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Relationship of steady-state photosynthesis to fluorescence in eucaryotic algae

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The change in fluorescence yield ($\Delta\phi$) was measured in five species of eucaryotic algae using a 'pump and probe' flash technique. The half-time for the oxidation of Q, which was measured by varying the delay time between actinic (pump) and measuring (probe) flashes, averaged 400 μs and was unaffected by background irradiance between 10^{12} and 10^{16} quanta $\cdot$ cm^{-1} $\cdot$ s^{-1}. The absorption cross-section of PS II traps was measured by varying the intensity of the actinic flash. These cross-sections did not change ($\pm$ 10%) with background irradiance. The cross-section data can be fitted to a cumulative one-hit Poisson distribution. In the steady state, the relationship between $\Delta\phi$ and photosynthetic oxygen evolution was highly nonlinear and cannot be explained by energy transfer between PS II units. Using the criteria of $\Delta\phi$, about 15% of the PS II traps remain open at light saturation of O_2 evolution. Conversely, at low irradiance levels, capable of stimulating much less than 1% of the maximum steady-state photosynthetic rate, the fluorescence yield decreases by as much as 25% from the dark-adapted value. Furthermore, the data suggest that a long-lived quencher of fluorescence is formed at moderate to continuous irradiance levels, at least 10^{16} quanta $\cdot$ cm^{-2} $\cdot$ s^{-1}. Our results suggest that cyclic electron flow around PS II occurs under normal physiological conditions and is especially pronounced in chlorophytes.

Introduction

Chlorophyll fluorescence has served as a valuable but indirect probe of the primary biophysical events in photosynthesis [1–4]. As a first approximation, fluorescence yield and photochemical reactions in PS II, as reflected by oxygen evolution, vary inversely and have been interpreted, along with nonradiative decay, as competing for excitation energy within the antennae Chl of PS II traps [1,3,5]. The early interpretation of fluorescence induction led to the postulation of a primary electron acceptor, Q, in PS II [1]. When Q is oxidized, excitation energy is absorbed by the 'open' PS II traps and fluorescence is quenched. When Q is reduced, the trap becomes 'closed' and photons arriving at PS II are dissipated as fluorescence or heat. Subsequent investigations suggested

Abbreviations: $\Delta\phi_t$, change in fluorescence yield ($=(F_s - F_p)/F_p$), measured at delay time, t, between 'pump' and probe flash; F_p, fluorescence yield of a weak probe flash; F_s, fluorescence yield of a weak probe flash following a 'pump' flash; F_b, fluorescence yield from the continuous background light; J, continuous irradiance level; P, steady-state photosynthetic oxygen evolution at any irradiance; P_{max}, maximum steady-state photosynthetic oxygen evolution at saturating continuous irradiance; RC, reaction center; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

that, in algae and higher plant chloroplasts, Q is a membrane-bound plastoquinone which mediates electron transfer to a secondary plastoquinone via a two-electron 'gate' (see Ref. 6 for a review). It is assumed that the fraction of open/closed PS II traps is directly proportional to Q/Q^-, and can be estimated by following changes in the variable fluorescence yield [1,5–7].

Most investigators have used fluorescence induction as a means of measuring variable fluorescence yields. In this method, cells, chloroplasts, or thylakoid membranes are preadapted to some condition (dark, far-red light, etc.) and then quickly exposed to a continuous actinic source. The initial fluorescence yield (F_0) and the maximum fluorescence yield (F_m) obtained with a saturating photon flux (or in the presence of inhibitors such as DCMU) are measured. The difference ($F_m - F_0$) is taken to be the variable fluorescence (F_v). The kinetics of the induction curve often are used to interpret relative pool sizes of electron carriers on the acceptor side of PS II [7,8], the flux of electrons into the pools following the onset of illumination [7,9,10], the relative absorption cross-sections of PS II [11], as well as the extent of energy transfer between reaction centers [9,12–14].

During steady-state photosynthesis, fluorescence induction techniques are not generally useful in analyzing the redox state of Q. Both F_0 and F_m may vary with irradiance (and therefore redox) conditions and induction curves do not have an inherent reference measurement (i.e., they are difficult to normalize). Modifications of the induction curve have been made to apply the technique to kinetic analyses [10,15,16]. One technique is to superimpose saturating flashes or a second light on the induction curve. These methods may provide information about the relative changes in F_m, and the fraction of unreduced Q remaining during induction and during steady state; however, the results from such experiments are often difficult to interpret [17].

An alternative method of following changes in fluorescence yield light intensities utilizes the 'pump and probe' technique [4,18]. In this approach the change in fluorescence yield of a weak 'probe' flash is measured following a 'pump' flash, which can be made sufficiently intense to saturate the observable effect (e.g., O_2, fluorescence yield, etc.). By varying the delay time between the pump and probe flashes, the kinetics of recovery of fluorescence yields can be followed (i.e., the fast turnover time). By varying the intensity of the pump flash, apparent absorption cross-sections can be measured. Unlike fluorescence induction methods, the pump and probe technique allows the convenient, non-destructive measurement of the fluorescence yield and cross-section as the physiological state changes under different steady-state conditions [19].

