

## II - CHLOROPHYLL A FLUORESCENCE AND O<sub>2</sub> -FLASH YIELD AS TOOLS FOR PHOTOSYNTHESIS RESEARCH

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### Introduction

In the last few years our knowledge of the different substances, which cause a toxic effect and those which stimulate the light induced electron transport of photosynthesis in green plants and algae has increased significantly. According to Duysens and Sweers (1963), every limitation of the photosynthetic electron transport between PSII and PSI does increase the PSII-fluorescence yield, whereas a block of electron transport at the oxidizing side of PSII, e.g., between the water – splitting enzyme system and the PSII reaction center pigment P<sub>680</sub>, is indicated by a decrease of PSII fluorescence. On the other hand, the development of sensitive polarographic techniques (Joliot and Joliot, 1965) allowed precise measurement of the small amounts of O<sub>2</sub> evolved in very weak continuous illumination or by single brief (10<sup>-5</sup> sec) flashes. Both techniques are repeatedly used today in photosynthesis research for understanding the mechanism of action of some inhibitors or activators in the photosynthetic electron transport chain.

In this part we will explain in some details the two phenomena (chlorophyll a fluorescence and O<sub>2</sub> – flash yield) and their application in the photosynthesis research.

### Chlorophyll a fluorescence

Plant Photosynthesis is driven by two series connected photoacts (Fig.1). One of these is PSII which is directly connected with the oxygen generating system. The primary electron acceptor of PSII, Q, has a midpoint potential close to ~0V, (Joliot and Kok, 1975) so that the energetic efficiency of the conversion would be ~ 50%. A small flash induced absorption decrease at 680 nm which rapidly returns in subsequent darkness (t<sub>1/2</sub> ~ 0.2 msec), has been interpreted as the bleaching of Chl a II which sensitizes the primary charge transfer of PSII (Joliot and Kook, 1975). An often used indicator of the early events in PSII is the yield of Chl a fluorescence. When the all traps are open, we find a residual emission yield (F<sub>0</sub>) which implies that not all singlet excitations reach the traps. Whereas, when all traps are closed the yield reaches a maximum (F<sub>max</sub>) and under many conditions the yield of the variable part of the fluorescence (F – F<sub>0</sub>) is inversely proportional to the degree of opening of the traps (Fig.2).

Generally the fluorescence yield (F – F<sub>0</sub>) reflects the redox state of the primary reductant Q (Duysens and Sweers, 1963) ( see Papageorgiou, 1975). F becomes maximal (Q reduced) under reduced conditions and minimal (F<sub>0</sub>) upon oxidation.

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### Abbreviations:

Chl- Chlorophyll; DBMIB- 2,5 dibromo-3-methyl-6-isopropyl-p-benzoquinone;  
DCMU-3-(3,4)-dichlorophenyl-1,1-dimethyl urea;  
DCPIP-2,6-dichlorophenol indophenol; PS-Photosystem.

