The Inhibition of Photosynthetic Electron Transport by DBMIB and its Restoration by *p*-Phenylenediamines; Studied by Means of Prompt and Delayed Chlorophyll Fluorescence of Green Algae

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Dedicated to Prof. U. F. Franck *on the Occasion of his 60th Birthday*

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Photosynthetic Electron Transport, Variable Chlorophyll Fluorescence, *p*-Phenylenediamines, Dibromothymoquinone

The effect of the plastohydroquinone antagonist dibromothymoquinone (DBMIB) on photosynthetic electron transport reactions was studied in the presence and absence of *p*-phenylenediamines by means of measurements of prompt and delayed chlorophyll fluorescence induction of the green alga *Scenedesmus obliquus*.

Prompt and delayed chlorophyll fluorescence induction phenomena are valid indicators for the native presence of and cooperation between the two photosynthetic light reactions. Their kinetics reflect the balancing of electron exchange reactions in the chain of coupled redox-systems between the two photosystems upon sudden illumination.

From distinct alterations of the short-term (sec) light induced changes in the yield of prompt and delayed chlorophyll fluorescence it is concluded that DBMIB inhibits the photosynthetic electron transport in the chain of redox-systems between the two light reactions. There is evidence to show that upon illumination of DBMIB treated cells only the reduction of primary electron acceptor pools of photosystem II (*i. e.* Q and PQ) is still possible. After their reduction the further electron transport through photosystem II is blocked.

The addition of *p*-phenylenediamines to DBMIB-treated cells abolishes the typical DBMIB-affected prompt and delayed fluorescence inhibition curves and the normal induction curves reappear qualitatively in all their important features. From these measurements it is suggested that the redox properties of *p*-phenylenediamines allow an electron transport bypass of the DBMIB inhibition site which results in a fully restored photosynthetic electron transport from water to NADP.

Introduction

In the last few years our knowledge about the topography of the redox-systems in the functional membrane of photosynthesis has much increased.

It seems now amply documented that the photosynthetic electron transport in the thylakoid-membrane has vectorial properties which leads to the generation of an electrochemical potential difference across this membrane [1].

The membrane potential is built up during illumination of the photosynthetic apparatus by four protolytic reactions: Two proton-uptake reactions at the outerside of the membrane (that is the plastoquinone and NADP reduction) and two proton releasing reactions in the inner phase of the membrane (that is the water-splitting reaction and the plastohydroquinone oxidation) (Fig. 1).

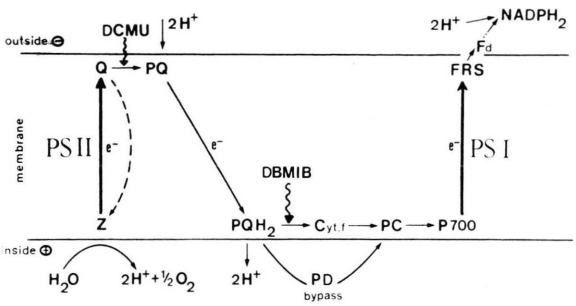

Fig. 1. Scheme of the vectorial electron transport across the thylakoid membrane (after Witt *et al.* [2]) with the most probable inhibition site of DBMIB and a possible bypass of the inhibition site *via* *p*-phenylenediamines (after Trebst *et al.* [3]).

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine; PD, 1,4-phenylenediamine-dihydrochlorid; PS I, PS II, photosystem I resp. II; PQ, Plastoquinone; Q, quencher of fluorescence (primary acceptor of PS II).

According to a generally accepted model the millisecond delayed chlorophyll fluorescence emission depends upon this functional membrane and occurs in the dark after switching off the light upon the reverse reaction of Q^- and Z^+ the primary photoproducts of the reaction centers of PS II [4]. It is reasonable to suggest that the activation energy necessary for the reverse flow of electrons from Q^- (over Chl a_{II}) to Z^+ is partially delivered from the membrane potential [5]. The PS II-driven delayed fluorescence emission is specifically influenced by the interaction of the primary PS II electron acceptor Q with the redox chain leading to PS I and therefore reflects the native presence of and the cooperation between the two photosystems, that means the delayed fluorescence emission indicates how PS I and PS II reactions are balanced via the electron transport chain between the two light reactions [6].