In this study we investigated the effects of continuous background irradiance on oxygen evolution and the fluorescence yield in intact, unicellular eucaryotic algae under normal physiological conditions. We examined how irradiance influences the turnover time and apparent absorption cross-sections of PS II. Finally, we examined the relationship between oxygen production and fluorescence yields under study-state conditions.

Materials and Methods

Culture conditions

Four species of marine unicellular algae were used in these experiments: two diatoms, *Thalassiosira weisflogii* and *Skeletonema costatum,* a haptophyte *Isochrysis galbana,* and a chlorophyte *Dunaliella tertiolecta.* All cultures were grown at 18°C in natural seawater enriched with f/2 nutrients [20]. Light was provided by continuous cool white HO fluorescent tubes and cells were maintained in exponential growth at $4 \cdot 10^5$ quanta $\cdot$ $cm^{-2} \cdot s^{-1}$. Cultures were diluted every 6 h with fresh media to maintain optically thin suspensions and the flasks were bubbled with sterile air which served as an additional source of CO_2 as well as a means of mixing. In addition, the fresh-water chlorophyte, *Chlorella vulgaris,* was grown in batch cultures as described [21].

Fluorescence measurements

For fluorescence measurements, 4–5 ml samples, containing 5–10 ng Chl *a*/ml (approx. 10^3 cells/ml) were placed in 1×1 cm fluorescence cuvettes in the thermostatically controlled, magnetically stirred holder of an Aminco DW-2a spectrophotometer. The cuvette holder was positioned in front of a beam scrambler and a Hamamatsu

R562 photomultiplier tube operated at 750 to 1300 V. The photomultiplier tube was protected with a filter package containing a 670 and a 700 nm interference filter (5 nm half-bandpass) and a Corning 2-64 sharp-cut filter. Continuous irradiance was supplied by a 150 W tungsten iodide source through a fiber-optic light guide. The continuous source was filtered through a 'hot' dichroic mirror (Meles Griot O3MHG007) and blue-green (Corning 4-96) filters. Intensities were measured with a Lambda LiCor 190S cosine corrected quantum sensor. The fiber optic bundle terminated at the axis of a Unibliz 26L electronic shutter mounted in front of the cuvette holder.

Xenon flashes were introduced through an access port at 90° to both the photomultiplier tube and the continuous irradiance source. Two flashes, filtered through a Corning 5-60 and a Corning 4-96 filter, were focused on the sample with lenses and a branched light guide. The saturating flash (EG&G 9B-3), triggered from a TTL circuit in a microprocessor, had a duration of 3 μs at 0.5 peak intensity with a 5 J output. The probe flash (GenRad 1539A), triggered at selected delay times after the saturating flash with a digital clock controlled circuit, had a duration of 1.2 μs at 0.5 peak intensity. Flash pairs were triggered at a rate of 0.5 s^{-1}. For measurements of absorption cross-sections the intensity of the first flash was attenuated with neutral-density filters.

The fluorescence signal, from the 1 kΩ load resistor on the photomultiplier tube's anode, was recorded on a Tektronix 468 digital storage oscilloscope. The maximum anode current from the flash was 120 μA. Between 16 and 64 flash pairs were averaged on the oscilloscope and the data were transmitted and stored on a Hewlett-Packard HP85 computer equipped with a dual disc drive. Flash-induced fluorescence measurements were made after at least 30 min of dark adaptation and at least 5 min of continuous illumination and are steady-state fluorescence yields.

The changes in fluorescence yields are calculated from the equation:

$$\Delta\phi = (F_s - F_p)/F_p \quad (1)$$

where F_p is the fluorescence yield induced by the probe flash alone and F_s is the yield of the probe flash following a pump flash. In this nomenclature, when F_p and F_s are measured without a background irradiance, they are analogous to F_0 and F_m used to describe induction curves measured with a saturating beam. The difference ($F_s - F_p$) is the variable fluorescence. The variable fluorescence yield is normalized to the probe flash under the experimental conditions; $\Delta\phi$ is the change in the variable fluorescence yield, not the variable fluorescence per se (F_v). We define $\Delta\phi$ to be the change in fluorescence yield at any time delay, while $\Delta\phi_{70}$ is at 70 μs time delay.

Oxygen measurements

Steady-state oxygen production and consumption was measured in a specially constructed poly(vinyl chloride) chamber with a YSI Clark-type polarographic oxygen electrode (Yellow Springs Instruments). The chamber has a flat glass front face through which water continuously flows, and a clear rear window behind which a Lambda Licor 190S quantum sensor is mounted. The 7 ml sample chamber has a 1 cm optical pathlength and the sample is magnetically stirred and maintained in the thermostatically controlled chamber at 18 ± 0.05°C. Light, supplied from a 150 W tungsten-iodide source, was filtered through a Schott Glass RG-5 heat filter and a Corning 4-96 blue-green filter. Intensities were adjusted with neutral-density filters. Samples were illuminated for 5–10 min at each irradiance level, and changes in oxygen were detected by the electrode, amplified and recorded on a strip chart recorder. Dark respiration was taken as the initial steady-state rates of oxygen consumption prior to exposure to irradiance in the oxygen chamber; there is an increase in dark respiration following intense illumination [22,23].