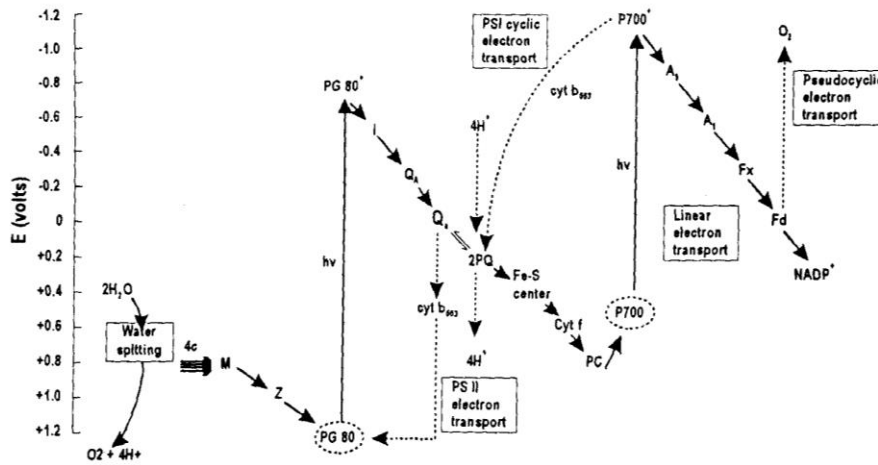


Fig. 1: The Z-scheme of linear photosynthetic electron transport from water to NADP<sup>+</sup> (—); cyclic electron flow in PSI diverting electrons from Q<sub>a</sub> to cytochrome b<sub>559</sub> and back to P<sub>680</sub> (---), cyclic electron transport around PSI through cytochrome b<sub>633</sub> and PQ and pseudocyclic electron transport from ferredoxin to O<sub>2</sub>. All of these electron transport chains can operate in the thylakoid membranes of the chloroplasts, indicating the redox potentials of the participants. Components from water to PQ, as in Figure 1, Fe-S, Rieske iron-sulfur center; cyt. *f*, cytochrome *f*; PC, plastocyanin; P<sub>700</sub>, reaction center chlorophyll a of PSI; A<sub>0</sub>, A<sub>1</sub>, F<sub>x</sub>, electron acceptors of PS I; Fd, ferredoxin; cyt b<sub>559</sub>, cytochrome b<sub>559</sub>; cyt b<sub>633</sub>, cytochrome b<sub>6</sub>. Also shown is H<sup>+</sup> accumulation in the lumen associated with water and plastoquinol oxidations.

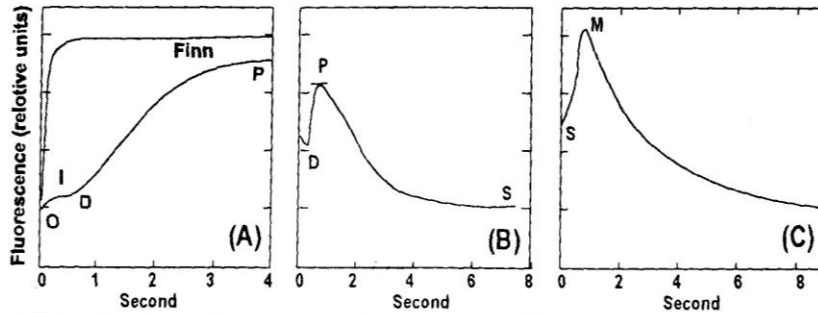


Fig. 2: The various stages of the fluorescence time course of Chl *a* *in vivo*. (A) The fast rise in isolated class II) spinach chloroplasts in the absence (lower curve) and in the presence (upper curve) of the electron transport inhibitor DCMU (B). The fast rise, and the ensuing fast decay of Chl *a* fluorescence in the red alga *Porphyridium cruentum* (re-drawn from Mohanty *et al.*, 1971 b) (C). The slow fluorescence change in green alga *Chlorella pyrenoidosa* (Papageorgiou and Govindjee, unpublished experiment).

On the reducing side of PSII, a pool (A) of about 10 equiv. of plastoquinone per trapping center functions as the electron acceptor. One of these is responsible for O<sub>2</sub> - evolution, which can be observed at the onset of illumination. Cytochrome b<sub>559</sub> appears to be closely associated with PSII. Its normally high midpoint potential of ~ 0.37 V. PSII is intimately connected with the O<sub>2</sub>- evolving system. The O<sub>2</sub> - evolving mechanism is

far more labile than the PSII trapping centers themselves (Joliot and Kok, 1975). Treatments such as aging, mild heating, high pH, or poison by dioxylamine destroy the capability to evolve O<sub>2</sub> while the photooxidation of artificial donors can still proceed with high efficiency (Papageorgiou, 1975).

