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CHLOROPHYLL FLUORESCENCE INDUCTION IN ANAEROBIC *SCENEDESMUS OBLIQUUS*

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SUMMARY

Fluorescence time curves (Kautsky effect) were studied in anaerobic *Scenedesmus obliquus*, with an apparatus capable of simultaneous recording of O_2 exchange, and far-red actinic illumination. Results, as interpreted in terms of electron transport reactions, suggest: In the course of becoming anaerobic, fluorescence induction undergoes a series of changes, indicating at least three different effects of the absence of O_2 on electron transport. (1) Immediately on removal of O_2, once the pool of intermediates between the two photo-systems is reduced by light reaction II, electron flow stops, resulting in high fluorescence yield and a cessation of O_2 evolution. O_2 appears to regulate linear electron flow and cyclic feedback of electrons to the intermediate pool. (2) An endogenous reductant formed anaerobically reduces the System II acceptors in the dark. The time course of this reduction is at least biphasic, indicative of inhomogeneity of the primary acceptor pool. Prolonged dark anaerobic treatment induces maximal initial fluorescence which decays rapidly in light and with a System I action spectrum. (3) Anaerobic treatment eventually results in deactivation of the oxidizing side of System II, limiting System II even when the acceptors are oxidized by System I pre-illumination.

INTRODUCTION

Much present knowledge of photosynthesis has been achieved by the use of chlorophyll fluorescence as a sensitive indicator of energy conversion at System II (for a review, cf. ref. 1). First kinetic evidence for the cooperation of two independent photoreactions in photosynthesis was presented by Franck [2] and Kautsky and Franck [3] in the early 1940s on the basis of the kinetic analysis of a fluorescence transient best observed under anaerobic conditions and now referred to as the "I-D dip" [4]. Many different types of investigations have led to the conclusion that there are two different photoreactions sensitized by two different pigment systems, but, as is recently pointed out [1] there are still uncertainties concerning the significance

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate.

of the I-D transient (for notations see Fig. 2). Our earlier data show that I-D reflects System I action [5–8], although how System I activity leads to fluorescence quenching at System II reaction centers remained uncertain. As an I-D decay persisted with conditions under which electron transport should be blocked, we suggested a direct interaction between the two pigment systems. Experiments on anaerobic fluorescence induction reported here provide more evidence that the I-D decay is sensitized by System I, but also suggest that the transient, at least in part, does reflect reoxidation of System II acceptors via the electron transport chain.

Whether or not molecular O_2 is required for photosynthesis has long been vigorously disputed. (An early review of Rabinowitch deals with this problem [9, 10].) Some investigators, among them Kautsky and Warburg, demonstrated extreme inhibition of photosynthesis when O_2 was missing, whereas other investigators, among them Gaffron and J. Franck, reported that photosynthesis can very well proceed without O_2. Some of the past arguments are now outdated because of a general increase in knowledge about photosynthesis. The last comprehensive review about anaerobic effects on photosynthesis is that of Kessler [11],written before the existence of two light reactions was generally accepted. At that time a strong argument against any O_2 requirement for photosynthesis was that H_2-adapted algae and purple bacteria carry out photoreduction best without O_2. From our present point of view, this fact argues for the most efficient functioning of System I with low O_2 tensions.

While the data presented here indicate that System I indeed functions optimally anaerobically, there is at the same time a deactivation of System II. The extent to which it is inhibited is comparable to the effect of mild heat treatment [6]. But contrary to heat treatment, anaerobic treatment does not irreversibly damage the algae. Recovery occurs with the addition of O_2, and even traces of O_2 lead to possibly autocatalytic reactivation of photosynthesis. What is usually referred to as "anaerobic conditions" in the literature appears to us as only a first step preceding an anaerobic adaptation process. This adaptation involves profound metabolic changes within the cells, possibly including the induction of hydrogenase activity [12]. To achieve the adapted state, increased temperatures and longer incubation times are as important as the lowering of the O_2 partial pressure. In a recent paper Diner and Mauzerall [13] presented a model for feed-back control by O_2 and an endogenous reductant over electron transport and O_2 evolution. We believe that the reduction of intermediates observed by these authors is light-induced and much too fast ($t_{\frac{1}{2}} =$ 160 ms) to reflect a dark reduction of System II acceptors during anaerobic treatment. A scheme is proposed below which is in agreement with both their data and ours.

The mechanism of fluorescence quenching has been shown to be far more complex [14] than in the original schemes of Kautsky and Franck [3], Kautsky et al. [15] and Duysens and Sweers [16]. Evidence presented here confirms that part of the complexity arises from inhomogeneity of the System II acceptors. Despite this complexity, for the sake of convenience, a complementary relationship between energy conversion and fluorescence yield as a constant portion of all non-photochemical losses is assumed.

MATERIALS AND METHODS

Scenedesmus obliquus, strain D3 (Gaffron) from the Alga Collection of Indiana State University, was grown on a mineral medium at 25 °C in continuous light of

30 mJ · cm^{-2} · s^{-1}, provided by a system of cool-white fluorescent lamps. Algae were harvested approx. 4 days after inoculation.

