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THE PHOTOCHEMICAL AND FLUORESCENCE PROPERTIES OF WHOLE CELLS, SPHEROPLASTS AND SPHEROPLAST PARTICLES FROM THE BLUE-GREEN ALGA *PHORMIDIUM LURIDUM*

ELISHA TEL-OR and SHMUEL MALKIN

Biochemistry Department, The Weizmann Institute of Science, Rehovot (Israel)

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SUMMARY

The photochemical activities and fluorescence properties of cells, spheroplasts and spheroplast particles from the blue-green alga *Phormidium luridum* were compared. The photochemical activities were measured in a whole range of wavelengths and expressed as quantum yield spectra (quantum yield vs. wavelength). The following reactions were measured: Photosynthesis (O_2 evolution) in whole cells; Hill reaction (O_2 evolution) with $Fe(CN)_6^{3-}$ and NADP as electron acceptors (Photosystem II and Photosystem II+Photosystem I reactions); electron transfer from reduced 2,6-dichlorophenolindophenol to diquat (Photosystem I reaction). The fluorescence properties were emission spectra, quantum yield spectra and the induction pattern.

On the basis of comparison between the quantum yield spectra and the pigments compositions the relative contribution of each pigment to each photosystem was estimated. In normal cells and spheroplasts it was found that Photosystem I (Photosystem II) contains about 90 % (10 %) of the chlorophyll *a*, 90 % (10 %) of the carotenoids and 15 % (85 %) of the phycocyanin. In spheroplast particles there is a reorganization of the pigments: they loose a certain fraction (about half) of the phycocyanin but the remaining phycocyanin attaches itself exclusively to Photosystem I (!). This is reflected by the loss of Photosystem II activity, a flat quantum yield vs. wavelength dependence and a loss of the fluorescence induction.

The fluorescence quantum yield spectra conform qualitatively to the above conclusion. More quantitative estimation shows that only a fraction (20–40 %) of the chlorophyll of Photosystem II is fluorescent. Total emission spectrum and the ratio of variable to constant fluorescence are in agreement with this conclusion.

The fluorescence emission spectrum shows characteristic differences between the constant and variable components. The variable fluorescence comes exclusively from chlorophyll *a*; the constant fluorescence is contributed, in addition to chlorophyll *a*, by phycocyanine and an unidentified long wavelength component.

The variable fluorescence does not change in the transition from whole cells to spheroplasts. However, the constant fluorescence increases considerably. This indi-

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPIP, 2,6-dichlorophenol indophenol; DQ, diquat-1,1-ethylene-2′-dipyridylium-dibromide.

cates the release of a small fraction of pigments from the photosynthetic photochemical apparatus which then become fluorescent.

INTRODUCTION

Blue-green algae have efficient photosynthetic apparatus involving pigment systems composed of chlorophyll *a* and accessory pigments. Electron micrographs of red and blue-green algae [1, 2] reveal fundamental difference between these algae and the chloroplasts of plants and green algae. Chlorophyll *a* is located in the photosynthetic lamellae, while the water-soluble accessory pigments phycocyanin and pycoerythrin, form particles (phycobilisomes) attached to the outer side of the lamellae.

Absorption of light by phycocyanin in blue-green algae results in a very efficient energy transfer to chlorophyll *a* [3–5] as evidenced by the observation of chlorophyll *a* fluorescence. The estimation is that out of a hundred quanta absorbed by phycocyanin only about 1–2 are emitted as fluorescent light from this pigment in the intact cell, while about 90 are transferred to chlorophyll *a* [4]. Similar efficiency of transfer was estimated by fluorescence studies in red algae [6], in which energy is transferred from phycoerythrin to chlorophyll *a* via phycocyanin.

The now well established concept of two photochemical systems associated, respectively, with two different pigment systems got a specially clear and strong support from studies done on blue-green and red organisms [5, 7–9]. The two pigment systems were distinguished on the basis of the cooperation between chlorophyll *a* and the accessory pigments: pigment system 2 which is involved in oxygen evolution contains fluorescent chlorophyll *a* and a high proportion of phycobilins, while pigment system 1 contains non-fluorescent (or very weakly fluorescent) chlorophyll *a* and less phycobilins.

To make a more profound study of the photochemical reaction of blue-green algae it is necessary to measure action spectrum of partial electron transport reactions. This can be done either in spheroplasts (cells which lost their outer envelope) or in active particles obtained from spheroplasts by osmotic shock. Spheroplasts retain most of their phycocyanin, while spheroplast particles usually lose much of the phycocyanin, which becomes solubilized in the aqueous medium.

In this work the three kinds of material (whole cells, spheroplasts, and spheroplast particles) of the alga *Phormidium luridum* were examined and compared with regard to their fluorescent and photochemical activities in order to get an insight into the accessory pigment role and distribution in the two photosystems. The data here are presented as quantum yield spectra, rather than the more customary action spectra. This enables a straightforward analysis of the role and distribution of the various pigments in the two photosystems.

METHODS

Algae. P. luridum var. olivaceae was obtained from the culture collection of algae at Indiana University. The cells were grown in modified medium C of Kratz and Myers [10] at 25 °C, aerated with 5 % CO_2 in air, and illuminated with 160 ft. candle

fluorescent light. Cells were harvested during the late logarithmic phase of growth, and washed once with mannitol/phosphate (0.5 M mannitol, 30 mM phosphate, pH 7.5).

Preparation of spheroplasts and spheroplast fragments. Spheroplasts were prepared by a modified method of Biggins [11]. The washed cells were resuspended in mannitol/phosphate and sufficient solid egg white lysosyme (Miles, grade 1) was added to a final concentration of 0.025 % (w/v). The suspension was incubated on a shaker for 40 min, at 37 °C, illuminated with 500 ft. candle light. The suspension was filtered through several layers of glass wool and spheroplasts were collected by centrifugation at $1000 \times g$ for 7 min, washed in mannitol/phosphate, resedimented, and resuspended in mannitol/phosphate, at a final concentration of 1–2 mg chlorophyll *a* per ml.

Spheroplasts were fragmented by a 10-fold dilution with 30 mM phosphate buffer, pH 7.5, the suspension was centrifuged at $10\,000 \times g$ for 10 min, and resuspended in mannitol/phosphate. Most phycocyanin was released by this hypotonic lysis.

Isolation of phycocyanin. Phycocyanin was released from *P. luridum* cells (300 g wet weight) suspended in phosphate buffer, by freezing and thawing. The lysate was sedimented ($20\,000 \times g$ for 30 min). The supernatant was brought to 50 % saturation with $(NH_4)_2SO_4$, and centrifuged as before. The precipitate was dissolved in phosphate buffer and dialyzed overnight against the same buffer. All steps were carried out at 4 °C (absorption spectrum in Fig. 10a).

Spectroscopy. Absorption spectra were obtained in Cary 14 or Cary 16 spectrophotometers. Light scattering was decreased according to Shibata [12]. The absorption at certain individual wavelengths, isolated by interference filters, was also measured by an integrating sphere photometer made in our laboratory to check the results of the former method. The two methods agreed within 5 %.