Examples of such artificial electron donors are hydroxylamine, ascorbate, hydroquinone, phenyl carbazide. Other poisons, notably DCMU, specifically inhibit electron flow through PSII, by blocking the oxidation of primary acceptor Q by its secondary acceptor.

### Flash yields of O<sub>2</sub>

When we give a sequence of short flashes of bright light, separated by darkness, to algae previously adapted in darkness, the yield of O<sub>2</sub> per flash should depend on the position of the flash in the sequence, Joliot and Kok (1975) (see Diner and Joliot, 1977) showed with refined rapid measurements that O<sub>2</sub> was evolved with characteristic oscillations (Fig. 3). The first two flashes evolved little or no O<sub>2</sub>, the third a large gush and the fourth a smaller amount of O<sub>2</sub> than the third but more than the first, that is a periodicity of four. Oscillations were damped and after some 20 flashes yield per flash was constant.

This pattern is characteristic of algae and chloroplasts. Oxygen production is slower just after illumination than with longer illumination and this lag period is inversely proportional to light intensity x time, showing that activation of an intermediate of water splitting is needed for O<sub>2</sub> - production. The states induced by light flashes are not stable. If two flashes are separated from a sequence of flashes by a long dark period, the characteristic sequence starts again as if after darkness. Light captured by one photosystem cannot contribute to another photosystem, O<sub>2</sub>-evolution would be proportional to light intensity if co-operation were possible. O<sub>2</sub>-evolution uses only light captured by PSII. The characteristics of the system do not change in the flash sequence as the optical cross section (area of pigment available for photon absorption) and the quantum yield are similar at all stages of water splitting.

Now we will give some examples for the application of these phenomena in the photosynthesis research.

### A- Application for understanding the effect of inhibitors and activators on the photosynthetic electron transport of cyanobacteria and algae.

#### 1. Influence of copper as heavy metals on cyanobacteria

To investigate the effect of copper on the photosynthetic electron transport of cyanobacteria, Osman et al. (1996) used *Anabaena cylindrica* and *Nostoc muscorum* as objects for such investigation. They observed that the PSII - mediated electron transport (measured as O<sub>2</sub>- evolution) using 2,6-dichloro-P-benzoquinone as electron acceptor was severely inhibited in presence of relatively high concentration of copper (3 μM - 4 μM) (Table 1). They also observed that PSII mediated electron transport was more sensitive against toxic copper concentrations than that of PSI (Table 2). In order to localize the action sites of copper in PSII, they studied the fluorescence induction kinetics at room temperature of both organisms, they observed that relatively low Cu<sup>+2</sup> concentration increased both initial fluorescence (F<sub>0</sub>) and maximum fluorescence intensity (F<sub>m</sub>) (Fig. 4a and 4b). Since the F<sub>0</sub> - value represents the fluorescence level at a physiological state in

which the primary acceptor  $Q_B$  is oxidized (dark adapted cells), and the  $F_m$  represents the equilibrium between the oxidized and reduced  $Q$  after a given time of light excitation, the increase of both values at low  $Cu^{+2}$  concentrations may indicate either an increase of the excitation energy transfer from the light harvesting antennae to the reaction center of PSII or a higher amount of reduced  $Q$  due to the inhibition of the electron transport at the reducing side of PSII (Duysens and Sweers, 1963). According to their results, the increase in the fluorescence intensities was accompanied by a reduction in DBMIB-insensitive  $O_2$ -evolution, which localized the inhibition site of low  $Cu^{+2}$  concentrations in photosynthetic electron transport before the plastoquinone pool. This was also the view of Mohanty *et al.* (1989), Singh and Singh (1987), Yruela *et al.* (1991) and Yruela *et al.* (1992). The fluorescence kinetics of DCMU-treated cells did not change in presence of low  $Cu^{+2}$  concentrations (up to  $2 \mu M$  which obviously indicated that the site of action of low  $Cu^{+2}$  concentrations on photosynthetic electron transport was between the DCMU and DBMIB inhibition sites. On the other hand, they found that higher amounts of  $Cu^{+2}$  ( $>3 \mu M$  in *A. cylindrica* and  $>4 \mu M$  in *N. muscorum*) reduced the fluorescence intensities of both DCMU treated and untreated cells, indicating that these  $Cu^{+2}$  concentrations inhibited the electron flow at the donor site of PSII as reported by Shioi *et al.* (1978b) and Hsu and Lee (1988).

