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The relationship between heat-stress and photobleaching in green and blue-green algae[1]

DAVID C. FORK,[2] ARINDAM SEN[3] and W. PATRICK WILLIAMS[4]

Carnegie Institution of Washington, Department of Plant Biology, 290 Panama Street, Stanford, CA 94305, USA

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Abbreviations: PSI, photosystem I; PSII, photosystem II, Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; DCMU, 3-(3'4' dichlorophenyl)-1'1'-dimethylurea, PC, plastocyanin; APC, allophycocyanin.

Abstract. Two characteristic temperatures were identified from measurements of the temperature dependence of O_2 evolution by *Chlorella vulgaris* and *Anacystis nidulans:* T_1, the threshold temperature for inhibition of O_2 evolution under saturating light conditions, and T_2, the upper temperature limit for O_2 evolution. Measurement of delayed light emission from photosystem II (PSII) showed that it passed through a maximum at T_1 and was virtually eliminated on heating the samples to T_2. Related changes were observed in low-temperature (77K) fluoresence emission spectra. Heat-stress had little effect on the absorption properties of the cells at temperatures below T_1 but incubation at higher temperatures, particularly under high-light conditions, resulted in extensive absorption losses. An analysis of these measurements suggests that this increased susceptibility to photobleaching is triggered by an inhibition of the flow of reducing equivalents from PSII that normally serves to protect the light-harvesting apparatus of the cells from photo-oxidation. Adaptation to higher growth temperatures resulted in increases in the values of T_1 and T_2 for *Anacystis nidulans* but not for *Chlorella vulgaris.*

Introduction

Incubation of leaves, chloroplasts isolated from higher plants and algal cells at temperatures 20–30 °C above their normal growth temperatures leads to a marked inhibition of their CO_2 fixation and O_2 evolution capabilities [3, 18].

It is well established that the primary site of heat-damage in isolated chloroplasts is the PSII light-harvesting apparatus. Photosynthetic O_2 evolution has long been known to be particularly sensitive to heat damage [13].

[1] CIW-DPB Publication No. 887.
[2] To whom reprint requests should be addressed.
[3] Present address: Department of Biophysics, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263, USA.
[4] Present address: Department of Biochemistry, Kings College London, (KQC), Campden Hill, University of London, London W8 7AH, UK.

The relative inefficiency of substitute electron donors to restore electron transport through PSII [7, 13], indicates that the primary lesion lies close to the PSII reaction center rather than in the O_2 evolution enzyme system. Detailed analyses of heat-induced changes in chl *a* fluorescence emission performed by Schreiber and his co-workers [24–26], have led to the suggestion that heat-stress leads to a breakdown of interactions between components of the light-harvesting apparatus of PSII. This view has subsequently been confirmed by freeze-fracture electron microscopy of heat-stressed chloroplasts which indicate the occurence of a physical dissociation of the supramolecular complex constituting this apparatus [2, 8].

Exposure of chloroplasts [11, 12, 21–23] or algae [1, 6, 9, 28, 29] to excessive light intensities leads to a photo-inhibition of PSI and PSII activity and to a photobleaching of pigments. Detailed studies indicate that two or more processes are involved. PSII inhibition has been shown to proceed equally efficiently in the presence or absence of O_2 while PSI inhibition and photobleaching requires the presence of O_2 [22]. Recent measurements in this laboratory [30] show that the susceptibility of isolated chloroplasts to photobleaching is dramatically increased by incubation at higher temperatures. The threshold temperature for this increase coincides closely with that for thermal inhibition of PSII. Measurements of absorption losses on bleaching and changes in the electrophoretic pattern of the unbleached Chl-protein complexes, however, revealed the preferential bleaching of PSI-related pigments. Similar changes are seen in chloroplasts in which electron transport through PSII has been inhibited by the addition of DCMU [20, 30], suggesting that PSI is normally protected from photo-oxidative damage by the flow of reducing equivalents from PSII and that damage is triggered by the cessation of this flow.

In this paper, we demonstrated that a similar correlation exists for algal cells and that the threshold temperature for photobleaching, in the case of *Anacystis nidulans* at least, can be raised by adaptation of the algae to higher growth temperatures.

Materials and methods

Algae cultures

Anacystis nidulans (*Synechococcus leopolensis*) TX20 (UTEX 625, Collection of Algae, University of Texas, Austin, TX, 78712) was grown at 26° and 39 °C in the nutrient medium described by Siva et al. [27]. *Chlorella vulgaris* (high temperature strain, 11 h) from the Collection of Algae of the University of Göttingen, Germany was grown in the medium of Hogetsu and Miyachi [10]. In both cases, the cells were aerated with 3% CO_2/air and the cells harvested in the logarithmic growth phase.

Incubation procedures

4 ml aliquots of algal cells (20–30 $\mu g \cdot ml^{-1}$ chl) suspended in growth medium were incubated for 15 min at different temperatures and then cooled rapidly to room temperature for assay purposes. Photobleaching was carried out at the desired incubation temperature using a 300 W Xenon lamp (Cermax Illuminator, ILC Technology, Sunnyvale, CA) as an actinic source. Balzers neutral-density filters were used to attenuate the incident intensity of the actinic beam and a Balzers Calflex C heat-reflecting filter, in combination with a series of water filters, used to remove infra-red radiation. Light intensities were measured using a quantum radiometer (Licor, Inc. Model LI-185B).