Photochemical action spectra. O_2 evolution and uptake were obtained polarographically by a Yellow Spring Instrument Clark type oxygen electrode connected to a Rikadenki recorder. The samples were illuminated by the monochromatic light source of the spectrofluorimeter (bandwidth 12 nm). Incident light intensity was approx. 10 nEinstein $\cdot$ $cm^{-2} \cdot s^{-1}$. Linearity checks to ensure that the rate of the reaction is proportional to the light intensity were made at several chosen wavelengths, representing different regions of absorption. It was found that light saturation was achieved at about 3–4-fold stronger intensity at the wavelengths which are absorbed strongly. An insertion of a neutral density filter of about 50 % transmission led to identical quantum yield spectra.

Fluorescence measurement. These were done in a locally built spectrofluorimeter, provided with three alternative light sources: A projector equipped with 500 W incandescent lamp; 200 W mercury lamp, or 1000 W xenon lamp. The exciting light passed either through suitable interference filters or monochromator. The emitted fluorescence was analyzed by another monochromator to obtain the emission spectrum, or alternatively isolated by filters: usually a narrow band (Baird-atomic/B-11) 685 nm interference filter which passed mainly chlorophyll *a* fluorescence. The same apparatus was provided with fast shutters and used to record fluorescence induction as reported previously [13]. The excitation and emission beams were at 90° to each other. The cuvette was a standard cell of 1 cm optical path. The sample was maximally diluted to prevent self-absorption effects. The emission spectra were uncorrected for the wavelength-dependent response of the apparatus.

Light intensity measurements. For wavelength longer than 600 nm a silicon photocell was used as a linear detector. For wavelength shorter than 600 nm Rhadmin-B fluorescence screen, which has the same sensitivity at all wavelengths shorter than 600 nm [14], was used. Both were calibrated by a commercial bolometer (YSI Radiometer-calibrated against NBS standards) or alternatively by Ferrioxalate actinometry [15] which gave identical results.

RESULTS AND DISCUSSION

Estimation of absorption of the pigment components

An elementary requirement to discuss results of action spectrum is to estimate, at least in an approximate way, the contribution of each pigment to the total absorption at any wavelength. The results of such study are in accord with numerous such analyses reported previously (cf. e.g. ref. 3). The relevant pigments are chlorophyll *a*, phycocyanin and the carotenoids. We measured (Fig. 1) the absorption spectra of the following: (a) Extract of spheroplasts in 80 % (v/v) acetone/water, which contains (as simple comparison of the spectra shows) chlorophyll *a*, the carotenoids, and also a little bit of phycocyanin. (b) Chlorophyll *a* (Sigma) solution in 80 % acetone/water. This spectrum was manipulated and redrawn in Fig. 1 shifting the red and the blue

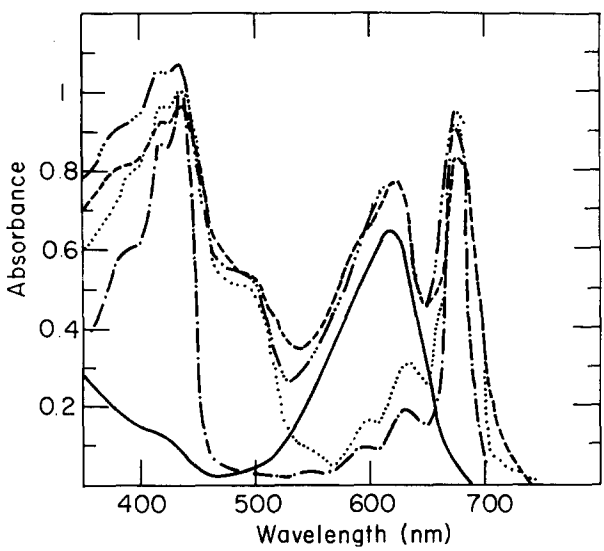

Fig. 1. Absorption spectra of spheroplasts and extracts from *Phormidium luridum*. – – –, spheroplasts suspended in mannitol phosphate (see: Methods). Spheroplasts were extracted in equal volumes either with 80 % acetone (· · · · ·) for chlorophyll *a* plus carotenoids extraction, or with phosphate buffer for phycocyanin extraction (water extract, —). The spectrum of pure chlorophyll *a* (Sigma) (· — ·) was normalized to match the peak absorption of the acetone extract at the red peak. Both the acetone extract spectrum and chlorophyll *a* spectrum were modified in drawing by slightly shifting the spectra independently in the red and the blue regions, so that the position of the peaks would match the in vivo chlorophyll peaks. The reconstructed total absorption (– · · · –) was found for $\lambda > 550$ nm by the sum of absorbancies of chlorophyll *a* solution and the water extract; for $\lambda < 550$ nm by the sum of the acetone extract, corrected for a trace of phycocyanin, and the water extract.

peaks to match those of spheroplast absorption. (c) Water extract, which contains phycocyanin. These spectra are compared to the absorption spectrum of the spheroplasts. The sum of the absorbancies of the solution of pure chlorophyll *a* and the water extract agrees with the spectrum of spheroplasts in the range 550-650 nm to within around 15 %; larger deviations occur at the falling or rising edges of the absorption. The spectrum of the acetone extract of spheroplasts shows, however, deviations from the pure chlorophyll *a* spectrum: in the range 550–650 nm the deviations are, very probably, due to the existence of a small amount of phycocyanin. From the size of the deviations one can estimate the phycocyanin content in the acetone/water extract to be around 20 % of the amount in the water extract. In the range < 550 nm the carotenoids contribute also to the absorption of the spheroplasts and thus the sum of chlorophyll *a* and water extract absorbancies is smaller than the total spheroplasts absorbance. In this range the addition of the spectrum of 80 % acetone extract, from which the phycocyanin contribution is subtracted, and the spectrum of the water extract, reconstructed the total absorbance of the spheroplasts. The agreement between this reconstruction and the observed spectrum is within 10 % in most of the range; only between 520 and 550 nm the matching is poorer (within around 30 %). Thus we infer that essentially all the carotenoids are extracted together with the chlorophyll in the 80 % acetone. There is a mismatch between the spheroplasts spectrum and the spectrum of chlorophyll *a* in the long wavelength region (> 650 nm) due to the existence of long wavelength forms of chlorophyll *a* in vivo.