Chlorophylls were extracted with 90% acetone by homogenizing cells filtered on Gelman AE glass-fiber filters, in a glass tissue grinder with a motor-driven poly(tetrafluoroethylene) pestle. Pigment concentrations were determined from absorption spectra following the equations of Jeffrey and Humphrey [24].

Results and Discussion

Following a short flash of light (less than 10 μs duration), the fluorescence yield of the photosyn-

thetic apparatus exhibits a complex series of changes which can be roughly described in terms of three time domains. During the first few microseconds following the absorption of light, the fluorescence yield increases to the final maximal value produced by the flash [4]. The kinetics of this increase are particularly complex, since they depend on past illumination conditions, the specific number of photons absorbed and the rate of photon absorption [4,18]. Fluorescence quenchers existing on this time-scale include short-lived products of reaction-center photochemistry (e.g., P^+-680, $t_{1/2}$ 25 ns) and much longer-lived (up to 4 μs) components arising from multiple hits on a photosynthetic unit during the flash. Multiple hits are the inevitable consequences of flashes of even moderate energies given the random nature of light absorption by the photosynthetic apparatus [18].

In the time range from approx. 10 μs to approx. 100 μs following the flash, the fluorescence yield is relatively constant at its maximum value for the flash. This time domain provides a useful window during which fluorescence quenchers produced by the flash are absent and the reaction center remains 'closed' (Q remains reduced) [6,18]. This time domain is optimal for the use of fluorescence yield as a simple measure of reaction centers closed by the flash.

Finally, at times longer than 100 μs following the flash, the fluorescence yield declines to its initial pre-flash value. The kinetics for this decay are complex and follow the oxidation of Q by the secondary quinone electron acceptor, Q_B [6,26–29].

Fig. 1 shows the kinetics of the decrease in the relative fluorescence yield (determined as $\Delta\phi$, see Materials and Methods) measured at times greater than 50 μs following a saturating flash of light. The data shown in Fig. 1 were obtained using the diatom *T. weisflogii* and are typical of all our measurements using eucaryotic algae. Previously it has been shown that the oxidation of Q proceeds via a two-electron gate [6], the kinetics of which have been followed by measuring the decay in $\Delta\phi$ [27,28]. In the dark, with a flash pair frequency of 2 Hz, it can be expected that the cells establish an equilibrium between Q and Q_B, so that at any given moment there are populations of Q^-Q_B,

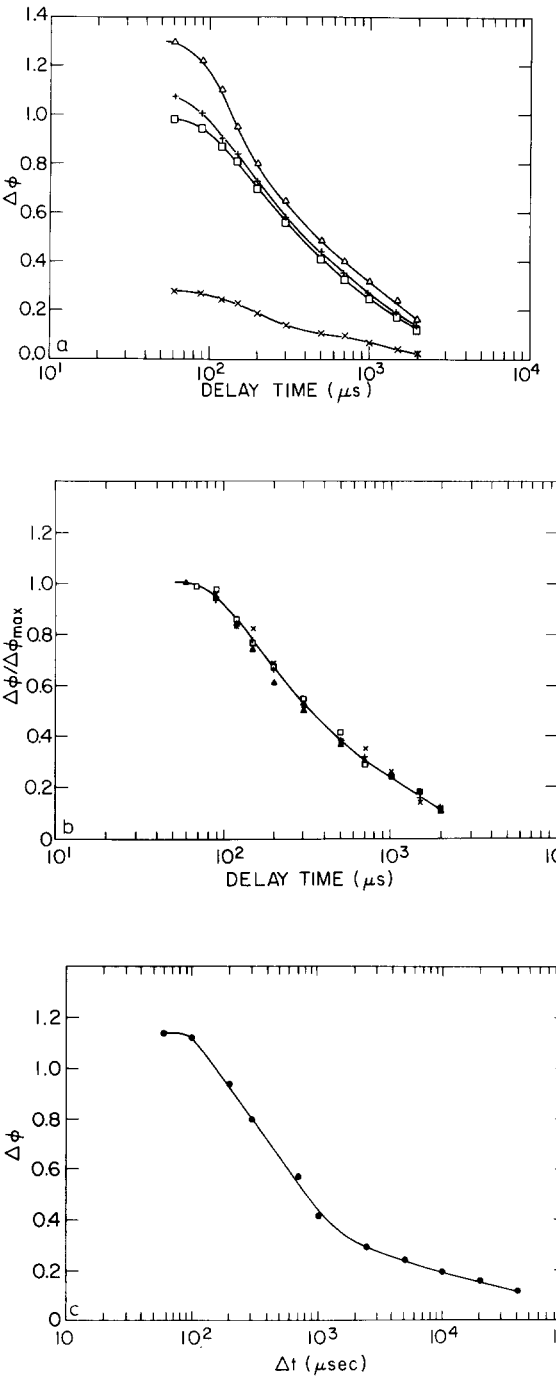

Fig. 1. Effects of continuous background irradiance on fluorescence yield. (a) Dark (△), $3 \cdot 10^{14}$ (+), $3 \cdot 10^{15}$ (□), $3 \cdot 10^{16}$ (×) quanta·cm^{-2}·s^{-1}. (b) Data from (a) normalized. (c) In a separate experiment, decay of $\Delta\phi$ in *T. weisflogii* without background light extended to the millisecond time domain. Note the small long-lived component of $\Delta\phi$ which decays with $t_{0.5} \approx 10$ ms.

