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# STUDIES ON THE PATHWAY OF CYCLIC ELECTRON FLOW IN MESOPHYLL CHLOROPLASTS OF A $C_4$ PLANT

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SUMMARY

1. Cyclic photophosphorylation driven by white light, as followed by $^{14}CO_2$ fixation by mesophyll chloroplast preparations of the $C_4$ plant *Digitaria sanguinalis*, was specifically inhibited by disalicylidenepropanediamine (DSPD), antimycin A, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDAC), and KCN suggesting that ferredoxin, cytochrome $b_{563}$, plastoquinone, cytochrome *f*, and plastocyanin are obligatory intermediates of cyclic electron flow. It was found that 0.2 μM DCMU and 40 μM *o*-phenanthroline blocked noncyclic electron flow, stimulated cyclic photophosphorylation, and caused a partial reversal (40–100%) of the inhibition by DBMIB and antimycin A, but not DSPD.

2. Cyclic photophosphorylation could also be activated using only far-red illumination. Under this condition, however, cyclic photophosphorylation was much less sensitive to the inhibitors DBMIB, EDAC and antimycin A, but remained completely sensitive to DSPD and KCN. Inhibition in far-red light was not increased by preincubating the chloroplasts with the various inhibitors for several minutes in white light.

3. The striking correspondence between the effects of photosystem II inhibitors, DCMU and *o*-phenanthroline, on cyclic photophosphorylation under white light and cyclic photophosphorylation under far-red light (in the absence of photosystem II inhibitors) suggests that electrons flowing from photosystem II may regulate the pathway of cyclic electron flow.

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Abbreviations: $C_4$ plant, plant having the $C_4$ dicarboxylic acid pathway of leaf photosynthesis; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; EDAC, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide; DSPD, disalicylidenepropanediamine. DABS, diazonium bezenesulfonate.

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## INTRODUCTION

Studies of photophosphorylation in chloroplasts often measure $^{32}P$ esterification into ATP. Such approaches necessitate the use of chloroplasts without an intact outer envelop ("broken"), since the rate of $^{32}P$ fixation by intact chloroplasts is very low [1]. Studying photophosphorylation with broken chloroplasts is somewhat an artificial system, since certain soluble components, e.g., ferredoxin and NADP, must be added back. Whether this approximates the conditions found inside an intact chloroplast is questionable at best. Simonis and Urbach [2] have reviewed methods which have been used to study photophosphorylation in vivo with various algae. We have devised a procedure to study cyclic photophosphorylation by intact chloroplasts, by following $^{14}CO_2$ fixation that requires only ATP and not NADPH, although conditions can also be obtained where there is a demand for NADPH as well as ATP.

$CO_2$ fixation in $C_4$ mesophyll chloroplast preparations requires the $\beta$-carboxylation reaction and the photochemical production of ATP [3]. The following partial reactions result in $CO_2$ fixation in our system [4]:

$$\text{(a) pyruvate} + 2\,\text{ATP} + P_i \xrightarrow[\text{adenylate kinase}]{\text{pyruvate, } P_i \text{ dikinase}} \text{phosphoenolpyruvate} + 2\,\text{ADP} + \text{PP}$$

$$\text{(b) phosphoenolpyruvate} + CO_2 \xrightarrow[\text{carboxylase}]{\text{phosphoenolpyruvate}} \text{oxaloacetic acid} + P_i$$

$$\text{(c) oxaloacetic acid} + \text{NADPH} \xrightarrow[\text{dehydrogenase}]{\text{malate}} \text{malate} + \text{NADP}$$

Only reactions (a) and (b) are strictly required for $CO_2$ fixation, although reaction (c) can operate concurrently if oxaloacetic acid is allowed to accumulate during the course of the reaction, or alternatively, if exogenous oxaloacetic acid is added. Hence, we can impose differing energy requirements on the chloroplasts; if only reactions (a) and (b) are operating there is a high demand for only ATP while if reaction (c) is also operating, a demand for NADPH is also imposed. Since the enzymes catalyzing the above reactions are present in excess [4, 5], $^{14}CO_2$ fixation is a valid indication of total photophosphorylation. Direct measurements of ATP utilization show 2 ATP required per $CO_2$ fixed with pyruvate induced $CO_2$ fixation in $C_4$ mesophyll chloroplast preparations [6]. One of our interests has been to determine the source of the ATP used in reaction (a) under differing experimental conditions, and we have found that cyclic, noncyclic [7], and pseudocyclic [8] electron flow are potential sources of ATP. We have also attempted to determine which electron transport components are involved in cyclic electron flow, by the use of several specific inhibitors: antimycin A, which is thought to block the oxidation of cytochrome $b_{563}$ [9]; 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), which prevents the oxidation of plastohydroquinone [10]; 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDAC), which probably blocks the reduction of cytochrome *f* [11]; and disalicylidenepropanediamine (DSPD), which blocks photosystem I at the site of ferredoxin reduction [1, 2].