Oxygen evolution measurements

O_2 evolution rates were measured using a Clark-type O_2 electrode (Rank Bros., Cambridge, England). All measurements were made under light-saturating conditions using a 500 W tungsten-filament lamp providing an incident intensity of 6000–7000 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Absorption and fluorescence measurements

Absorption spectra were collected and stored using a computer-based system connected to a Cary 17 spectrophotometer fitted with a scattered transmission accessory. Low temperature (77 K) fluorescence emission spectra were measured using a micro-processor based spectrophotometer equipped with a trifurcated fiber-optic bundle [4]. Fluorescence was excited using blue-green actinic light defined by using two Corning CS 4-96 filters and a Balzers Calflex C heat-reflecting filter. PC extracted from *Synechococcus lividus* was added to the samples as a fluoresecent standard in measurements made on *Chlorella* cells. The fluorescence spectra were corrected on a quantum basis using values obtained with a standard lamp.

Delayed light emission (DLE) in the millisecond range was measured as a function of temperature using a Becquerel type phosphoroscope as described previously [5]. Blue light (442 nm) from a HeCd laser (Liconix, Model 4240) provided the actinic beam which was interrupted by a rotating sector that produced light and dark cycles of 0.8 and 5.9 ms respectively. The samples were illuminated by the actinic light for about 20 min to allow DLE to reach a stationary level before the temperature was increased from low to high at a rate of about 1 °C/min.

Results

Oxygen evolution

Samples of a high-temperature strain of the green alga *Chlorella vulgaris*, cultured at 23 °C or 33 °C, and the thermophilic blue-green alga *Anacystis*

nidulans, cultured at 26 °C or 38 °C, were incubated for 15 min at elevated temperatures. The algae were then cooled to temperatures close to their original growth temperatures and their rates of O_2 evolution measured under light-saturating conditions. The results of these measurements are presented in Figure 1 and 2. In each case, a critical temperature can be identified below which incubation had little or no effect and above which there is a dramatic reduction in O_2 evolution efficiency. A second characteristic temperature can also be identified, usually about five degrees higher, above which the algae show a complete loss of O_2 evolution capability. For convenience, we shall refer to these two temperatures as T_1 and T_2 respectively. The estimated values of T_1 for *Chlorella* grown at 23 ° and 33 °C were 45 ° and 43–45 °C respectively while T_2 was estimated to be 49 °C for both growth temperatures. The corresponding values for *Anacystis* grown at 26 ° and 38 ° were 48–49 ° and 51–51 °C for T_1 and 53–54 ° and 56 °C for T_2.

Photobleaching

Typical absorption spectra of *Chlorella* and *Anacystis* samples exposed to high intensity white light ($6{,}700\,\mu E\,.\,m^2\,.\,s^{-1}$) at different temperatures are shown in Figures 3 and 4.

Chlorella cells, grown at 23 °C, showed little change in absorption on illumination at temperatures below about 50 °C. However, as the incubation temperature is raised above this value, there is a progressive loss of absorption, particularly in the region of the red absorption maximum (Figure 3a). Subtraction of the absorption spectra of such samples from those of unheated controls indicates that photobleaching is most marked at longer wavelengths (Figure 3b). The difference spectra calculated in this way reflect a combination of absorption changes arising from the heat treatment and photobleaching. In order to separate these two effects, further difference spectra were calculated. The absorption spectra of samples heated in the dark were first subtracted from those of unheated controls to yield the changes attributable to heat alone (Figure 3c). The absorption spectra of samples heated in the light were subtracted from those of the corresponding samples heated in the dark to yield the effects attributable to light alone (Figure 3c).

The samples incubated in the dark above preferential losses of absorption by a long-wavelength Chl *a* component with an absorption maximum at about 680 nm. Exposure to strong white light during incubation resulted in additional losses of absorption by a shorter wavelength Chl *a* component, absorbing maximally at about 670 nm, and Chl *b* absorbing maximally at about 650 nm.

In the case of heat-stressed *Anacystis,* the main absorption changes are associated with PC, absorbing maximally at about 625 nm, and Chl *a*, absorbing maximally at about 680 nm (Figure 4). Exposure of the samples to strong white light at temperatures above 50 °C, however, led to marked losses of both Chl *a* and PC absorption (Figure 4b). Difference spectra obtained