The data of Fig. 1 were used to obtain the fraction of light absorption, contributed by each pigment, as shown in Fig. 2. The fractions of chlorophyll *a* or phycocyanin absorption is given from the ratio of the spectrum of chlorophyll *a* or the water extract, respectively, to the total reconstructed absorption. The fraction of the caro-

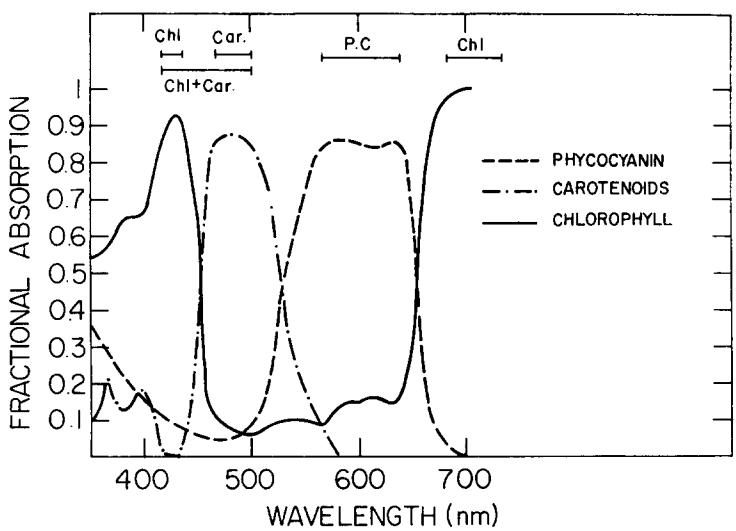

Fig. 2. The fractions of absorptions into each of the photosynthetic pigments as a function of wavelength. These are calculated from Fig. 1 by dividing the absorbance of each pigment by the total absorbance.

tenoids was calculated by subtracting from 1. In Fig. 2 the regions where the absorption of one pigment predominates over the others are indicated by horizontal bars.

In view of the many factors which distort the in vivo spectrum from the sum of the components it is felt that we have a reasonable accuracy in estimating the fractions of each pigment, as shown by the agreement of the reconstructed and spheroplasts absorptions.

Quantum yield spectra of electron transport reactions

(*a*) *General.* Intact algae cells evolve molecular oxygen which might be measured polarographically [16]. The quantum yield spectrum of this oxygen evolution obtained in the present work (Fig. 3a) is similar to results reported by Emerson et al. [3, 17] who found that light absorbed by phycocyanin in the range of 500–650 nm is most active in driving oxygen evolution. In addition we show a reasonable activity driven by light absorbed by pycocyanin below 400 nm. Both "red" drop and "blue" drop in activity in the range of chlorophyll *a* absorption are present.

In whole and fragmented spheroplasts three different activities were measured: (a) Photosystem II activity of oxygen evolution in the presence of potassium ferricyanide; (b) oxygen consumption by Photosystem I reaction, in which DQ is reduced by artificial donor in presence of DCMU and reoxidized by oxygen and (c) oxygen evolution in the presence of NADP, ferredoxin and flavoprotein, representing the cooperation of both photosystems.

(*b*) *Analysis of pigment distribution in the two photosystems. Spheroplasts* (*Fig. 3b*). The results with spheroplasts can be subjected to the following quantitative analysis:

The electron transport from water to potassium ferricyanide follows qualitatively the fraction of absorption of phycocyanin, as shown in Fig. 2. We may conclude that this reaction, which is either activated by Photosystem II alone, or is limited by Photosystem II, is sensitized mainly by phycocyanin. Phycocyanin is therefore the main component of Photosystem II. More quantitative inspection shows that in the blue wavelength region where chlorophyll absorption predominates there is an increase of Photosystem II quantum yield compared to the dip at 480–510 nm. This is not accounted by the absorption of phycocyanin. We may, therefore, conclude that a small fraction of chlorophyll adds a contribution to the absorption of Photosystem II. To account for this contribution, as well as for a possible contribution from the carotenoids we may write:

$$\text{Quantum yield}_{(\text{PS II})} = \Phi_{\text{II}} [\alpha_{\text{II}}(\text{PC}) + \beta_{\text{II}}(\text{Chl}) + \gamma_{\text{II}}(\text{Car})] \quad (1)$$

where (PC), (Chl) and (Car) are the fractional absorption of phycocyanin, chlorophyll and the carotenoids, respectively, and α_{II}, β_{II}, γ_{II} are coefficients connecting the contribution of each pigment to absorption with the resulting electron transport activity of Photosystem II (PS II). These coefficients represent the probability of energy transfer from each pigment respectively to the reaction center. The quantum yield depends also on the fraction of undamaged photosynthetic units and the condition of the unit; factors which certainly do not depend on the wavelength and therefore are common to α_{II}, β_{II} and γ_{II}. This is included in Φ_{II}.

Assuming that the unknown coefficients α_{II}, β_{II} and γ_{II} are wavelength independent, one may write Eqn. 1 at any wavelength, substituting the experimental values of