The measuring apparatus was designed for the detection of fluorescence yield at actinic wavelengths overlapping the fluorescence emission. In order to achieve a high intensity of narrow-band monochromatic light the measuring chamber used was shaped as a narrow cuvette, on opposite sides of which the slit images from two monochromators (M1 and M2) (Jarrel Ash, Model 82-410) were focussed. Light from M1 normally was the fluorescence excitation beam and could be modulated by a chopper (Princeton Applied Research Corp.). Light sources were a microscope illuminator for M1 and a projector lamp (Sylvania, Type DLG) for M2. Maximum actinic light intensity from M2 was 50 mJ · cm^{-2} · s^{-1} at 680 nm, half bandwidth 2 nm. Higher intensities were obtained with larger entrance and exit slits of M2. Light intensity was measured with a PIN-Photodiode (Hewlett Packard, 5082-4204) calibrated against a YSI-Kettering Radiometer (Model 65). Fluorescence was collected at 90° to the light beams by a fiber light guide (Crofon, Du Pont) with a front surface comparable to the monochromator slits and leading to an EMI 9558B phototube behind a 4 mm far-red cut-off filter (Corning, CS 7-69). With blue actinic light, fluorescence transients at the 685 nm peak proved identical to those at $\lambda > 715$ nm. Action spectra (see Figs 6–7) were determined, using a low intensity (0.2 μJ · sec^{-1} · cm^{-2}) modulated measuring beam. To avoid photomultiplier artifacts with the onset of the much stronger actinic beam, stray light was minimized by additional filtering of the actinic light through interference filters (Balzers B-40) and a Didymium filter with a strong absorption at 750 nm (Corning, CS 1-60) in combination with a 750 nm interference filter (Balzers B-40) protecting the phototube. The phototube signal, developed across a 1 MΩ resistor, was fed into a lock-in amplifier (Princeton Applied Research Corp., Model HR-8 with a type A preampiifier). Rapid fluorescence time courses were recorded with a storage screen oscilloscope (Tektronix, Type RM 564) and slow ones by a pen recorder (Riken-Denshi, Model SP-J2). The cuvette assembly minimizes preillumination of the sample by a preceding experiment, since the volume of the measuring chamber is only about 1/1000 of the dark reservoir. This is particularly important with anaerobic experiments, where preillumination tends to have long-lasting effects. With the stopcock (S) closed, the measuring chamber and the reservoir were flushed with N_2, whereas with S open a bypass for the N_2 flow is created and a fresh batch of algal suspension can enter the chamber by gravity. O_2 was removed from the samples by a stream of water saturated with N_2 plus 4% CO_2 (Liquid Carbonic Corp.), which contained less than 5 ppm O_2. Flow was controlled by a Rotameter (Matheson Co.) and kept constant at 100 ml · min^{-1}.

For some experiments a Haxo-Blinks type bare platinum-electrode polargraph, as described by Vidaver [17], was modified for simultaneous O_2 and fluorescence transients monitoring. Bifurcated fiberoptics were used, directing actinic light through one branch to the sample and leading fluorescence, collected from the sample surface, through the other branch to the phototube. The Pt cathode was polarized −0.65 V versus the Ag/AgCl anode. Electrode current was measured by a High Speed Picoammeter (Keithley, Type 417) and recorded along with fluorescence emission on the storage screen of the dual beam oscilloscope.

RESULTS AND DISCUSSION

The change of fluorescence time curves with anaerobic treatment

Removal of O_2 from *Scenedesmus* samples results in characteristic changes of the fluorescence–time curves. Depending on temperature and the duration of anaerobic dark storage, three basic types of curves appear, which are related to three different effects of anaerobic treatment on photosynthesis [6, 8]. In Fig. 1 these phases are depicted for 25 °C:

(1) Phase 1, reached in 5–10 min, shows no change in the O and I levels, but a large change occurs in the P and S levels (see Fig. 2 for notation).

(2) Phase 2 occurred after 30–40 min and shows both raised O and I levels, but with I more than O; the I-D dip is very pronounced and the P and S levels are only slightly higher than in Phase 1.

(3) Phase 3, which at 25 °C was fully developed only after 7 h, shows a very high O-level, which is no longer distinguishable from the I-level. The initial fluorescence yield is higher than the maximum yield in the presence of DCMU or sodium dithionite [6]. The O-level measured represents the true initial fluorescence, as the shutter rise can be clearly distinguished from the O-I rise. The rate of the O-I rise is a function of light intensity; at low intensities clear resolution of the O-level is facilitated (see legend to Fig. 3). Furthermore the O-level measured is proportional to light intensity. With illumination, the yield decays rapidly to a level near the stationary aerobic level. The D-P-S transient has totally disappeared.

With different anaerobic times or temperatures, intermediate types of curves appear. The time to reach phases 1, 2 and 3 can be correlated to actual O_2 concentrations of 10^{-1}, $3 \cdot 10^{-2}$ and less than 10^{-5} vol.%, respectively [8]. Allowing sufficient time for equilibration and reactivation (approx. 20 min at 25 °C) addition of appropriate amounts of O_2 to a sample in phase 3 results in transformation to phases 2 and 1 and finally to restoration of the normal aerobic transient.

Fig. 2 shows the effect of temperature on the curves after 20 and 60 min anaerobic dark time. While after 20 min at 15 °C the curve is still in phase 1, at 35 °C phase 2 is well developed, and at 25 °C an intermediate curve occurs. After 60 min

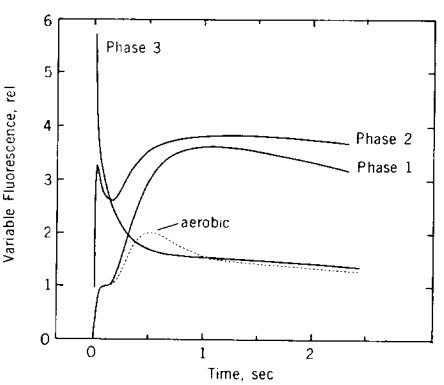

Fig. 1. The three basic types of fluorescence–time curves occurring with various anaerobic dark times. Phase 1 after 10 min, phase 2 after 40 min and phase 3 after 8 h. Temperature, 25 °C; light intensity, 50 mJ · cm^{-2} · s^{-1} at 480 nm, 10 nm half-bandwidth.

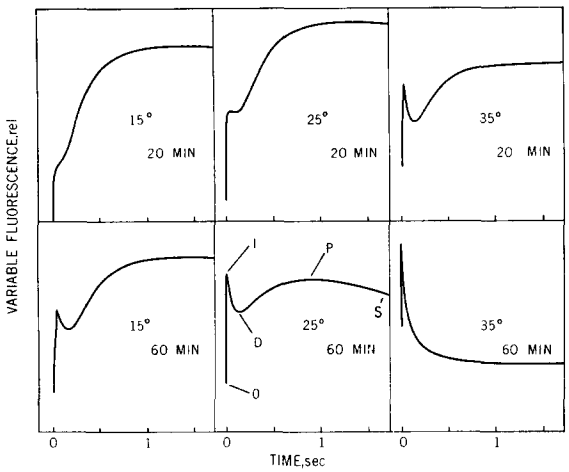

Fig. 2. Effects of temperature and anaerobic dark times on the fluorescence time curves. Conditions as in Fig. 1. The notations of the characteristic fluorescence levels are: O = initial fluorescence yield; I = intermediate level in aerobic curve or first spike in anaerobic curve; D = dip; P = peak; and S = so-called "stationary yield", which can differ from the steady state yield after prolonged illumination.

phase 2 is reached at 15 °C, while at 25 °C progress is made towards phase 3, and at 35 °C phase 3 is nearly attained.

