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Enhancement of Energy Conservation by Hill Reaction Inhibitors in Isolated Spinach (*Spinacia oleracea*) Chloroplast Fragments¹

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Abstract. The effect of diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea], desmedipham [ethyl *m*-hydroxycarbanilate carbanilate(ester)], propanil (3',4'-dichloropropionanilide), and dibromothymoquinone (DBMIB) (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) on proton translocation and photophosphorylation in isolated spinach (*Spinacia oleracea* L.) chloroplast fragments was investigated. In the absence of added cofactors, O₂, or artificial electron acceptors, cyclic electron transport occurred, which was coupled to energy conservation. Under aerobic conditions O₂ acted as the terminal acceptor in non-cyclic electron transport. Proton translocation and photophosphorylation in the cyclic process were enhanced by diuron, desmedipham, and propanil, while in the non-cyclic process they were inhibited by all three herbicides. DBMIB inhibited proton translocation and photophosphorylation in both processes. Proton translocation and its enhancement increased with increasing light intensities. The finding that the plastoquinone (PQ) antagonist DBMIB disrupted cyclic as well as noncyclic electron flow, while diuron enhanced the cyclic and inhibited the noncyclic process, indicated that the acceptor site for endogenously-cycling electrons must lie between the active site of diuron inhibition and PQ. The close similarity in the behavior of diuron, desmedipham, and propanil suggests that their site of action is the same.

INTRODUCTION

Until recently, cyclic electron transport in isolated chloroplasts was assumed to be possible only after addition of cofactors (29). However, in the last few years, reports of endogenous, cyclic electron transport, leading to ATP synthesis, have appeared (6, 21, 25). The pathway followed by electrons transported cyclically in thylakoid membranes and the associated energy conversion sites are still controversial. A special cyclic coupling site linked to a separate electron transport chain (2, 30) has been proposed to account for the energy requirements of the chloroplast. More recently a photosystem II (PS II)-linked energy conservation site has been discovered (8, 18, 27, 32) which, together with the previously known coupling site between PQ and cytochrome *f*

(1, 4) appears sufficient to account for all the ATP requirements of photosynthetic cells. According to this latter concept a separate cyclic system would not be needed, and cycling electrons would return directly to the main electron transport chain (3, 8, 14, 31).

Of the two known native energy conservation steps (proton releasing sites) in the thylakoids (12, 26), one located in the H₂O-splitting portion of the pathway (20) and the other in the electron transport chain connecting the two photosystems, only the latter step is likely to apply to cyclic electron transport. It involves the translocation of protons across the thylakoid membrane by PQ (28). In the absence of artificial cofactors that might create additional conservation sites (13), proton translocation (19) due to cyclic electron transport can therefore occur only as the electrons pass through the reduction-oxidation step at PQ. Diuron, which prevents reduction of PQ by electrons from PS II, and DBMIB (5, 11) which blocks the oxidation of PQ by cytochrome *f*, may be used to approximate the return site of endogenously cycling electrons to the electron transport chain.

The objective of this study was to determine the response of proton translocation and photophosphorylation to: O₂ concentration, the presence of three Hill reaction inhibitors (diuron, desmedipham, and propanil) and of the PQ antagonist, DBMIB, and light intensity and light quality.

MATERIALS AND METHODS

Plant materials. Chloroplast fragments were prepared from market-purchased spinach according to Hind and Jagendorf (17) with the following modifications: 30 g of de-veined leaves were ground in a Virtis 45 homogenizer for 5 s at full speed and 15 s at half speed in .05 M, pH 7.8, Tricine buffer. The homogenate was filtered through two layers of Miracloth (Calbiochem). The fragmented chloroplast pellet was suspended in 70 ml of a medium containing 10 mM NaCl and 1 mM MgCl₂, centrifuged at 8000 g and washed once using the same procedure. The final pellet was suspended in a minimal

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volume of the NaCl-MgCl₂ medium. Chlorophyll (Chl) content was determined according to Walker (33).

