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The Mechanism of Hydrogen Photoproduction by Several Algae

II. The Contribution of Photosystem II* **

Tim S. Stuart and Hans Gaffron

Institute of Molecular Biophysics and Department of Biological Science, Florida State University, Tallahassee, U.S.A.

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Summary. The contribution of PS II to H_2 photoproduction by several unicellular green algae was measured both when O_2 evolution and photophosphorylation were unimpaired and also when these processes had been eliminated by Cl-CCP. As judged by the effects of DCMU, a PS II contribution was found under both sets of experimental conditions for several strains of *Chlorella*, *Ankistrodesmus* and *Scenedesmus*. However, H_2 photoproduction by *Chlamydomonas moewusii* was insensitive to DCMU and thus was entirely due to PS I. With cells treated with Cl-CCP, the relative amount of PS II contribution varied from zero in autotrophically grown *Chlamydomonas reinhardii*, to $\approx 20\%$ in photoheterotrophically grown and $\approx 50\%$ in autotrophically grown cells of *Ankistrodesmus braunii*, *Chlorella fusca*, *Chlorella vulgaris* and *Scenedesmus obliquus*. The dehydrogenation of reduced H-donors by PS II of *Scenedesmus* treated with Cl-CCP showed the same biphasic kinetics previously described for H_2 photoproduction by PS I of this alga.

Introduction

From earlier work it is known that hydrogen photoproduction by green algae (*e.g.*, *Ankistrodesmus*, *Chlamydomonas*, *Scenedesmus*) does not require PS II or photophosphorylation and is due to non-cyclic electron flow through PS I to hydrogenase, where hydrogen is released (Stuart and Kaltwasser, 1970; Healey, 1970b; Stuart, 1971; Stuart and Gaffron, 1972a, b). However, although not required for H_2 photoproduction, PS II appears to donate electrons for this process under certain conditions.

We define a contribution of PS II as that portion of H_2 photoproduction which disappears upon the addition of 10^{-5} DCMU. As mentioned elsewhere (Stuart and Gaffron, 1972b), this concentration of DCMU completely inhibited O_2 evolution by these algae. This compound has long been known to inhibit electron transport near PS II (see Avron,

* *Abbreviations:* Cl-CCP = carbonyl cyanide *m*-chlorophenylhydrazone; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ICC = Indiana Culture Collection; PS = photosystem; SAL = salicylaldoxime; SIO = Marine Botany Culture Collection, Scripps Institution of Oceanography.

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1967; Hind and Olson, 1968). Recent work (see Cheniae, 1970) strongly supports the earlier proposal of Duysens and Sweers (1963) that DCMU blocks electron flow between "Q" and PS I. Therefore, as in the oxidation of artificial electron donors by PS II (see Cheniae, 1970), any contribution of electrons by organic substrates to PS II during H_2 photoproduction should be sensitive to DCMU.

As judged from the effect of DCMU, the relative amount of contribution by PS II is known to vary from zero in mutants Nos. 8 and 11 of *Scenedesmus* (Stuart and Kaltwasser, 1970) and several species of *Chlamydomonas* (Healey, 1970a), to $\approx 20\%$ in heterotrophically grown cells of *Scenedesmus* (Stuart and Kaltwasser, 1970; Stuart, 1971; Stuart and Gaffron, 1971) and 50% or more in autotrophically grown *Chlorella* (Healey, 1970a) and *Scenedesmus* (Bishop and Gaffron, 1963; Stuart and Kaltwasser, 1970; Healey, 1970a; Stuart and Gaffron, 1971).

In this paper we present evidence that in *Chlorella vulgaris*, *Chlorella fusca* and *Ankistrodesmus braunii*, as in *Scenedesmus obliquus* (Stuart, 1971; Stuart and Gaffron, 1971), PS II can contribute electrons to H_2 production even in the absence of O_2 evolution. We shall also show that the relative amount of PS II contribution to H_2 production varies with the growth conditions.