QQ_B^-, $Q^-Q_B^-$ and QQ_B^{2-}. In this 'flash steady-state' the kinetics of the first electron transfer are not distinguishable from the second, and the decay of $\Delta\phi$ is presumably an average due to the contributions from $Q^-Q_B \rightarrow QQ_B^-$ and $Q^-Q_B^- \rightarrow QQ_B^{2-}$. In all cases, the decay of $\Delta\phi$ has multiple components but has a halftime of about 400 μs. This halftime compares well with the 'fast' turnover time for oxygen production reported for a great variety of photosynthetic organisms [30].

There is a significant component of $\Delta\phi$ displaying a long decay time; on the order of 10 ms (Fig. 1c). The origin of this long-lived component is not clear. The decay may reflect a heterogeneity [11] or multiplicity in PS II acceptors [12,31] or may result from the discharge of local electrochemical potentials [32].

When cells are illuminated with increasing levels of continuous background light, an increasing fraction of the PS II reaction centers will be closed at the instant of an assaying flash. The actual fraction of closed RC II will depend on turnover times, absorption cross-sections, and irradiance. In Fig. 1 the effects of background irradiance on the decay of $\Delta\phi$ are shown. As background irradiance is increased, the magnitude of $\Delta\phi$ declines markedly throughout the entire course of the decay (Fig. 1a), reflecting the decrease in F_s relative to F_p. However, the kinetics of the decline in $\Delta\phi$ are unchanged by background irradiance. When the data shown in Fig. 1a are normalized to give equal values for $\Delta\phi_{70}$, all four decay curves are coincident (Fig. 1b). Thus, the rate at which $\Delta\phi$ relaxes to its steady-state level (set by background irradiance) following a saturating flash is independent of the magnitude of that steady-state. If the decay of $\Delta\phi$ reflects the oxidation of Q^-, this result indicates that the rate of oxidation of Q^- does not change as the fraction of RC II open in the steady-state declines.

Fig. 2 shows flash energy saturation curves for $\Delta\phi_{70}$ measured in the presence of continuous background irradiance. The data were obtained using cells of *T. weisflogii* exposed to four levels of background irradiance having fluences between 0 and $3 \cdot 10^{16}$ quanta $\cdot$ cm$^{-2} \cdot$ s^{-1}. Over this range of background illumination, $\Delta\phi_{70}$ decreased from about 1.2 to about 0.2. For these measurements we determined $\Delta\phi_{70}$ following attenuation of the pump flash with calibrated neutral-density filters. All four sets of data have been normalized so that $\Delta\phi_{70}$ produced by an unattenuated pump flash is unity.

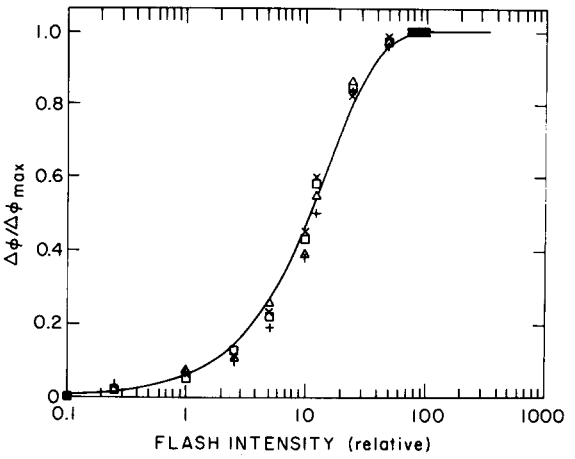

Fig. 2. Effects of continuous background irradiance on the relative apparent cross-sections of PS II in *T. weisflogii*. $\Delta\phi$ measured after a 70 μs delay time. Curve is best-fit Poisson function, $\Delta\phi/\Delta\phi_{max} = 1 - e^{-\sigma I}$; dark (+), $3 \cdot 10^{14}$ (□), $3 \cdot 10^{15}$ (×), $3 \cdot 10^{16}$ (△) quanta $\cdot$ cm$^{-1} \cdot$ s^{-1}.