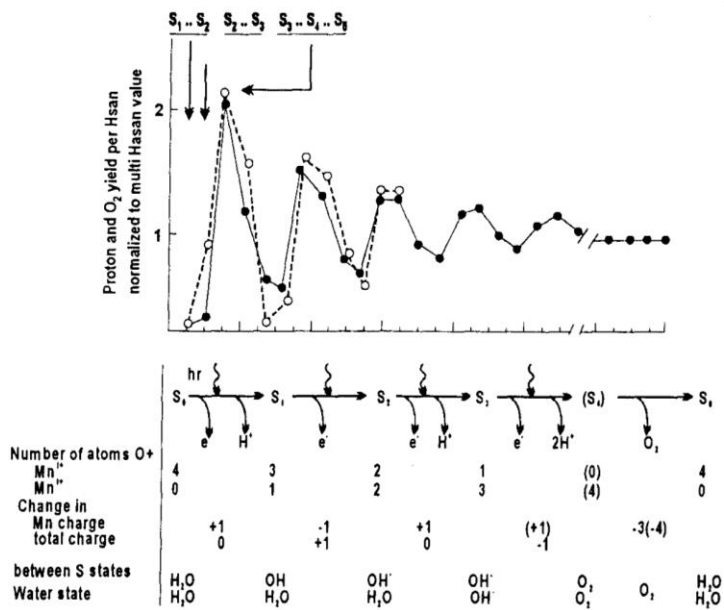


Fig. 3: The (a) Oxygen evolution and proton release by chloroplasts given short ( $2 \mu s$ ) intense flashes of light separated by darkness. The number per flash is expressed relative to the production after many flashes.  $\bullet$  —  $\bullet$   $O_2$  evolution;  $\circ$  —  $\circ$   $H^+$  evolution, (After Kok, 1976). (b) An hypothetical model of the arrangement of manganese and chloride at the water-splitting site and the associated proton and electron movement. (After Critchley and Sargeson, 1984).

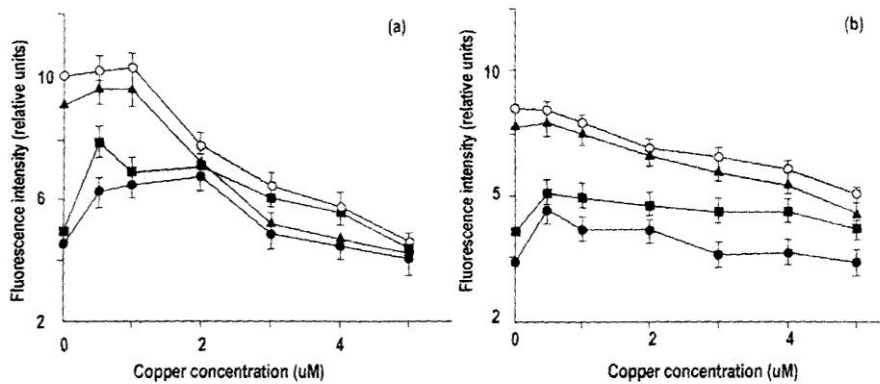


Fig. 4: Fluorescence induction kinetics of *A. cylindrica* (Figure 2 a) and *N. muscorum* (Figure 2 b) at different CuSO<sub>4</sub> concentrations, F<sub>0</sub>-DCMU, ●; F<sub>m</sub>-DCMU, ■; F<sub>0</sub> + DCMU, ▲; F<sub>m</sub> + DCMU, ○. Reaction mixture contained 40 mM phosphate buffer (pH 6.8), 18-22 µg Chl/ml and 5x10<sup>-6</sup> M DCMU.

