Biochimica et Biophysica Acta, 459 (1977) 47–57

BBA 47217

515 nm ABSORPTION CHANGES IN *CHLORELLA* AT SHORT TIMES (4–100 μs) AFTER A FLASH

PIERRE JOLIOT, RENÉ DELOSME and ANNE JOLIOT

Service de Photosynthèse, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris (France)

(Received June 21st, 1976)

SUMMARY

Using *Chlorella*, three types of absorption changes at 515 nm have been studied in the 4–100 μs time range following a flash.

(1) The absorption change observed when both photoreactions are blocked, probably due to the formation of the triplet state of a carotenoid, is shown to depend on Photosystem II excitation only.

(2) The absorption increase induced by photoreaction I is biphasic; a first phase, complete in less than 4 μs, is followed by a slower phase with a half-rise time of 15–20 μs.

(3) On the other hand, photoreaction II induces only a fast absorption increase (< 4 μs).

The time course of the biphasic 515 nm absorption increase induced by photoreaction I is similar to the biphasic absorption decrease previously observed at 480 nm by Cox and Delosme (1976, C. R. Acad. Sci. Paris 282D, 775-778).

No significant absorption change is observed at 490 nm.

These results suggest that the transmembrane electric field induced by photoreaction I rises to its maximum value in at least two phases within 100 μs following flash excitation.

INTRODUCTION

The illumination of photosynthetic material from green plants causes a characteristic absorption change, with an absorbance increase at 515-520 nm, and an absorbance decrease at 480 nm [1]. This spectral change is generally attributed to the response of the chloroplast pigments to an electric field generated across the thylakoid membrane by vectorial electron transport (electrochromic effect) [2]. The time course of the absorption change following a flash was first studied by Witt [3]. Using chloroplasts, Wolff et al. [4] measured a rise time shorter than 100 ns for the absorbance

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

increase at 515 nm. However, on *Chlorella*, a slower increasing phase is observed in the 1 to 50 ms time range [5, 6]. This phase, labelled "phase b" in ref. 6, is linked to Photosystem I activity and disappears after illumination by several flashes. The difference spectrum of phase b is that of the electrochromic effect.

Between the very fast nanosecond rise and the subsequent phases studied by several authors at times longer than 300 μs, the time range from 1 to 200 μs remained practically unexplored. In this time range, another phenomenon causes absorbance changes in the same region of the spectrum, appearing rapidly during the flash, and decaying in the dark with a half-time of a few microseconds [7]. This component was ascribed to the formation of a triplet state of carotenoid [8] as a way of dissipating excess light energy absorbed by chlorophyll ("valve reaction", cf. ref. 9). The difference spectrum of the formation of this metastable state has a positive band very similar to that of the electrochromic effect in chloroplasts [7, 9] and *Chlorella* [10], with a maximum absorbance increase at 510–520 nm. But the two difference spectra differ strongly at 480 nm, where the contribution of the metastable state is negligible compared to the absorbance decrease due to the electric field. Cox and Delosme [10] studied the time course of the absorption changes induced at 480 nm by a saturating flash. They observed a biphasic absorption decrease induced by photoreaction I: a first phase is completed within the time response of the apparatus (3 μs) and is followed by a second phase of larger amplitude ($t_{\frac{1}{2}} \cong 25$ μs). Photoreaction II, on the contrary, generates only a fast decrease (< 3 μs). These authors attempted the same study at 515 nm, but were unable to draw clear-cut conclusions, because of interference by the metastable state of carotenoid generated during the actinic flash.

A more refined analysis was necessary to evaluate the respective contributions of the electrochromic effect and the carotenoid triplet to the absorption changes at 515 nm in the 4–100 μs following the actinic flash.

MATERIAL AND METHODS

The differential spectrophotometric method described in a previous paper [6] has been improved in sensitivity and time response, with the collaboration of D. Béal and B. Frilley. A suspension of *Chlorella pyrenoidosa* is dark adapted for more than 3 min and then excited by a single actinic flash (Stroboslave, General Radio, type 1539A, 2 μs duration at half height). The amplitude of the absorption change is determined by comparing the absorption of detecting flashes given before and a variable dark time t after the actinic flash (the time t is measured between the peaks of the actinic flash and the following detecting flash). The signal-to-noise ratio is improved by averaging 4 to 8 measurements.

The wavelength of the detecting flash is determined by an interference filter (Microphysics 5150 Å, spectral bandwidth 34 Å at half height; or Seavom 4800 Å, spectral bandwidth 56 Å at half height), and that of the actinic light by Wratten filters (no. 24 exciting both photosystems, or no. 24 plus 97 exciting essentially Photosystem I).

In some experiments, photoreaction II is blocked according to Bennoun [11]: the algae are treated with DCMU plus hydroxylamine, preilluminated and then incubated a few minutes in the dark before measurement.

To determine the decay of the carotenoid metastable state in the absence of any activity of the reaction centers, inhibited algae are illuminated with a continuous red light (from a 2500 W Xenon arc lamp, with a Wratten filter no. 24) during the measurements. The intensity of the continuous light is strong enough to maintain *P*-700 in an oxidized state (blocking photoreaction I), photoreaction II being blocked as above by DCMU plus hydroxylamine.

Algae (20 μg chlorophyll/ml) were suspended in 0.1 M phosphate buffer, pH 7.0, plus Ficoll 7 %. All the experiments were performed at room temperature.