The data shown in Fig. 2 are typical of all our results using eucaryotic algae. The flash energy saturation behavior of $\Delta\phi_{70}$ appears to be completely unaffected by background irradiance. The solid curve shown in Fig. 2 is a least-squares fit to the data of the exponential saturation function:

$$\Delta\phi_{70}/\Delta\phi_{70\,max} = 1 - \exp(\sigma \cdot E)$$

where E is the relative pump flash energy and σ is the relative effective absorption cross-section. Relative effective absorption cross-sections obtained from such fits were not significantly different at any background irradiance (analysis of covariance; the F statistic with three degrees of freedom in the numerator and 27 degrees of freedom in the denominator = 0.317). We conclude that, within the uncertainty of the experimental measurements ($\pm 10\%$), the relative effective absorption cross section for PS II is not changed by background irradiance; even at levels approaching the light-saturation of steady-state photosynthesis. A similar conclusion was reached by Ley and Mauzerall based on measurements of oxygen flash yields and fluorescence yields in *Chlorella* (see Ley, A.C. and

Mauzerall, D., unpublished results).

Fig. 3 shows in detail the effects of continuous background irradiance on F_p, F_s and $\Delta\phi_{70}$ in all five of the algae studied. In addition, Fig. 3 presents the relative rates of steady-state photosynthetic oxygen production (P/P_{max}) over the same range of continuous irradiance. When cells are first exposed to a new background irradiance, they exhibit a transient period, lasting 2–3 min, during which F_p rises and then falls to a new steady-state level. Transients are observed even when cells previously exposed to irradiance levels which saturate steady-state oxygen production are exposed to still greater irradiance. All the data shown in Fig. 3 were obtained following the transient period.

Although the relationships among F_p, F_s, $\Delta\phi_{70}$, and background irradiance are complex, four general irradiance regimes are apparent. We shall refer to these as the low, moderate, high and very high light regimes and will discuss each in turn.

The low-light regime ($J < 10^{13}$ quanta per cm^2 per s)

At irradiances less than about 10^{13} quanta $\cdot$ cm$^{-2} \cdot$ s^{-1}, steady-state oxygen production is not detectable with a Clark O_2 electrode, but $\Delta\phi_{70}$ declines by as much as 25% from the dark-adapted state. The decline in $\Delta\phi_{70}$ results from changes in both F_p and F_s and occurs within 1 s of the onset of illumination. The process is completely reversible; $\Delta\phi_{70}$ reverts to its original dark level in less than 1 s following the end of continuous illumination. The effect is not produced by the pump and probe flash pairs for frequencies between 0.2 and 2 Hz. Since flash pairs excite all RC II once every second (at 1 Hz) and produce no effect, while continuous illumination at 10^{13} quanta $\cdot$ cm$^{-2} \cdot$ s^{-1} excites RC II at a similar rate, we conclude that the absorption cross-section for this effect is very large, at least 10-times that of PS II. The relatively short life-time of the state (under 0.5 s) argues against its arising from the longer-lived photosynthetic intermediates such as Q_B^- or the higher oxidation states of the oxygen-evolving apparatus (e.g., S_2 or S_3).

The low-light, reversible decrease in $\Delta\phi$ was also observed in thylakoid membranes isolated from *D. tertiolecta* in 50 mM Tris-HCl buffer (pH 8.0)/5 mM $MgCl_2$. At $1.8 \cdot 10^{13}$ quanta per cm^2 per s, the change in $\Delta\phi$ was not associated with the phosphorylation of the light-harvesting chlorophyll *a/b* protein by [γ-^{32} P]ATP, nor was it inhibited by 5 mM NaF (Clark, R., personal communication). At 100-fold higher irradiance, a thylakoid protein with an apparent molecular weight of 31 kDa was phosphorylated, which corresponds to the molecular mass of one apoprotein of the light-harvesting chlorophyll proteins in this species. These results suggest that, in *D. tertiolecta* at least, the change in $\Delta\phi_{70}$ at very low continuous irradiance is not directly associated with phosphorylation or dephosphorylation of a chlorophyll-*a/b*-binding protein, a process which is implicated in the State I-State II transition [34,35].

We feel the most likely candidates for the observed low-light-induced decrease in $\Delta\phi_{70}$ are electrochemical potentials (see below), exerted over entire thylakoid membranes, produced by the low rates of light-driven electron transport [36,37].

The moderate-irradiance regime (J ranges from 10^{13} to 10^{15} quanta per cm^2 per s)

Over this 100-fold range of irradiance, steady-state rates for oxygen production are low; increasing to no more than 20% of the light-saturated rate in *Chlorella,* and to less than 5% in the other organisms. We observe two basic types of fluorescence behavior which seem to be related to taxonomic differences among the algae. In the chlorophytes (*Dunaliella,* Fig. 3d; *Chlorella,* Fig. 3e), values for F_p remain constant while values for F_s either decline slightly or are also unchanged. As a result, $\Delta\phi_{70}$ varies by less than 5% over this range of background irradiance.

In contrast, a different fluorescence behavior appears to be typical of the diatoms (*Skeletonema,* Fig. 3a; *Thalassiosira*, Fig. 3b), chrysophytes (*Isochrysis,* Fig. 3c), and dinoflagellates (data not shown) (i.e., the chlorophyll-*c*-containing organisms). In these algae, both F_p and F_s increase with increasing irradiance. The rise in F_p is more rapid than the rise in F_s so that $\Delta\phi_{70}$ declines substantially over the range of background irradiance.