Previously we reported [13] that 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) can reverse the inhibition of cyclic photophosphorylation by antimycin A and DBMIB. In this report, we show that both DCMU and *o*-phenanthroline can partially reverse the inhibition of cyclic photophosphorylation under white light by

DBMIB, antimycin A and EDAC, but not inhibition by DSPD and KCN. Also, the effect of these inhibitors on cyclic photophosphorylation driven by far-red light was studied. On the basis of these results, it is proposed that electrons from photosystem II may regulate the pathway of ferredoxin-mediated cyclic electron flow in intact chloroplasts.

## MATERIALS AND METHODS

*Preparation of mesophyll chloroplasts. Digitaria sanguinalis* was grown and mesophyll protoplasts were enzymatically isolated and purified as previously described [14, 15]. Mesophyll protoplasts were mechanically ruptured in a medium consisting of 0.3 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EDTA, 1 mM $KH_2PO_4$ and 50 mM Tricine-KOH (pH 7.8) to yield a suspension of 85–98 % intact chloroplasts [3, 16] and extrachloroplastic components, which were used in all experiments ("mesophyll chloroplast preparations"). The extrachloroplastic components were retained, since phosphoenolpyruvate carboxylase, which is required for $CO_2$ fixation, is a cytoplasmic enzyme [3].

*$CO_2$ fixation assays.* All assays were performed in 0.3 M sorbitol, 1 mM $MgCl_2$, 1 mM $KH_2PO_4$, 50 mM Tricine-KOH (pH 7.8), 2 mM $NaH^{14}CO_3$ (1–2 $\mu$Ci/$\mu$mol), 2 mM pyruvate (potassium salt) and 2–4 $\mu$g of chlorophyll in a total volume of 0.25 ml. Where indicated, oxaloacetic acid was added to give a final concentration of 0.5 mM. At time intervals, 50 $\mu$l aliquots of the reaction mixture were acidified, and acid-stable cpm were determined by liquid scintillation counting. Rates, expressed as $\mu$mol $CO_2$ fixed/mg chlorophyll per h, were calculated from the linear phase of $CO_2$ fixation. Since 2 ATP are required per $CO_2$ fixed in pyruvate-induced $CO_2$ fixation by $C_4$ mesophyll preparations [6], the rate of ATP synthesis would be at least twice the rate of $CO_2$ fixation.

Where used, white light was provided by a General Electric Lucalox sodium discharge lamp (400 W) giving an irradiance of $1.5 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s$^{-1}$ at the reaction cuvette, as measured with a YSI Model 65 radiometer. Far-red light was provided by passing white light from a Sylvania direct light incandescent bulb (150 W) through two 2 mm Schottgen filters, giving $6.6 \cdot 10^4$ ergs $\cdot$ cm$^{-2} \cdot$ s$^{-1}$ at the reaction cuvette. Reactions were run in closed vials that had been pre-equilibrated with 2 % oxygen, by mixing commercially prepared $N_2$ and $O_2$.

*Chlorophyll determination.* Chlorophyll was determined by the method of Wintermans and De Mots [17].

## RESULTS AND DISCUSSION

### *Effect of photosystem II inhibitors on cyclic photophosphorylation*

Strong noncyclic electron flow can be induced in $C_4$ mesophyll chloroplast preparations by the addition of pyruvate plus oxaloacetic acid [3, 7]. The pyruvate rapidly utilizes the ATP formed by the pyruvate, $P_i$ dikinase reaction while the oxaloacetic acid, by reduction to malate, continually oxidizes the NADPH generated (see Introduction) allowing noncyclic electron flow to proceed. Both oxygen evolution and $CO_2$ fixation by $C_4$ mesophyll preparations in the presence of pyruvate plus oxaloacetic acid are inhibited strongly by 0.2 $\mu$M DCMU [7]. Another photosystem II

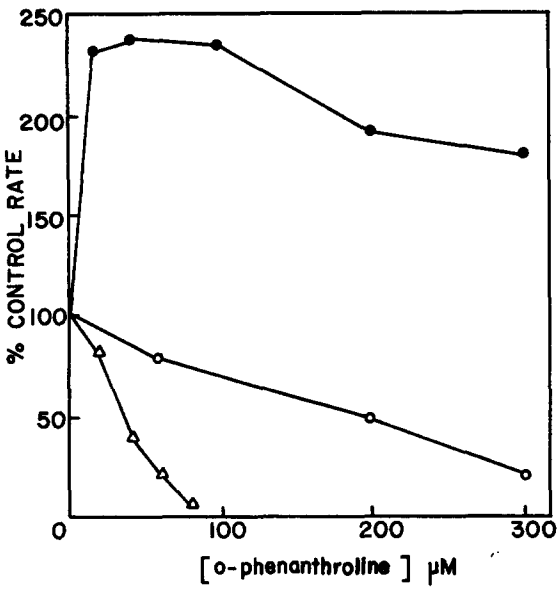


Fig. 1. The effect of *o*-phenanthroline on $CO_2$ fixation and oxygen evolution by mesophyll chloroplast preparations of *D. sanguinalis*. $^{14}CO_2$ fixation induced by pyruvate, (●—●) and pyruvate plus oxaloacetic acid, (○—○); oxygen evolution induced by pyruvate plus oxaloacetic acid, (△—△). Control rates were 26, 196 and 165 μmol/mg chlorophyll per h, respectively. All reactions were run in white light under 2% oxygen. Oxygen evolution was measured in a separate experiment using a YSI polarograph and conditions similar to those used for $CO_2$ fixation studies.