In Fig. 3, the characteristic levels of fluorescence yield at O, I and $S_{2\,s}$ are plotted versus anaerobic dark time at 25 °C. Four different processes, the significance of which will be discussed later (see section below, "The action of an endogenous

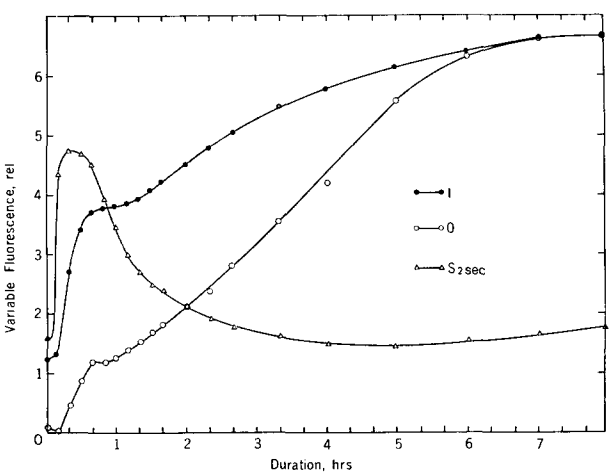

Fig. 3. Change of the characteristic fluorescence levels with anaerobic dark times at 25 °C for a single experiment. Culture conditions may alter these responses to some extent; some experiments indicate a third phase in the O-level rise. $S_{2\,s}$ = fluorescence yield after 2 s illumination. Conditions as in Fig. 1. The O-level was measured, for better resolution, with one-tenth of the light intensity and 10-fold sensitivity of the detecting system used for the other determinations.

reductant"), are reflected in the following changes: (1) A rapid rise in the S_{2s} level ($t_{\frac{1}{2}} = 5$ min); (2) after a delay of approx. 10 min a somewhat slower first rise of the O-level ($t_{\frac{1}{2}} = 10$ min); (3) a decay of the S_{2s} level ($t_{\frac{1}{2}} = 45$ min); (4) after a transitory delay, a second rise in the O-level ($t_{\frac{1}{2}} = 160$ min). The rise in the I-level is biphasic too, the first rise being slightly faster than that of the O-level.

Changes in the characteristic fluorescence levels with switching from air to O_2-free atmosphere were previously reported by Munday and Govindjee [4]. Their finding of an approx. 10–20 min delay in the rise of the I-level agrees with our results. However, these authors using *Chlorella* did not report a rise in O-level. Their anaerobic adaptation does not appear to exceed our phase 2, possibly because the algal strain used lacks a functional hydrogenase system [12].

From the changes of the O-, I- and S_{2s}-levels with anaerobic dark storage, their contributions to the development of the different phases of the fluorescence–time curves become apparent. S_{2s} has reached its maximum value after 10 min, while O and I are still low (phase 1); O and I complete their first rise, while S_{2s} is still high (phase 2); O and I approach their maximum values when S_{2s} has already reached a minimum value (phase 3).

Fig. 4 shows the temperature depencence of the O-level with anaerobic dark time. The first rise in the O-level has a Q_{10} of about 2; the delays at the beginning and after the first rise are longer at lower temperatures; the yield after 8 h is lower with lower temperature. When a sample which has reached maximum anaerobic adaptation is reaerated, the O-level decays biphasically with $t_{\frac{1}{2}} = 2$ s at 25 °C. This decay is difficult to evaluate quantitatively, since the time for equilibration of the sample with air is a disturbing factor. If such a pre-adapted sample is made anaerobic again, the rate of the O-level rise increases (see Fig. 4, upper curve). This explains why, in some previously reported work, phase 3 was reached much earlier than would be expected with a fresh sample. The rise in the O-level appears to reflect the reduction of System

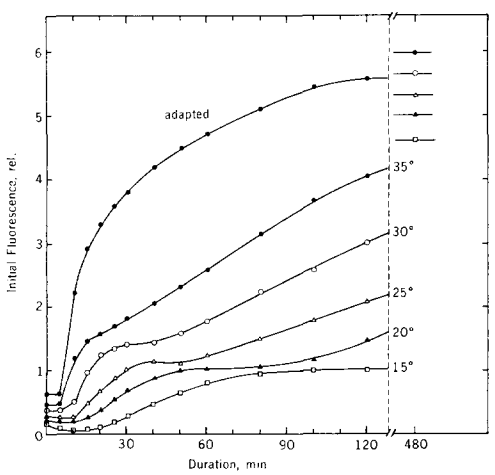

Fig. 4. Temperature dependence of the change in the O-level with anaerobic treatment. Fresh samples of algae were used for each temperature, except for the upper curve at 35 °C which was recorded after 8 h anaerobic dark storage followed by 30 min darkness in air. Other conditions the same as in Fig. 3.

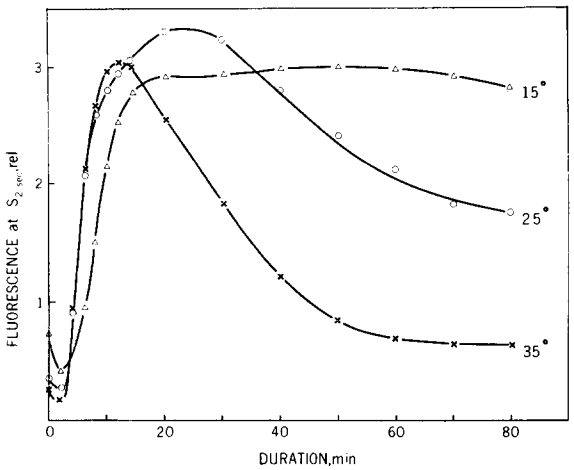

Fig. 5. Temperature dependence of the change in the $S_{2\,s}$-level with anaerobic treatment. Conditions as in Fig. 4.

II acceptors by a pool of reductants formed during anaerobic adaptation. This pool seems to withstand oxidation by air for some time. Stimulated rise of the O-level persisted for several hours in air following 8 h of anaerobic pre-adaptation. The reductant does not interfere seriously with photosynthesis, as normal induction kinetics reappear within minutes after readmission of air. No effect of the reductant after 12 h in air on a second anaerobic adaptation was detected.

