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RELATIONS BETWEEN FLUORESCENCE AND THYLAKOID STRUCTURE IN *PORPHYRIDIUM CRUENTUM*

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SUMMARY

The increase in chlorophyll *a* steady-state fluorescence, induced by high NaCl concentration in *Porphyridium cruentum* in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, is directly correlated with a significant decrease in thylakoid thickness. It does not appear affected either by alteration of light absorption due to configurational change or by electron transport processes. Oxygen evolution occurs only in intact structures. The interrelationship between membrane structure, oxygen evolution and chlorophyll *a* steady-state fluorescence is discussed.

INTRODUCTION

Changes in the membrane thickness of the thylakoid of the red alga *Porphyridium cruentum* correlated with changes in the chlorophyll *a* fluorescence intensity have been reported previously [1]. The intensity of chlorophyll fluorescence is found to depend not only upon the redox state of the electron transport components during photosynthesis, but also upon the distance between the pigment molecules and their mutual orientation, thus providing an internal indicator of the physical state of the photosynthetic apparatus.

Chlorophyll *a* molecules in the red alga *P. cruentum* are embedded in the thylakoid membrane while the major accessory pigments, the phycobiliproteins, are localized externally in large granules "phycobilisomes", on the outer surface of the chlorophyll bearing lamellaes [2–5]. As is well substantiated at present, the structure and function of isolated chloroplasts of higher plants depend upon their cationic environment [6–10]. Several reports have appeared recently which correlate conformational changes in chloroplasts [11, 12] or whole cells [13], with changes in chlorophyll fluorescence due to the effect of salts. The results obtained with whole intact cells appear inconsistent with those obtained using chloroplast or chloroplasts fragments.

Abbreviations: DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCIP: 2-6, dichlorophenolindophenol, PMS: phenazine methosulfate, diaminodurene: 2,3,5,6-tetramethyl-1,4-phenylene diamine.

In this communication we report observations on the relationship between the dependence of the rate of the electron flow, the excitation transfer, the solute concentration of the medium, chlorophyll *a* fluorescence and conformational changes in the thylakoid structure.

METHODS

Axenic cultures of *P. cruentum* Naegeli (Indiana University Algal Culture Collection 161) were grown as previously described [14], harvested in the middle of the exponential growth phase and washed with phosphate buffer 0.1 M, pH 7.4. Such cells have a phycoerythrin ($A_{545\ nm}$)/chlorophyll ($A_{676\ nm}$) ratio of 1.3. The following procedure was used for glutaraldehyde fixation. A suspension containing 10^8 cells/ml was fixed with glutaraldehyde (2%) (Fisher Scientific or Polysciences EM grade) for 15 min, at 0 °C. The cells were washed once in 0.2 M methylamine hydrochloride (pH 7.4) to remove unreacted glutaraldehyde and then twice in phosphate buffer. Control experiments showed that this procedure produced material with the same, 2-6, dichlorophenolindophenol (DCIP)-reduction activity and P_{700} light-induced changes, as did the short glutaraldehyde fixation method of Hallier and Park [15].

Cells (either glutaraldehyde fixed or unfixed) were suspended at a concentration sufficient to give an absorbance of 0.3 at 676 nm, the red absorption maximum in vivo of chlorophyll *a*. In our experiments, cells were maintained for 60 min at 0 °C either in distilled water or in NaCl solutions of several different concentrations. The effects of different treatments on the functional parameters to be described below were complete after 60 min of incubation, as tested by incubation periods as long as 120 min.