Reaction conditions. The reaction vessel of a Clark-type (Rank Brothers, Bottisham, Cambridge, England) oxygen electrode was used in all experiments. Temperature was kept constant at 20 C. The actinic red light used in most experiments was provided by an incandescent 150-watt flood lamp filtered through 7 cm of water and a cellulose acetate red transmitting filter (10% cut-off at 436 nm, 1% cut-off at 527 nm). Light intensity in the photosynthetically active range was measured with a Lambda Instruments LI-185 quantum sensor. White and far-red light were provided either by omitting the red transmitting filter or by substituting a far-red transmitting filter (10% cut-off at 690 nm, 1% cut-off at 680 nm). The output of the flood lamp was the same for the three light conditions and the light intensity at the chloroplast suspension depended on the type of filter interposed. Stock solutions of the photosynthetic inhibitors were prepared in 95% acetone or absolute ethanol. The final concentration of the stock solutions after addition to the chloroplast fragment suspensions was less than .02%, which was determined to have no effect on the reactions.

The level of activity varied with the source of biological materials. Most chloroplast-fragment suspensions retained their activities undiminished for up to 8 h. All experiments were repeated at least four times and consistently showed the response patterns reported. When experiments were repeated on the same day, using the same preparation, results were identical under the carefully controlled experimental conditions. However, a variation of up to 30% was introduced with plant materials purchased on different days. Variety, age, and storage condition prior to purchase apparently affected the absolute magnitude of the responses. Statistical analyses were not performed since they would have evaluated only the quality of the biological materials rather than the effectiveness of the treatments. In these experiments the physical unit is the thylakoid, and one can estimate that each ml of suspension contains many millions of thylakoids. This large number of independent units assures a negligible standard error.

Proton translocation measurements. The oxygen electrode vessel was modified to admit a combination pH electrode (Thomas 4094 L15) into the chloroplast-fragment suspension which contained 50 μg Chl/ml in 5 ml of a medium containing 10 mM NaCl and 1 mM MgCl₂. Light-induced pH changes were registered with an extended-scale pH Meter. Oxygen concentration and pH were monitored simultaneously with two potentiometric recorders. The quantity of protons translocated into the thylakoids was measured by titrating the suspension with 10 mM NaOH. The pH of the suspension rose in the light to a maximum and returned to its original value when the light was turned off. Base was added at this time, and the amount of NaOH required to raise the pH back to the maximum level was recorded. Results were expressed as μmoles of H⁺ translocated (lost from the medium) per μmole of Chl. The oxygen electrode was calibrated between 0% attained by adding Na₂S₂O₄ to the suspension, and 21% attained by saturating with air. Oxygen concentrations in solution are expressed in this paper as per cent O₂ in the

atmosphere which is in equilibrium with the solution. Oxygen concentrations in H₂O may be converted to molarities by means of the formula: $M_{\text{O}_2} = B \times \% \text{O}_2 / 22.4$ where B is the Bunsen coefficient (the solubility of O₂ in H₂O given as ml/ml referred to standard conditions). The desired O₂ concentrations were achieved by bubbling N₂ (99.997% pure) through the suspension, and maintained throughout the reaction by the air-tight configuration of the reaction vessel.

Photophosphorylation measurements. ATP synthesized was trapped in the presence of glucose and hexokinase, and the resulting glucose-6-phosphate was determined according to Lamprecht and Trautschold (23). Traces of O₂ were evolved during the course of the reaction in the presence of organic buffers (Tricine, Bicine, TAPS), consequently 0.033 M phosphate buffer, pH 8.3 was used. No deviation from the initial pH of 8.3 was noted during the 15-min duration of the reaction. The reaction mixture consisted of broken chloroplasts containing 210 μg Chl, 1.27 mg MgCl₂, 5.35 mg ADP,