Table (1): Effect of CuSO<sub>4</sub> concentration, which induces 50% inhibition O<sub>2</sub>-evolution (1.2 µM for *A. cylindrica* and 3 µM for *N. muscorum*) on DBMIB-insensitive O<sub>2</sub>-evolution.

Organism	O <sub>2</sub> -evolution (%): Control	Plus Cu <sup>2+</sup>	Inhibition* (%)
<i>A. cylindrica</i>	100	27 ± 4	73 ± 3
<i>N. muscorum</i>	100	33 ± 3	67 ± 2

\* Inhibition (%) of DBMIB insensitive O<sub>2</sub>-evolution, PSI activity (100%) equaled 20 and 27 µM O<sub>2</sub> mg Ch<sup>-1</sup> h<sup>-1</sup> for *A. cylindrica* and *N. muscorum*, respectively. Experimental conditions as described in Materials and methods. Results given as mean ± standard error (SE). The control is in the presence of 1µM DBMIB and 1 mM DCQ.

Table (2): PSI-activity of *A. cylindrica* and *N. muscorum* membrane (measured as O<sub>2</sub> uptake) in the presence of different Cu<sup>2+</sup> concentrations.

Species	Cu <sup>2+</sup> (µM)	PSI-activity (%)
<i>A. cylindrica</i>	0.0	100.0
	0.4	100.0
	0.8	85 ± 3
	1.0	73 ± 4
<i>N. muscorum</i>	1.4	60 ±
	0.0	100.0
	1.0	95 ± 4
	2.0	80 ± 3
	4.0	58 ± 2
	5.0	50 ± 2

Data given as percentage of control; 100% activity = 21 and 28 µM O<sub>2</sub> mg Ch<sup>-1</sup> h<sup>-1</sup> for *A. cylindrica* and *N. muscorum*. Experimental conditions as described in Materials and methods. Results given as mean ± SE.

## 2. Influence of nickel as heavy metals on green algae.

As a model for this study, El-Naggar (1998) used the unicellular green alga *Chlamydomonas reinhardtii* to investigate the effect of nickel on the photosynthetic electron transport of the organism. She found that the O<sub>2</sub>- evolution capacity of this alga measured as O<sub>2</sub>-evolution both polarographically and under flash light conditions decreased with increasing nickel concentrations (Fig. 5). She observed also that nickel inactivated PSII activity without affecting PSI-mediated electron transport (Fig. 6).

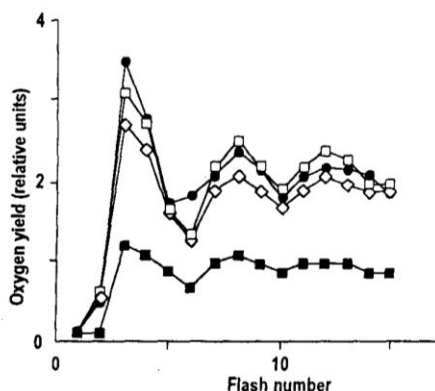


Fig. 5: Effect of different concentrations of NiSO<sub>4</sub> on flash-induced yields of intact cells of *C. reinhardtii* after incubation with Ni<sup>2+</sup>. The cells were suspended in 50 mM phosphate buffer at pH 6.5 and in 50 mM KCl and incubated for 3 min in the dark on the electrode surface prior to taking measurements, ■, Control; □, 1 ppm; ◇, 2 ppm; ●, 3 ppm.