Materials and Methods

The algae used were *Scenedesmus obliquus* strain D_3 (Gaffron), *Chlorella vulgaris* f. *tertia* Fott et Novakova 211-31, *Chlorella fusca* Shihira et Krauss (= *pyrenoidosa*) 211-15, *Ankistrodesmus braunii* (Naegeli) Collins 202-7c, and *Chlamydomonas reinhardii* Dangeard (ICC No. 89). Autotrophic and photoheterotrophic cultures of these algae were grown, harvested and tested for H_2 production as described in the preceding paper.

The p-benzoquinone Hill reaction of whole cells was followed with the oxygen electrode using the method of Cheniae and Martin (1969). In this assay, the reaction mixture (3.0 ml) contained 8 µl of autotrophically grown cells, 100 µmoles of Na-phosphate buffer, pH 6.5, 3 µmoles of freshly sublimated p-benzoquinone and 6 µmoles of $K_3Fe(CN)_6$. The oxygen electrode, which has been described elsewhere (Stuart *et al.*, 1972) was also used for measurements of photosynthesis.

Cl-CCP, DCMU and SAL were obtained from the same sources as in Stuart and Gaffron (1972b).

Results

The contribution of PS II to H_2 photoproduction, as judged from the effect of DCMU, was measured under two different experimental conditions. In the first case measurements were made with unpoisoned cells, *i.e.*, O_2 evolution and photophosphorylation were unimpaired. In the second case, O_2 evolution and photophosphorylation were blocked by Cl-CCP. As discussed below, the rates measured under the former conditions may have been somewhat depressed by photoreduction and O_2 evolution.

Table 1. Effect of DCMU upon hydrogen photoproduction by unpoisoned cells

	μl H_2/h/100 μl cells			PS II contribution as %
	Control A	+DCMU B	A–B	
Autotrophically grown cells:				
Ankistrodesmus braunii Mb 7	25	12	13	54
Chlorella vulgaris 211–31	15	12	3	20
Chlorella fusca 211–15	12	8	4	33
Scenedesmus obliquus D_3	10	6	4	40
Chlamydomonas reinhardii Dangeard	19	18	1	7
Photoheterotrophically grown cells:				
Ankistrodesmus braunii Mb 7	27	25	2	6
Chlorella vulgaris 211–31	15	12	3	20
Chlorella fusca 211–15	13	17	−4	—
Scenedesmus obliquus D_3	14	11	3	23

100 μl cells/vessel adapted either 2 h (*Ankistrodesmus*, *Scenedesmus* and *Chlamydomonas*) or 14 h (*Chlorella*) under H_2, then flushed with N_2. Illumination = 1.6×10^2 μW cm^{-2} (= 400 lux). Alkaline pyrogallol was present in the side arm in order to absorb any oxygen evolved. DCMU (final concentration 10^{-5} M), when present, was added before adaption. Values are the average of 4 determinations.

The Photosystem II Contribution in Unpoisoned Cells. In experiments with unpoisoned autotrophically and photoheterotrophically grown cells, we found that DCMU partially blocked H_2 photoproduction by strains of *Ankistrodesmus*, *Chlorella* and *Scenedesmus*, but did not significantly inhibit this reaction in *Chlamydomonas reinhardii* (Table 1). In agreement with these results, similar work (Healey, 1970a) with autotrophically grown cells has shown that, unlike H_2 production by *Chlamydomonas moewusii* and *Chlamydomonas dysosmos*, DCMU inhibited H_2 production by several strains of *Chlorella* and *Scenedesmus*.

The apparent stimulation of H_2 production by DCMU in photoheterotrophically grown *Chlorella fusca* 211-15 was probably due to the inhibition of the hydrogenase by O_2 in the control without DCMU (cf. Fig. 1 of Healey, 1970b).