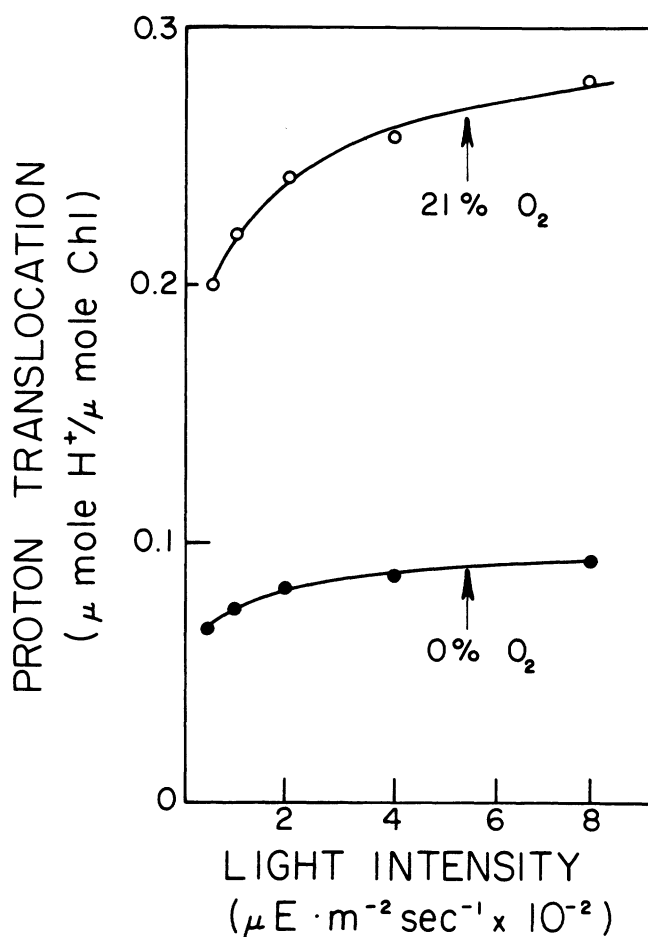


Figure 1. Proton translocation in chloroplast fragments as a function of light intensity. The reaction mixture consisted of broken chloroplasts containing 50 μg chlorophyll per ml of 10 mM NaCl and 1 mM MgCl₂, and was illuminated by red light with a 1% short wavelength cut-off at 527 nm. Proton translocation was determined by titration with 10 mM NaOH. The reaction mixture was either air saturated (\circ) or O₂-free (\bullet). μE : microeinstein.

1.25 mg hexokinase, and 25 mg glucose in 4 ml of 10 mM NaCl. The concentration of photosynthetic inhibitor was 7.5 μM ; DBMIB, when used, was 0.5 μM .

Reagents. Analytical grade diuron, technical grade desmedipham and technical grade propanil were employed. Desmedipham and propanil were crystallized twice from aqueous acetone. Grade 1 ADP, Sigma grade monosodium salt of NADP⁺, and type XII lyophilized glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. Type D hexokinase used (Nutritional Biochemicals Co.) had an activity of 28,000 K.M. units/g.

RESULTS

Proton translocation by illuminated chloroplast-fragment suspensions was a function of both light intensity and O₂ concentration. At the zero O₂ level proton translocation increased less rapidly with increasing light intensity than at the 21% level, and approached saturation at lower light intensity (Figure 1). In the presence of O₂, diuron inhibited proton translocation and inhibition decreased with increasing light intensity (Figure 2). This enhancement increased with light intensity and with diuron concentrations up to 6 μM (Figure 3). Above this concentration no further increase was observed. Diuron stimulated proton translocation up to an O₂ concentration of 0.5% (Figure 4); above 0.5% O₂ diuron was inhibitory. Typical recorder tracings of pH response to light and diuron are shown in Figure 5. With O₂ concentrations greater than 0.5%, a rapid collapse of the pH plateau occurred