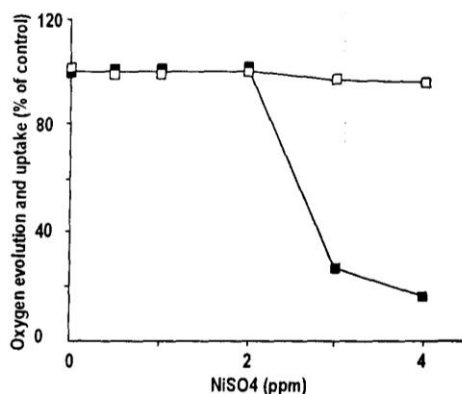


Fig. 6: Effect of various concentrations of NiSO<sub>4</sub> on different partial transport rate reactions in *C. reinhardtii*, oxygen evolution for whole chain electron transport. PSI activity was measured in terms of oxygen uptake from 40 μM DCPIP to 2 mM MV in the presence of 10 μM DCMU and 2 mM sodium ascorbate. The control rate (100%) for the whole chain was 110 μmol O<sub>2</sub> evolved mg Chl<sup>-1</sup>h<sup>-1</sup> and for DCPIP to MV was 200 Mmol O<sub>2</sub> uptake mg Chl<sup>-1</sup>h<sup>-1</sup>. Values are the means of three replicates, ■, O<sub>2</sub>-evolution; □, O<sub>2</sub> uptake.

The fluorescence induction kinetics at room temperature of the Ni-treated cells showed a rise in maximum fluorescence yield in absence and presence of DCMU (Fig. 7<sub>A</sub> and 7<sub>B</sub>), which indicate an accumulation of Q in the reduced state (Q<sup>-</sup>) suggesting an inhibition site of nickel at the reducing side of PSII between Q<sub>A</sub> and Q<sub>B</sub>. The nickel effect in this case is similar to the DCMU type effect (Buttler, 1977 and Renger *et al.*, 1984). Similar results were recorded by El- Sheekh (1993 a) and Singh *et al.* (1989) using other algal species.

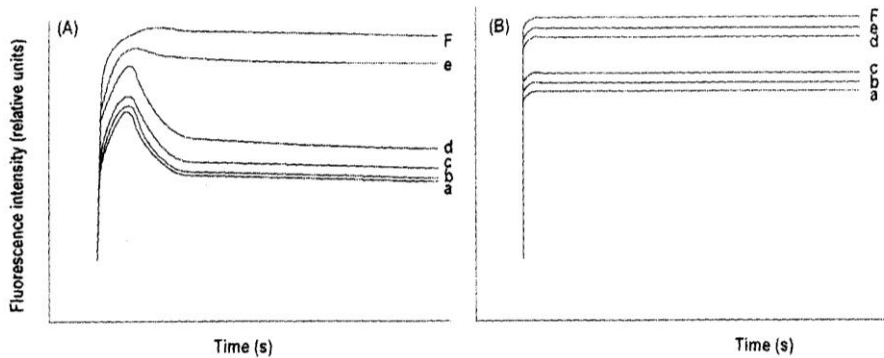


Fig. 7: Fluorescence induction kinetics of untreated and Ni<sup>2+</sup>-treated cells of *C. reinhardtii*, (A) in the absence of DCMU, and (B) in the presence of DCMU, (a) Controle; (b) 0.5 ppm; (c) 1 ppm; (d) 2 ppm; (e) 3 ppm; and (f) 4 ppm.

## B- Use of algae to investigate the role of some anions in the photosynthetic electron transport chain

### 1. The role of bicarbonate

El- Shintinawy *et al.* (1990) demonstrated inhibition of O<sub>2</sub>-evolution in HCO<sub>3</sub><sup>-</sup>-depleted cells of *Chlamydomonas reinhardtii*. They suggested two inhibition sites in the photosynthetic electron transport chain, one after and the other before Q<sub>A</sub>, where Q<sub>A</sub> is the bound plastoquinone electron acceptor of PSII. The long term depletion process slows the rate of oxidation of Q<sub>A</sub>, measured by variable chlorophyll a fluorescence after an actinic flash. Concomitant with 2 fold increase in amplitude of the slow component, the amplitude of the fast component decreases at both pH 6.5 and 7.5 (Fig. 8<sub>A</sub> and 8<sub>B</sub>).