Measurements of photosynthetic oxygen production were made with a YSI 53 Biological oxygen monitor. Measurements of the light-mediated reduction of DCIP were performed as described by Hallier and Park [15] with the modifications that the concentration of DCIP was monitored at 600 nm, and reaction mixtures were contained in a total volume of 3 ml, pH 7.0, DCIP 0.05 μmole, phosphate buffer 0.1 M 1 ml, and chlorophyll *a* 30–50 μg. Fluorescence emission spectra were measured in steady-state conditions with the instrument built by Dr M. Iwatsubo [16], eliminating the variations arising from fluorescence induction. Emission spectra were scanned using a 8 nm half-band width and corrected for the spectral sensitivity of the photomultiplier and the transmission efficiency of the monochromator, except in Fig. 5. Control experiments showed that suspensions with a tenfold lower absorbance at 676 nm gave spectra of identical shape. Absorption spectra were measured with a Cary 14 spectrophotometer. Scattering errors were minimized by the Shibata [17] technique using opal glass plates; residual absorption at 720 nm was applied as a uniform scattering correction at all wavelengths. Chlorophyll *a* fluorescence time course was measured with the instrument built in our laboratory by Drs J. Lavorel and C. Vernotte. Samples for electron microscopic examinations were fixed with 2% glutaraldehyde (v/v) in phosphate buffer (0.1 M, pH 7.4) at 2 °C for 120 min, followed by fixation with 1% (w/v) osmium tetroxide for 60 min, and then post-stained with lead citrate. They were then dehydrated with ethanol and embedded in Epon–Araldite. Sections were examined in a Hitachi HU 11 A with a high resolution device.

In electron micrographs of cells which contained transversely sectioned thylakoids, the thickness of the thylakoids was measured on negatives with a recording double-beam microdensitometer (MK IIIC, Joyce Loebl and Co). The average unit membrane thickness characteristic of each sample was based on at least 30 separate measurements.

RESULTS AND DISCUSSION

Fig. 1 shows the fluorescence emission spectra of unfixed cells, following excitation with light absorbed by phycoerythrin (544 nm). Irrespective of the ionic environment, two major peaks appeared, one at 656 nm (attributable to phycocyanin) and one at 683 nm (attributable to the chlorophyll *a* in System II); these were always accompanied by a minor peak at 705 nm (attributable to the chlorophyll *a* in System I) [18, 19]. The emission spectra obtained with cells suspended in 0.4, 0.6 and 0.8 M NaCl were identical, and corresponded closely to other fluorescence emission spectra of this organism already published [20]. Since the range of ionic strengths in question corresponded roughly to that in medium of customary growth for this organism, we considered this type of emission spectrum to represent the norm, against which all variations were to be evaluated. In all subsequent experiments, 0.6 M NaCl was used as a standard reference environment.

It can be seen that changes of the ionic environment, outside the range of 0.4–0.8 M NaCl produced major effects of the relative fluorescent peak heights of phycocyanin and chlorophyll *a*. In the curves obtained with cells suspended in distilled water or in 0.2 M NaCl, the fluorescence attributable to phycocyanin was greatly

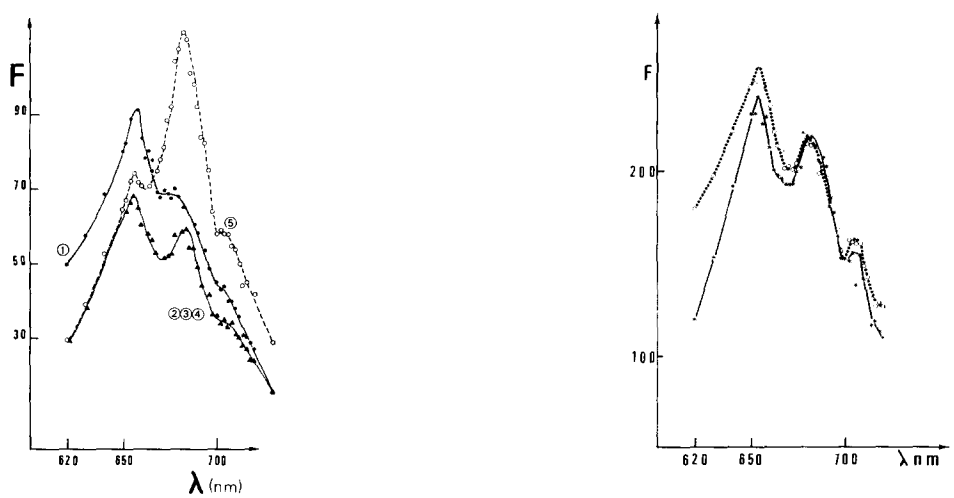

Fig. 1. Fluorescence emission spectra for *P. cruentum* cells ($A^{1\,cm}_{676\,nm} = 0.3$) incubated at 22 °C in: (1) distilled water; (2) 0.4 M NaCl; (3) 0.6 M NaCl; (4) 0.8 M NaCl; (5) 4 M NaCl. λ excitation = 544 nm; excitation intensity = $2.1 \cdot 10^4$ ergs cm^{-2} s^{-1}. F = relative fluorescence intensity in arbitrary units.