The Photosystem II Contribution in Cl-CCP-Treated Cells. Cl-CCP is known to inhibit both photophosphorylation and O_2 evolution (see De Kiewiet *et al.*, 1965; Homann, 1971; Kimimura *et al.*, 1971). As shown in Fig. 1, 10^{-4} M Cl-CCP strongly inhibited O_2 evolution by the various algae tested. However, O_2 evolution by *Ankistrodesmus* cells was less sensitive to Cl-CCP and, in this case, the contribution of electrons from water cannot be ruled out completely.

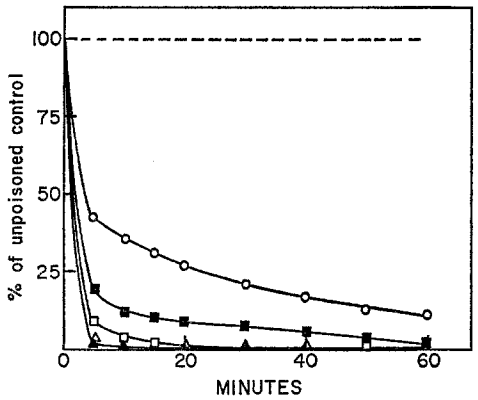

Fig. 1. Inhibition of the p-benzoquinone Hill reaction by 10^{-4} M Cl-CCP. The reaction mixture (see Materials and Methods) was flushed for 5 min with N_2 before closing the O_2 electrode vessel. Cl-CCP (final concentration 10^{-4} M) when present, was added in the dark at time zero. Immediately after time zero, the rate of O_2 evolution (*i.e.*, the Hill reaction) was determined for a one min period of illumination (light intensity $\sim 1.8 \times 10^4$ μW cm^{-2}). The reaction mixture was then kept dark until the next one min period of illumination. In this manner, the samples were tested periodically for Hill activity for the duration of the experiment (*i.e.*, 60 min). Values are the average of 3 determinations and have been corrected for parallel controls assayed without Cl-CCP. ○ *Ankistrodesmus braunii*, △ *Chlamydomonas reinhardii*, ■ *Chlorella fusca* 211-15, □ *Chlorella vulgaris* 211-31; ▲ *Scenedesmus obliquus*

Like H_2 photoproduction via PS I (Stuart and Kaltwasser, 1970; Healey, 1970b; Stuart and Gaffron, 1972a, b), the contribution of electrons via PS II was generally enhanced by the addition of Cl-CCP (Tables 1, 2). Also in contrast to unpoisoned cells (Table 1), the variation of the magnitude of PS II contribution with the growth conditions was clear-cut, in that PS II was generally more active in autotrophically grown cells (Table 2). The large contribution of PS II found for *Ankistrodesmus* also may be due to the oxidation of water, since O_2 evolution in this organism was rather insensitive to Cl-CCP (Fig. 1). Unlike the other algae tested, neither unpoisoned nor Cl-CCP-treated *Chlamydomonas* cells showed a PS II contribution to H_2 production.

The Kinetics of H_2 Photoproduction by Cl-CCP-Treated Cells. In earlier work (Stuart and Gaffron, 1971), we found that H_2 photoproduction by PS I of *Scenedesmus* occurs in two phases: a rapid initial phase which depends upon the dehydrogenation of a "pool" of H donors and a later and slower second phase which is limited by the flow of electrons from fermentation. As shown in Fig. 2, the same two phases were also

Table 2. Effect of DCMU upon hydrogen photoproduction in the presence of Cl-CCP

	µl H_2/h/100 µl cells			PS II contribution as %
	Control A	+ DCMU B	A–B	
Autotrophically grown cells:				
Ankistrodesmus braunii MB 7 ($n=8$)	93	30	63	68
Chlorella vulgaris 211-30 ($n=7$)	19	12	7	39
Chlorella fusca 211-15 ($n=8$)	14	7	7	53
Scenedesmus obliquus D_3 ($n=6$)	48	25	23	47
Chlamydomonas reinhardii ($n=5$)	19	19	0	0
Photoheterotrophically grown cells:				
Ankistrodesmus braunii ($n=7$)	65	52	13	20
Chlorella vulgaris 211-31 ($n=7$)	48	45	3	17
Chlorella fusca 211-15 ($n=7$)	58	45	13	22
Scenedesmus obliquus D_3 ($n=6$)	38	33	5	15