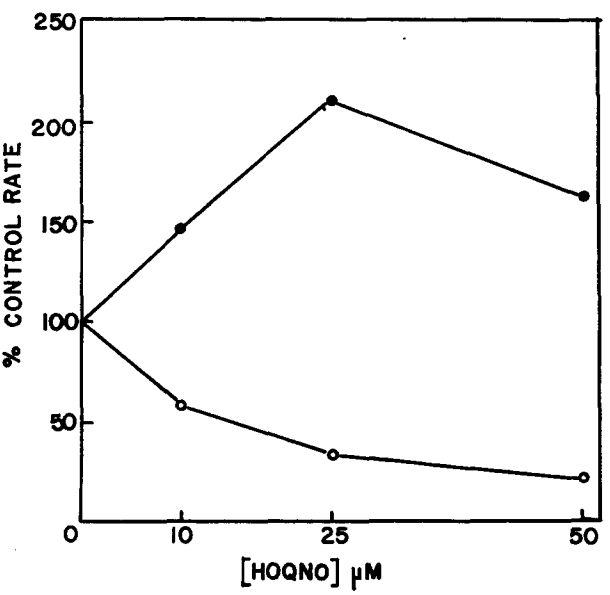


Fig. 2. Effect on HOQNO on $^{14}CO_2$ fixation by mesophyll chloroplast preparations of *D. sanguinalis*, induced by pyruvate, (●—●) and pyruvate plus oxaloacetic acid, (○—○). Control rates were 36 and 220 μmol/mg chlorophyll per h, respectively. All reactions were run in white light under 2% oxygen.

inhibitor, *o*-phenanthroline, was also observed to inhibit pyruvate plus oxaloacetic acid induced oxygen evolution and $CO_2$ fixation (Fig. 1). In contrast, $CO_2$ fixation induced by pyruvate alone, which is dependent on cyclic photophosphorylation, was stimulated several-fold by DCMU [7] and *o*-phenanthroline (Fig. 1). Another photosystem II inhibitor, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), acted in a similar fashion by inhibiting noncyclic-dependent $CO_2$ fixation and stimulating cyclic-dependent $CO_2$ fixation (Fig. 2).

The strong inhibition of pyruvate plus oxaloacetic acid induced $CO_2$ fixation by these inhibitors is due firstly to a direct inhibition of noncyclic electron flow and perhaps partial inhibition of any cyclic electron flow by the drainage of electrons, from a closed system, due to oxaloacetic acid reduction. This latter effect is directly analogous to the ferricyanide inhibition of cyclic photophosphorylation in chloroplasts where photosystem II had been inactivated by removal of chloride ions [18]. The stimulation of $CO_2$ fixation induced by pyruvate alone (cyclic photophosphorylation) is probably related to the "overreduction" phenomenon described by Hauska et al. [19] where blocking the flow of electrons from photosystem II increases the potential for a cyclic electron flow around photosystem I. Such a stimulation of ferredoxin-mediated cyclic photophosphorylation by DCMU has been previously reported in systems with broken [20] and intact [21] chloroplasts of spinach, a $C_3$ species.

*Inhibitors of cyclic electron flow*

The electron transport components involved in cyclic electron flow can be studied by the use of specific inhibitors. We previously reported [13] that $CO_2$ fixation induced by pyruvate, under conditions where only cyclic photophosphorylation was operating, was inhibited by DBMIB and antimycin A, suggesting that plastoquinone and cytochrome $b_{563}$ are obligatory intermediates. Cytochrome *f* is probably also a required electron carrier, as cyclic photophosphorylation was sensitive to EDAC (Table I), which blocks the reduction of cytochrome *f* [11]. 3 mM EDAC also was strongly inhibitory to $CO_2$ fixation induced by pyruvate plus oxaloacetic acid

TABLE I

EFFECT OF EDAC AND DSPD ON $^{14}CO_2$ FIXATION BY $C_4$ MESOPHYLL CHLOROPLAST PREPARATIONS OF *D. SANGUINALIS*

$CO_2$ fixation assays were performed as described in Materials and Methods, under 2 % oxygen. Actual rates of $CO_2$ fixation are given in parentheses as μmol $CO_2$ fixed/mg chlorophyll per h. DSPD stocks were prepared in absolute ethanol and added to give less than 1 % ethanol, final concentration.