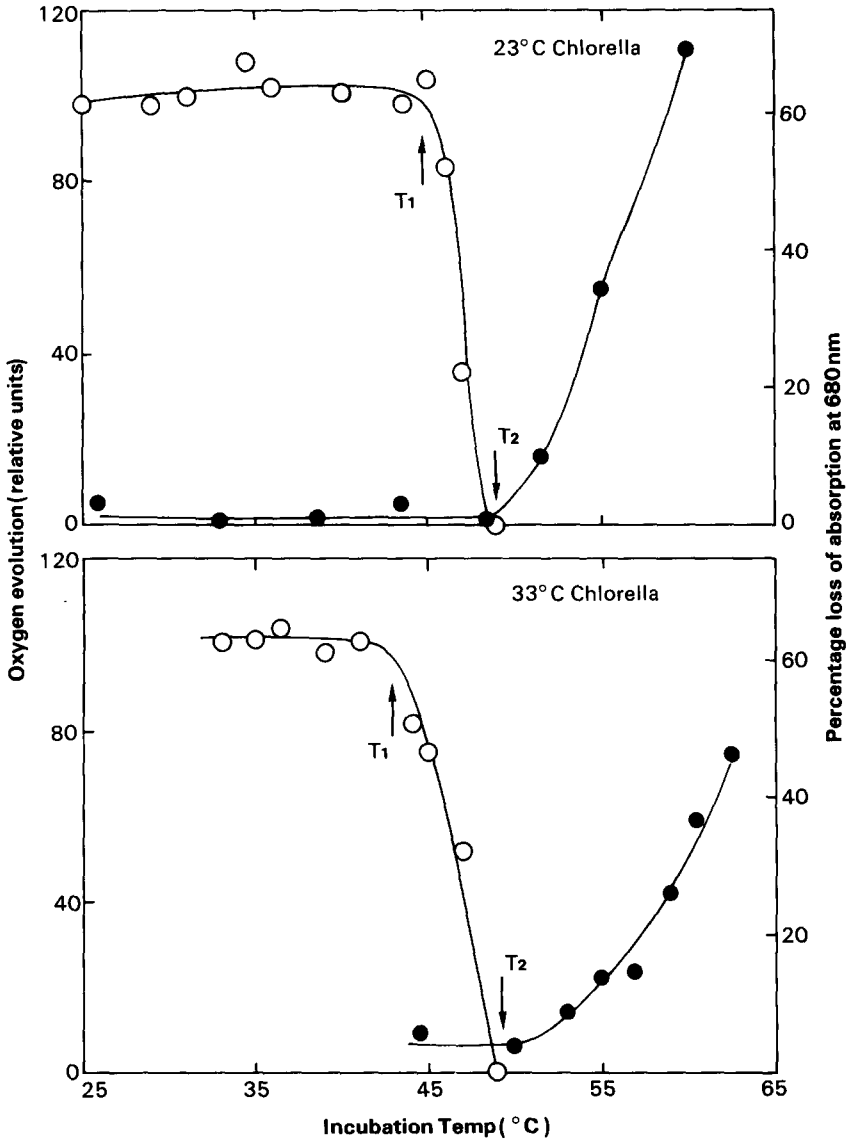

Figure 1. The dependence of O_2 evolution (open circles) and the percentage loss of absorption at 680 nm (closed circles) in *Chlorella*, cultured at 23° and 33 °C, as a function of incubation temperature. See text for measurement details.

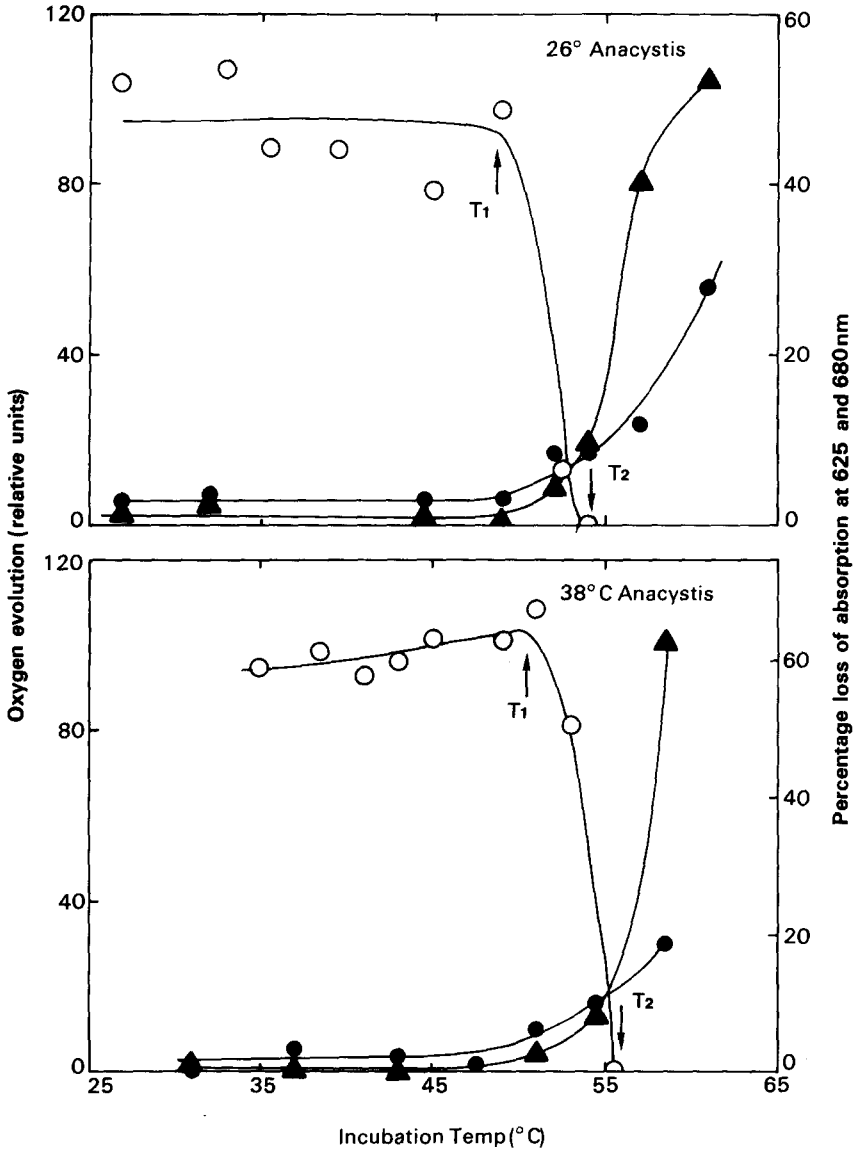

Figure 2. The dependence of O_2 evolution (open circles) and the percentage loss of absorption at 680 nm (closed circles) and 625 nm (open triangles) in *Anacystis*, cultured at 26° and 38 °C, as a function of incubation temperature. See text for measurement details.