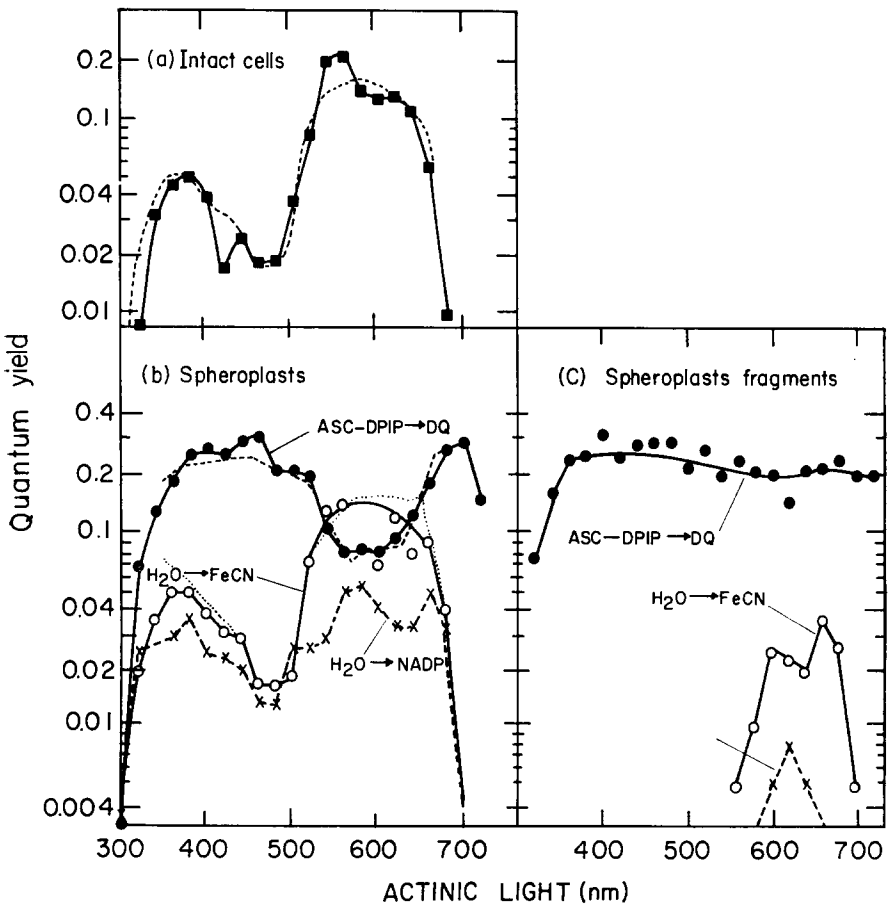

Fig. 3. Quantum yield spectrum of photochemical activities. (a) Intact cells: O_2 evolution activity was measured as follows: cells containing 40 μg chlorophyll *a* were suspended in 2 ml mannitol phosphate, and illuminated for 2 min at each wavelength with the spectrofluorimeter light source. (b) Spheroplasts: O_2 evolution with spheroplasts ($H_2O \rightarrow$ FcCN or $H_2O \rightarrow$ NADP) was measured as follows: spheroplasts containing 66 μg chlorophyll *a* were suspended in 2 ml mannitol phosphate with either 1 mM FeCN or 0.5 mM NADP plus saturating amounts of Swiss-Chard ferredoxin and flavoprotein. O_2 uptake (DPIP/Asc $\rightarrow$ DQ) was measured in 2 ml mannitol phosphate solution containing: DQ, 0.05 mM; sodium azide, 1 mM; DPIP, 0.05 mM; sodium ascorbate, 10 mM; DCMU, 10^{-3} mM and spheroplasts containing 42 μg chlorophyll *a*. (c) O_2 evolution and O_2 uptake in spheroplasts particles measured as in (b). O_2 uptake was measured with a sample containing 46 μg chlorophyll *a* and oxygen evolution with a sample containing 91 μg chlorophyll *a*. The dotted curves in the intact cell quantum yield spectrum and in the spheroplasts quantum yield spectrum which closely match those of the ASC-DPIP $\rightarrow$ DQ and $H_2O \rightarrow$ FeCN curve are the theoretical curves for each case, calculated on the basis of the proportion of each pigment in each photosystem (cf. text).

the quantum yield from Fig. 3, and the fractional absorptions from Fig. 2. One can set a system of three equations for the three unknown $\Phi_{II}\,\alpha_{II}$, $\Phi_{II}\,\beta_{II}$, and $\Phi_{II}\gamma_{II}$ and solve for them. The best wavelengths to be chosen are in the three different regions where each one of the pigments has its highest contribution to absorption. When the data at

430, 485 and 580 nm were thus applied the following values of the coefficients were found:

$$\Phi_{II}\,\alpha_{II} = 0.18 \qquad \Phi_{II}\,\beta_{II} = 0.018 \qquad \Phi_{II}\,\gamma_{II} = 0.010 \qquad (\pm 15\,\%) \tag{2}$$

Substitution of Eqn. 2 back into Eqn. 1 gives a theoretical dependence of the quantum yield upon the wavelength, which may be compared with the experimental one. Such a comparison is quite satisfactory, as can be inspected in Fig. 3b, and is much more satisfying than a comparison to the absorption of phycocyanin alone.

In an exactly similar way we analyzed the electron transport from reduced dichlorophenolindophenol to diquat, measured by the concomitant O_2 uptake, which is presumably sensitized by Photosystem I. Again, in a qualitative way, the quantum yield follows the combined fraction of absorption of chlorophyll and the carotenoids, with perhaps somewhat less efficiency for absorption into the carotenoids. Further inspection shows, however, that the dip in the quantum yield profile in the range of phycocyanin absorption is not as big as would be predicted by the fraction of chlorophyll absorption in this region. This implies that phycocyanin also contributes to some extent to Photosystem I, either by direct association with Photosystem I or by a limited amount of energy transfer from Photosystem II. One may make a similar analysis, as made before, and write a relation between the quantum yield and the fractional absorption for Photosystem I.

$$\text{Quantum yield}_{(\text{PS I})} = \Phi_I\,[\alpha_I(\text{PC}) + \beta_I(\text{Chl}) + \gamma_I(\text{Car})] \tag{3}$$

where PS I means Photosystem I and the other abbreviations are defined above. Again, the same three wavelengths were chosen as before to write a system of three equations with the three unknowns $\Phi_I\alpha_I$, $\Phi_I\beta_I$, and $\Phi_I\gamma_I$, The values of the solved parameters were:

$$\Phi_I\alpha_I = 0.05 \qquad \Phi_I\beta_I = 0.3 \qquad \Phi_I\gamma_I = 0.2 \qquad (\pm 15\,\%) \tag{4}$$

The theoretical calculation of the quantum yield profile agrees well with the experiment (Fig. 3b).

To see the possible significance of the above parameters let us introduce the reasonable assumption that the energy transfer yield to the reaction center is close to 1 for both phycocyanin and chlorophyll, and that the pigments belong only to Photosystem I and Photosystem II (i.e. no significant loss of non-attached pigments). This implies that:

$$\begin{aligned} \alpha_I + \alpha_{II} &= 1 \\ \beta_I + \beta_{II} &= 1 \end{aligned} \tag{5}$$

These equations, after substitution of the values of the parameters from Eqns. 2 and 4 form equations for the unknown Φ_I and Φ_{II}, the solution of which is: $\Phi_I = 0.30$ and $\Phi_{II} = 0.20$. From these values one can give values to α_I, α_{II}, β_I and β_{II}. The result is:

$$\begin{aligned} &\alpha_I \text{ fraction of phycocyanin in Photosystem I} &&\cong 15\,\% \\ &\alpha_{II} \text{ fraction of phycocyanin in Photosystem II} &&\cong 85\,\% \\ &\beta_I \text{ fraction of chlorophyll in Photosystem I} &&\cong 90\,\% \\ &\beta_{II} \text{ fraction of chlorophyll in Photosystem II} &&\cong 10\,\% \end{aligned} \tag{6}$$

With regard to γ_I and γ_{II}, it can be calculated that $\gamma_I+\gamma_{II} < 1$ and is around 0.7. This may indicate a loss in the energy transfer from the carotenoids, the efficiency of which is around 70 %. The significance of this number is especially meaningful for Photosystem I, wherein essentially is the main contribution of the carotenoids. Summarizing:

$$\begin{aligned} &\gamma_I \text{ effectiveness of the carotenoids in Photosystem I } \cong 65\,\% \\ &\gamma_{II} \text{ effectiveness of the carotenoids in Photosystem II } \cong 5\,\% \end{aligned} \tag{7}$$

From these numbers, assuming the same transfer efficiency for each photosystem, it follows that the fractions of the carotenoids in Photosystems I and II are 93 and 7 %, respectively.

The reaction of water → NADP electron transfer behaves in a similar way to the water → potassium ferricyanide reaction, but the activity is less and the results are much less quantitative. In the region where Photosystem I is more limiting the activity of this reaction falls off much more considerably, compared to the water → potassium ferricyanide reaction. This indicates a shortage of a factor in Photosystem I segment of electron carriers, which is required for NADP reduction.

Comparing the activity of whole cells to spheroplasts (Figs. 3a and 3b) it is seen that the quantum yield profile of photosynthesis in whole cells roughly follows that of the water → potassium ferricyanide activity in spheroplasts. One can conclude that in our case photosynthesis is limited by Photosystem II in most of the wavelength region, except perhaps at the peak of phycocyanin where the activities of the two photosystems may be comparable*.