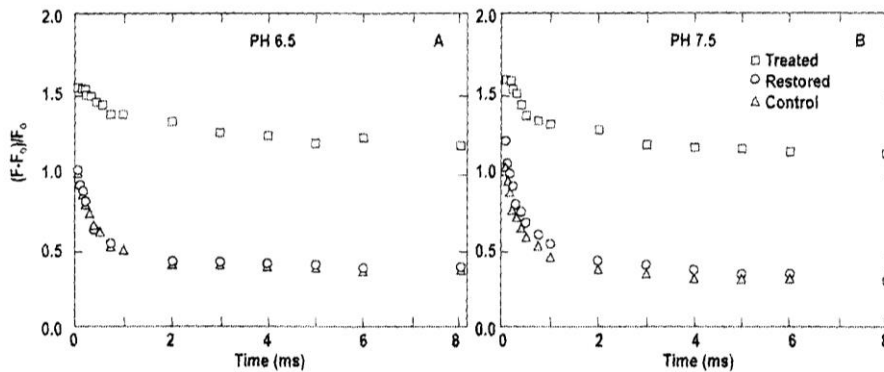


Fig. 8: The effect of long-term formate treatment on the decay of Chl a fluorescence yield after the third actinic flash in control (Δ), formate treated (◻, 25 mM, 3 h) and bicarbonate-restored (◊, 2.5 mM) *Chlamydomonas* cells at pH 6.5 (A) and at pH 7.5 (B). F = fluorescence yield at time t after the actinic flash; F<sub>0</sub> = fluorescence yield before the actinic flash, i.e., when (Q<sub>A</sub>) is maximum.

Addition of 2.5 mM  $\text{HCO}_3^-$  to the depleted cells fully reverses this effect. The anion effects on the electron flow from  $Q_A^-$  to  $Q_B$  (where  $Q_B$  is the second plastoquinone acceptor) were accompanied by effects on Hill reaction. A 4-fold stimulation was observed upon addition of 20 mM  $\text{HCO}_3^-$  to  $\text{HCO}_3^-$ -depleted *Chlamydomonas* cells. Since this stimulation was observed in the presence of 2,5-dibromo-6-isopropyl-p-benzoquinone, which inhibits intersystem electron flow, it is unrelated to  $\text{CO}_2$  fixation in intact system. Similar results were obtained by using spinach chloroplasts as experimental materials (El-Shintinawy and Govindjee, 1990).

## 2. Nitrate effect on photosynthetic electron transport of *Chlorella vulgaris*

The enhanced  $\text{O}_2$ -evolution of irradiated *Chlorella* cells by addition of  $\text{NO}_3^-$  was first observed by Warburg and Negelein (1920). This enhancement was attributed to an accelerated  $\text{NO}_3^-$ -reduction (Losada and Guerrero, 1979). Using thylakoid preparations, which under the chosen experimental condition could not reduce nitrate any more, the involvement of  $\text{NO}_3^-$  ions at the donor side of PSII was demonstrated (Osman *et al.* 1982). This conclusion disagrees with the interpretation of Warburg *et al.* (1965), who considered nitrate as possible electron acceptor of Hill reaction. In 1999, Osman and El-Naggar reinvestigated the nitrate effect in  $\text{NO}_3^-$ -depleted *Chlorella vulgaris* as intact