The effect of temperature on the rise in $S_{2\,s}$ with anaerobic dark time is very small (Fig. 5), but there is a strong stimulation of the decay of $S_{2\,s}$ with higher temperature. Higher temperatures, up to 40 °C, shorten the time required for anaerobic adaptation and do so, in the case of *Scenedesmus* [6], with no irreversible damage. With temperatures exceeding 45 °C, even in air, the O-level is raised to a value which is close to the maximum anaerobic level; O returns to a low level upon recooling only when O_2 is present [6, 8].

At 20–25 °C and without preceding anaerobic adaptation, it takes many hours to come close to the isolated fluorescence decay of phase 3. This may be one reason why the occurrence of this transient has been obscured for a long time.

Kautsky and Franck [3] studied anaerobic fluorescence transients in *Ulva lactuca*, which even after prolonged anaerobiosis does not show phase 3 [6, 8]. Attempts to induce a dark rise in the O-level and the elimination of the D-P-S transient by increased temperature fail, as this treatment leads to irreversible damage in *Ulva*. The O-I rise in anaerobic *U. lactuca* is extremely steep, comparable to the purely photochemical O-I rise in the presence of DCMU and phenylurethan [6]. Due to the limited time resolution of their apparatus, Kautsky and Franck [3] could not resolve this rise so that their curves only seem to start at the maximum level, a phenomenon which actually occurs in *Scenedesmus*. Gingras and Lavorel [18] first demonstrated an increase of the O-level with anaerobic adaptation in *Scenedesmus*, paralleled by a slight decrease in the P-level. From their Fig. 3 in ref. 18 we conclude, though, that their anaerobic treatment was still not long enough to reach the maximum O-level, which exceeds P even at very high light intensities. Kessler [12] recently reported

on a comparative study of algae with and without hydrogenase, showing that the dark rise in fluorescence yield requires this enzyme system, apparently present in the *Scenedesmus* strain used here, but not in *Ulva*.

Fluorescence decay from an extreme value F_{max} close to the minimum value F_0 in the light under the conditions described above provides a helpful tool for the study of the different quenching mechanisms, all of which apparently are first blocked and then resume during illumination.

An action spectrum for the anaerobic fluorescence decay

It is well known that light absorbed by System I tends to lower fluorescence yield after reduction of System II acceptors by light, strong artificial reductants or anaerobic treatment. Govindjee et al. [19] first reported that far-red light is most effective in lowering fluorescence yield. On the other hand, Munday and Govindjee [4] found a slightly faster I-D drop in 705 nm light than in 650 nm light. Govindjee and Papageorgiou [1] assume that System I and System II are equally effective in causing the drop and speculate that the I to D decay originates from a fast reoxidation of part of A^- by oxygen produced during the gush. Recently Duysens *et al.* [20] detected a fluorescence decay from F_{max} to $F_{max/2}$ in a saturating flash within microseconds, which is much too fast to reflect reoxidation of Q. This extremely rapid decay, mediated by pigment System II, is favoured by low oxygen tension and may involve carotene triplets. Additional doubts about the significance of fluorescence decay like the I-D and P-S transients arise from the concept of "spill-over" of System II absorbed energy to System I. There is now evidence from various groups that energy transfer from System II to System I pigments is possible [21–24]. Murata [22] discusses the P to S decline in terms of a "state 1" to "state 2" transition. While any

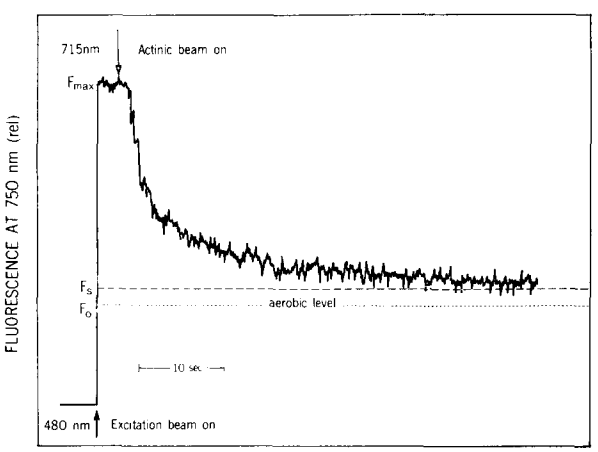

Fig. 6. Anaerobic fluorescence decay in 715 nm light measured with the modulated beam method. Excitation beam, 0.2 $\mu J \cdot cm^{-2} \cdot s^{-1}$ at 480 nm, 20 nm half-bandwidth, modulated at 80 cycles/s; continuous actinic beam 20 $\mu J \cdot cm^{-2} \cdot s^{-1}$ at 715 nm, 2 nm half-bandwidth; time constant at amplifier, 300 ms; fluorescence yield measured at 750 nm, 8 nm half-bandwidth; temperature, 30 °C; anaerobic dark storage, 8 h before beginning of series. Similar curves were recorded for more than 40 different wavelengths between 420 and 720 nm to determine the action spectrum in Fig. 7. Readmission of air results in a constant fluorescence yield indicated by the aerobic level.

or all of the foregoing could be related to the I-D dip, a determination of the action spectrum should help to resolve the question of its cause.

We determined the action spectrum for the anaerobic decay, using a technique similar to that applied by Duysens and Sweers [16]. Fig. 6 shows a typical measurement of fluorescence excited by a modulated 480 nm beam of extremely low intensity ($0.2\ \mu J \cdot cm^{-2} \cdot s^{-1}$). Even with prolonged anaerobiosis, when F_{max} is reached this excitation beam does not lead to any appreciable fluorescence decay. When the actinic beam is switched on, there is a short lag phase before the fluorescence yield drops, with obviously biphasic kinetics, to a low stationary value which is close to the minimal fluorescence yield F_0 observed in air. We measured such fluorescence decay curves over the wavelength region from 420 to 720 nm with a high degree of precision. Equal quantum fluxes of monochromatic light (half-bandwidth 2 nm) were used and the maximum rate of the decay taken as the measure of activity. A study of the decay kinetics shows that the intensity–rate relationship is linear even with intensities far higher than the ones used here (Schreiber, U. and Vidaver, W., unpublished).