100 µl cells/vessel adapted either 2 h (*Ankistrodesmus*, *Scenedesmus* and *Chlamydomonas*) or 14 h (*Chlorella*) under H_2, then flushed with N_2. Cl-CCP (final concentration 10^{-4} M) was added 1 h before illumination (1.67×10^3 µW cm^{-2}) in order to prevent oxygen evolution and photophosphorylation. DCMU (final concentration 10^{-5} M), when present, was added before adaptation.

found for H_2 photoproduction by both photosystems, *i.e.*, in the absence of DCMU. Since DCMU inhibited both phases of H_2 photoproduction by Cl-CCP-treated cells (cf. Stuart and Gaffron, 1971), we may conclude that PS II was able to dehydrogenate both a new "pool" of H-donors and also compounds formed during fermentation.

The amount of H_2 derived from the dehydrogenation of the PS I and PS II "pools" may be estimated from the type of manometric data shown in Fig. 2. If we assume that the slower second phase continues during the oxidation of the pool, then the size of the pools is given by simply extrapolating back to the ordinate. Thus, for three separate experiments, the average pool size was 13.3 µl of H_2 in the presence of DCMU and 38 µl of H_2 in the absence of DCMU. Therefore, about 25 µl of H_2 apparently originated from the PS II pool. At least under these experimental conditions, the PS II pool is thus about twice as large as the PS I pool. Calculating the µmoles of H_2 produced per µmole of chlorophyll present, gave values of 0.49 µmole H_2/µmole chlorophyll in the presence of DCMU and 1.44 µmoles H_2/µmole chlorophyll in the absence of DCMU.

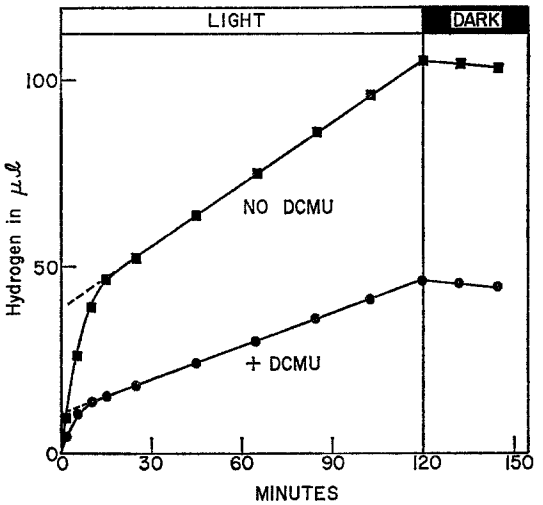

Fig. 2. Effect of DCMU on H_2 production by Cl-CCP-treated *Scenedesmus*. 100 μl autotrophically grown cells/vessel adapted 2 h under H_2, then flushed with N_2. DCMU (final concentration 10^{-5} M), when present, was added before adaptation. Cl-CCP (final concentration 10^{-4} M) was added 1 h before illumination (3.4×10^3 μW cm^{-2}) in order to prevent O_2 evolution and photophosphorylation. Values are the average of 3 determinations. Other experimental conditions as in Materials and Methods

Discussion

In place of the mechanisms outlined by Bishop (1966) and Healey (1970a), our results suggest the following mechanisms for H_2 photoproduction by green algae:

1. Hydrogen Photoproduction by Photosystem I. This reaction, which occurs in all of the hydrogenase-containing green algae tested thus far (except perhaps for an unidentified marine species of *Chlorella*, see Healey, 1970a), is evidently due to non-cyclic electron flow from organic H donors through PS I to hydrogenase, where molecular H_2 is released (Stuart and Kaltwasser, 1970; Stuart, 1971; Healey, 1970b).