Prompt chlorophyll fluorescence changes during the first few seconds of beginning illumination (called after its discoverer Kautsky-effect) (Fig. 2) are brought about by energy acceptors in the immediate neighbourhood of the chlorophylls in the reaction centers of PS II [7].

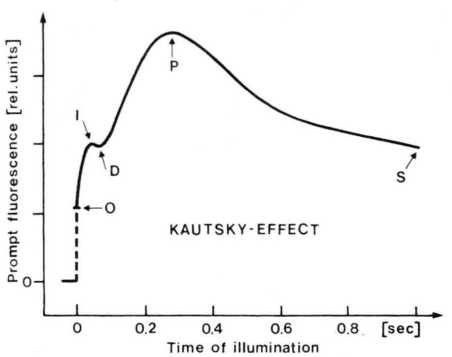

Fig. 2. Typical chlorophyll fluorescence induction curve of *Scenedesmus obliquus* at the onset of illumination. Temperature: 28 °C, Light intensity: $8 \cdot 10^4$ erg·cm^{-2}·sec^{-1}. Notations: O = initial fluorescence measured after the shutter opening time of 0.5 msec, I = level of the initial peak, D = dip, P = peak, S = almost steady fluorescence level. The different transients of the induction curve are characteristic for all photosynthesizing green plants.

It is generally accepted that photochemical PS II-reactions compete with fluorescence for quanta: When energy acceptors are receptible quanta are converted in electron transport reactions and are unavailable for fluorescence, when they are not receptible (*i. e.* if they are reduced or separated from the reaction centers) fluorescence is high, because the absorbed light energy must now be dissipated by the reaction centers.

According to the classical concept of Duysens and Sweers [8] the redox-state of the primary electron acceptor pool of PS II – Q – is mainly responsible for the temporal changes of fluorescence intensity during light-driven electron transport from water to NADP.

The fluorescence is quenched by Q only in its oxidized state. If Q is reduced to a certain extent by endogenous or exogenous electron donors, equal amounts of reaction centers in PS II are closed and fluorescence increases because the absorbed light energy can not be utilized chemically by reaction centers containing electron saturated Q-pools.

Because Q is reduced by PS II and oxidized by defectelectrons (holes) produced in the light by the electron consuming light-reaction I, the slope of the prompt fluorescence induction curve indicates at any point of time which of the both light-reactions is the more effective one.

A temporal increase of fluorescence therefore corresponds to a predomination of PS II and a decrease to a predomination of PS I activity.

Extrema and horizontal sections of the fluorescence curve are accordingly the result of a kinetic balance of both light reactions.

Without to discuss further details (for reviews see refs. [9–11]) it can be accepted, that prompt and delayed chlorophyll fluorescence yield changes reflects the kinetic cooperation of both light reactions during their initial balancing and therefore can be utilized as a valid monitor of the native primary photochemistry in the photosynthetic apparatus of green plants and algae.

Disposition

By means of measurements of prompt and delayed fluorescence we try to discuss a hypothesis of Trebst *et al.* [3] concerning the inhibition of the photosynthetic electron transport by DBMIB and its restoration after the addition of *p*-phenylenediamines.

Trebst *et al.* postulate that DBMIB acts in isolated chloroplasts (already at low concentrations) as a potent competitive antagonist of plastohydroquinone and interrupts in this capacity the photosynthetic electron transport at the reducing site of

plastoquinone [12]. They give evidence that in DBMIB-treated spinach chloroplasts only the PS II-mediated reduction of the primary and secondary electron acceptor pools, that is Q and plastoquinone should be possible, whereas their reoxidation by PS I-produced defectelectrons should be inhibited.

However the inhibition of the electron transport by DBMIB could be restored by the addition of artificial redox-systems which are able to mediate an electron transport bypass of the inhibition site.