The high-irradiance regime

This irradiance regime extends from about 10^{15} to about $2 \cdot 10^{16}$ quanta $\cdot$ cm$^{-2} \cdot$ s^{-1} in the chloro-

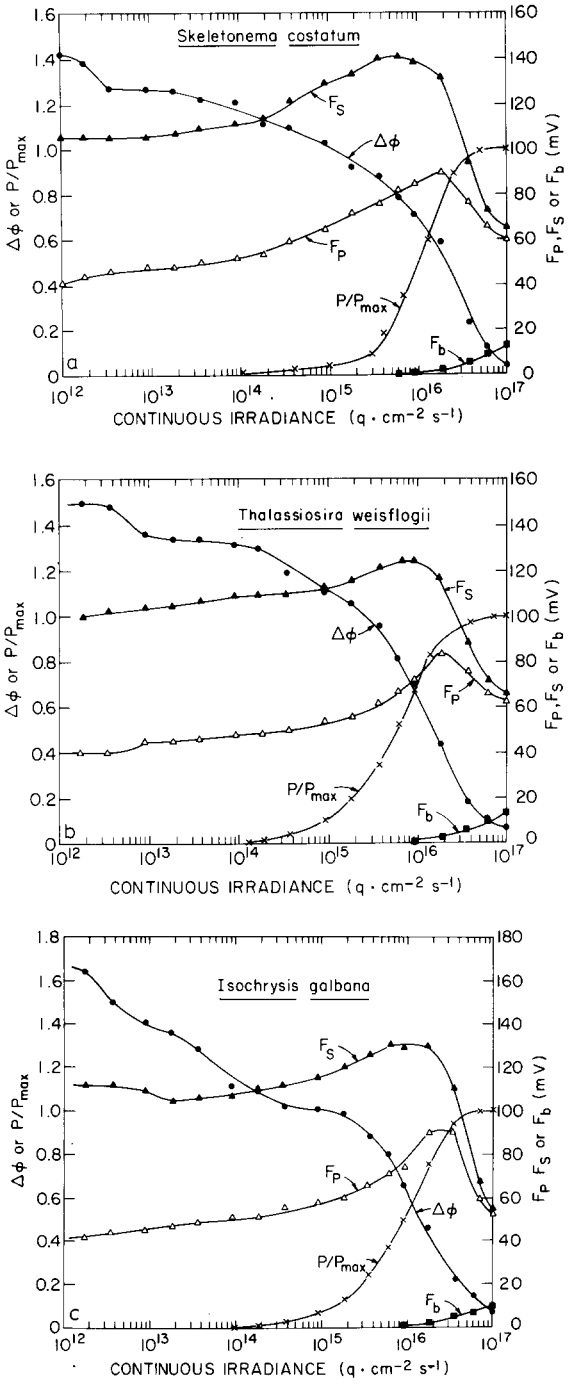

Fig. 3. Effects of continuous background irradiance on F_p (△), F_s (▲), $\Delta\phi$ (●) and P/P_{max} (×) in various species. Fluorescence from the background source (F_b) is also shown (■). $\Delta\phi$ was measured at 18°C with a 70 μs time delay.

phyll-*c*-containing cells or to about $5 \cdot 10^{16}$ quanta $\cdot\, cm^{-2} \cdot s^{-1}$ in the chlorophytes. The difference in the upper boundary of this regime between the two types of cell is probably a reflection of a greater absorption cross-section for the blue background light enjoyed by the chlorophyll-*c*-containing algae.

Over this range of irradiance, the steady-state rates for oxygen production increase from less than 10% to more than 90% of their final light-saturated values. At the same time, $\Delta\phi_{70}$ decreases, primarily due to the rapid increase in F_p relative to F_s. In the chlorophyll-*c*-containing organisms (and perhaps in *D. tertiolecta* as well), F_s continues to show a slow increase as background irradiance increases.

The changes in the fluorescence properties of the cells are perhaps easiest to interpret in this high-irradiance regime. We attribute the precipitous decline in $\Delta\phi_{70}$ and rise in F_p with increasing background irradiance as reflecting prim-

arily an increasing probability of finding RC II in the closed state at the time of the probe flash. Superimposed on this, however, are smaller changes, particularly evident in F_s, suggesting changes in fluorescence yields which are unrelated to the redox state of the acceptor of RC II, Q.

Using the inhibitor of the oxygen-evolving complex, NH_2OH, and the uncoupler, CCCP, we examined the effects of electrochemical fields on $\Delta\phi$ and its components. Addition of 1 mM NH_2OH reduced $\Delta\phi_{70}$ to zero but decreased F_p at high background irradiance levels to the value obtained with fully dark-adapted cells. Addition of 100 μM CCCP also restored F_p observed in the high-light regime to the dark-adapted level. Unlike NH_2OH, addition of the uncoupler did not markedly influence $\Delta\phi_{70}$. We conclude from these observations that the light-induced increases in F_p and F_s we observe in the chlorophyll-*c*-containing organisms arise from changes in fluorescence yield resulting from additional changes in ΔpH or $\Delta\Psi$ across the thylakoid membranes in response to changes in light-driven electron transport [15,17].