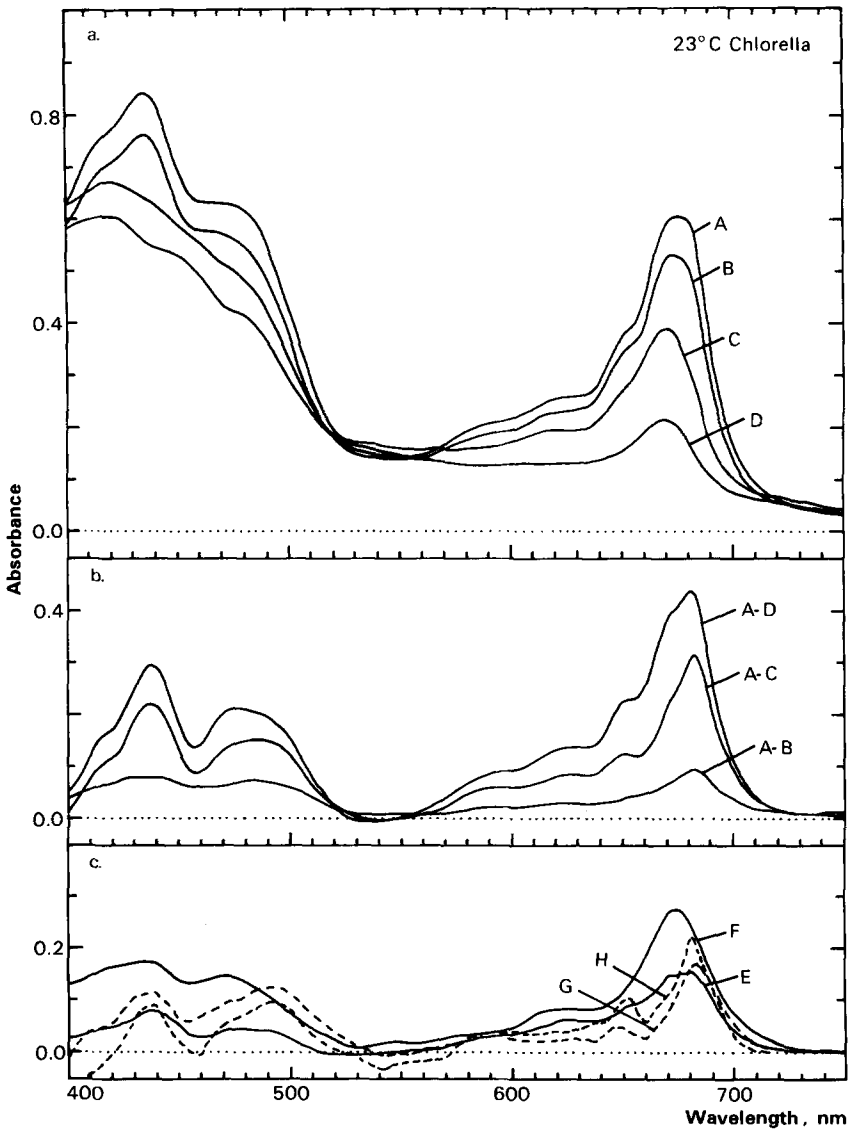

Figure 3. (a) Absorption spectra of *Chlorella*, cultured at 23 °C, measured after 15 min heat treatment in the dark at 51.5 °C (A), in the light at 51.5 °C (B), in the light at 55 °C (C) and in the light at 60 °C (D). (b) Difference spectra calculated by subtraction of the samples incubated in the light from the dark control. (c) Difference spectra calculated by subtraction of the spectra of samples heated in the dark at 55 °C (E) and 60 °C (F) from those of unheated controls and the spectra calculated by subtraction of the spectra of samples heated in the light from those of samples heated in the dark at 55 °C (G) and 60 °C (H).