So for example the lipophylic *p*-phenylenediamine (PD) and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) can enter the membrane and drain off in their oxidized form electrons from reduced plastoquinone before the inhibition site of DBMIB. After their reduction by plastohydroquinone the reduced phenylenediamines can serve as electron donors and feed back electrons in the electron transport chain close before PS I (see Fig. 1).

This bypass of the DBMIB-inhibition site by *p*-phenylenediamines restores the electron flow between water and NADP and oxygen evolution returns anew [3].

We tried to check up whether the restored electron flow reactions in such treated cells could be verified by means of prompt and delayed fluorescence of green algae.

Materials and Methods

The prompt fluorescence measuring apparatus was in principle the same as described previously [13]. Blue exciting light beyond 460 nm was separated from the emission spectrum of a xenon lamp (150 W) by means of broadband filters (BG 28 and BG 38, Schott, Mainz, Germany); the intensity was $8 \cdot 10^4$ erg·cm^{-2}·sec^{-1}. The photomultiplier (S-20 characteristic) was provided with a Schott filter RG 695 to separate the red fluorescence light from the blue exciting light.

The millisecond delayed fluorescence was measured as an induction curve by means of a modified Becquerel-type phosphoroscope. The time-course curves were obtained in the following way: Two four-fold segmented chopper-discs provided alternating light pulses of 6.5 msec and dark periods of 1 msec (Fig. 3).

During the dark periods the amount of delayed fluorescence was monitored always at 0.5 msec of each decay-curve. The integration of the single measuring points of the decay-curves leading to the resulting time-course curve measured during the first few seconds of illumination is shown schematically in Fig. 4.

The source of excitation was a 7 mW HeNe-laser ($\lambda = 632.8$ nm). Its intensity at the sample surface during measurements was $2 \cdot 10^5$ erg·cm^{-2}·sec^{-1}. The electronic equipment for measuring the delayed fluorescence signal was in principle the same as for prompt fluorescence measurements [6, 13].

All experiments were carried out with the unicellular green alga *Scenedesmus obliquus*, strain Gaffron D_3.

The algae were grown homocontinuously in aerated (3% CO_2 in air) liquid cultures (inorganic medium as described by Kessler *et al.* [14]) in a light

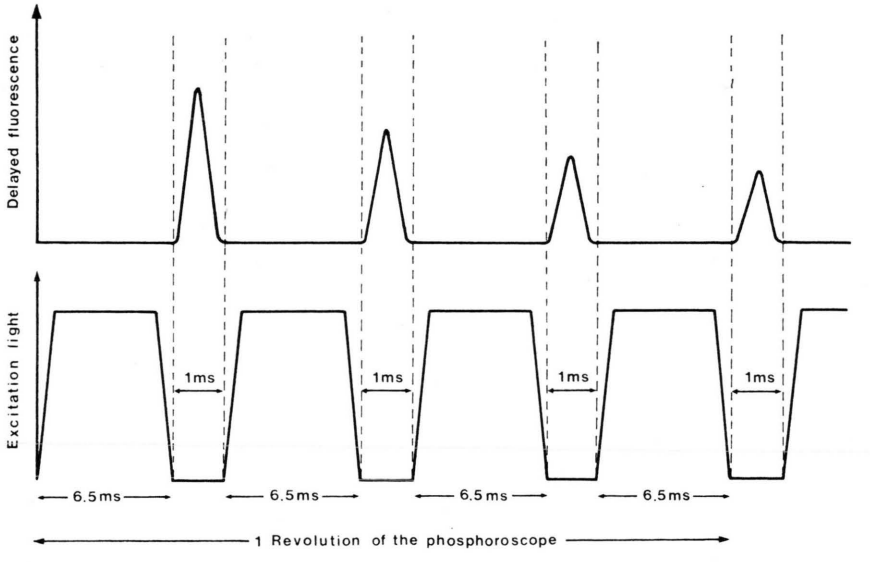

Fig. 3.
Pulse program for measuring delayed chlorophyll fluorescence emission.