| Inhibitor concentration (mM) | | Substrates | |
|---|---|---|---|
| | | Pyruvate | Pyruvate plus oxaloacetic acid |
| EDAC | 0 | 100 (29) (% control) | 100 (160) |
| | 1.5 | 6 | 20 |
| | 3.0 | 10 | 10 |
| DSPD | 0 | 100 (19) | 100 (230) |
| | 0.1 | 17 | 10 |
| | 0.5 | 4 | 2 |

(noncyclic photophosphorylation) which is consistent with the proposed role of cytochrome *f* in noncyclic electron flow as an electron donor to plastocyanin [22]. Electron flow through plastocyanin can be reduced severely by treatment of chloroplasts with 30 mM KCN for 90 min at 4 °C [23]. Such treatment reduced the rate of pyruvate-induced $CO_2$ fixation to 15 % of the rate obtained with control chloroplasts that were held at 4 °C for 90 min (data not shown).

Various cofactors of cyclic electron flow such as phenazine methosulphate, pyocyanine, menadione and ferredoxin, have been studied in vitro [24]. All cofactors can induce high rates of cyclic photophosphorylation. The physiological cofactor, however, is thought to be ferredoxin [20]. A role of ferredoxin in cyclic electron flow in mesophyll chloroplasts of *D. sanguinalis* is suggested by sensitivity to the ferredoxin antagonist, DSPD (Table I). $CO_2$ fixation induced by pyruvate plus oxaloacetic acid (noncyclic photophosphorylation) was also completely sensitive to DSPD, which is consistent with the role of ferredoxin as an electron donor to NADP.

The reaction which we are actually measuring in this study, i.e., the fixation of $H^{14}CO_3$ by phosphoenolpyruvate carboxylase, is several steps removed from the process of photophosphorylation. It is important, therefore, that the inhibitors we are using are specific for the light reactions and are not blocking one or more of the dark reactions (see Introduction). This has been checked by determining the effect of the inhibitors on $^{14}CO_2$ fixation which is driven by exogenous ATP. $C_4$ mesophyll chloroplasts apparently have a high activity ATP translocator which is capable of transporting ATP at rates ranging from 40–70 μmol ATP/mg chlorophyll per h [6]. In a previous study [6], we reported that the inhibition of $CO_2$ fixation in the light by DBMIB, DCMU and antimycin A could be overcome by exogenous ATP suggesting that these inhibitors were specific for the light reactions. As a result of the ATP translocator, $CO_2$ fixation can be driven in the dark by exogenous ATP [6]. In one experiment with such a system, the addition of 2.5 mM EDAC or 0.25 mM DSPD, or pretreatment of the chloroplasts with 30 mM KCN for 90 min was without effect on the rate of $CO_2$ fixation (30 μmol/mg chlorophyll per h; data not shown). Since photophosphorylation is the only reaction not required for ATP-dependent fixation in the dark, it can be concluded that the inhibitors we are using act specifically at the level of the light reactions involved in photophosphorylation.

*Reversal of blocks in cyclic pathway*

In a previous communication [13], we reported that the inhibition of $CO_2$ fixation induced by pyruvate (cyclic photophosphorylation) by DBMIB and antimycin A could be reversed by DCMU. As shown in Fig. 3, *o*-phenanthroline and DCMU, but not HOQNO, can reverse the DBMIB block. Since DCMU and *o*-phenanthroline both cause the reversal and since the inhibition sites have not been distinguished [25], it suggests that the site of action rather than the chemical nature of the photosystem II inhibitor is the important factor. While the site of action of HOQNO is unknown, the exact site of inhibition may be different than that of DCMU [26]. All three photosystem II inhibitors, however, stimulate cyclic photophosphorylation (Figs. 1 and 2, ref. 13) which suggests that the reversal of the DBMIB block is more than simply a change in rate limiting steps. That DCMU is not acting by preventing the uptake of DBMIB is shown in the recovery experiment of Fig. 4. Inhibition of cyclic photophosphorylation by DBMIB can be demonstrated for several minutes and the release

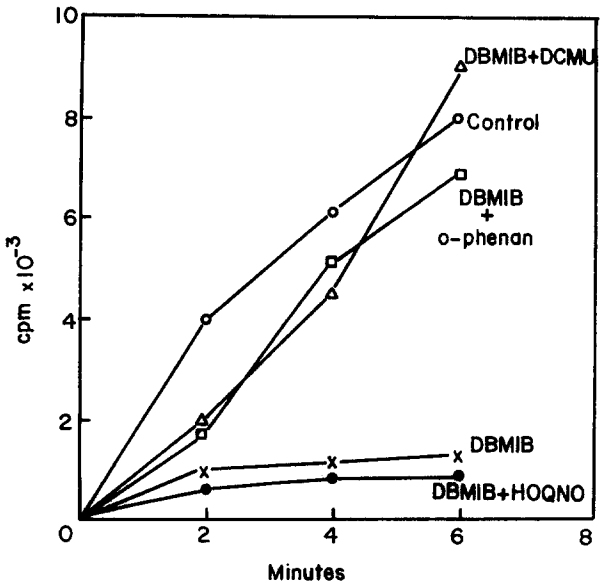


Fig. 3. Effect of the photosystem II inhibitors, DCMU, *o*-phenanthroline and HOQNO on the kinetics of pyruvate-induced $^{14}CO_2$ fixation inhibited by DBMIB. The control rate of $CO_2$ fixation was 26 $\mu$mol/mg chlorophyll per h, based on cpm fixed after 6 min. Additions were: 2 $\mu$M DBMIB, 0.2 $\mu$M DCMU, 40 $\mu$M *o*-phenanthroline and 25 $\mu$M HOQNO. All reactions were performed in white light under 2 % oxygen as described in Materials and Methods.