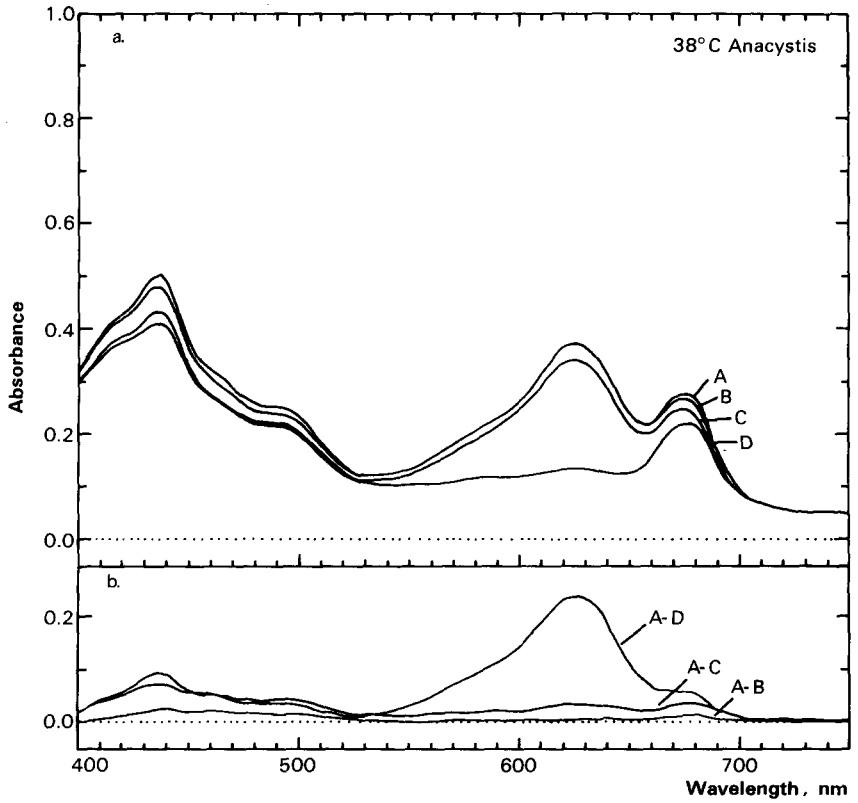

Figure 4. (a) Absorption spectra of samples of *Anacystis*, cultured at 38 °C, following 15 min heat treatment in the dark at 51.5 °C (A), in the light at 51.5 °C (B), in the light at 54.5 °C (C) and in the light at 58.5 °C (D). (b) Difference spectra calculated by subtraction of the samples incubated in the light from the dark control.

by subtracting dark heated samples from dark controls showed no significant effects of heat alone.

The temperature dependencies of absorption loss at 680 nm of *Chlorella* and *Anacystis* samples exposed to high light intensities are compared to the corresponding temperature dependencies for loss of O_2 evolution ability in Figures 1 and 2. There is a close correlation between the threshold temperatures for absorption loss and those defining the upper limits for efficient O_2 evolution for both algae. In the case of *Chlorella*, little or no absorption loss is seen below T_2, the temperature above which O_2 evolution completely ceases. A sharp rise in absorption loss at T_2 is also seen for *Anacystis*. There

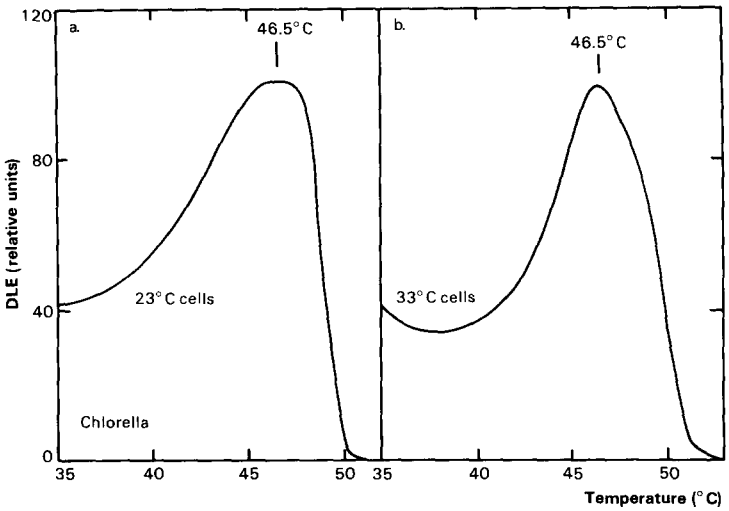

Figure 5. The temperature dependence of DLE in *Chlorella* cells grown at (a) 23 °C and (b) 33 °C.

is, however, some indication that T_1, the temperature at which O_2 evolution efficiency begins to decline, correlates rather better with the threshold temperature for absorption loss in the blue-green algae.

Delayed light emission (DLE)

Earlier studies performed in this laboratory [5] have shown that the ability of higher plant chloroplasts and algal cells to resist heat stress can be estimated from measurements of the temperature dependence of their DLE. Typical examples of such measurements for the *Chlorella* and *Anacystis* cells used in the present investigation are shown in Figures 5 and 6. In each case, the DLE signal shows a high temperature maximum followed by a dramatic reduction in the signal to nearly zero. The temperatures at which these maxima occur correspond closely to the values for T_1 for the different samples and DLE is, in all cases, effectively eliminated at temperatures above T_2.

Low temperature (77K) fluorescence emission

Typical low-temperature fluorescence emission spectra of *Chlorella* and *Anacystis* cells incubated at elevated temperatures are presented in Figures 7 and 9. Both algae show the three Chl emission peaks [16]; two shorter wavelength peaks near 687 and 696 nm (F687 and F696), associated with PSII, and a longer wavelength peak associated with PSI that is centered