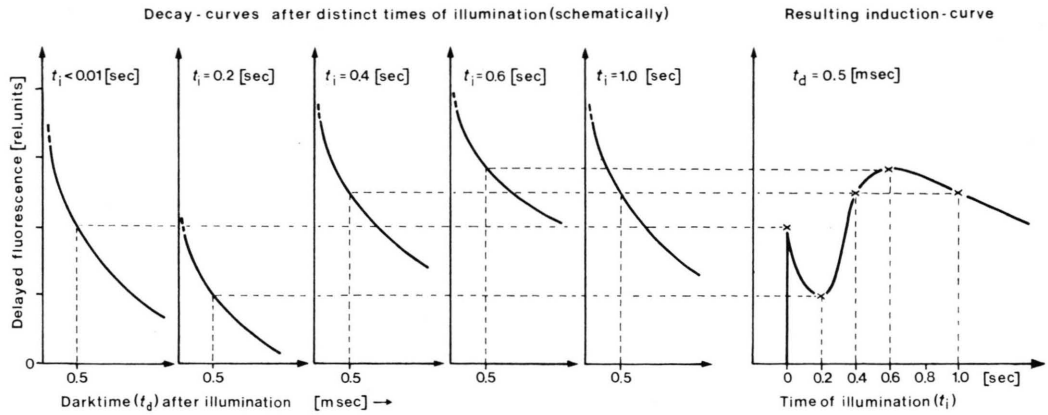

Fig. 4. Schematical illustration which shows how a time-course curve originates from single points of delayed fluorescence decay-curves.

thermostate (at 20,000 lx) with an automatic dilution apparatus (Senger *et al.* [15]) at 30 °C.

The *Scenedesmus mutant No. 8* (with a block in PS I) was grown photoheterotrophically according to Bishop [16].

DCMU and DBMIB (oxidized form) were dissolved in pure ethanol and prepared at appropriate concentrations such that the final concentration of ethanol in the algae suspensions never exceeded 1%. PD was added in the dark in the reduced form (1,4-phenylenediamine-dihydrochlorid). The dark incubation time before illumination was always 10 min. During this time PD obviously is oxidized to *p*-phenylenediimine after penetrating the algae. The endogenous oxidation in the cells occurs either by oxidizing redox-systems or possibly by enzymes (phenol-oxidase?). TMPD also was added in the dark in the reduced form. But in contrast to PD it forms upon oxidation a quite stable cation-radical.

Results and Discussion

Fig. 5 shows the alteration of the normal prompt fluorescence induction curve of *Scenedesmus obliquus* with increasing amounts of added DBMIB. It is obvious, that with increasing concentrations of DBMIB the PS I-caused fluorescence declines occuring in untreated algae [13, 17], that is the ID-decline and the PS-decline (notations see Fig. 2) are progressively suppressed.

At a concentration of $5 \cdot 10^{-5}$ M which nearly completely abolishes photosynthetic oxygen evolution in *Scenedesmus* cells only a slow increase of fluorescence occurs to a constant high stationary level.

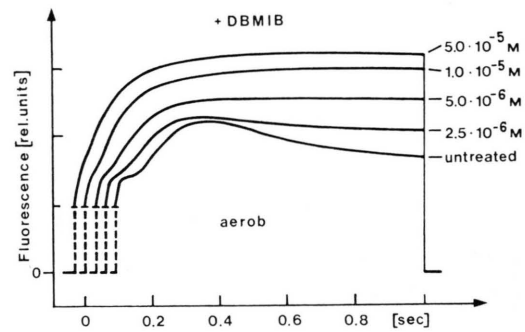

Fig. 5. Effect of different concentrations of DBMIB on the fluorescence transient of *Scenedesmus obliquus*. Temperature 25 °C. *Note!* The initial fluorescence level 0 (marked by little crosses) is not affected by DBMIB.

The slow fluorescence increase indicates that in the presence of DBMIB a large pool of native primary PS II electron acceptors is still reducible at the onset of illumination.