(c) *Spheroplast fragments* (*Fig. 3c*). In spheroplasts fragments we observe an unusual phenomenon: Photosystem I activity is essentially constant throughout all the wavelength range from 340 to 720 nm and is approximately equal to the maximal activity in spheroplasts. Photosystem II activity is completely negligible. About half of the phycocyanin still remains (Fig. 4) but now all the light which is absorbed by it is diverted to Photosystem I, in contrast to what happens in spheroplasts. It seems that the phycocyanin particles rearrange during fragmentation: they are dissociated from Photosystem II and become associated with Photosystem I. Alternatively we may consider that the pigment complexes of the two photosystems which are separated enough in spheroplasts, to prevent strong energy transfer interaction, become much closer in spheroplast fragments. This increases the quantum yield of energy transfer to Photosystem I from phycocyanin, while Photosystem II is depleted during the particles preparation, and does not show any activity.

Fluorescence properties

The quantum yield data for the electron transport reactions were supplemented by fluorescence measurements illuminating further aspects of the role and the arrangement of the various pigments.

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* In a first glance it seems paradoxical that Photosystem II will limit (or at the best be approximately equal in activity to Photosystem I) in the range of phycocyanin absorption. A closer inspection shows, however, that the ratio of yield factors Φ_I and Φ_{II} could be such as to produce this result. For example a 2-fold higher value of Φ_I in whole cells compared to spheroplasts, with the same value of Φ_{II} would produce the observed result.

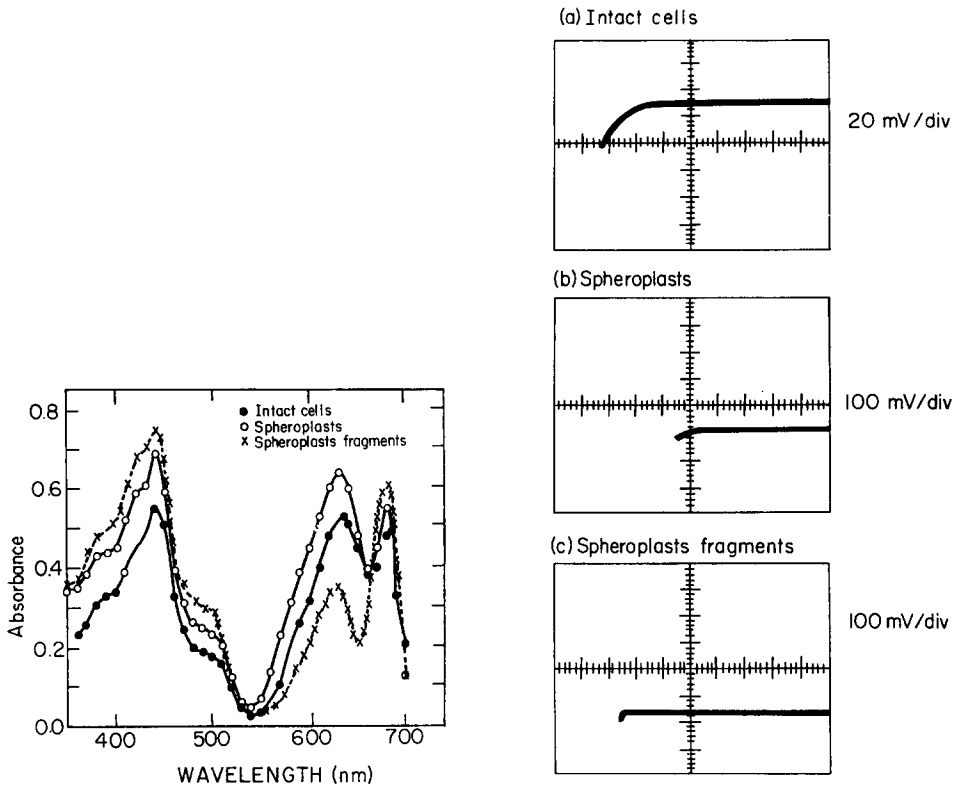


Fig. 4. Absorption spectra of the different cell preparates. All three preparations were suspended in mannitol phosphate. Small changes occur upon the transition from intact cells to spheroplasts. The most pronounced change is the decrease in the phycocyanin absorption in spheroplasts particles showing a loss of phycocyanin by around 50 %.

Fig. 5. Comparison of chlorophyll *a* fluorescence induction in three different cell preparations. Cell preparations were suspended in 1 ml mannitol-phosphate. Exciting light, 600 nm, $I = 11$ nEinstein/$cm^2 \cdot s$. (a) Intact cells containing 2 μg chlorophyll *a*. 20 mV/div., 1 s/div. (b) and (c), spheroplasts and spheroplast fragments containing 4 μg chlorophyll *a*. 100 mV/div., 1 s/div. Note the different sensitivity in (a) compared to (b) and (c).

(*a*) *Emission spectrum of the constant and variable fluorescence whole cells.* *P. luridum* cells exhibit fluorescence induction transients composed mainly of a rising phase, similar to isolated chloroplasts. An example of such transient is shown in Fig. 5a. The ratio variable fluorescence/constant fluorescence was always relatively small ($\approx 1/3$) compared e.g. to what is usually obtained from lettuce chloroplasts (2–3), but nevertheless it was distinct and characteristic. This transient was used to obtain, point by point, the emission spectrum of both the variable part of the fluorescence (F_v) and the initial constant part (F_o). To obtain more accurate results for the variable part alone the sensitivity of the oscilloscope was increased 2-fold and the zero line suppressed. One sample could be used for repetitive on-off cycles while the wavelength of emission was selected and changed. Fig. 6 shows such emission spectrum for F_o and F_v excited by light mainly absorbed by phycocyanin (570 nm),

which gave optimal extent the variable fluorescence. For comparison we bring also the emission spectra of chlorophyll *a* in vivo (spinach chloroplasts) and of extracted phycocyanin. It is seen that F_o and F_v are different in their emission spectra: F_o is composed of contributions from chlorophyll *a*, phycocyanin and also of a third component emitting between 690 and 740 nm, which was calculated by the subtraction of the sum of contributions of the first two components.

A similar spectrum to F_o was originally observed by Duysens [5] who measured the total emission spectrum for the blue-green alga oscillatoria. He did not separate, however, between F_o and F_v. F_v, in contrast to F_o, is contributed mainly, if not all, by chlorophyll.