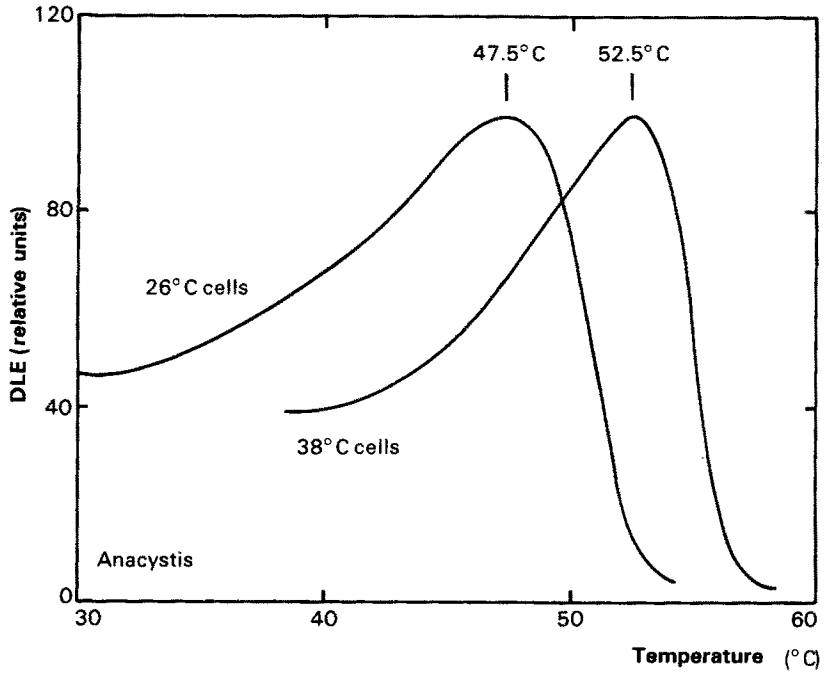

Figure 6. The temperature dependence of DLE in *Anacystis* cells grown at 26 °C and 38 °C.

near 728 nm (F728) in *Chlorella* and 720 nm (F720) in *Anacystis*. In the case of *Anacystis*, there is an additional peak at about 650 nm (F650) associated with PC and APC.

Incubation of samples of *Chlorella* in the dark at temperatures below about 45 °C had little or no effect on their low temperature fluorescence emission. Exposure of the samples to high-intensity white light, however, leads to marked reductions in F687, F696 and F728 (Figure 7). Measurements of the temperature dependences of F687 and F728 emission are presented in Figure 8. They indicate that the effect of illumination tends to reduce as the incubation temperature approaches T_1. Incubation at temperatures above T_1 leads to dramatic reductions in F687 but F728 only begins to fall at incubation temperatures above T_2.

A broadly similar pattern of changes is seen for *Anacystis*. As with *Chlorella*, exposure to light intensities at temperatures below T_1 leads to losses in emission in all three chl *a* bands (Figure 9). Increasing the incubation temperature leads to dramatic increases in F687 which more than doubles its value over the very narrow temperature range between T_1 and T_2 (Figure 10).

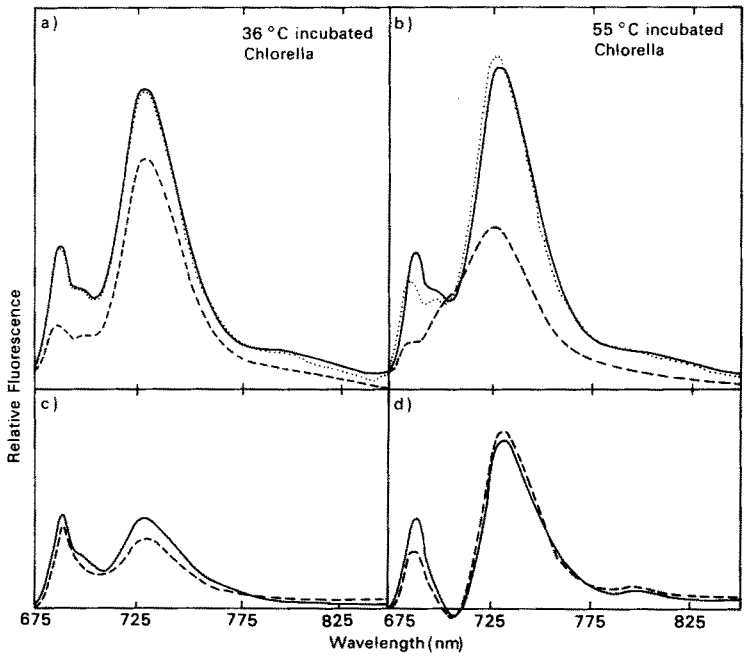

Figure 7. Typical low temperature (77K) fluoresence emission spectra of *Chlorella* cultured at 33 °C, before and after 15 min heating at (a) 36 °C and (b) 55 °C. Solid lines, unheated controls; dotted lines, cells heated in the dark; dashed lines, cells heated in the light. The difference spectra given in panels c and d are for unheated controls minus heated in the light (solid lines) and heated in the dark minus heated in light (dashed lines).

At the same time, the F696 band is shifted to slightly shorter wavelengths. PC/APC emission (F650) increases over the same temperature range but less sharply. Exposure to strong light rapidly reverses these increases resulting in a bleaching at all wavelengths.

Discussion

The present experiments demonstrate that algal cells show characteristic temperatures above which their photosynthetic pigments become increasingly susceptible to thermal and light-induced damage. They also show that these threshold temperatures are closely related to the temperature at which the efficiency of O_2 evolution becomes impaired by heat-stress (Figures 1 and 2) suggesting that this increased susceptibility might be associated with damage to PSII.