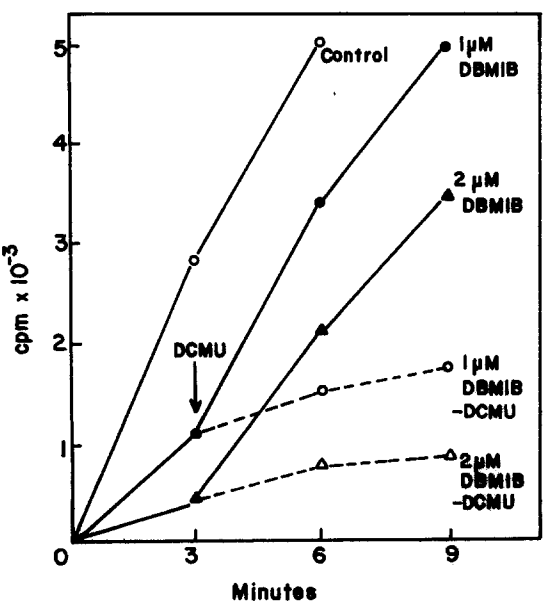


Fig. 4. Reversal of the DBMIB block of pyruvate-induced $CO_2$ fixation by mesophyll chloroplast preparations of *D. sanguinalis* by DCMU. The control received no inhibitors, and fixed $CO_2$ at a rate of 25 $\mu$mol/mg chlorophyll per h. DBMIB was added to give a final concentration of either 1 $\mu$M or 2 $\mu$M, as indicated. After 3 min of fixation in the presence of DBMIB, 0.2 $\mu$M DCMU was added (solid lines) or an equivalent amount of water was added (dashed lines) to serve as the control. All reactions were run in white light under 2 % oxygen.

of that inhibition is evident after the addition of DCMU. Without DCMU to reverse the inhibition, $CO_2$ is fixed slowly for a few minutes and then ceases altogether.

Similar results were obtained of the EDAC block of cyclic electron flow. As shown in Fig. 5, both DCMU and *o*-phenanthroline were effective in reversing the inhibition, whereas HOQNO was not. Because the uptake of EDAC is light driven [11], all experiments with EDAC were performed similar to the experiment of Fig. 4: chloroplasts were preincubated with EDAC for 2 min in white light after which reactions were initiated by the addition of pyruvate and the photosystem II inhibitor. If the photosystem II inhibitors were present during the preincubation period, subsequent inhibition was greatly reduced, possibly as a result of blocking the uptake of EDAC (data not shown).

We previously reported [13] that DCMU could partially reverse the antimycin A block of cyclic photophosphorylation. This finding is confirmed and extended in Fig. 6, which shows that both DCMU and *o*-phenanthroline were effective in the reversal, while HOQNO was not.

The other inhibitor we have studied is DSPD, which inhibits photosystem I at the site of ferredoxin reduction. Previous studies have shown that DSPD inhibits $CO_2$ fixation by spinach chloroplasts [27] and ferredoxin-mediated cyclic photophosphorylation [12]. In our cyclic system, DSPD was also inhibitory, and the three photosystem II inhibitors DCMU, *o*-phenanthroline and HOQNO were found not to relieve this inhibition (data not shown). Similarly, neither photosystem II inhibitor could overcome the inhibition by KCN treatment, which blocks electron flow through

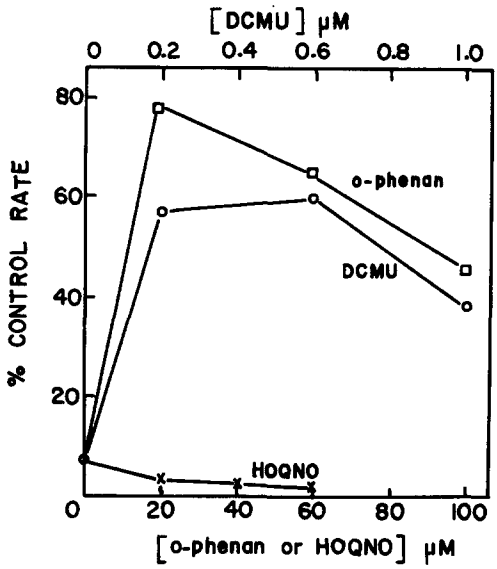


Fig. 5. Effect of the concentration of DCMU, *o*-phenanthroline and HOQNO on pyruvate-induced $CO_2$ fixation inhibited by 1.5 mM EDAC. Chloroplasts were preincubated with the EDAC for 3 min in white light; reactions were initiated by the addition of pyruvate and the second inhibitor. The control rate was 29 μmol/mg chlorophyll per h and refers to the rate of pyruvate induced fixation in the absence of EDAC. All reactions were performed in white light under 2 % oxygen.