The conventional thinking is that the fluorescence transient reflects the event of the photoconversion of reaction centers in Photosystem II, form the initial "open" to a "closed" form, by the reduction of the primary acceptor Q [18]. The result that F_o is contributed by different pigments than F_v may be interpreted in one of two ways, or by a combination of both: (a) much of F_o has nothing to do with trapping probabilities in Photosystem II, and can be regarded as a background fluorescence [19, 20] contributed either by pigments which are disconnected, and photochemically inactive or by Photosystem I pigments (e.g. the 690–740 nm component could be due to Photosystem I). (b) The energy transfer pathway from phycocyanin to chlorophyll *a* and finally to the pigments closely associated with the reaction center is largely irrevers-

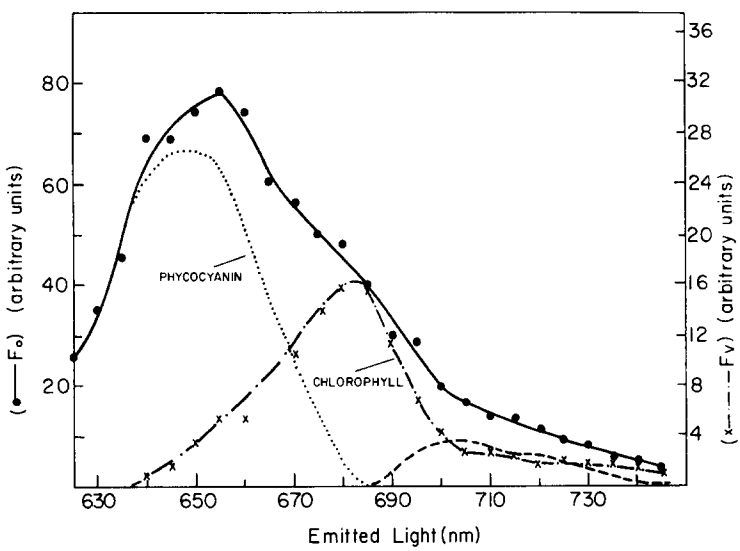


Fig. 6. Emission spectra of the constant fluorescence F_o (●) and the variable fluorescence F_v (×), which were obtained point by point from fluorescence induction oscilloscope traces, of whole cells (cf. Fig. 5a). For comparison, the emission spectra of phycocyanin (· · · · ·) and chlorophyll *a* in vivo (· — ·) from lettuce chloroplasts are also drawn. The chlorophyll *a* emission spectrum matches the spectrum of the variable fluorescence. The chlorophyll and phycocyanin spectra were each normalized in such a way that their sum matched the constant fluorescence as close as possible in the range < 685 nm. The emission at 685 nm is almost exclusively contributed by chlorophyll *a*. The difference between the constant fluorescence in the range > 685 nm (——) and chlorophyll *a* fluorescence reveals an extra far-red emission component (– – –) for the constant fluorescence.

ible. The contribution to F_0 includes therefore pigments in the energy transfer chain. However, the additional fluorescence, caused by the conversion of reaction centers to a non-trapping form comes mainly from pigments around the reaction center itself, which probably consists mainly of chlorophyll *a*. According to this conclusion the pigments are spatially ordered according to their single-excited state energy (Scheme 1A).

Fig. 7 shows how the emission spectrum changes drastically at different wavelengths of excitation. For 440 nm excitation the ratio of the phycocyanin emission peak to the chlorophyll emission peak is about half compared to a ratio of about 2.5 or more* at 600 nm excitation. This is essentially not a new observation [21], but it has interesting quantitative aspects which will be discussed.

(*b*) *Quantum yield spectrum for chlorophyll a fluorescence whole cells.* We attempted to measure the quantum yields for both F_0 and F_v emitted at the chlorophyll *a* band, as measured at 695 nm (Fig. 8). However, while the result for F_0 is apparently correct the result for F_v is probably an artifact due to the low level of exciting light intensity, which at the wavelengths below about 550 nm cannot drive a full extent of Q reduction and hence of the variable fluorescence**. To estimate the true extent of F_v, Fig. 9 shows the ratios F_v/F_0 obtained at a few selected wavelengths of a high intensity mercury arc, which provides sufficient intensity to drive a full extent of F_v. The true ratio F_v/F_0 does not decrease at the shorter wavelengths where chlorophyll absorption predominates (405 and 436 nm) but even rather increases compared to the ratio at the wavelength of phycocyanine absorption.

Conclusions from the fluorescence data; comparison to the results obtained from electron transport data

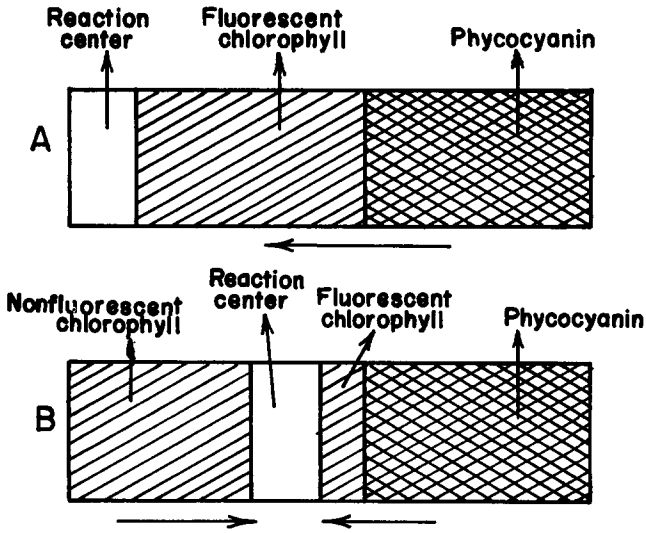
The data for F_0 (Fig. 8) are qualitatively in agreement with the old observation by Duysens [5] that two populations of chlorophyll molecules exist: fluorescent and non-fluorescent, and that phycocyanin interacts with the fluorescent chlorophyll which exists in relatively small amount. Light absorbed by chlorophyll itself (at approx. 430 nm) gives very little fluorescence compared to light absorbed by phycocyanin.

A quantitative analysis of Fig. 7 (for F_0) will, however, introduce some problems: according to a simplified proposition the chlorophyll of Photosystem II, which is in connection with phycocyanin, is fluorescent and that of Photosystem I is not. Assuming thus two separate homogenous populations of chlorophyll we could write:

$$F_{(\mathrm{Chl})} = \frac{\Phi_T I_{(\mathrm{Pc\ II})} + I_{(\mathrm{Chl\ II})}}{I} \cdot \Phi_{(\mathrm{Chl\ II})} \qquad (8)$$

* Fig. 7 was obtained by continuous scanning of the steady-state fluorescence spectrum; hence it is essentially F_0+F_v spectrum. However, as is shown in Fig. 8 (see also text), the contribution of F_v under our conditions is small for 440 nm excitation, hence at this wavelength the spectrum is mainly due to F_0. The contribution of F_v at other wavelengths of excitation is moderate (one-third of F_0 for chlorophyll *a* emission at 685 nm; 0 for emission at 640 nm at the phycocyanine emission peak). One can thus apply a correction to the peak at 685 nm to subtract this F_v contribution for 600 and 640 nm excitations. This will result in an increase of the ratio phycocyanine peak/chlorophyll peak.

** The use of DCMU was tried, to slow down the reoxidation of Q, and thus to drive a full extent of the variable fluorescence. In this case, however, complications arose and the results were not quite reproducible.