Further evidence for the breakdown of the PSII light-harvesting apparatus

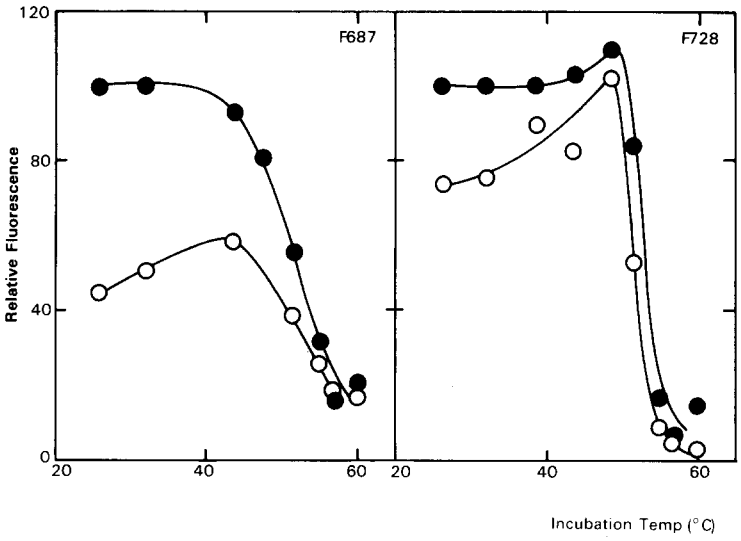

Figure 8. Fluoresence emission at 687 and 728 nm measured at 77K in *Chlorella* cultured at 33 °C. Cells were heated at different temperatures for 15 min in the dark (solid circles) or in the light (open circles) prior to freezing and measurement of fluorescence.

in heat stressed algal cells is provided by measurements of changes in DLE shown in Figures 5 and 6 and the low-temperature fluorescence emission measurements presented in Figures 7–10. The correlation seen between the fluorescence changes seen at 77K and the changes in DLE presumably reflects the fact that a major rearrangement of PSII occurs at a critical temperature for each plant studied. DLE, which originates from PSII [14], peaks at temperatures close to T_1, the upper limit of efficient O_2 evolution, and then decreases dramatically at higher temperatures in parallel with the losses of O_2 evolution, finally disappearing at temperatures close to T_2, the upper temperature limit for O_2 evolution. Similar correlations are seen for low-temperature fluorescence emission. In general, exposure to high light intensities at temperatures below T_1, leads to a reduction in F687 and F696 in both *Chlorella* and *Anacystis*, probably as a result of the formation of quenching centers. As the incubation temperature approaches T_1, this trend is reduced. In the case of *Anacystis*, there is even a net increase in F687 emission with respect to unheated samples (Figure 10). These increases probably reflect a dissociation of the bulk Chl *a* pigment from the PSII reaction center. They are rapidly reversed at higher temperatures where emission from both photosystems drops as the photosynthetic pigments become increasingly sensitive to thermal and light-induced degradation. The increase of F687 in *Anacystis* may also reflect an interruption of energy

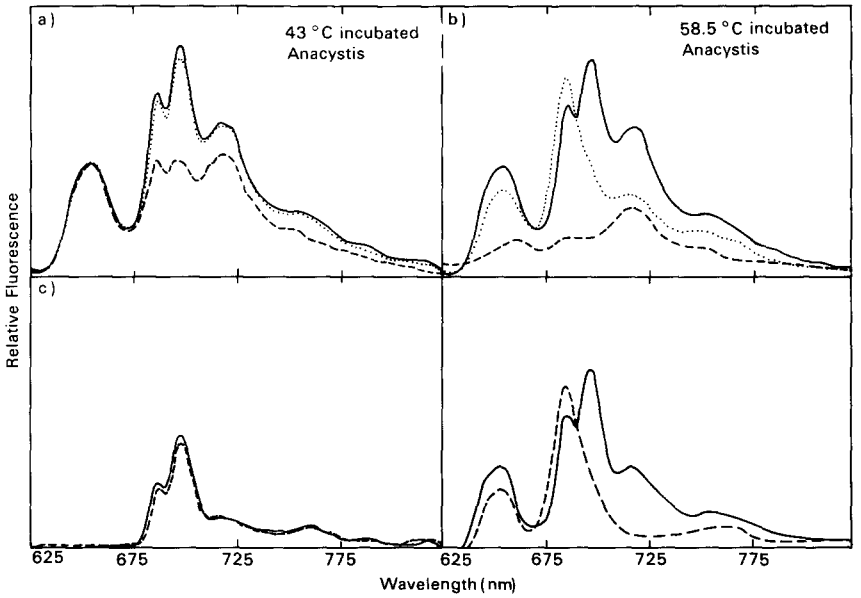

Figure 9. Typical low temperature (77K) fluorescence emission spectra of *Anacystis*, cultured at 38 °C, before and after heating at (a) 43 °C and (b) 58.5 °C. Solid line, unheated controls; dotted line, cells heated in the dark; dashed line, cells heated in the light. The corresponding difference spectra calculated as described in the legend to Figure 8, are shown in panels (c) and (d).

Table 1. Temperature characterizing heat-induced changes in *Chlorella* grown at 23° and 33 °C

Parameter	Temperature	
	23 °C *Chlorella*	33 °C *Chlorella*
Onset of inhibition of O_2 evolution (T_1)	45°	43–45°
Cessation of O_2 evolution (T_2)	49°	49°
* DLE maximum	46.5°	46.5°
DLE Abolition	50°	52°
Photobleaching threshold	48°	50°
F687 loss	40–43°	41–43°
F728 loss	48°	49°

* DLE = delayed light emission

flow from phycobilins to Chl *a* most probably at the level of the long wavelength form of APC [15].

Summaries of the critical temperatures for inhibition of O_2 evolution, loss of absorption and the changes in DLE and low-temperature fluorescence emission reported here are given in Tables 1 and 2 for *Chlorella* and *Anacystis* respectively.