Fig. 7 shows the action spectrum of the anerobic decay. For comparison we also plotted the action spectrum of the aerobic fluorescence rise in the presence of DCMU, which was determined in the same way as described above for the decay. Both action spectra possess maxima centered around the well-known absorption peaks of chloroplasts *a* and *b*, at 440, 480, 650 and 675 nm. In addition, only the anaerobic spectrum has a pronounced peak at 685 nm and a relatively high activity reaching into the far-red region with a shoulder around 695 nm. Both spectra also show shoulders around 667, 620 and 590 nm, which can be considered real as they are reproducible. The anaerobic decay displays the features of a typical System I action spectrum.

Taking the aerobic DCMU rise as characteristic for Photosystem II action and calculating the ratio between the two actions produces a ratio spectrum almost

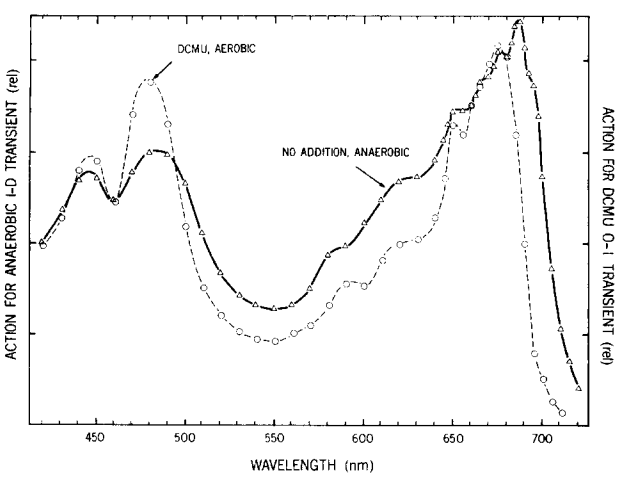

Fig. 7. Action spectra of the anaerobic fluorescence decay and the aerobic fluorescence rise in the presence of DCMU. Conditions as in Fig. 6. The procedure for determination of the DCMU ($5 \cdot 10^{-5}$ M) O-I rise was identical to that for the anaerobic decay. Curves are normalized at 680 nm.

identical to that of Joliot et al. [25], if we assume equal active absorption of the two photosystems at 680 nm. The most prominent feature in the ratio spectrum is a pronounced rise at $\lambda > 680$ nm. In the anaerobic action spectrum, 650 nm light is about two times more effective than 706 nm light. Assuming a ratio between absorption at 650 and 705 nm in the order of 10, we arrive at an approximate 5-fold greater activity of 705 nm light, compared with 650 nm light for the same number of absorbed quanta.

We conclude that the anaerobic decay, as well as the I-D dip in the aerobic sample from which the decay clearly derives (see Figs 1 and 2), does reflect System I activity. Moreover, we can say that by System I activity exclusively almost all quenchers or quenching mechanisms, which are at first blocked after sufficiently long dark anaerobic storage, can be regenerated (see Fig. 6). The small difference between the aerobic level F_0 and the stationary level F_s may reflect the only type of quenching which actually does depend on molecular oxygen. These data rule out earlier speculations [6] that molecular oxygen is required at System II reaction centers. As we will show below, the action sites of O_2 appear rather to be on the oxidizing side of System II and on the reducing side of System I.

An oxygen requirement for linear electron transport

The O and I levels during phase 1 are unchanged from the aerobic sample and there is obviously no change in the pool sizes of the primary acceptor Q and the secondary acceptor pool A. On the other hand, as P is raised and the P-S decline practically abolished, some other essentially infinitely large electron acceptor pool is cut off. Massive electron uptake by O_2 contradicts the concept of efficient photosynthetic electron transfer. Thus the function of O_2 should be considered more as that of a cofactor or regulator, rather than an essential acceptor.

The only large pool of oxidant, except O_2 itself, that can be visualized as cut off by the mere removal of O_2 seems to be Photosystem I, which in the light actually does show an infinite pool-size. It is hard to understand, though, why (a) electron transfer to System I should depend on the presence of oxygen and (b) a pronounced I-D dip and a blocked P-S decline is observed in the same curve as, for example, in phase 2 of anerobic cells (see Fig. 1) or in the presence of sodium dithionite after preillumination [6]. The problem is how in one and the same curve there can be indications of electron transfer to System I being speeded up (I-D) and of electron transfer to System I being blocked (P-S).

Simultaneous measurements of O_2-exchange transients and fluorescence transients were carried out anaerobically in order to check whether, during the time fluorescence yield stays at a high P-S level, electron transport and water-splitting really are inhibited. The result is shown in Fig. 8. There is a complementary correlation between fluorescence yield and O_2 evolution rate throughout the D-P-S transient. Once the A-Q acceptor pool is filled up during the "gush", the O_2 evolution rate stays low until after a delay which depends on the anaerobic pretreatment, then it slowly recovers and is paralleled by a simultaneous P-S decline in fluorescence. The acceptor pool of System II can be totally oxidized by far-red preillumination, but without O_2 being evolved. The effect of such a preillumination during phase 2 is seen in a comparison of curves c and d: O and I fluorescence levels are lowered to about their air values, but P- and S-levels are not affected. There is a

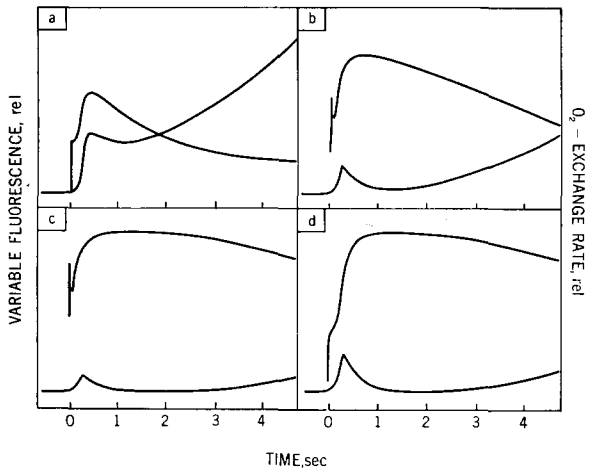

Fig. 8. Simultaneous recordings of O_2-exchange rates and fluorescence induction. For details see Methods. (a) After 15 min with 4 % O_2 in N_2+CO_2; (b) after 15 min with 0.4 % O_2 in N_2+CO_2; this sample was preilluminated by the actinic light, 5 s light +2 s dark; (c) after 30 min N_2+CO_2, without preillumination; (d) after 30 min N_2+CO_2, with 5 s far-red preillumination. Temperature 30 °C; light intensity, 0.1 J · cm^{-2} · s^{-1} at 680 nm, 20 nm half-bandwidth; intensity of far-red light, 10 mJ · cm^{-2} · sec^{-1} at 701 nm, 2 nm half-bandwidth.

stimulation of the first gush of O_2, but no effect on the second rise.