Scheme 1. (a) Possible spatial order of Photosystem II pigments around the reaction center, from emission spectrum data alone. (b) Possible pigment composition and order in Photosystem II, taking into account the data of the fluorescence changes with the wavelength of excitation.

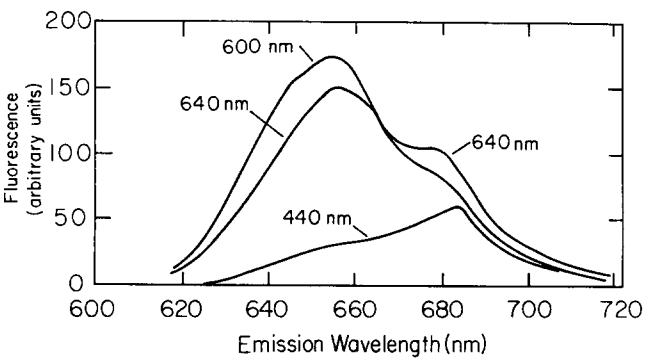


Fig. 7. Fluorescence emission spectra, from intact cells excited in three different wavelengths. Cells containing 5 μg chlorophyll *a*, were suspended in 1 ml mannitol phosphate. The exciting intensities were comparable, around 10 nEinstein/cm^2 · s but the sensitivity of the 440 nm excitation spectrum was around 10 times higher compared to the other spectra.

where $F_{(\mathrm{Chl})}$ is the observed fluorescence yield emitted from the chlorophyll in Photosystem II. $I_{(\mathrm{Pc\ II})}$ and $I_{(\mathrm{Chl\ II})}$ are the light intensities which are absorbed, respectively, by the phycocyanin and the chlorophyll of Photosystem II, and I is the total light absorbed. Φ_{T} is the yield of energy transfer from phycocyanin to chlorophyll in Photosystem II, and $\Phi_{(\mathrm{Chl\ II})}$ is the inherent emission yield from excited chlorophyll in Photosystem II.

Assuming for a while a constant value of 1 for Φ_{T}, as well as a constant value for $\Phi_{(\mathrm{Chl\ II})}$ we shall compare the prediction from Eqn. 8 to the experimental result for the chlorophyll fluorescence at two characteristic wavelengths: 430 and 570 nm. Using

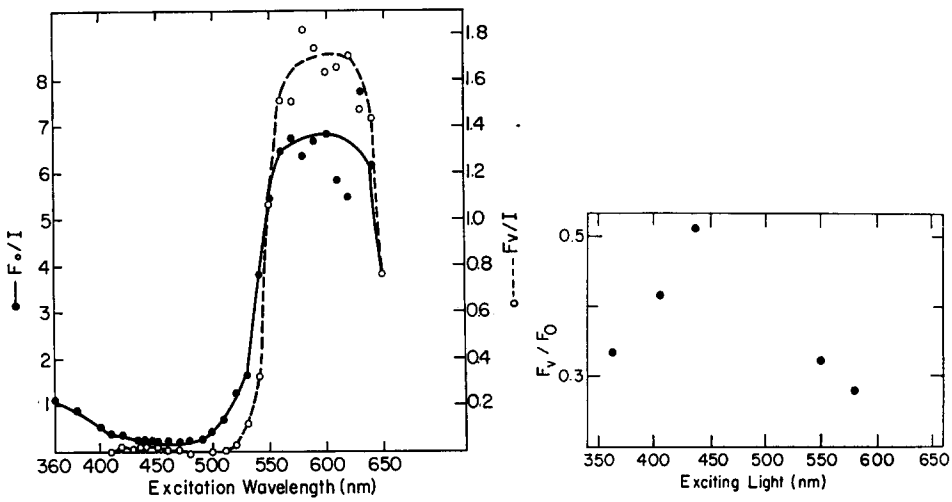


Fig. 8. Relative quantum yield spectrum (fluorescence signal/absorbed light intensity) for the constant and variable chlorophyll *a* fluorescence in whole cells, measured at 685 nm. Other conditions as in Fig. 7, except that the light intensities at the blue region were smaller by a factor of 3 at 450 and 10 at 400 nm.

Fig. 9. The ratio of variable to constant fluorescence in saturating light at different wavelengths of light from a mercury arc with whole cells. Light intensities were in the range 30–70 nEinstein/cm^2 · s.

the values obtained before for the relative contributions of phycocyanin and chlorophyll for the absorption in each photosystem we obtain for the theoretically expected ratio:

$$\frac{F_{\text{Chl(430 nm excitation)}}}{F_{\text{Chl(570 nm excitation)}}} = \frac{\alpha_{II}(\text{PC})_{430} + \beta_{II}(\text{Chl})_{430}}{\alpha_{II}(\text{PC})_{570} + \beta_{II}(\text{Chl})_{570}} \cong \frac{0.17}{0.73} = 0.23 \qquad (9)$$

This number may be compared to the actual observed ratio of 0.03(!).

This discrepancy shows that the assumptions that Φ_{Chl} and/or Φ_T are constants must be rechecked. However, it will be shown that mainly the assumption that the entire population of chlorophyll in Photosystem II is homogeneous with respect to its fluorescence properties must be replaced.

With regard to Φ_{Chl}, the measurements of chlorophyll *a* fluorescence yield in vitro do not show variation with respect to wavelength (e.g. chlorophyll *a* in hexane, ref. 22). These results were reconfirmed by us for chlorophyll *a* in vivo (for lettuce chloroplasts). It is therefore very likely, although by no means absolutely proved, that Φ_{Chl} is essentially wavelength independent.

With regard to Φ_T, it is quite possible that Φ_T indeed changes considerably, decreasing at the shorter wavelength region (approx. 430 nm) compared to the longer wavelength region (approx. 550 nm). Indeed, measurement of phycocyanin fluorescence quantum yield (Fig. 10) shows a considerable drop at the blue region. This indicates non-efficient radiationless conversion to the first excited singlet state at this wavelength (or alternatively the presence of an additional absorbing chromophore or species), which ultimately results in a reduction of the overall transfer efficiency Φ_T (since the energy transfer is presumably from the first excited singlet state). However,

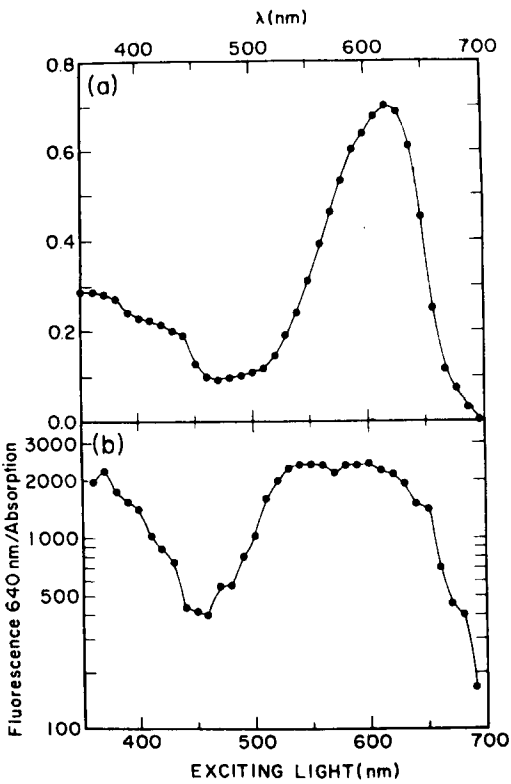


Fig. 10. Absorption and fluorescence spectra of purified phycocyanin. (a) Absorption spectrum. (b) Fluorescence quantum yield spectrum. For emission spectrum see Fig. 6.