This process apparently is not ATP-dependent, since inhibiting photophosphorylation with 2,4-dinitrophenol (Gaffron and Rubin, 1942; Healey, 1970b), Cl-CCP (Kaltwasser *et al.*, 1969; Stuart and Kaltwasser, 1970; Healey, 1970a; Stuart and Gaffron, 1972a, b), and SAL (Stuart, 1971; Stuart and Gaffron, 1972a, b), or a heat treatment (Stuart, 1971) either stimulated or did not significantly inhibit the photoproduction of hydrogen.

In *Scenedesmus*, this DCMU-insensitive process occurs in two distinct phases: a rapid dehydrogenation of a "pool" of H donors followed by

the slow "leak" of electrons from fermentation. Hydrogen photoproduction by PS I depends upon the level of reduced organic compounds in the cell, since the rate was depressed by starvation and was enhanced by added H donors (*i.e.*, acetate for *Chlamydomonas moewusii* (Healey 1970b), glucose for *Scenedesmus obliquus* (Kaltwasser *et al.*, 1969). Furthermore, with *Scenedesmus*, a quantitative relationship was found between the amount of glucose added and the resulting stimulation of H_2 photoproduction, with one μmole of glucose giving about 0.5 μmole of hydrogen gas (Stuart and Gaffron, 1971).

2. Hydrogen Photoproduction by Photosystem I and Photosystem II. In all strains of green algae tested (except perhaps an unidentified marine *Chlorella*, see Healey, 1970a), PS II is apparently not required for H_2 photoproduction (see literature quoted above and Tables 1–3). Although earlier work (Bishop and Gaffron, 1963) suggested that both photosystems were necessary for H_2 photoproduction by *Scenedesmus*, further study, as discussed above, clearly showed that PS II is not required for H_2 production by this alga. Yet in some organisms this photosystem appears to contribute electrons for this reaction. As mentioned above, the contribution of PS II has been judged by the effect of DCMU, which apparently blocks electron flow between "Q", the primary electron acceptor on the reducing side of PS II, and PS I (see Cheniae, 1970). This contribution was assayed both under conditions where oxygen evolution and photophosphorylation were unimpaired and also under conditions where both of these processes were blocked with Cl-CCP.

When the effect of DCMU was determined in entirely unpoisoned cells, this inhibitor depressed H_2 photoproduction in several strains of *Ankistrodesmus*, *Chlorella* and *Scenedesmus*, but did not affect H_2 production by *Chlamydomonas* (Healey, 1970a; Tables 2, 3). Unlike experiments done in the presence of Cl-CCP (Table 2) the relationship between the growth conditions and the amount of PS II contribution was not clear-cut. The stimulation of H_2 production by DCMU found for photoheterotrophically grown *Chlorella fusca* may indicate that, in the absence of this inhibitor, O_2 formation by PS II partially inactivated the hydrogenase and thus depressed H_2 photoproduction (cf. Fig. 1 of Healey, 1970b).

The ultimate source of the electrons evolved under these experimental conditions is not clear. Although in the absence of DCMU the evolved H_2 may have derived from water, the low rates found could also have been due to the dehydrogenation of the same H donors utilized by PS II in the presence of Cl-CCP (cf. Table 2). Alternatively, these low rates may have been due to the partial inactivation of the hydrogenase by evolved O_2 or to the reconsumption of the evolved H_2 via photoreduction (see Kaltwasser *et al.*, 1969; Healey, 1970b; Stuart and Gaffron, 1972a).

Table 3. Summary of recent studies on the contribution of photosystem II to hydrogen photoproduction

Alga	Reference	Effect of DCMU (% of control without DCMU)			
		Autotrophically grown cells		Photoheterotrophically grown cells	
		No Cl-CCP	+ Cl-CCP	No Cl-CCP	+ Cl-CCP
Ankistrodesmus braunii	A	46	32±6	94	80±11
Chlamydomonas dysosmos ICC 342	B	100	—	—	—
Chlamydomonas moewusii ICC 97	B	100	—	—	—
Chlamydomonas reinhardii	A	93	102±9	—	—
Chlorella fusca 211-15	A	67	47±7	128	78±8
Chlorella pyrenoidosa ICC 251	B	40	—	—	—
Chlorella vulgaris SIO	B	10	—	—	—
Chlorella vulgaris 211-31	A	78	61±5	82	93±7
Chlorella sp. (Marine)	B	0	—	—	—
Scenedesmus obliquus D_3	A, B, C	60	53±8	77	85±9
Scenedesmus quadricauda SIO	B	40	—	—	—