Fig. 2. Comparison of fluorescence emission spectra for fixed (○–○) and unfixed (*–*) *P. cruentum* in reference conditions: 0.6 M NaCl; other conditions same as Fig. 1.

increased. On the other hand, in the curves obtained with cells suspended in 1–4 M NaCl, there was a great accentuation of the chlorophyll *a* sensitized fluorescence, increasing with the salt concentration. Fixation of the cells with glutaraldehyde completely abolished the variations in sensitized fluorescence due to NaCl, although fixation did not affect the emission spectrum in the normal conditions, as shown on Fig. 2.

Table I shows the results of densitometric measurements of mean thickness of membrane thylakoid in cells, both fixed and unfixed, that had been incubated in distilled water, in 0.6 and 4.0 M NaCl.

TABLE I

EFFECT OF INCUBATION CONDITIONS UPON THE THICKNESS (HALF-WIDTH), OF THE THYLAKOID MEMBRANE OF UNFIXED OR GLUTERALDEHYDE FIXED *P. CRUENTUM* CELLS.

Average (± S.D.) of at least 30 traces.

Incubation conditions	H_2O	0.6 M NaCl	4 M NaCl
Unfixed cells	60 ± 14 Å	62 ± 9 Å	55 ± 12 Å
Glutaraldehyde fixed cells	63 ± 13 Å	61 ± 9 Å	64 ± 10 Å

Fig. 3 shows typical densitometric traces for two of the conditions listed in Table I and the actual electron micrographs from which these tracing were obtained. A significant difference was observed in the case of unfixed cells incubated in 4.0 M NaCl, the only instance in which the thickness of the thylakoid membrane was reduced significantly.

To establish a direct correlation between the change in the thylakoid structure as measured on the electron microscopic negatives and the increase in the steady-state chlorophyll fluorescence produced by high concentrations of NaCl, it would be necessary to discuss five possible mechanisms which can be related to the fluorescence yield: (a) Change in light absorption due to enhancement of flattening effect. (b) Inhibition of electron transport between Photoreaction II and NADP reduction site. (c) Inhibition of electron transport between Photoreaction II and O_2 evolving site. (d) Change in excitation transfer from phycobilins to chlorophyll *a*. (e) Modification of excitation transfer from Pigment System II to I.

The absorption spectra recorded at room temperature of the same initial concentration of algae after incubation in 0.6 M or 4 M NaCl (Fig. 4) shows a slight progressive "flattening effect" [21], which stabilizes after 1 h incubation. The highest observed decrease (1%) in light absorbed at 544 nm in the presence of 4 M NaCl cannot be responsible for the increase in sensitized chlorophyll *a* fluorescence. In the same way, the increased chlorophyll *a* fluorescence directly excited at 440 nm, (shown below), cannot be attributable to an increased light absorption at that wavelength.

Turning off excitation light for short or long periods during the time course failed to change fluorescence field; thus illumination had no influence on the fluorescence change induced by NaCl. In order to assess the influence of Photoreaction II on the yield of chlorophyll *a* fluorescence in normal cells incubated in high concen-