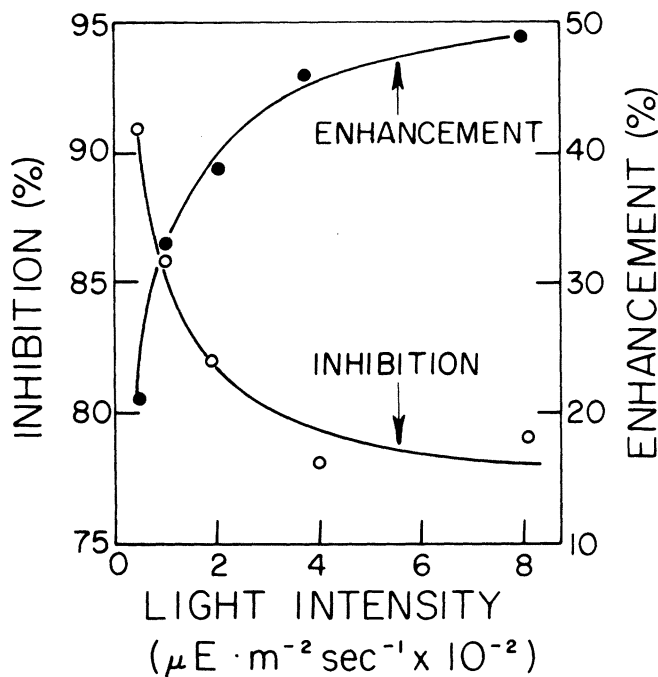


Figure 2. Effect of diuron on proton translocation as a function of light intensity; inhibition (\circ) in air-saturated chloroplast suspensions and enhancement (\bullet) O_2 -free chloroplast suspensions. Diuron concentrations was 6 μM . All other conditions were as in Figure 1. μE : microeinstein.

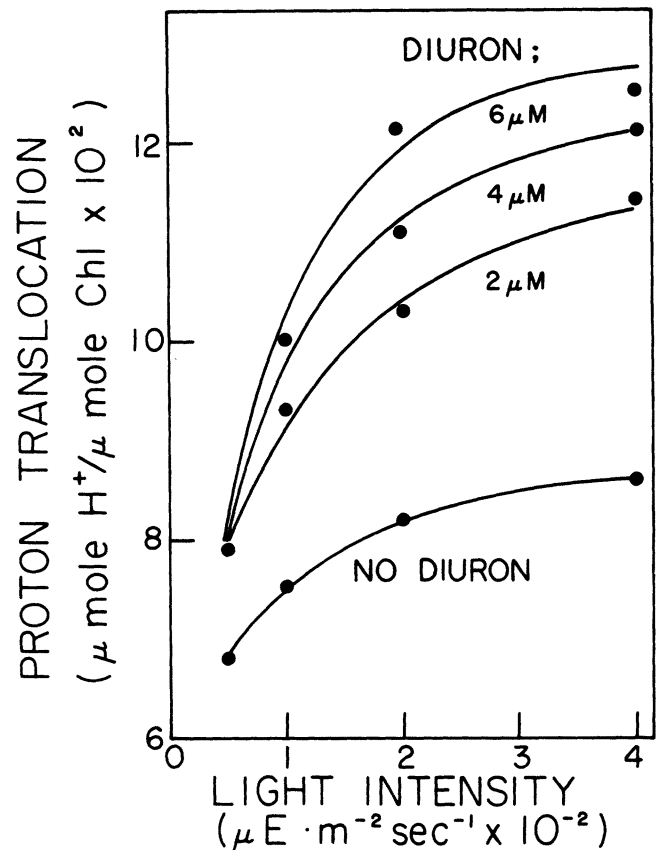


Figure 3. Proton translocation in O_2 -free chloroplast suspensions as a function of light intensity and diuron concentration. All conditions except for the addition of diuron were as in Figure 1. μE : microeinstein.

upon addition of diuron, desmedipham, or propanil, followed by gradual decay. The H^+ concentration expressing diuron inhibition was measured to the point where the pH trace