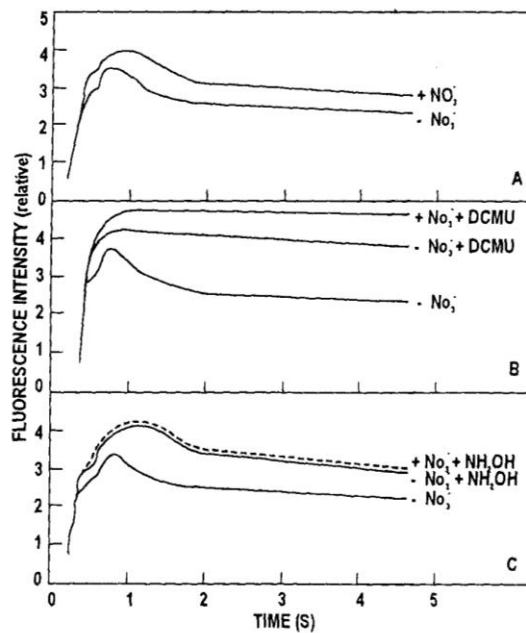


Fig. 9. Fluorescence induction kinetics of  $\text{NO}_3^-$  depleted *Chlorella vulgaris* cells: (A) Before and after addition of 10 mM  $\text{KNO}_3$ , (B) Nitrate-depleted cells,  $\text{NO}_3^-$  depleted + 10  $\mu\text{M}$  DCMU, and  $\text{NO}_3^-$  depleted + 10  $\mu\text{M}$  DCMU + 10 mM  $\text{KNO}_3$ , (C)  $\text{NO}_3^-$  depleted cells.  $\text{NO}_3^-$  depleted cells + 25 mM  $\text{NH}_2\text{OH}$  added before the dark adaptation period and  $\text{NO}_3^-$  depleted cells + 25 mM  $\text{NH}_2\text{OH}$  + 10 mM  $\text{KNO}_3$ . Experimental conditions as described in Materials and methods.



system to reevaluate the NO<sub>3</sub><sup>-</sup> effect on the photosynthetic electron transport. On the basis of the results obtained from the fluorescence induction studies, the low temperature fluorescence emission spectra and O<sub>2</sub> - evolution under flash light condition (Fig. 9, 10, and 11), they concluded that nitrate accelerates the light induced charge transfer from the intact O<sub>2</sub> - evolving system to the primary electron acceptor of PSII and stimulates the PSI-mediated electron transport.

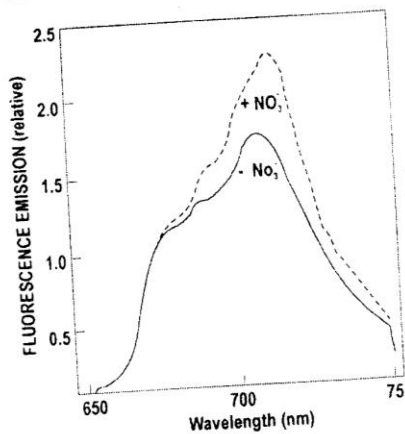


Fig. 10: Fluorescence emission spectra of NO<sub>3</sub><sup>-</sup> depleted *Chlorella vulgaris* cells (—) and of these cells after addition of 10 mM KNO<sub>3</sub> (---) at liquid nitrogen temperature. Excited by broad blue band at 470 nm. Experimental conditions as described in Materials and methods.

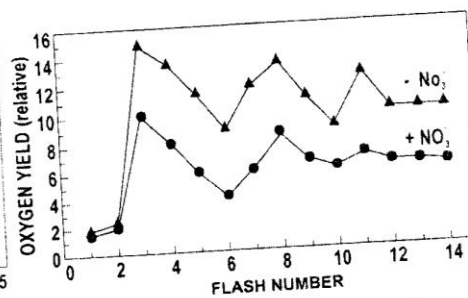


Fig. 11: Effect of NO<sub>3</sub><sup>-</sup> (10 mM KNO<sub>3</sub>) on the flash oxygen yields of NO<sub>3</sub><sup>-</sup> depleted *Chlorella vulgaris* cells. The cells were suspended in 50 mM phosphate buffer, pH 6.5 and incubated for 3 min in the dark on the electrode surface prior to measurements.

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