Systems II and I. First, the amount of DCIP reduced during the very slow reaction in darkness is diminished by addition of plastocyanin (Curve B, Fig. 9). Evidently, added plastocyanin speeds the drainage of electrons from the reduced pool, upon which the very slow reaction in darkness depends. In order for such a competition to occur, the pool (and hence the site of reduction) must lie on the System II side of plastocyanin. Secondly, with whole chloroplasts, a similar slow transient reduction of DCIP occurs upon darkening, and is completely abolished by methylamine–HCl. This also indicates a reduced pool which can be drained by System I and, furthermore, that both the pool and the site of the very slow reduction lie on the System II side of a phosphorylation-coupled step. Finally, the size of the reduced pool (1 equiv per 75 chl according to KOK *et al.* and 1 equiv per 60 ± 15 chl according to us) is fairly large: it is about 1/2 the size of the pool of internal oxidants of System II reported by JOILET[19,20] and KOK AND MALKIN[21,22].

DISCUSSION

The fast reversible transient observed in the presence of reduced plastocyanin we take as conclusive evidence of a rapid reduction of DCIP at a site close to the primary photoreduced product of System I. Insensitivity to DCMU, preferential sensitization by 710 nm, the presence of the phenomenon in system II — but not System I — deficient mutants all support this conclusion.

That the slow DCIP reduction in the absence of added plastocyanin takes place at a site close to the primary reduction of System II is harder to prove — indeed, the present evidence is not conclusive. The occurrence of the slow reaction both in the absence of plastocyanin, and in System I deficient mutants, points to a System II reduction site. But the alternative explanation, that there is a slow step, not requiring plastocyanin in parallel to the fast, plastocyanin-mediated transfer of electrons to System I, is difficult to disprove. One argument against this alternative is the finding of KOK *et al.*[9] that the slow reaction occurs in *Scenedesmus* No. 8 which presumably lacks the functional photochemical center of System I, not — as in Chlamydomonas ac-206 — just an electron carrier (cytochrome *f*) which transports electrons to System I. Also against the alternative is the coincidence that lack of plastocyanin in oat chloroplast fragments, of P_{700} in *Scenedesmus* No. 8 and of cytochrome *f* in Chlamydomonas ac-206 all leave the slow reaction proceeding at the same rate. With these two arguments, we tentatively conclude that the location of the site of a slow DCPIP reduction is close to System II. In this regard, a demonstration that fragments which sustain a slow DCPIP reduction do not reduce methylviologen would be important.

A closing point concerns the relative proximity to System II of the sites of the 'slow reduction' in light and the 'very slow reduction' which continues for 15 sec. in darkness. Of these, the 'low site' must be closer to Photoreaction II. Were the reverse true, one would expect the more rapid reduction of DCIP at the 'slow site' to drain the pool of reductant quickly and the 'very slow reduction' in dark would not be observed.

Finally, while the alternative of a slow transport to System I not requiring plastocyanin cannot be completely ruled out, we think the evidence for a three-site mechanism of reduction of DCIP is comparatively strong. It follows that just as plastocyanin loss can shift the site of reduction of DCIP from System I to System II, so other modification of the rate of electron transport (*e.g.* by uncoupling) can be expected to shift the site of reduction of other high potential Hill oxidants. Such shifts would have obvious bearing on matters such as the P/2e ratio in the $Fe(CN)_3$ Hill reaction.

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REFERENCES

1 J. B. THOMAS, O. H. BLAAUW AND L. N. M. DUYSENS, *Biochim. Biophys. Acta*, 10 (1953) 230.
2 R. B. PARK AND N. G. PON, *J. Mol. Biol.*, 3 (1961) 1.
3 M. J. BECKER, A. M. SHEFNER AND J. A. GROSS, *Plant Physiol.*, 40 (1965) 243.
4 D. W. KROGMAN AND A. T. JAGENDORF, *Plant Physiol.* 34 (1959) 277.
5 S. KATOH AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 99 (1965) 156.
6 J. GRESSEL AND M. AVRON, *Biochim. Biophys. Acta*, 94 (1965) 31.
7 S. IZAWA AND N. E. GOOD, *Biochim. Biophys. Acta*, 109 (1965) 372.
8 S. KATOH AND A. SAN PIETRO, *J. Biol. Chem.*, 241 (1966) 3575.

9 B. Kok, S. Malkin, O. Owens and B. Forbush, Brookhaven Symposium No. 19, 1967, pp. 446–459.
10 B. Ke, *Plant Physiol.*. 42 (1967) 1310.
11 A. L. Given and R. P. Levine, *Biochim. Biophys. Acta*, 189 (1969) 404.
12 Govindjee and M. Bazzaz, *Photochem. Photobiol.*, 6 (1967) 887.
13 S. Lien, Ph. D. thesis, University of Rochester, 1969.
14 D. E. Arnon, *Plant Physiol.*, 24 (1949) 1.
15 A. T. Jagendorf and M. Evans, *Plant Physiol.*, 23 (1957) 435.
16 S. Katoh and A. Takamiya, *Nature*, 189 (1961) 665.
17 Y. Fujita and J. Myers, *Arch. Biochem. Biophys.*, 112 (1965) 506.
18 S. Katoh, I. Shiratori and A. Takamiya, *J. Biochem.*, 51 (1962) 32.
19 P. Joliot, *Biochim. Biophys. Acta*, 102 (1965) 116.
20 P. Joliot, *Biochim. Biophys. Acta*, 102 (1965) 135.
21 S. Malkin and B. Kok, *Biochim. Biophys. Acta*, 126 (1966) 413.
22 S. Malkin and B. Kok., *Biochim. Biophys. Acta*, 126 (1966) 433.
23 K. Saner and R. B. Park, *Biochem.*, 4 (1965) 2791.
24 H. D. Gibbs, B. Cohen and R. K. Cannan, *U. S. Public Health Reports*, V. 4D (1925) 649.