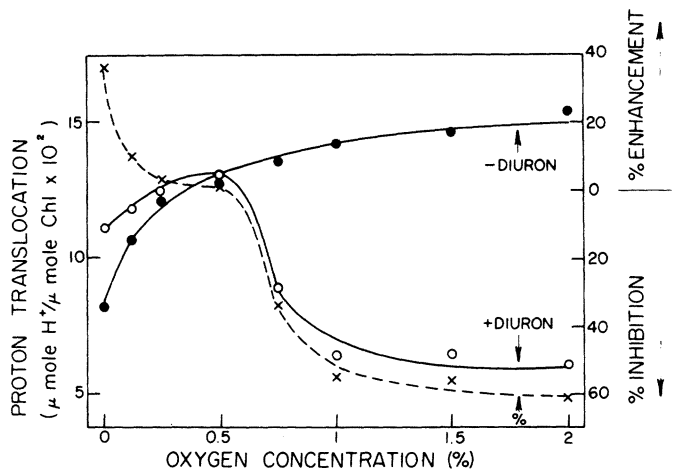


Figure 4. Proton translocation in chloroplast suspensions as a function of O₂ concentration with (\circ) and without (\bullet) 6 μM diuron. Light intensity was 40 $\mu\text{E} \cdot \text{m}^{-2} \text{sec}^{-1}$. The broken line (x) indicates the relative change in proton translocation caused by 6 μM diuron.

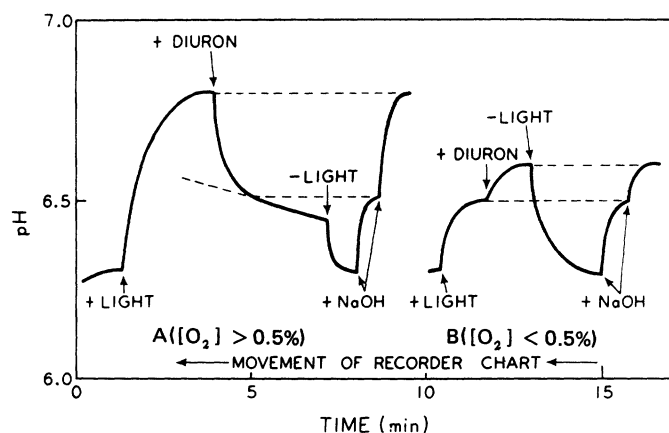


Figure 5. Representative recorder tracings of pH in chloroplast-fragment suspension as a result of proton translocation showing the effect of light and O_2 concentration on the diuron effect. A: O_2 concentration greater than 0.5%; B: O_2 concentration less than 0.5%. Titration with 10 mM NaOH was accomplished from the dark equilibrium level to respective pH levels. The amount of NaOH used was taken as a measure of H^+ translocated into the thylakoids.

became a straight line. After the light was turned off an equilibrium pH close to the pre-illumination level was re-established within a few minutes. With O_2 concentrations less than 0.5% a further increase in pH was brought about by diuron. In the presence of O_2 , oxygraph tracings (not shown) showed O_2 uptake, which stopped upon addition of the Hill reaction inhibitors. When catalase was added to the system, O_2 concentration returned to the original level indicating H_2O_2 formation by the Mehler reaction (24).

Inhibition of proton translocation by diuron in the presence of O_2 was considerably smaller in far-red light than in red or white light; while its enhancement in the absence of O_2 was greater in red and white light than in far-red light (Table 1).

The enhancement of proton translocation mediated by diuron, desmedipham, and propanil qualitatively showed the

Table 1. The influence of light quality on proton translocation as modified by diuron^a.

Reaction conditions		Light quality		
$[O_2]$	Diuron	White	Red	Far-red
(%)	(μM)	(μmoles H^+ /μmole chlorophyll)		
0	0	0.093	0.086	0.036
	6	0.132	0.125	0.039
	% change	+42	+45	+8
1	0	0.153	0.136	0.042
	6	0.086	0.079	0.032
	% change	-44	-42	-24

^aChloroplast-fragment suspensions containing 50 μg Chl per ml of 10 mM NaCl and 1 mM $MgCl_2$ were illuminated with white, red (1% cut-off at 527 nm), and far-red (1% cut-off at 680 nm) light. The same source output was used in all cases.

same light intensity dependence with all three compounds (Figure 6). Inhibitory effects by desmedipham and propanil on proton translocation in the presence of O_2 were similar to those of diuron. The effect of these three Hill reaction inhibitors on photophosphorylation confirmed that observed for proton translocation. In the absence of O_2 , ATP production was enhanced; in the presence of 1% O_2 , ATP production was inhibited (Table 2). In either case, photophosphorylation was inhibited by 0.5 μM DBMIB. In the unbuffered medium employed for proton translocation studies, the use of DBMIB did not appear meaningful in view of its pH-dependent properties (9).