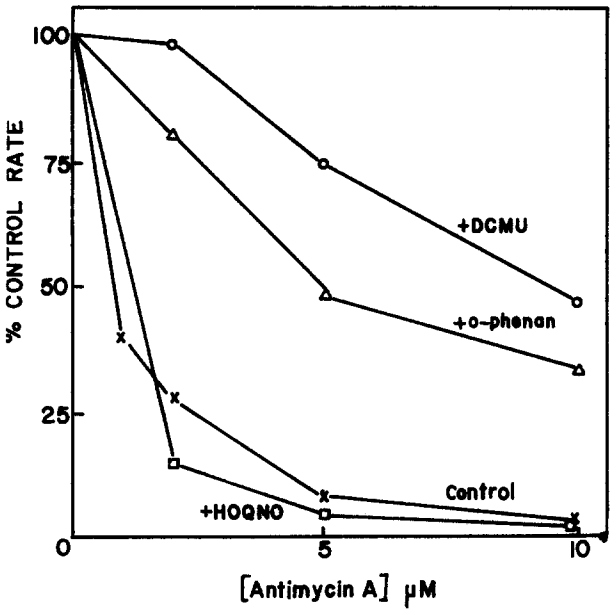


Fig. 6. The effect of antimycin A on pyruvate-induced $CO_2$ fixation. Additions were: 0.2 $\mu$M DCMU, 40 $\mu$M *o*-phenanthroline, 25 $\mu$M HOQNO. Control rate of $CO_2$ fixation was 35 $\mu$mol/mg chlorophyll per h in white light under 2 % oxygen.

plastocyanin. In sum, cyclic photophosphorylation can be inhibited by DBMIB, EDAC, and antimycin A and this inhibition can be reversed by DCMU and *o*-phenanthroline. The inhibition of cyclic photophosphorylation by DSPD or KCN was not reversed by either DCMU or *o*-phenanthroline. This may indicate that in the absence of photosystem II inhibitors, cyclic electron flow proceeds through cytochrome $b_{563}$, plastoquinone and cytochrome *f*, while in the presence of either DCMU or *o*-phenanthroline, electron flow bypasses cytochrome $b_{563}$, plastoquinone and cytochrome *f*.

*Cyclic photophosphorylation in far-red light*

In the experiments described above, cyclic electron flow was induced under white light and low $O_2$ by imposing a high demand for only ATP with pyruvate. Cyclic photophosphorylation can also be induced by selectively activating only photosystem I using far-red illumination. As shown in Fig. 7, far-red light supported considerable $CO_2$ fixation that is dependent on cyclic photophosphorylation. $CO_2$ fixation under far-red light, however, responded differently than $CO_2$ fixation in white light. Oxaloacetic acid, which stimulates pyruvate induced $CO_2$ fixation in white light [3], was inhibitory in far-red light. Presumably oxaloacetic acid is inhibiting by draining electrons from the cyclic system as it is reduced to malate (see Introduction). This is the same logic that was used in part to explain the inhibition of pyruvate plus oxaloacetic acid induced $CO_2$ fixation by photosystem II inhibitors. It appears that whenever electrons from photosystem II are blocked (either by an inhibitor or by activating only photosystem I) oxaloacetic acid inhibits. DCMU was also very

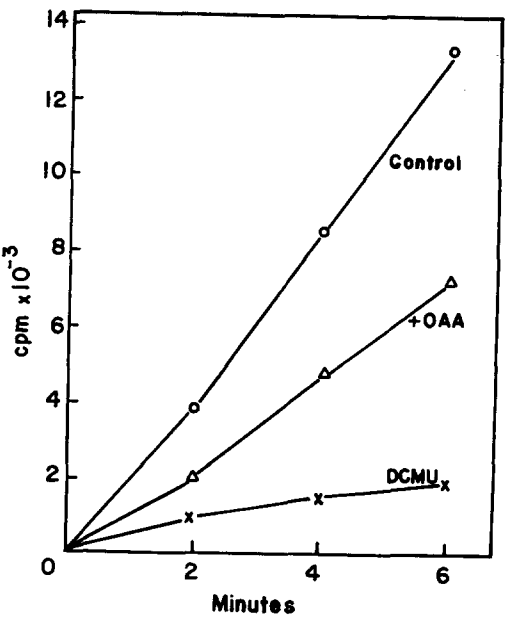


Fig. 7. Kinetics of pyruvate-induced $CO_2$ fixation by mesophyll chloroplast preparations of *D. sanguinalis* in far-red light. Additions were: 0.5 mM oxaloacetic acid, 0.2 $\mu$M DCMU. The rate of $CO_2$ fixation by the control was 20 $\mu$mol/mg chlorophyll per h. All reactions were under 2 % oxygen. See text for discussion.