A = See Tables 1, 2; B = Healey (1970a); C = Stuart and Gaffron (1971). ICC = Indiana Culture Collection (Starr, 1964); SIO = Marine Botany Culture Collection, Scripps Institution of Oceanography.

Although Spruit (1958) found that adapted cells of *Chlorella vulgaris* immediately produce both H_2 and O_2 upon illumination, his results do not rule out the possibility that the O_2 was formed via an internal "Hill reaction" while the H_2 was due to electron flow from organic donors through PS I to hydrogenase. In another study (Bishop and Gaffron, 1963) it was suggested that H_2 photoproduction by *Scenedesmus* requires the simultaneous production of O_2. However, further work (Kaltwasser *et al.*, 1969; Stuart and Gaffron, 1972a, and see above) has shown that the two gases are not necessarily released simultaneously. In addition, O_2 evolution could be specifically depressed merely by decreasing the concentration of CO_2, which indicated that the O_2 production was due to ordinary photosynthesis. Therefore, it is still not clear whether hydrogen-adapted algae are able to produce H_2 and O_2 simultaneously via the oxidation of water.

Photosystem II is also able to contribute electrons for H_2 photoproduction when O_2 evolution has been blocked with Cl-CCP or SAL (Stuart, 1971; Stuart and Gaffron, 1971; Tables 2, 3). When the oxidation of water was completely blocked by Cl-CCP, the relative amount of PS II contribution, as measured by the effect of DCMU, varies from

zero in *Chlamydomonas reinhardii* and mutants Nos. 8 and 11 of *Scenedesmus* to $\approx 20\%$ in photoheterotrophically grown cells and $\approx 50\%$ for autotrophically grown cells of *Ankistrodesmus braunii*, *Chlorella vulgaris*, *Chlorella fusca* and *Scenedesmus obliquus* (Stuart and Kaltwasser, 1970; Stuart, 1971; Tables 2, 3). Since, at least in *Scenedesmus* (Stuart, 1971), H_2 photoproduction was light-saturated (*i.e.*, substrate limited) under our experimental conditions, the effect of DCMU was most probably not due to a change in the distribution of light quanta between the photosystems (*i.e.*, "spillover", cf. Fork and Amesz, 1969). Another possibility is that DCMU inhibits electron flow in PS I of *Scenedesmus*. However, it has been reported (Izawa, 1968) that DCMU does not block electron flow in PS I of isolated chloroplasts. Since O_2 evolution was evidently not possible in the presence of 10^{-2} M SAL or high concentrations of Cl-CCP (Kaltwasser *et al.*, 1969; Stuart and Kaltwasser, 1970; and Fig. 1), the electrons must enter the electron transfer chain between the DCMU-sensitive site and the O_2-evolving mechanism (Stuart, 1971; Stuart and Gaffron, 1971).