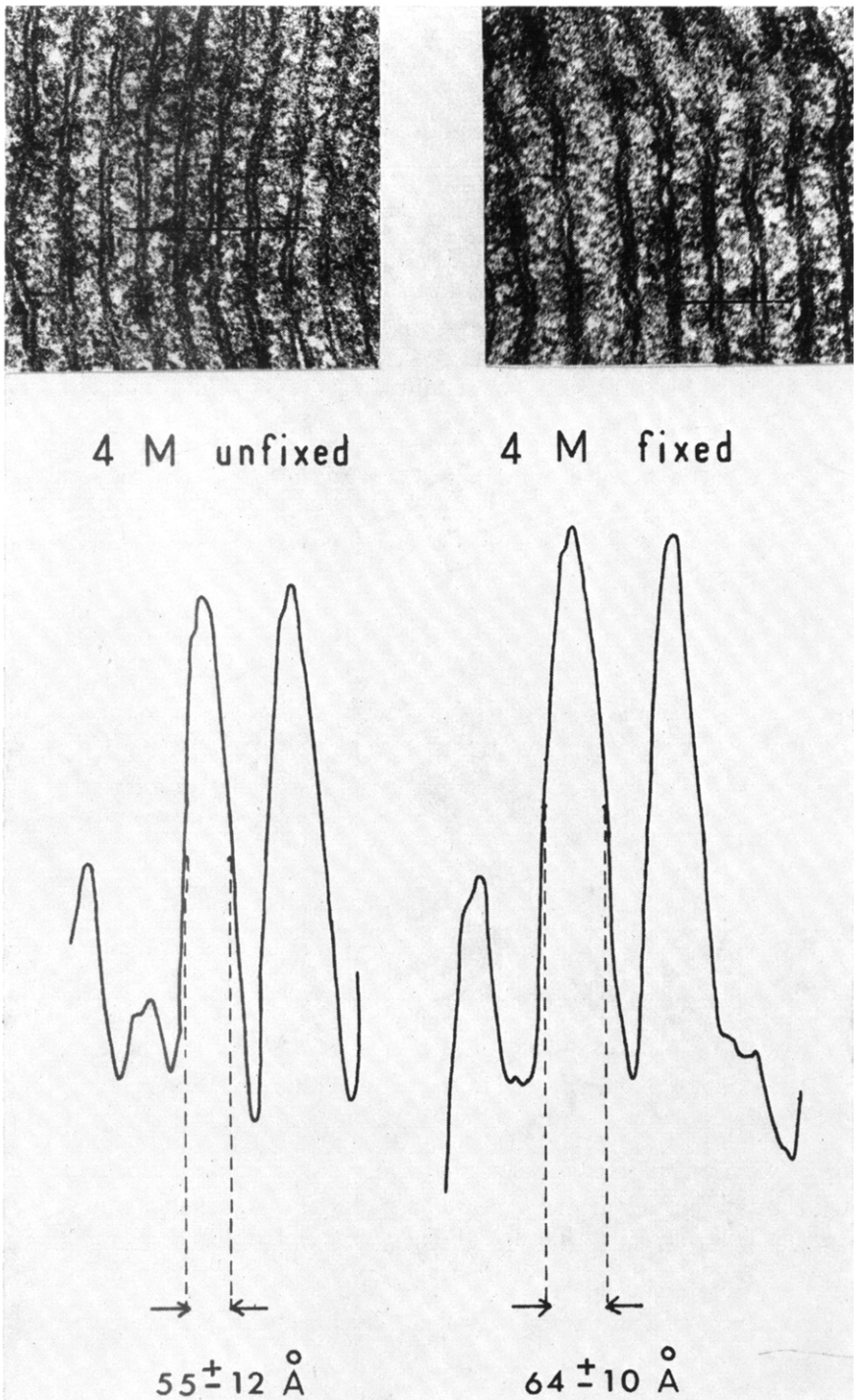

Fig. 3. Comparison of the thickness (half width of the peak of the densitometric curves) of the thylakoid membrane of *P. cruentum* for two of the conditions listed in Table I. Photodensitometric curves were scanned perpendicular to the direction of the membrane, by a micro beam of 50 μ diameter, along the dark line.

trations of NaCl, the experiments were repeated in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) which intercepts the electron flow on either side of Photosystem II (PS II) [22]. 2 to 4 M NaCl stops indeed, the oxygen evolution in *P. cruentum*. This inhibition is reversible as is the effect of salt on fluorescence. Nevertheless although photosynthesis is stopped at 2 M NaCl, fluorescence continues to increase with increasing salt concentration up to 4 M. To test the role of NaCl on the electron transport between Photoreaction II and NADP reduction site, we measured the photoreduction of DCIP, after glutaraldehyde fixation [15], and observed that NaCl also stops the electron flow, after a time which varies with the salt concention and the sample.