The constant high stationary fluorescence level and the fact that O_2-evolution is nearly totally diminished under these conditions indicates that the electron acceptors are kept reduced during further illumination. An accumulation of reduced electron acceptors results in the closure of PS II reaction centers which causes the high stationary fluorescence yield. If DBMIB, which was added in the oxidized form to the algae cells (see Materials and Methods) would act as an electron acceptor itself as was found for isolated spinach chloroplasts by Lozier and Butler [18] and Gould and Izawa [19], we should expect a quenching of the chlorophyll fluorescence. However the fact that this did not occur indicates that either a. DBMIB acts in *Scenedesmus* cells in

the reduced as well as in the oxidized state as an electron transport inhibitior or b. DBMIB inhibits only in the reduced form and if added to *Scenedesmus* cells in the oxidized form there is enough dark reduction power inside the cells to convert all oxidized DBMIB into the only inhibiting reduced form.

In our opinion the last possibility is the most probable one. To characterize more precisely the inhibition site of DBMIB we added DCMU to previously DBMIB-inhibited cells.

From DCMU it is known that it interrupts the electron transport on the reducing side of PS II between the primary electron acceptor Q and the secondary electron acceptor pool which obviously is plastoquinone (see Fig. 1).

Upon illumination of DCMU-treated, dark adapted algae cells, only the PS II-driven reduction of the primary acceptor pool Q is possible, whereas the normally occuring rapid reoxidation of Q^- by the secondary electron acceptor pool of plastoquinone is prevented by this inhibitor [8].

Because DCMU blocks electron flow at a site closer to PS II than DBMIB we should expect a faster fluorescence rise to the same high stationary level as by DBMIB treated algae. Fig. 6 shows the effect of DCMU when added to *Scenedesmus* cells previously inhibited by DBMIB. A fast fluorescence rise occurs which follows the progressive reduction of Q in the light. The same DCMU rise-curve was obtained when DCMU was added alone or when added before the addition of DBMIB. This indicates that the two inhibitors act very specifically and that there is no overlapping of their inhibition regions.

Assuming that in the presence of DCMU only the Q pool and in the presence of DBMIB only the Q *plus* plastoquinone pools are reducible, the *active* size of the two acceptor pools can be calculated from the area above the inhibition curves. As shown previously by Murata *et al.* [20] and Malkin and Kok [21] the area above fluorescence inhibition curves bounded between the ordinate and the parallel to the abscissa on the level of the high stationary fluorescence is proportional to the number of electrons which can be fed by PS II reactions into available acceptor pools.

Estimates of this type are allowed if light induced fluorescence changes are *exclusively* mediated by PS II reactions and if they occur *strictly antiparallel* to the effectiveness of photochemical electron transport through PS II reaction centers. On these premises the area above the DCMU-inhibition curve (dotted area in Fig. 6) is a measure for the electron capacity of the Q-pool and the area bounded between the DBMIB-inhibition curve and the DCMU-inhibition curve (shadded area in Fig. 6) is a measure of the electron capacity of secondary electron acceptors present between the DCMU and the DBMIB inhibition site.

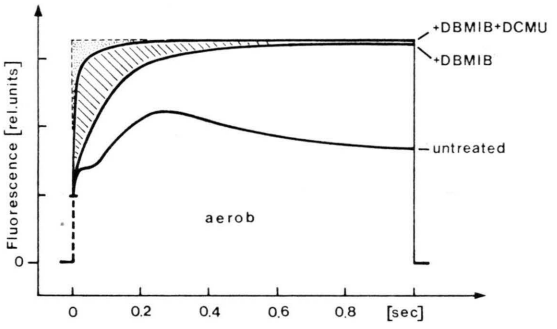

Fig. 6. Effect of DBMIB ($5 \cdot 10^{-5}$ M) and DBMIB *plus* DCMU ($5 \cdot 10^{-6}$ M) on the fluorescence transient of *Scenedesmus obliquus*. Temperature 25 °C.

Under our experimental conditions (see legend of Fig. 6 and Materials and Methods), the ratio of the two characterized areas and thereby the electron capacity of the Q-pool and the pool between the DCMU and DBMIB inhibition site was estimated to be 1 : 6.