Table 2. Temperatures characterizing heat-induced changes in *Anacystis* grown at 26 °C and 38 °C

Parameter	Temperature	
	26 °C *Anacystis*	38 °C *Anacystis*
Onset of inhibition of O_2 evolution (T_1)	48–49 °	51–52 °
Cessation of O_2 evolution (T_2)	53–54 °	56 °
* DLE maximum	47.5 °	52.5 °
DLE abolition	53–54 °	57 °
Photobleaching threshold	47–48 °	47–48 °
F687 increase	49–50 °	48–52 °

* DLE = delayed light emission

In the case of *Chlorella,* there appears to be a very good correlation on the one hand between T_1 and the temperature for maximal DLE and, on the other hand, between T_2, the cessation of DLE and the threshold temperatures for inhibition of low temperature fluorescence from PSI and pigment degradation.

In the case of *Anacystis,* there is a similar correlation between losses of O_2 evolution and changes in DLE to that seen for *Chlorella.* The threshold temperature for pigment damage, however, tends to correlate rather better with the value of T_1 than T_2.

Measurements of the absorption losses occurring in heat-stressed cells (Figures 3 and 4) indicate that algae contain a long-wavelength Chl *a* component, absorbing maximally at 680 nm, that is particularly susceptible to thermal and light-induced damage. Similar measurements performed on higher-plant chloroplasts [Sen, Fork and Williams, unpublished observations; 30] have shown the preferential bleaching of a Chl *a* component with the same absorption characteristics which electrophoretic studies show to be a P700/Chl *a* protein, the main light-harvesting pigment of PSI. In general, the changes seen in the algal systems resemble those seen for higher-plant chloroplasts with the exception, in the case of *Chlorella* at least, that the pigments show an enhanced thermal degradation in the dark.

The bleaching seen in algae and chloroplasts is known to reflect oxidative damage and can be largely eliminated by anaerobiosis [22]. The absense of such bleaching at temperatures below T_1 strongly supports the view that electron flow from PSII normally serves to protect PSI pigments from such damage and that it is the cessation of the flow at higher temperatures that triggers oxidation. The greater susceptibility of the photosynthetic pigments of algal cells as opposed to those of chloroplasts isolated from higher-plants probably reflects the presence of higher levels of endogenous oxidants.

The factors governing the thermal stability of PSII, and hence that of the photosynthetic apparatus as a whole, are still not fully understood.

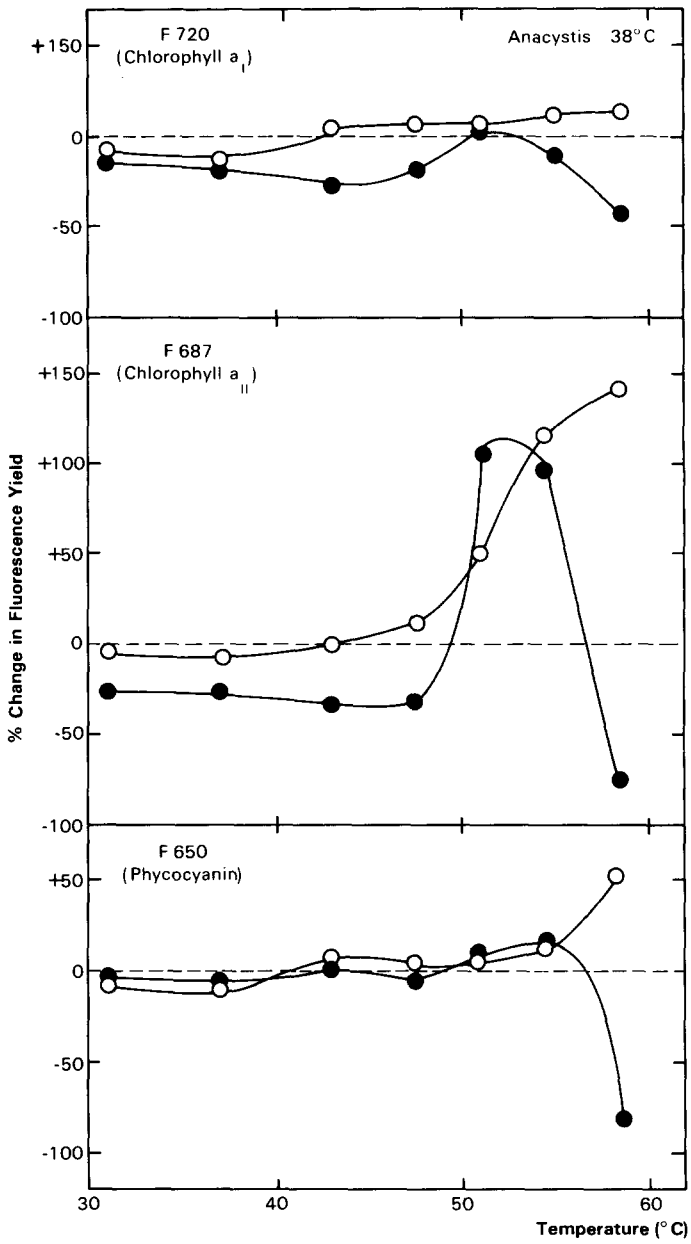

Figure 10. Fluorescence emission at 720, 687 and 650 nm measured at 77K in *Anacystis* cultured at 38 °C. Cells were heated at different temperatures for 15 min in the dark (solid circles) or in the light (open circles) prior to freezing and measurement of fluorescence.

Resistance to heat stress in certain desert plants is known to be improved by adaptation to higher growth temperatures [3, 17, 19]. It is interesting to note in this context that increasing the growth temperature leads to increased values of T_1 and T_2 in the case of *Anacystis* but that the corresponding values for *Chlorella* remain unchanged.

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