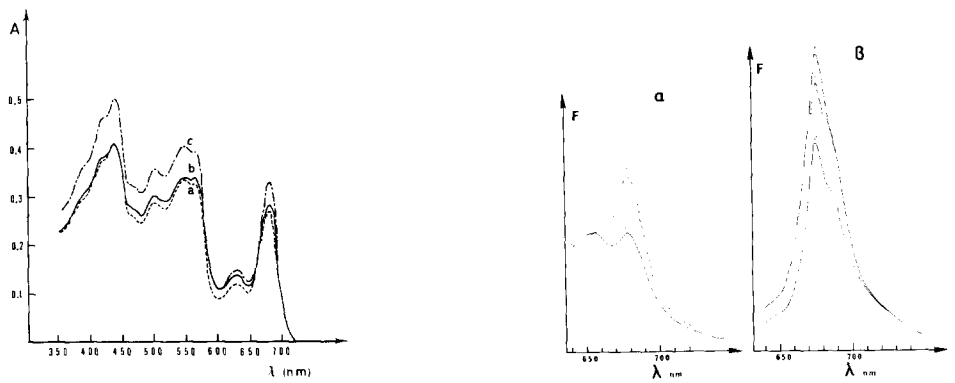

Fig. 4. Absorption spectra of *P. cruentum* cells after 60 min incubation in: (a) 4 M NaCl; (b) 0.6 M NaCl; (c) distilled water.

Fig. 5. Non corrected fluorescence emission spectra for *P. cruentum* cells ($A^{1\,cm}_{676\,nm} = 0.3$) incubated at 22 °C in the presence of 10^{-5} M DCMU and (1) 0.6 M NaCl; (2) 2 M NaCl; (3) 4 M NaCl. λ excitation = (a) 544 nm; (b) 440 nm. F = relative fluorescence intensity in arbitrary units (ordinate in b is $\times 10$ that in a).

Fig. 5 shows fluorescence spectra measured in DCMU-treated cells. DCMU has the usual effect on fluorescence (Spectrum 1). When the cells are incubated in 2 M NaCl+DCMU the fluorescence levels are intermediate between that of the norm and that obtained with DCMU alone (Spectrum 2). When Chl *a* is excited at 440 nm and the cells incubated in 4 M NaCl the effect of the high salt concentration (in the presence of DCMU) is the same as, or higher than, that of DCMU alone (Spectrum 3). The effect of NaCl on the emission of fluorescence in the presence of DCMU has also been studied as a function of time, for two different excitation wavelengths, 544 and 440 nm; the former corresponds to sensitized fluorescence (excitation in phycoerythrin transfered to phycocyanin and chlorophyll *a*), the latter to the direct excitation of chlorophyll *a* in its Soret absorption band. The same results were obtained: addition of DCMU, after the fluorescence has reached the steady level, produces an important change in fluorescence yield, even at the high light intensity available in the fluorometer used ($1\text{–}2.10^4$ ergs $cm^{-2} \cdot s^{-1}$). In most cases addition of NaCl after DCMU induces a rapid decrease in steady-state fluorescence yield which nevertheless is maintained at levels far above the steady normal value. This lowering effect similar to that observed by de Kouchkovsky [13] is quite evident with 544 nm exci-

tation light and its magnitude depends upon the salt concentration. It is very much smaller, or unobservable with 440 nm light excitation. This type of quenching is quite different from the energy dependent quenching observed in DCMU poisoned chloroplasts in the presence of PMS [23] or diaminodurene [24] in that it is essentially insensitive to the effect of uncouplers of photophosphorylation. The addition of methylamine, HCl ($0.7 \cdot 10^{-3}$ M) has no effect on 2 to 4 M NaCl treated cells. Moreover it is, in *Porphyridium* as in *Chlorella* [13], independent of light intensity and therefore should have nothing to do with the electron transport chain or chains.