With the O_2 exchange curves presented here, the possibility of O_2 uptake cannot be excluded. However, uptake of evolved O_2 is not detected with anaerobic conditions [26], a fact which argues against the lag in anaerobic O_2 evolution being due to increased O_2 uptake.

Thus there appears to be an oxygen requirement for photosynthesis, a point which has been emphasized earlier on the basis of other experimental results [27]. But as the system produces its own oxygen, photosynthesis can hardly be blocked for long periods by the initial lack of O_2. Fig. 9 shows a slow recovery from anaerobic pretreatment. After a few minutes in strong light, approximately the same steady rate is achieved as in the aerobic sample.

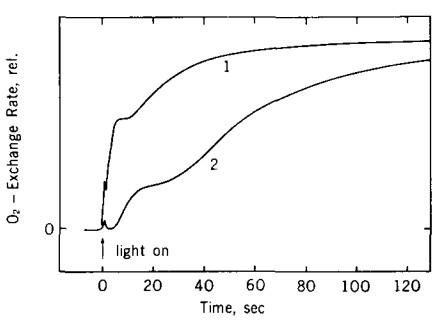

Fig. 9. Induction transients of O_2 production: (1) under aerobic conditions; (2) with 30 min anaerobic dark time. Temperature, 30 °C; light intensity, 0.1 J · cm^{-2} · s^{-1} at 680 nm, 20 nm half-bandwidth.

The question arises as to where to locate the site of an oxygen requirement for electron transport. As possible sites, System II acceptor pool A and System I acceptor X can be visualized. It is well known that O_2 can oxidize pool A in the dark, but any appreciable reaction in the light has not been reported. O_2 uptake shows a clear System I action spectrum (Vidaver and French) [28], which favours a reaction of O_2 at X. Munday and Govindjee [4] reported elimination of the D-P-S fluorescence transient by methylviologen in *Chlorella*, which would argue for X^- causing the "traffic jam" reflected at P, but such an effect in *Scenedesmus* has not yet been found. There is experimental evidence that the rate limiting step during aerobic steady state photosynthesis is between A, which accumulates in the reduced form, and P700, which accumulates in the oxidized form [29, 30]. This does not exclude, though, the possibility that during the first phase of induction, reflected by O-P, the electron transfer from X to NADP is limiting, as the Calvin cycle is not yet operating. In the presence of O_2 there is an alternate "pseudocyclic" electron transfer to O_2, which can not occur anaerobically. Heber and French [31] and Heber [32] gave evidence for an electron transfer to O_2 in whole cells, which competitively suppresses cyclic flow in air. Anaerobically cyclic flow is strongly stimulated, a fact which is important in connection with the anaerobic fluorescence curves. Urbach and Fork [33] reported "a dramatic effect of short periods of anaerobiosis" on absorption changes of cytochrome *f* and plastocyanin in the electron transport chain. In anaerobic *Chlorella* cells plastocyanin was reduced by System II but not oxidized by System I, whereas cytochrome *f* was oxidized by System I but not reduced by System II. These findings led the authors to locate the O_2 requirement between cytochrome *f* and plastocyanin and to suggest a competition between cyclic and linear electron flow at this point, resulting in a block of electron flow from System II to System I. Putting things together, it is clear how O_2 acts as an acceptor after System I and its removal indirectly causes the block of flow between the systems, while System I is not limiting. The O_2 concentration in the sample will regulate the ratio between pseudocyclic and cyclic electron transfer. This will also determine the rate of linear electron transport from System II to System I by controlling the electrons fed back into the chain. With aerobic conditions, we expect a minimal amount of feedback, as electrons end up either at NADP or O_2, so that O_2 production will be high and fluorescence yield low. With the removal of O_2, feedback of electrons through cyclic flow will be stimulated, resulting in decreased linear electron flow, high fluorescence and cessation of O_2 evolution. Resumption of electron

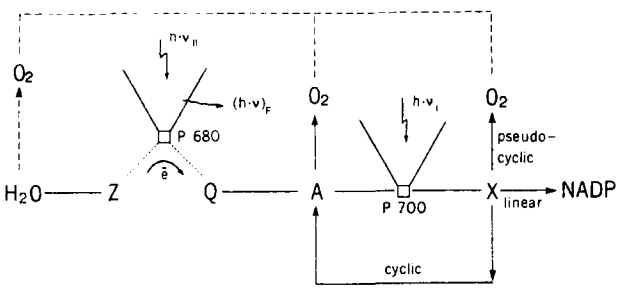

Fig. 10. Schematic interpretation of the function of molecular O_2 as a regulator of electron flow between the two photosystems.

flow, which is observed after a delay (see Figs 8 and 9), may result from a reoxidation of NADP by Calvin cycle activity or by some autocatalytic effect of traces of O_2 still in the medium or released during the small gush. In Fig. 10, this situation is depicted schematically.

Recently, Diner and Mauzerall [13] proposed a model with a "cross reaction" specified by a reductant "R" and the oxidant O_2 with the pool A, which fits their data from anaerobic flash-yield measurements. Their finding that PMS, a promoter of cyclic electron transport, speeds up the "deactivation" of linear electron transport from System II to System I fits well in the scheme of Fig. 10. The difference between their scheme and ours is that they assume direct oxidation of A by O_2, whereas we see only an indirect O_2 control over the redox state of A. Furthermore, we prefer to distinguish between the anaerobic reduction of A in the light, which seems to be partly due to cyclic feedback or to their R process, and the reduction of A by an endogenous donor during anerobic incubation in the dark, which is reflected by the increase in the O and I levels. The R mechanism is extremely fast ($t_{\frac{1}{2}} = 160$ ms at 24 °C) and certainly not related to the anaerobic adaptation process.