DISCUSSION

Our broken chloroplast preparations exhibited proton translocation and photophosphorylation in the absence of any added factors that promote cyclic electron flow. These endogenous energy conservation phenomena were sensitive to the presence of dissolved O_2 and to changes in light intensity. In the presence of O_2 , a net light-dependent transfer of electrons from H_2O to O_2 took place with the formation of H_2O_2 . Proton translocation (Figures 2, 4, Table 1) and photophosphorylation (Table 2) associated with this process were inhibited by diuron. With increasing light intensity,

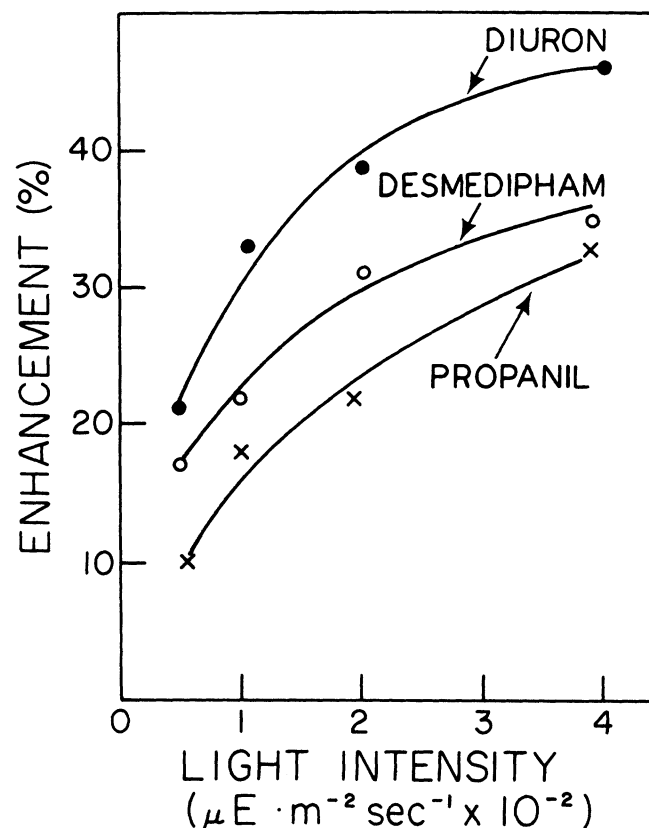


Figure 6. Enhancement of proton translocation by diuron, desmedipham and propanil in O_2 free chloroplast-fragment suspensions as a function of light intensity. Herbicide concentrations were 6 μM . All other conditions were as in Figure 1.

Table 2. The effect of diuron, desmedipham, propanil and DBMIB on ATP synthesis and the presence or absence of O₂.^a

[O ₂]	Incubation conditions (%)	Inhibitors				
		None	Diuron	Des-medipham	Propanil	DBMIB
0	(μ mole ATP/ μ mole Chl) % change	3.10	3.88 +25	3.73 +20	3.22 +7	1.88 -25
1	(μ mole ATP/ μ mole Chl) % change	5.51	3.20 -42	3.25 -41	3.21 -42	3.91 -31

^aThe reaction mixture consisted of chloroplast-fragments containing 210 μ g Chl, 1.27 mg MgCl₂, 5.35 mg ADP, 1.25 mg hexokinase, and 25 mg glucose in 4 ml of 10 mM NaCl and 33 mM phosphate buffer at pH 8.3, and was illuminated for 15 min by red light at 400 μ Em⁻²sec⁻¹. Herbicide concentrations were 7.5 μ M, DBMIB concentration was 0.5 μ M. The ATP synthesized was determined with hexokinase and glucose-6-phosphate dehydrogenase.