Fig. 6 shows the relationship between the salt concentration and the fluorescence yield under various conditions. In the absence of DCMU, the fluorescence yield depends on both the excitation transfer and the electron transport. In the presence of DCMU, the fluorescence yield is directly related to the excitation transfer.

By comparing the two sets of experiments, the dependences on NaCl concentration of the excitation transfer can be discriminated. It seems clear from Fig. 6a that the changes in fluorescence yield upon addition of high concentrations of NaCl are the sum of an increase due to the stop in electron transport ($\uparrow$) and a decrease due to suppression of the excitation transfer from phycoerythrin to chlorophyll *a* ($\downarrow$).

Two cases are considered for the change in energy transfer. In the first, the

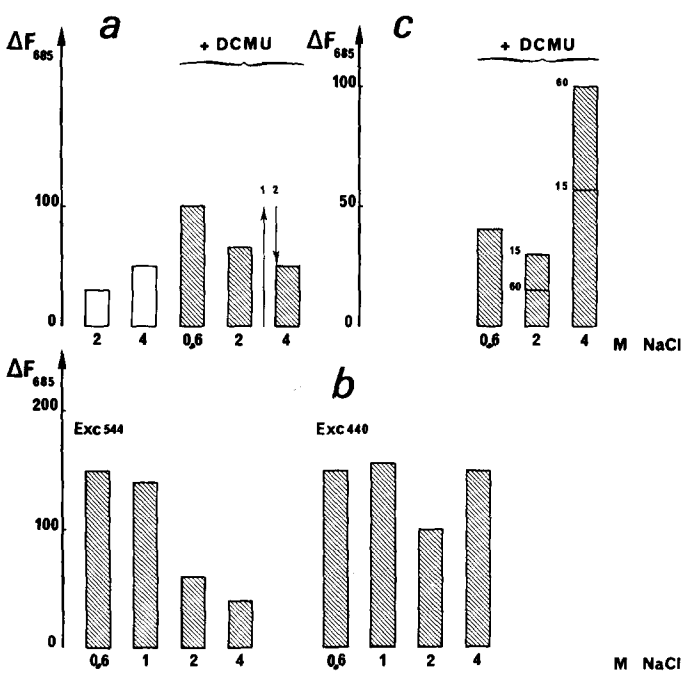

Fig. 6. Increase in chlorophyll *a* steady state fluorescence yield (685 nm) due to NaCl concentration. Expressed as % of the normal value (0.6 M NaCl). (a) Excitation at 544 nm. Experiments were made in the presence ■ or absence □ of DCMU $3 \cdot 10^{-5}$ M. Excitation intensity 10.3 ergs $cm^{-2} \cdot s^{-1}$. (b) In the presence of DCMU, comparison of the increase in fluorescence yield at 685 nm, excited at 544 nm or 440 nm. Excitation intensity 440 nm 9.3 ergs $cm^{-2} \cdot s^{-1}$. (c) Effect of time on the increase in chlorophyll *a* fluorescence yield excited at 544 nm in the presence of DCMU, 15 or 60 min after mixing.

excitation transfer from phycoerythrin to chlorophyll *a* is suppressed by NaCl: the decrease in chlorophyll *a* fluorescence should be observed when phycoerythrin is excited but not when chlorophyll *a* is directly excited. In the second case, the transfer from chlorophyll *a* in System II to chlorophyll *a* in System I is stimulated: the chlorophyll *a* fluorescence should be decreased whatever wavelength of excitation light is used. As shown on Fig. 6b, 4 M NaCl decreases the excitation transfer between phycobilins and chlorophyll, and has no effect whatever on the excitation transfer from Photoreaction II to Photoreaction I. The situation is more complicated for 2 M NaCl, where there seems to be at one and the same time a decrease in excitation transfer from phycoerythrin to chlorophyll and a stimulation of excitation transfer from Photosystem II to Photosystem I. However, when it exists, the lowering effect is very often transitory (Fig. 6c). The situation resembles closely that described by Murata [10] using spinach chloroplasts; the fast quenching of fluorescence upon the addition of NaCl is followed by a large slow increase. The only difference is that the variations in fluorescence yield are much more rapid in the former case, all changes being completed in 5 min. The variation in salt concentration is 10-fold that of the medium normal concentration in Murata's experiments and about 7-fold in ours.