Assuming that Q is an one-electron acceptor [22] present in a concentration approximately equal to the number of PS II reaction centers [7], and the fact that plastoquinone functions as a two-electron acceptor [23] the ratio of the *active reducible* Q to plastoquinone molecules can than be calculated to be 1 : 3.

This value is about one-half of the *total* pool-size of plastoquinone determinated for spinach chloroplasts and whole *Chlorella* cells by Schmidt-Mende *et al.* [24]. They measured directly the absorption changes of plastoquinone at 254 nm and estimated under specific experimental conditions a *total* pool size of about 10 – 14 electron equivalents *i. e.* 5 – 7 molecules of plastoquinone per electron transport chain.

But they observed too, that under normal conditions only 50% of the light-reduced plastoquinone molecules are reoxidized in the following dark-period [25].

If we assume that this is valid also under our experimental conditions for the plastoquinone reoxidation of *Scenedesmus* cells, we have to double our value because by means of fluorescence measurements we can detect only that part of plastoquinone *reducible* at the onset of illumination. So we can calculate the total pool of plastoquinone to be 6 molecules (12 electron equivalents) per reaction center of PS II. This estimate is in agreement with the pool sizes measured by Schmidt-Mende *et al.* [24, 25] and *other groups* [26, 27].

The result furthermore gives evidence concerning the inhibition site of DBMIB because it predicts that DBMIB blocks indeed behind the *total* pool of plastoquinone molecules of each electron transport chain.

In order to get further certainty in this assumption we investigated the influence of DBMIB on the fluorescence behaviour of mutant No. 8 from *Scenedesmus obliquus*.

From this mutant it is known that its Hill-reaction – including the plastoquinone reduction – is still intact.

However PS I reactions which could lead to an oxidation of reduced plastoquinone are defect [16].

Hence the fluorescence behaviour of this mutant is very simple. At the onset of illumination only a slow fluorescence increase occurs. As shown previously [28] this slow fluorescence increase reflects only the *reduction* of the primary electron acceptor pools Q and plastoquinone by PS II. This is supported by the finding [29] that the reduction of the primary electron acceptors is accompagnied by a short-term oxygen outburst of the water-splitting reaction. DBMIB has no effect on the slow fluorescence increase, whereas DCMU causes a fast increase of fluorescence which is typical for the reduction of Q only (Fig. 7). After these findings which favoured our assumption that DBMIB inhibits the electron transport behind the total pool of plastoquinone we added phenylenediamines to DBMIB-inhibited wildtype-cells of *Scenedesmus*.

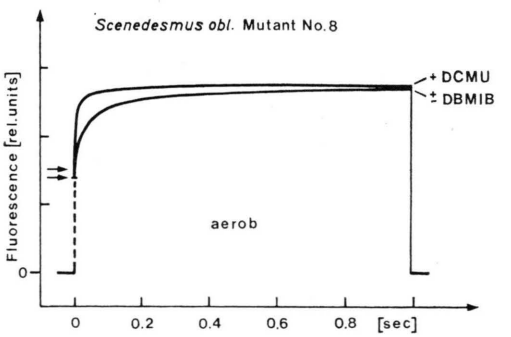

Fig. 7. Effect of DCMU ($5 \cdot 10^{-6}$ M) and DBMIB ($5 \cdot 10^{-5}$ M) on the fluorescence rise of mutant No. 8 of *Scenedesmus obliquus*. Temperature 25 °C.

Fig. 8 clearly demonstrates that after the addition of $5 \cdot 10^{-4}$ M *p*-phenylenediamine the normal fluorescence induction curve is restored *qualitatively* in all its important features. (TMPD-addition

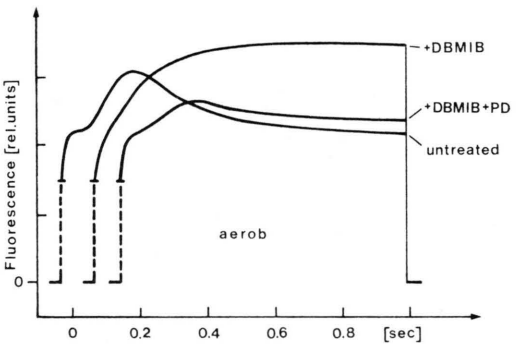

Fig. 8. The restoration of the Kautsky-effekt after the addition of PD ($5 \cdot 10^{-4}$ M) to DBMIB inhibited ($5 \cdot 10^{-5}$ M) *Scenedesmus* cells. Temperature 25 °C.