inhibitory in far-red light (Fig. 7) whereas under white light, DCMU stimulated cyclic photophosphorylation. Similar effects of DCMU were recently reported by Arnon et al. with ferredoxin-catalyzed cyclic photophosphorylation in spinach chloroplasts [28]. These results are also in agreement with the findings of Kaiser and Urbach [29] that low concentrations of DCMU inhibit cyclic electron flow under far-red light in spinach chloroplasts. These data may suggest that while strong noncyclic electron flow is inhibitory to cyclic electron flow due to the overreduction phenomenon (i.e., DCMU stimulates under white light), some flow of electrons from photosystem II is absolutely required. This proposal, however, needs further consideration.

Oxygen was also very inhibitory to cyclic photophosphorylation in far-red light. In one experiment, the rate of $CO_2$ fixation was reduced to 46 and 15 % of the rate under $N_2$ by 21 and 100 % oxygen, respectively. This is likely due to the direct removal of electrons from the cyclic system by autooxidation of an electron transport component, perhaps ferredoxin, since a strong potential for pseudocyclic electron flow in intact mesophyll chloroplasts of *D. sanguinalis* has recently been suggested [8]. Presumably the sites of oxygen reduction would be the same during pseudocyclic electron flow and cyclic electron flow. We previously reported that pyruvate induced $CO_2$ fixation in white light was stimulated by oxygen apparently due to the extra ATP formed from pseudocyclic electron flow. Due to the potential for oxygen reduction by photosystem I, it would be expected that oxygen should inhibit cyclic photophosphorylation in far-red light, which was observed. Also, $CO_2$ fixation in the presence of pyruvate plus DCMU in white light was inhibited by oxygen (data not shown);

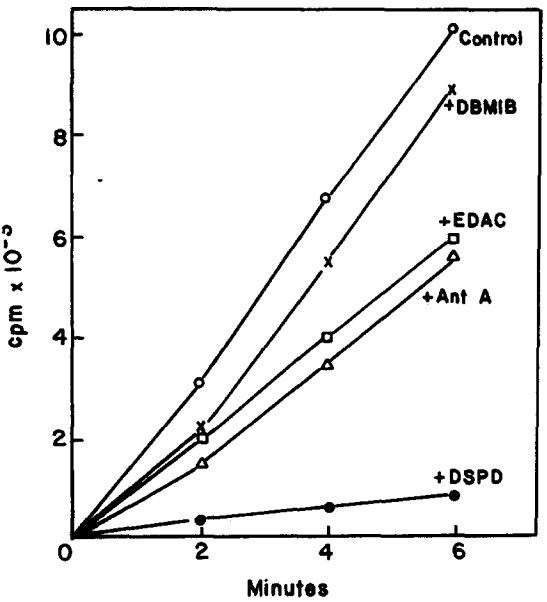


Fig. 8. Effect of various inhibitors on pyruvate-induced $CO_2$ fixation in far-red light. Concentrations used were: 3 $\mu$M DBMIB, 1.5 mM EDAC, 10 $\mu$M antimycin A and 0.25 mM DSPD. The control rate of $CO_2$ fixation was 31 $\mu$mol/mg chlorophyll per h. All reactions were run under 2 % oxygen.

here again, inhibiting electron flow from photosystem II by DCMU or activating only photosystem I behaves similarly. In opposition to our finding that oxygen inhibits cyclic photophosphorylation in far-red light, are the recent results of Arnon et al. [28] with a reconstituted cyclic system which suggested that ferredoxin was a more efficient catalyst of cyclic photophosphorylation in far-red light under 21 % oxygen as opposed to pure $N_2$. Working with intact spinach chloroplasts, Kaiser and Urbach [21] reported no effect of oxygen on cyclic photophosphorylation in far-red light. The basis for these discrepancies cannot be ascertained at present due to differences in techniques and plant materials.

The inhibitors DBMIB, EDAC, antimycin A and DSPD used to study the pathway of cyclic electron flow in white light were also tested on cyclic flow in far-red light. As shown in Fig. 8, DBMIB, EDAC and antimycin A were significantly less inhibitory at concentrations that gave near complete inhibition in white light (Table I, Figs. 3 and 6), while DSPD remained inhibitory. Inhibition by DBMIB, EDAC and antimycin A was not increased by a preincubation in white light (data not shown). $CO_2$ fixation also remained completely sensitive to KCN treatment that blocks electron flow through plastocyanin (data not shown). Further evidence that inhibitor uptake in far-red light was not a factor is shown in Fig. 9, where chloroplasts are exposed to alternate cycles of white and far-red light. $CO_2$ fixation induced by pyruvate alone proceeds well in both light regimes. However, in the presence of either oxaloacetic acid or DCMU, $CO_2$ fixation proceeds only in white light, and promptly ceases after transfer to far-red light. In contrast, when DBMIB is present, $CO_2$ fixation dependent on cyclic photophosphorylation, is inhibited severely in white light, but the inhibition