The very high irradiance regime

At these very high irradiance levels (about $2 \cdot 10^{16}$ quanta $\cdot$ cm$^{-2} \cdot$ s^{-1} for the chlorophyll-*c*-containing organisms and about $5 \cdot 10^{16}$ quanta $\cdot$ cm$^{-2} \cdot$ s^{-1} for the chlorophytes), steady-state rates of oxygen evolution reach their light-saturated levels. We find no evidence of photoinhibition at these illumination levels. $\Delta\phi_{70}$ continues to decline but is still non-zero at the highest irradiances shown in Fig. 3. We could not provide background irradiance levels high enough to drive $\Delta\phi_{70}$ to zero.

Remarkably, at these highest irradiances, in all five organisms, both F_p and F_s decline precipitously. F_p does not decrease as rapidly as F_s and as a result $\Delta\phi_{70}$ continues to decline. We can qualitatively interpret this fluorescence behavior as the result of a quenching state generated in RC II which have been multiply hit during the time-course of primary photochemistry (vide infra). We emphasize that, although in all cases the onset of this fluorescence quenching corresponds to onset of light saturation of oxygen production, the quenching state is not related to any kind of photoinhibition of oxygen production.

At these high irradiance levels, addition of the herbicide DCMU has an interesting effect. If cells are incubated for 5 min in absolute darkness with 10 μM DCMU both F_p and F_s levels are increased by 20–30% of the values obtained with dark-adapted cells in the absence of the herbicide. If DCMU is added during illumination at irradiances chosen to give the maximum possible values for F_s, F_s measured in the presence of the inhibitor is increased by an additional 30%. When the herbicide is added at irradiance levels where marked quenching of F_s is observed, the values for F_s then observed is the same high value found if DCMU is added at lower, nonquenching irradiance levels. It appears that the fluorescence quenching state seen at very high irradiance requires multiple turnover by RC II.

We urge caution in the interpretation of fluorescence yields measured in the presence of DCMU. In many measurements, variable fluorescence yields are often taken as $F_{DCMU} - F_0$. In induction measurements, F_0 is often difficult to measure in the presence of DCMU and is therefore often measured prior to the addition of the herbicide, while F_{DCMU} is determined after incubation with the inhibitor. Under such circumstances the variable yield can be overestimated since F_{DCMU} is significantly larger than F_s. In addition, while DCMU is known to bind to the Q_B protein, it may have other binding sites which can affect fluorescence yields. Additionally, even in the presence of DCMU, Q may turn over, albeit at much lower rates (about 1000-fold) than in uninhibited systems. Finally, incubation with DCMU prevents the reduction of plastoquinone and may cause cells to become 'State I', which is a high-fluorescence-yield state [37].

Fluorescence quenching by continuous light at high irradiance

Fig. 4 outlines a simple kinetic scheme which as a working model qualitatively describes the fluorescence quenching we observe at very high irradiance. Light energy is received by RC II at a rate σJ, where σ is the effective absorption cross-section and J is the incident irradiance. Initial excitation of RC II rapidly produces the charge-separated state, Z^+PIQ^-, which can return to ZPIQ via the intermediate states $ZPIQ^-(k_1, k_3)$ or $Z^+PIQ(k_2, k_4)$. Excitation of Z^+PIQ^- or $ZPIQ^-$

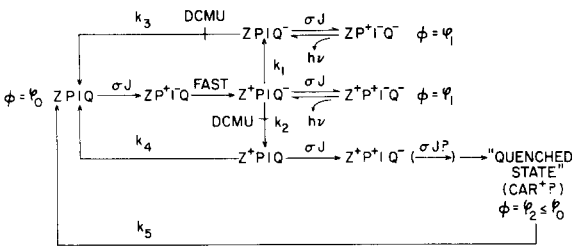

Fig. 4. RC II is represented as ZPIQ where: Z = secondary donor; P = primary donor (P-680); I = intermediate acceptor (phaeophytin); Q = primary acceptor (plastoquinone). Light energy is received by RC II at a rate σJ, where σ is the effective absorption cross section and J is the incident irradiance. Initial excitation of RC II rapidly produces the charge separated state, Z^+PIQ^-, which can return to ZPIQ via the intermediate states $ZPIQ^-$ (k_1, k_3) or Z^+PIQ (k_2, k_4). Excitation of Z^+PIQ^- or ZPIQ results in the high-fluorescence-yield state ($\phi = \psi_1$) due to recombination luminescence from long-lived P^+I^- and a fluorescence-quenching state ($\phi = \psi \leq \psi_0$) is generated in subsequent reactions, perhaps requiring the input of a third exciton. The 'quenched state' may involve the oxidation of a carotenoid associated with RC II [39]. The quenched state eventually returns to ZPIQ (k_5). Note that in the presence of DCMU, the state Z^+PIQ cannot be formed.

results in the high-fluorescence-yield state ($\Phi = \phi_1 > \phi_0$) due to recombination luminescence from long-lived P^+I^- [38]. Excitation of the state Z^+PIQ leads to a fluorescence quenching state ($\Phi = \phi_2 \leqslant \phi_0$) in subsequent reactions, perhaps requiring the input of a third excitation (see below). The quenching state eventually returns to ZPIQ (k_5).