CONCLUSION

The effect of NaCl on the steady-state fluorescence of *P. cruentum* is quite complex: it is the sum of almost four different effects. (1) Inhibition of electron transport between Photoreactions II and I and Photoreaction II and O_2 evolution. (2) Inhibition of excitation transfer from phycocyanin to chlorophyll *a*. (3) Stimulation of excitation transfer from Photoreaction II to Photoreaction I. (4) Changes in the thylakoid structure.

The increase in fluorescence yield caused by the action of NaCl cannot be attributed to an alteration in the intensity of light absorption. Slow changes in the long term chlorophyll *a* fluorescence yield in intact algal cells have been shown to be independent of the redox state of the primary electron acceptor of Photosystem II [25, 26]. Mohanty and co-workers [27] have also shown the constancy of steady state chlorophyll *a* fluorescence yield in *Porphyridium cruentum* with intensity, in the same range as used in this work: 0.9 to $9.2 \cdot 10^3$ ergs $cm^{-2} \cdot s^{-1}$. The fluorescence variations in the presence of DCMU are independent of intensity of exitation, irreversible in the dark and independent of energy coupling. This would appear to eliminate Photosystem I activity as a factor. If one assumes a localization of pigments which seems quite reasonable at present [5] i.e. phycobiliproteins outside the thylakoid and chlorophyll *a* in the chloroplast lamellae, one is justified in considering that the evidence for a correlation between modification of the thylakoid thickness and the increase in chlorophyll *a* fluorescence follows from the data on fluorescence excitation at 440 nm. This evidence is given by Fig. 5. Similarly the increase observed in Fig. 6c for 4 M NaCl cannot be explained by the above causes. The striking difference of behaviour between unfixed and glutaraldehyde fixed cells is due to structural causes.

It is clear there is an interrelationship between oxygen evolution, thylakoid structure and steady-state chlorophyll *a* fluorescence. Fixed cells can still reduce DCIP and methyl viologen to a large extent, and bleach P_{700} (the reaction center of

Photosystem I) but they can no longer evolve oxygen. They do not show the large increase in Chlorophyll *a* fluorescence induced by NaCl. It may be noted the effect of high NaCl concentration on fixed cells is quite different [1], that is, there is a small decrease in energy transfer from Photosystem II to Photosystem I totally masked in unfixed cells by the great increase in chlorophyll *a* fluorescence due to modification of the thylakoid thickness. Neither the fixed structure nor altered (treated with high salt concentration) evolve oxygen.

Water has an effect on the absorption spectrum, the cell volume and the emission of fluorescence, quite different from the effect of salt. As the cell volume increases one can observe the corresponding increase of the maxima ("sieve" effect) on the absorption (Fig. 4c). A correlative increase in phycocyanin fluorescence emission (Fig. 1) is accompanied by an important decrease in chlorophyll *a* fluorescence at 683 nm, most likely corresponding to a decrease in efficiency of transfer energy from the phycobilisomes to the thylakoid. As seen in Table I, water has no effect on the thylakoid thickness. As previously mentioned, oxygen evolution is normal in water-incubated cells; 440 nm excitation gives fluorescence emission spectra almost the same as in 0.6 M NaCl, taking into account the increase in absorption. The decrease in energy transfer from phycocyanin to chlorophyll *a* is thus attributable either to a direct effect of water on the phycobilisome or to an increase in the distance between the latter and the thylakoid.

Hoch and Randles [28] emphasized the role of permeability agents in controlling the fluorescence emission in *Porphyridium cruentum*. Variable permeability of the cells from one sample to another is very likely to be responsible for the variable final levels of steady-state fluorescence observed on Figs 6a and 6c, especially their evolution with time. Indeed there is a variable response to DCMU during the life cycle of some green algae [29]. Nevertheless its use in this work confirms that changes in chlorophyll *a* steady-state fluorescence intensity are correlated with changes in the thylakoid structure.

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