The scheme proposed in Fig. 10 accounts for the discrepancy apparent in phase 2 fluorescence curves, where one and the same curve suggested System II to System I electron transfer in I-D and the blocking of this transfer in P-S. As cyclic flow comprises dark steps and probably is not even initiated before the reduction of NADP is saturated, it is conceivable that the fast reoxidation of Q by System I is not affected and only the much later occurring P-S decline is.

The action of an endogenous reductant

The significance of the different steps in the changes of fluorescence levels with anaerobic storage can now be pointed out by a reconsideration of the data shown in Figs 3 and 4.

Four different processes occurring with anaerobic treatment are indicated: (1) Removal of molecular O_2 as a regulator between linear, cyclic and pseudocyclic electron flow (see previous section). (2) After a delay, the reduction of a readily reduced fraction of the electron acceptor pool by an endogenous electron donor. (3) A deactivation at the oxidative side of System II by either an endogenous reductant or merely by the lack of O_2. (4) After another delay the reduction of the other, less readily reduced fraction of the electron acceptor pool.

As 3 and 4 proceed more or less simultaneously, the four processes are distinguished only by three typical "phases" in the anaerobic fluorescence–time courses (see Fig. 1). Phase 1, as pointed out above, is entirely due to the nonavailability of O_2 and does not indicate any dark reduction of System II acceptors. An identical fluorescence–time curve results from the addition of sodium dithionite ($Na_2S_2O_4$) [6, 8] which suggests that this strong reductant, at least in whole cells after short incubation, only scavenges O_2 without reducing any intermediates.

Fig. 4 shows that there is not much temperature effect on the anaerobic rise in the $S_{2\,s}$-level, whereas there is a strong effect on the decrease in $S_{2\,s}$ and the increase in the O-level during the second phase. Some hastening of the rate at which O_2 is removed by an N_2 stream is to be expected with increasing temperature as O_2 has to diffuse out of the cells. The high temperature dependancy and slow development of phases 2 and 3 suggest that the reduction of the intermediates is caused by an enzy-

matic or metabolic adaption to the treatment. A reasonable possibility which arises from the work of Kessler [12] is the induction of hydrogenase, which reaches its full activity only slowly and under strictly anaerobic conditions (Gaffron) [34]. As pointed out earlier this endogenous electron donor system, which leads to the anaerobic dark reduction of A-Q, cannot possibly be identical with Diner and Mauzerall's R [13]. R is demonstrated by the use of a repetitive flash technique: steady-state O_2 yield per flash decays with $t_{\frac{1}{2}} = 160$ ms at 24 °C, when t is the time between two saturating flashes. It seems that this effect is induced by the flashes and probably, as suggested above, due to cyclic feed-back from X^- to A-Q (see Fig. 10).

The dark reduction of System II acceptors observed with anaerobic adaptation clearly proceeds in at least two steps, a fact which is in agreement with other observations, that the acceptor pool is inhomogeneous [14, 35–39]. Cramer and Butler [36] reported two transitions with midpoint potentials of about −20 mV and −320 mV upon reductive titration of the fluorescence yield of spinach chloroplasts; the readily reduced compound contributing about 40 % to the total quenching if oxidized. Anaerobic adaptation could induce a similar reductive "titration" of System II acceptors by increasing the reduction potential of an endogenous reducing agent. R. Govindjee et al. [38] and Joliot and Joliot [39] suggested the intervention of two different fluorescence quenchers: a quencher Q_1 which is difficult to reduce (low redox potential) and a quencher Q_2 of higher potential. Joliot proposed that in the dark, quenching is mainly of the Q_1 type and correlated to the S_0 and S_1 state in Kok et al.'s [40] model of charge cooperation, whereas in the light, S_2 and S_3 are essentially associated with Q_2. It would follow that the equilibrium constant between the primary acceptors of System II, Q_1 and Q_2, and A (probably plastoquinone with a redox potential close to −20 mV) is practically infinite in the dark and approx. 1 in the light. One would conclude that our "titration curves" reflect reduction of plastoquinone and the associated quencher Q_2 (A-Q_2) during the first transition, and after all the A-Q_2 has become reduced, the reduction of Q_1 during the second transition.

Radmer and Kok [37] suggest that A rather than Q is inhomogenous, a fraction A_1 being linked by a high K and A_2 by a low K to the primary acceptor Q. Although their model does not directly contradict our results, it seems to conflict with Cramer and Butler's [36] observation of two transitions even when electron transfer from Q to A is blocked by DCMU.

From recent work of Joliot and Joliot [14], it might be concluded that there are even more types of quenching possible at System II reaction centers. The possibility must be considered that the first transition may reflect the elimination of Q_R quenching (terminology of Joliot and Joliot) and that the second transition results from the suppression of Q_F quenching, while a suspected third transition, for which we have recent indication (Schreiber, U. and Vidaver, W., unpublished), could represent the loss of Q_s quenching.

The deactivation of System II during anaerobic adaptation

All System II reaction centers after prolonged anaerobic treatment are in an inactive state which is indicated by the maximum fluorescence yield of the O-level. This block is rapidly removed by System I action in light. In the experiment described in Fig. 11, fluorescence curves were recorded depending on the duration of anaerobic storage where each sample was far-red preilluminated sufficiently long to oxidize

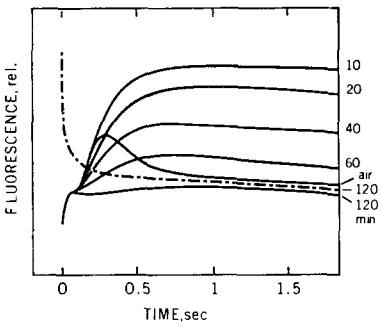

Fig. 11. Effect of anaerobic dark time on fluorescence induction after far-red preillumination. Each sample was kept for the indicated period in N_2 and preilluminated for 30 s with 701 nm light (2 nm half-bandwidth) of 20 $\mu J \cdot cm^{-2} \cdot s^{-1}$ before the curves were recorded (except the dotted curve). Temperature, 30 °C; light intensity, 50 mJ $\cdot cm^{-2} \cdot s^{-1}$ at 680±10 nm. 60 min and 120 min curves are shifted slightly downwards to normalize initial fluorescence. The dotted curve shows for comparison the fluorescence decay without far-red preillumination after 120 min anaerobic treatment.