even if we take an extreme position and assume Φ_T to contribute nothing ($\Phi_T = 0$) at 430 nm, the ratio F_{Chl} (430 nm)/F_{Chl} (570 nm) should still be much higher ($\cong$ 0.12) compared to the low actual value observed ($\cong$ 0.03). This difficulty is avoided by assuming that the chlorophyll population of Photosystem II is divided into a fluorescent part which is closely attached to phycocyanine, and a non-fluorescent part, mainly detached from phycocyanine (Scheme 1B). In order to get experimental agreement the fraction of the fluorescent chlorophyll in Photosystem II must be around 20 %. Thus, light absorbed into phycocyanin itself at 570 nm will be transferred exclusively into the fluorescent chlorophyll population, while light absorbed by the chlorophyll at 430 nm will be distributed to the fluorescent chlorophyll according to its fraction, which is about 20 % × 10 % = 2 %.

This result is consistent with the following observations: (a) The change of the phycocyanin to chlorophyll fluorescence intensity ratio by changing the exciting wavelength from 600 to 440 nm (Fig. 7, see also the related text). From these results one can calculate the fraction of the fluorescent chlorophyll independently, by using Eqn. 6 together with a similar equation for phycocyanin fluorescence. In addition one should take into account a decrease of the radiationless transition efficiency to the first excited singlet state of phycocyanin by light absorbed into it at 440 nm (one-

fifth according to Fig. 10). This gives an estimation that the fraction of fluorescent chlorophyll is about 4 %, compared to 2 % estimated above. This discrepancy is not serious in view of the uncertainties involved in assuming that F_0 is contributed by a number of pigment pools (cf. below).

(b) The increase of the ratio F_v/F_0 at 430 nm compared to 578 nm (Fig. 9). Such increase is expected since at shorter wavelengths there is a considerable contribution by absorption into the non-fluorescent chlorophyll fraction of Photosystem II, which will not produce fluorescence as long as the reaction center is "open". When the reaction center is "closed" excitation may tunnel to the fluorescent chlorophyll fraction and will produce a significant contribution to the variable fluorescence. Quantitative estimations show, however, that a considerably larger increase in the ratio F_v/F_0 is expected at 430 nm, than actually is observed.

It seems that further elaboration of this point would be premature in the absence of additional data. The crucial problem in all the above estimations is that an unknown proportion of F_0 may be due to a small percentage of pigments detached from the reaction center, and having (for the "fluorescent" components) very high efficiency of fluorescence. The "true" F_0 may be thus masked by a non-relevant contribution.

Fluorescence properties; comparison of different preparations

As Fig. 5 shows the ratio F_v/F_0 decreases as one goes from intact cells to spheroplasts and spheroplast particles. Closer inspection shows that F_v (for the same amount of chlorophyll in the sample) remained the same for intact cells and spheroplasts but the background F_0 fluorescence increased considerably in spheroplasts. That F_v remained the same was checked directly several times by observing only the variable transient on a sensitive scale with zero suppression. The rise in F_0 is reflected also in Table I which shows that this increase is caused by contributions from both chlorophyll and phycocyanin. This increase in F_0 was not followed by significant changes in the yield of the photochemical reactions except perhaps for Photosystem I (cf. footnote to page 165). This means that relatively small amount of pigments from Photosystem II are detached from the photosynthetic apparatus, although the relative fluorescence increase may be quite large. In the case of spheroplasts F_0 is certainly contributed mainly by "non-relevant" fluorescence.

TABLE I

COMPARISON OF FLUORESCENCE YIELD OF THE THREE PREPARATIONS

Yield of emission calculated as fluorescence signal/absorption. Phycocyanin emission is given by the value of the intensity at 640 nm corrected for chlorophyll emission. Chlorophyll emission is given by the value of the intensity at 690 nm, where phycocyanin emission is negligible. The relative values at 640 and 690 nm cannot be compared because the difference in the sensor sensitivity. Chlorophyll *a* concentrations were 8 μg/2 ml in all three preparations.

Preparation	Relative yield of emission (excitation 550 nm)	
	Phycocyanin emission	Chlorophyll emission
Whole cells	70	18
Spheroplasts	250	50
Spheroplast particles	200	40

In contrast to cells and spheroplasts, spheroplast particles do not show any variable fluorescence, while retaining a high fluorescence yield (Table I). This change was paralleled to the loss of Photosystem II activity, as was shown in Fig. 3.

Interestingly, the transformation of the photochemical apparatus in the transition from spheroplasts to spheroplast particles affects relatively little the constant fluorescence intensity and the relative contribution to emission of chlorophyll and phycocyanin (Table I). Perhaps there is a complex of phycocyanin and fluorescent chlorophyll behaving as a single entity which moves from Photosystem II to Photosystem I when spheroplasts are broken in a response to the change of the medium. The existence of phycocyanin chlorophyll complexes, isolated from blue-green algae, was reported [23]. Such complexes can dissociate from the membrane and reattach again at different sites. Binder et al. [24] demonstrated such transformation and formation of new vesicles in spheroplasts fragments by electron-micrography.

CONCLUSIONS

Our results stress again the importance of phycocyanin, as a collecting pigment to Photosystem II. However, they also show that it can be easily detached from Photosystem II and become attached to Photosystem I (spheroplast particles). They show that a small fraction of phycocyanin is associated with Photosystem I in intact systems, and at the same time a small fraction of the chlorophyll is associated with Photosystem II. Of this chlorophyll it is conceivable that only a small part is fluorescent. Deduced from the emission spectrum of the variable fluorescence this fluorescent chlorophyll is closely associated with the reaction center of Photosystem II. The fluorescent chlorophyll is attached to phycocyanin in all the preparations, probably in a protein-pigment complex.

The transition from whole cells to spheroplasts did not alter the photochemical activity of Photosystem II considerably and photosynthesis was roughly parallel to Photosystem II activity, which in our case was the limiting factor. The transition to spheroplast particles changed however, the structure of the pigment systems drastically, and phycocyanin became attached to Photosystem I. Photosystem II activity was abolished, following not only the loss of phycocyanin but probably also the loss of a water-soluble Hill factor [25, 26]. The loss of Photosystem II activity in spheroplast particles was also indicated by the loss of the fluorescence induction.

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