The dehydrogenation of reduced H-donors by PS II of Cl-CCP-treated *Scenedesmus* showed the same biphasic kinetics previously described for H_2 photoproduction by PS I of this alga. Thus, the utilization of H donors by PS II depends upon the dehydrogenation of a new "PS II pool", followed by a later and slower second phase which is apparently limited by the flow of electrons from fermentation (Stuart and Gaffron, 1971). The calculations on the amount of H_2 produced from the PS I and PS II pools per μmole of chlorophyll lead to two conclusions. First, at least under our experimental conditions, the PS II pool is about twice as large as the PS I pool. Second, the large amount of H_2 produced per μmole chlorophyll means that the evolved H_2 cannot all be due to the dehydrogenation of the known intermediates of the photosynthetic electron transport chain. For example, the large "A" pool on the reducing side of PS II corresponds, on a molar basis, to only about 1/20 of the chlorophyll content (Malkin and Kok, 1966; Forbush and Kok, 1968; Homann, 1968), while several other known photosynthetic electron carriers, *e.g.*, P-700, cytochrome *f*, cytochrome *b* 559, are only present at a concentration of $\leqslant 1$ μmole/100 μmoles of chlorophyll (see Bishop, 1971; Boardman, 1970; Cheniae, 1970). Since a stoichiometric relationship between the amount of added glucose and the resulting stimulation of H_2 production has already been demonstrated (Stuart and Gaffron, 1971), it is quite likely that most of the evolved H_2 is ultimately derived from carbohydrates.

The two most probable pathways (a and b) for electron flow into PS II of Cl-CCP-treated algae are shown in Fig. 3. At this time we are unable to specify whether the electrons from reduced organic compounds

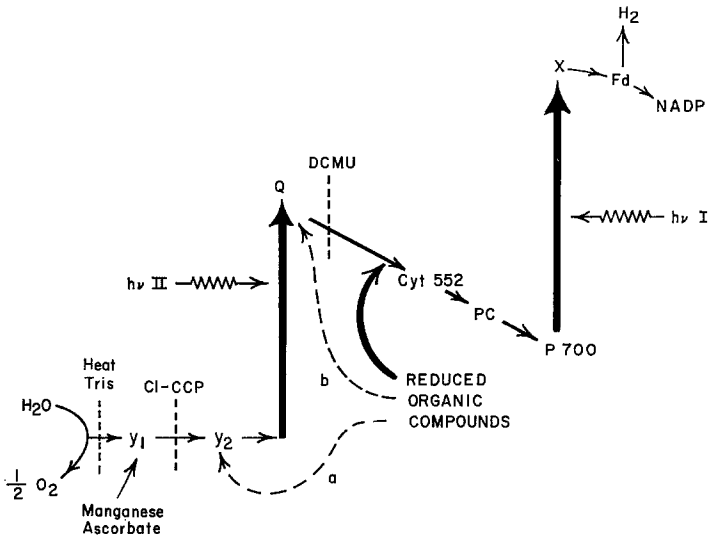

Fig. 3a and b. Scheme showing the two most probable pathways (a and b) of electron flow into PS II during H_2 photoproduction by *Scenedesmus* cells treated with Cl-CCP. The site of action of Cl-CCP shown in this figure is based upon the work of several authors (*e.g.*, Katoh and San Pietro, 1967; Itoh *et al.*, 1969; Kimimura *et al.*, 1971; also see Cheniae, 1970)

enter on the oxidizing (pathway a) or reducing (pathway b) side of PS II. On the one hand, several workers (Yamashita *et al.*, 1969, Itoh *et al.*, 1969; Inoue *et al.*, 1971) have found that chloroplasts treated with Cl-CCP, although unable to evolve O_2, are able to dehydrogenate endogenous carotenoids via PS II. Experiments are planned to determine if carotenoid oxidation occurs under our experimental conditions. On the other hand, work on the fluorescence of algal cells (*e.g.*, Gingras and Lavorel, 1965; Kessler, 1968, 1970) indicates that "Q" is reduced in the dark by prolonged anaerobiosis or, in H_2-adapted algae, by hydrogen gas. Measurements on the amount of "enhancement" found for H_2 photoproduction by Cl-CCP-treated algae should allow a decision between these two pathways.

The difference in the amount of PS II contribution to H_2 photoproduction by autotrophically and heterotrophically grown cells remains unexplained. Since the amount of contribution by PS II was much less in heterotrophically grown cells (Table 3), these cells must have formed a different substrate (which could not be dehydrogenated via PS II) and/or the access of the substrate to PS II was somehow blocked.

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Tim S. Stuart
Hans Gaffron
Institute of Molecular Biophysics
Florida State University
Tallahassee, Florida, 32306, U.S.A.