A-Q, as indicated by low O and I levels. The far-red preillumination affects the curve representing phase 1 (10 min) hardly more than the aerobic one, which confirms that during phase 1 of anaerobic adaptation, the dark reduction of A-Q has not yet begun. With increasing anaerobic periods there is a gradual elimination of the D-P-S transient. Thus, the intermediate pool is reoxidized, the centers are obviously reactivated by the preillumination and there is still some reaction which remains deactivated. There is a normal O-I rise, which indicates that a single electron transfer per reaction center does occur. Practically the same type of curve results after moderate heat treatment (5 min at 48 °C) of intact algae cells, which leads to the irreversible deactivation of H_2O-splitting [6]. Contrary to heat deactivation, anaerobic deactivation is reversible. Admission of air after several hours of anaerobiosis (data not shown in the figures) leads to a rapid decay of the O-level ($t_{\frac{1}{2}} = 2$ s at 25 °C) and to a slower restoration of the D-P-S transient ($t_{\frac{1}{2}} = 3$ min at 25 °C).

The cause of anaerobic deactivation of watersplitting remains uncertain. Simultaneous determinations of fluorescence and O_2 production with saturating flashes, currently in progress, may help to elucidate the problem. Molecular O_2 could play an active role in the watersplitting mechanism. On the other hand its absence may allow the formation of an inhibitory product, which is destroyed aerobically. Whatever the mechanism, further studies of the anaerobic deactivation phenomenon should be of help to the eventual understanding of photosynthetic watersplitting.

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REFERENCES

1 Govindjee and Papageorgiou, G. (1971) in Photophysiology (Giese, A. C., ed.), Vol. VI, pp. 1–46, Academic Press, New York
2 Franck, U. F. (1941) Dissertation, University of Leipzig, Germany
3 Kautsky, H. and Franck, U. F. (1943) Biochem. Z. 315, 139–232.
4 Munday, Jr, J. C. and Govindjee (1969) Biophys. J. 9, 1–21.
5 Franck, U. F., Hoffmann, N., Arenz, H. and Schreiber, U. (1969) Ber. Bunsenges. Phys. Chem. 73, 871–879
6 Schreiber, U. (1971) Dissertation, RWTH Aachen, Germany
7 Schreiber, U., Bauer, R. and Franck, U. F. (1971) Z. Naturforsch. 26b, 1195–1196
8 Schreiber, U., Bauer, R. and Franck, U. F. (1972) Proc. 2nd Int. Congr. Photosynth. Res., Stresa, 1971 (Forti, G., Avron, M. and Melandri, A., eds), Vol. 1, pp. 169–179, Dr W. Junk N. V. Publishers, The Hague
9 Rabinowitch, E. (1945) Photosynthesis and Related Processes, Vol. 1, pp. 326–330, Wiley, New York
10 Rabinowitch, E. (1956) Photosynthesis and Related Processes, Vol. 2, pp. 1364–1371, Wiley, New York
11 Kessler, E. (1960) in Handbuch der Pflanzenphysiologie (Ruhland, W., ed.), Vol. V, pp. 935–950, Springer, Berlin
12 Kessler, E. (1973) Arch. Microbiol. 93, 91–100.
13 Diner, B. and Mauzerall, D. (1973) Biochim. Biophys. Acta 305, 329–352
14 Joliot, P. and Joliot, A. (1973) Biochim. Biophys. Acta 305, 302–316
15 Kautsky, H., Appel, W. and Amann, H. (1960) Biochem. Z. 332, 277–292
16 Duysens, L. N. M. and Sweers, H. E. (1963) in Studies on Microalgae and Photosynthetic Bacteria (Miyachi, S., ed.), Special Issue of Plant Cell Physiol., Tokyo, pp. 353–372
17 Vidaver, W. (1964) Carnegie Inst. Wash. Year Book 63, 466–468
18 Gingras, G. and Lavorel, J. (1965) Physiol. Veg. 3(2), 109–120
19 Govindjee, Ichimura, S., Cederstrand G. and Rabinowitch, E. (1960) Arch. Biochem. Biophys. 89, 322
20 Duysens, L. N. M., Van der Schatte Olivier, T. E. and Den Haan, G. A. (1972) Abstr. VI Int. Congr. Photobiol., 277, Bochum, Germany
21 Murata, N. (1970) Biochim. Biophys. Acta 205, 379–389
22 Bonaventura, C. and Myers, J. (1969) Biochim. Biophys. Acta 189, 366–383
23 Duysens, L. N. M. (1972) Biophys. J. 12, 858–863
24 Mohanty, P., Braun, B. Z. and Govindjee (1973) Biochim. Biophys. Acta 292, 459–476
25 Joliot, P., Joliot, A. and Kok, B. (1968) Biochim. Biophys. Acta 153, 635–652
26 Vidaver, W. and Chandler, T. (1969) in Progress in Photosynthesis Research (Metzner, H., ed.), Vol. 1, pp. 514–520, H. Laupp, Jr, Tübingen
27 Vidaver, W. (1965) Carnegie Inst. Wash. Year Book 64, 395–397
28 Vidaver, W. and French, C. S. (1965) Plant Physiol. 40, 7–12
29 Stiehl, H. H. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1588–1598
30 Kok, B., Joliot, P. and McGloin, M. (1969) in Progress in Photosynthesis Research (Metzner, H., ed.), Vol. 2, pp. 1042–1059, H. Laupp, Jr, Tübingen
31 Heber, U. and French, C. S. (1968) Planta 79, 99–112
32 Heber, U. (1969) Biochim. Biophys. Acta 180, 302–319
33 Urbach, W. and Fork, D. C. (1965). Carnegie Inst. Wash. Year Book 64, 390–395
34 Gaffron, H. (1940) Am. J. Bot. 27, 273–283
35 Joliot, P. (1965). Biochim. Biophys. Acta 102, 116–134
36 Cramer, W. A. and Butler, W. L. (1969) Biochim. Biophys. Acta 172, 511–524
37 Radmer, R. and Kok, B. (1973) Biochim. Biophys. Acta 314, 28–41
38 Govindjee, R., Govindjee, Lavorel, J. and Briantais, J. M. (1970) Biochim. Biophys. Acta 205, 361–370
39 Joliot, P. and Joliot, A. (1971) in Proc. 2nd Int. Congr. Photosynth. Res., Stresa, Vol. 1, pp. 26–38, Dr. W. Junk N. V. Publishers, The Hague
40 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475