inhibition was partially reversed (Figure 2). In the absence of O₂ or any other electron acceptors, only cyclic electron transport can take place. Under such conditions diuron did not inhibit, but rather stimulated proton translocation (Figures 2, 3, Table 1) and photophosphorylation (Table 2). This stimulation was not reversed, but enhanced by increasing light intensity. The effect of the Hill reaction inhibitors desmedipham and propanil was similar to that of diuron (Figure 6). In the absence of photosynthetic inhibitors, proton translocation was also influenced by light intensity, and its saturation characteristics and magnitude were different in the presence or absence of O₂ (Figure 1).

These phenomena can be explained in the following manner. Under aerobic conditions electron flow is unidirectional towards the acceptor (O₂). Photosystems I and II act in concert and higher light intensities cause an increase in electron flow. Under aerobic conditions, electrons cycle around PS I, passing through PQ. In so doing, they compete for an acceptor site in the electron transport chain with electrons excited at PS II. At higher light intensities, overreduction of this site by PS II limits the cycling process, and therefore proton translocation by PQ. Non-cyclic electron flow is not subject to such a limitation; furthermore, it activates other proton translocation steps in addition to the one at PQ (34). These phenomena account for the greater magnitude and continued light intensity response of proton translocation connected to non-cyclic electron flow (Figure 1).

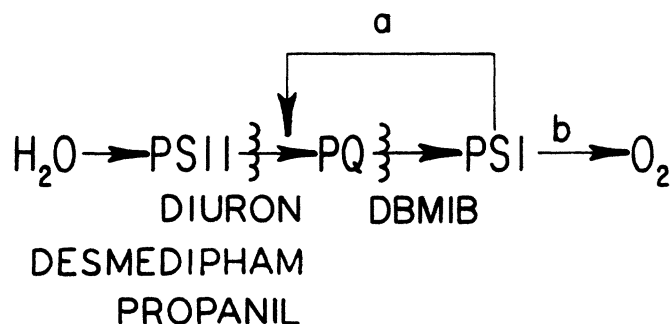


Figure 7. A scheme for electron transport pathways in (a) endogenous cyclic flow and (b) non-cyclic electron flow to O₂.

Addition of diuron to the aerobic system blocks electron flow to the acceptor; and this inhibition is partly overridden by increasing the light intensity. In the anaerobic system the same blockage decreases overreduction of the cyclic acceptor site thus facilitating the return of electrons to PS I. The concomitant enhancement of proton translocation is greater at higher light intensity, where the effect of PS II is more pronounced (15).

The increase in proton translocation upon addition of O₂ to the chloroplast-fragment suspension (Figure 4) above the maximum attainable anaerobically, indicated a shift from the cyclic process to a Mehler reaction (24). As O₂ concentration increased, the stimulating effect of diuron upon proton translocation decreased, until at 0.5% O₂ it was reduced to zero. At higher O₂ concentrations diuron became inhibitory (Figure 4). This transition indicates that above a certain O₂ concentration, non-cyclic electron flow to O₂ predominates over cyclic electron flow. The repression of cyclic electron flow by O₂ has been described (14, 16, 22). Enhancement of proton translocation by diuron under anaerobic conditions in far-red light was considerably smaller than in red or white light (Table 1). Light from the far-red source probably contained insufficient active radiation to energize PS II, and therefore caused less overreduction of the electron carriers.

The enhancement of ATP synthesis in the absence of O₂ by diuron, desmedipham, and propanil (Table 2) is ascribed to the stimulation of proton translocation by PQ, which results in increased phosphorylation potentials. The similarity of the responses of these three compounds as enhancers of cyclic and inhibitors of non-cyclic proton translocation and photophosphorylation indicates that their sites of action may be close or even identical. Certain common characteristics of their molecular structures have been identified, which are thought to be responsible for their biological activity (10). The observation that diuron inhibits only non-cyclic processes suggests that the acceptor site for endogenous, cyclic electron flow lies between the sites of diuron and DBMIB action (Figure 7).

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