The generation of the quenching state requires continuous light (or a long flash). It cannot be formed by even a supersaturating single-turnover flash because of the delay time $1/k_2$ to form Z^+PIQ. The formation of the latter state may be inefficient, as its yield is given by $k_2/(k_1 + k_2)$. Thus repeated excitation may be necessary to accumulate the quenching state. In addition, a series of short, saturating flashes will not generate the quenching state if the time interval between flashes exceeds $1/k_4$. Finally, in the presence of DCMU the state Z^+PIQ cannot form, and so neither can the long-lived quenching state. These properties are just those we observe in our experiments.

Calculations of the fluorescence yield from the probe flash alone (F_p) or from the probe flash preceded (70 μs) by a saturating pump flash (F_s) in the presence of continuous light using steady-state approximations for the various intermediates produces curves having the same general shapes as those shown in Fig. 3. However, the calculated curves tend to be too broad and, at high irradiances, decrease more slowly than do the data. Better fits can be obtained by assuming an I^2 dependence for the formation of the quenching state (Fig. 4), suggesting that two or more excitations in rapid sequence are required to form the quenching state from Z^+PIQ.

Relationship between photosynthesis and fluorescence

The relationship between normalized steady-state photosynthetic oxygen evolution and normalized $\Delta\phi_{70}$ is shown for all five organisms in Fig. 5 for comparison. The low-light-induced decline in $\Delta\phi$ varies between species and with length of dark-adaptation. In order to scale $\Delta\phi$ for all species objectively, we normalized $\Delta\phi$ to 1.0 at continuous irradiance levels supporting a P/P_{max} of 0.01 ($= \Delta\phi'$). Qualitatively, the relationship is the same for all the algae; however, there are some signifi-

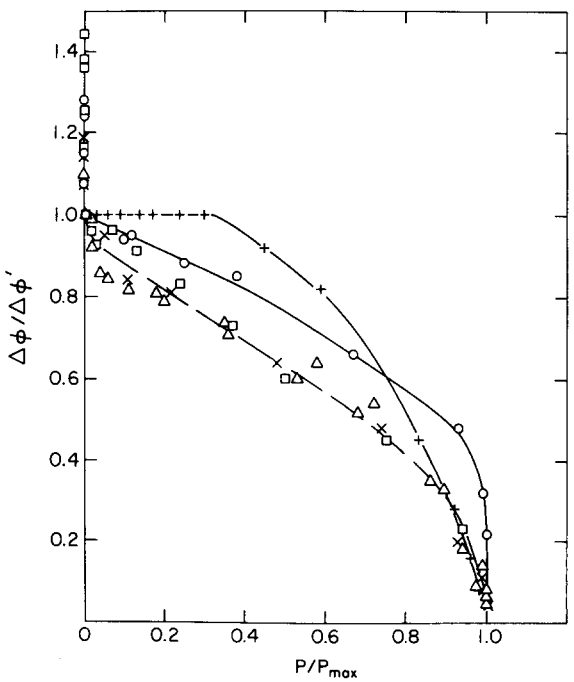

Fig. 5. The relationship between $\Delta\phi/\Delta\phi'$ and P/P_{max} under steady-state conditions at 18°C for the five algal species. $\Delta\phi$ was normalized to 1.0 at $\Delta\phi$ corresponding to $P/P_{max} = 0.01$. *S. costatum* (×), *I. galbana* (□), *T. weisflogii* (△), *D. tertiolecta* (○), *C. vulgaris* (+).

cant quantitative differences. The relationship between normalized $\Delta\phi_{70}$ and P/P_{max} is clearly non-linear. Between 5 and 30% of the change in variable fluorescence occurs at low irradiance, where photosynthetic oxygen production is negligible. We have argued that the low-light-induced changes in $\Delta\phi_{70}$ result primarily from changes in the inherent quantum yield of fluorescence brought about by the formation of transmembrane chemical or electrical potentials. At higher irradiances, particularly in the chlorophytes, $\Delta\phi_{70}$ remains larger than perhaps might be anticipated. The data shown in Fig. 5 suggest that as light intensities sufficient to saturate photosynthetic oxygen production, 10–20% of the total change in $\Delta\phi_{70}$ remains. If this residual $\Delta\phi_{70}$ represents the presence of Q in the oxidized state, it is possible that, at high irradiance, the oxidized precursors for oxygen production are reduced by something other than H_2O. In this case, the observation that RC II appear 'closed' on the donor side and 'open' on the acceptor side simultaneously could imply that reducing equivalents on the acceptor side of PS II compete with water for the reduction of electron carriers on the donor side of PS II; i.e., there is cyclic electron flow around PS II at high irradiance in vivo, which is especially pronounced in the chlorophytes [33].

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