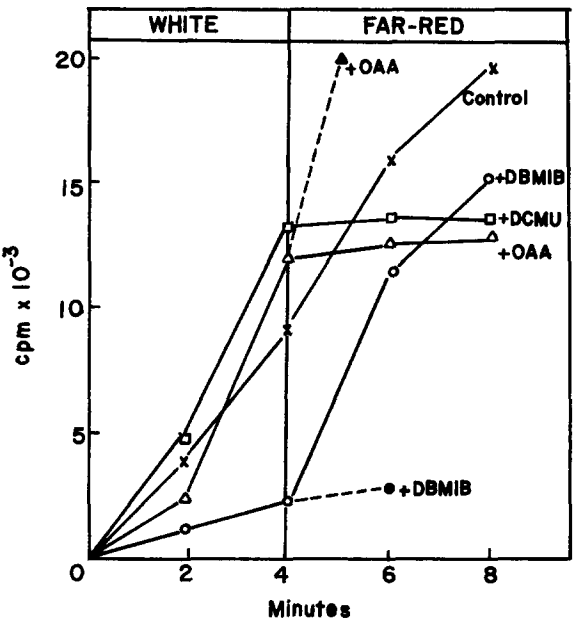


Fig. 9. Kinetics of $CO_2$ fixation by mesophyll chloroplast preparations of *D. sanguinalis* in alternating white and far-red light. The control mixture contained only pyruvate, and the rate of fixation was 39 $\mu$mol/mg chlorophyll per h. As indicated, additions were: 0.5 mM oxaloacetic acid, 0.2 $\mu$M DCMU, 3 $\mu$M DBMIB. The two dashed lines in the figure indicate the fixation that occurred if the reaction mixture were left in the white light. See text for discussion.

is released after transfer to far-red light. Similar results were obtained when DBMIB was replaced with either 10 $\mu$M antimycin A or 1.5 mM EDAC. This suggests that the cyclic electron flow in far-red light does not involve cytochrome $b_{563}$, plastoquinone or cytochrome *f* as obligatory intermediates, but includes ferredoxin and plastocyanin.

## CONCLUDING REMARKS

Cyclic photophosphorylation can operate in white or far-red light, but results have been presented which suggest that the electron transport components involved in $C_4$ mesophyll chloroplasts are different in the two cyclic systems. Cyclic photophosphorylation in white light is sensitive to the inhibitors DSPD, antimycin A, DBMIB, EDAC, and KCN suggesting that ferredoxin, cytochrome $b_{563}$, plastoquinone, cytochrome *f* and plastocyanin are involved. Two photosystem II inhibitors, DCMU and *o*-phenanthroline, were observed to significantly reverse the inhibition of cyclic photophosphorylation by DBMIB, antimycin A and EDAC. This may indicate that when electrons from photosystem II are blocked by an inhibitor, a secondary pathway of cyclic electron flow is operative that does not include cytochrome $b_{563}$, plastoquinone, or cytochrome *f* as intermediates. This secondary pathway also seems to be operative when photosystem I is selectively activated i.e. by far-red light when electron flow from photosystem II is limited.

On the basis of these results, it is proposed that in white light, electrons flowing from photosystem II past the site of action of DCMU are required for cyclic electron flow to include plastoquinone. If electron flow through this "critical region" is reduced, a shortened cyclic pathway will be operating that does not include cytochrome $b_{563}$, plastoquinone, or cytochrome *f*, as obligatory intermediates. This pathway can be induced by blocking electron flow through the "critical region" either by DCMU or *o*-phenanthroline action or by selectively activating only photosystem I. The manner by which electrons from photosystem II control the pathway of cyclic electron flow is open to speculation. Electrons flowing in the "critical region" may induce, or prevent, a conformation change in the thylakoids that determines the course of cyclic electron flow. It is interesting to note that in $C_3$ mesophyll chloroplasts, it has been postulated that electron flow between the site of action of DCMU and plastoquinone is involved in light-induced DABS binding [30] (indicative of a conformation change) and making Site II (at the level of water oxidation) phosphorylating [31]. Alternatively, electron flow in the "critical region" may alter redox potentials of certain components that determine which pathway will be operative.

The secondary, or shortened, cyclic pathway would appear to involve ferredoxin and plastocyanin. One of the most interesting features of this shortened cyclic pathway is the coupling site(s) involved. This pathway is phosphorylating, since it supports ATP dependent $CO_2$ fixation. Normal cyclic electron flow through plastoquinone is thought to share the non-cyclic coupling site between plastoquinone and cytochrome *f*. In addition to transferring electrons, plastoquinone is proposed to transport protons across the thylakoid membrane [24]. The secondary cyclic pathway, however, appears not to involve plastoquinone, and hence the nature of the coupling site is of prime importance, toward which current studies are being directed.

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