– not shown here – leads to similar results!) This indicates the restoration of primary electron transport reactions between water and NADP and amply documents the validity of Trebst's conception concerning the DBMIB-inhibition of photosynthetic electron transport reactions and its restoration by *p*-phenylenediamines.

Delayed fluorescence measurements lead to the same results. Fig. 9 shows in the upper part on the left the normal delayed fluorescence time-course curve of *Scenedesmus obliquus* at 25 °C and on the right after the addition of DBMIB. It can be seen that after the addition of DBMIB the normal induction curve is drastically altered.

Only the rapid initial spike of the normal delayed fluorescence induction curve remains at the beginning of illumination followed by a decline to a steady state level.

The initial spike obviously reflects a short-term and DBMIB unaffected luminescent back-reaction between reduced primary electron acceptors (*i. e.* Q and PQ) and oxidized primary electron donors of PS II in the dark periods of the beginning intermittent illumination.

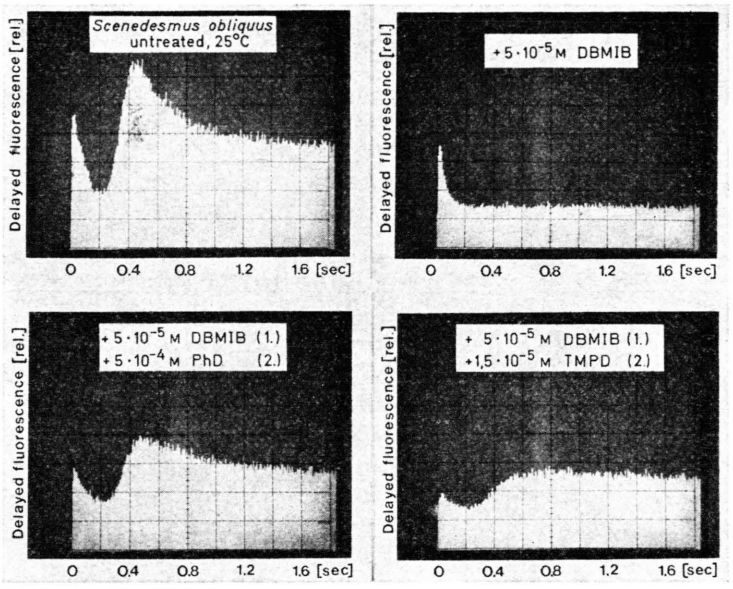

Fig. 9. Delayed fluorescence time-course curves of *Scenedesmus obliquus* after various treatments. Temperature 25 °C, HeNe-laser intensity: $2 \cdot 10^5$ erg·cm^{-2}·sec^{-1}.

Further variable induction features which normally still occur – so for instance the slow delayed fluorescence rise to the maximum – are suppressed because they are caused by the interaction of primary PS II electron acceptors with the redox-chain leading to PS I [6] – a reaction which now is inhibited by DBMIB.

The steady state delayed fluorescence emission following the initial spike of the DBMIB-curve reflects a constant rate of charge separation and charge recombination in the reaction centers of PS II mediated by the light-dark pulses of the rotating sector phosphoroscope.

This continious "*cycling*" of electrons around the reaction centers of PS II is possible because electrons are accumulated in front of the DBMIB-inhibition site.

In the lower part of Fig. 9 on the left and on the right it is shown what happens if *p*-phenylenediamine or TMPD are added in appropriate concentrations to DBMIB-inhibited samples. The normal time-course curve is restored *in all its important features*. This indicates anew that *p*-phenylenediamines can mediate an electron transport – bypass of the DBMIB-inhibition site and therefore can restore a functional electron transport between the two photosystems.

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