RESULTS

In Fig. 1, curve A, the time course of the 515 nm absorption change induced by a flash has been measured in *Chlorella* when both photoreactions are blocked by a continuous background light in the presence of DCMU. The actinic flash causes a rapid absorption increase probably due to the formation of a triplet state of carotenoid (cf. refs. 8 and 9), followed by a decay with a half-lifetime of around 6 μs. Mathis [7] and Wolff and Witt [9] reported a half-lifetime of 3 μs in isolated chloroplasts, and the discrepancy with our measurements is partially due to the longer duration of our actinic flash. We observed that the half-lifetime can differ appreciably from one algal culture to another (5–7 μs).

Fig. 1, curve B, shows that a high concentration of hydroxylamine (100 mM, incubated more than 10 min) increases the lifetime of the carotenoid triplet state in *Chlorella*. The same effect can be observed at a lower concentration of hydroxylamine, provided the incubation time is long enough (for instance 100 min with 1 mM hydro-

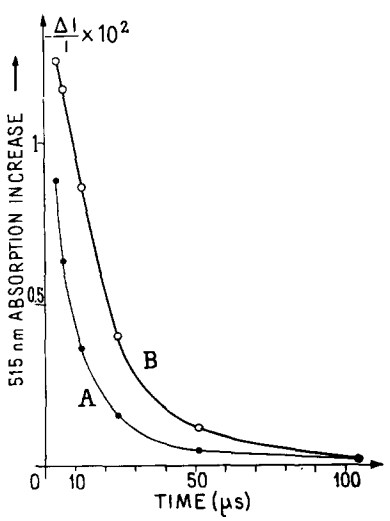


Fig. 1. 515 nm absorption change due to the carotenoid triplet state induced by a strong red flash (Wratten No. 24 filter). Algae are illuminated by a strong background of red light (Wratten no. 24) to inactivate both photosystems. (A), 20 μM DCMU; (B), 20 μM DCMU+100 mM hydroxylamine. $\Delta I/I$ (I, transmitted light intensity) when negative corresponds to an increase in absorption.

xylamine). In the experiments presented here (except Figs. 1 and 8), low concentrations of hydroxylamine and short incubation times are used to avoid this effect.

Relative contributions of the electrochromic effect and the carotenoid triplet to the absorption changes at 515 nm

The experiments of Cox and Delosme were performed only with actinic flashes of high energy (saturating the photochemical activity) and broad spectral bandwidth (exciting Photosystems I and II equally). In the present work, we varied the intensity and spectral bandwidth of the actinic flash, in order to modify the respective contributions of photochemistry and metastable state in the total absorption change.

In Fig. 2 are shown the 515 nm absorption changes induced by a red (Fig. 2, left) and a far red (Fig. 2, right) non-saturating actinic flash. The energy of the red and far red flashes has been adjusted to obtain the same electrochromic effect induced by photoreaction I, in a time range where the carotenoid metastable state has disappeared (cf. curves 2A and 2B at times longer than 50 μs).

In the same time range (> 50 μs), the contribution of photoreaction II to the electrochromic effect is equal to the difference between curves 1A and 2A and curves 1B and 2B. As expected, the signal due to photoreaction II is much larger (approx. 4 times) for the red flash than for the far red flash. As the flashes are not saturating, the amplitude of the signal is proportional to the number of photons absorbed by Photosystem II pigments.

The absorption changes due to the formation of the triplet state of carotenoid induced by the red and far red flashes are measured when both photoreactions are

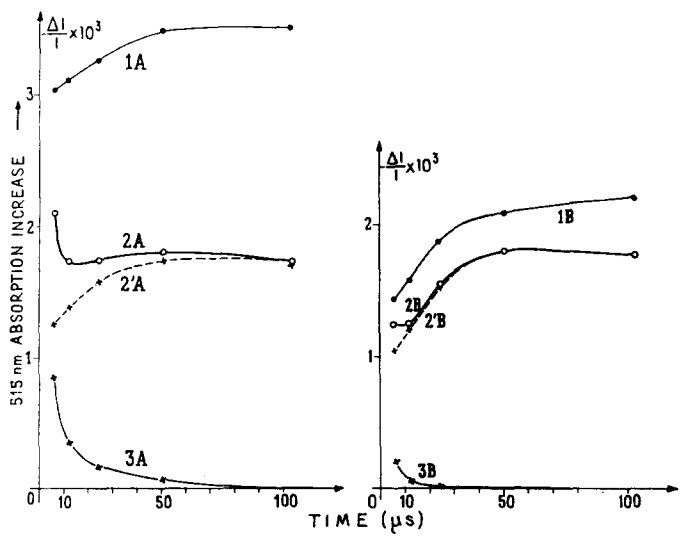


Fig. 2. 515 nm absorption change induced by non-saturating flashes. Left: red actinic flash (Wratten No. 24); Curve 1A: control; Curve 2A: 20 μM DCMU+300 μM hydroxylamine (the algae have been preilluminated to inactivate the Photosystem II centers, and kept in the dark 3 min before the actinic flash); Curve 2'A: difference between Curves 2A and 3A; Curve 3A: 20 μM DCMU+300 μM hydroxylamine (the algae are submitted to a strong red background light during the measurement). Right: same experiment as Fig. 2 left, but with a far red actinic flash (Wratten No. 24+97).

blocked by a continuous background in the presence of hydroxylamine plus DCMU (curves 3A and 3B). The amplitude of this signal measured at 6 μs is about 4 times higher for the red flash than for the far red one. This number is equal to the ratio of the numbers of photons absorbed by Photosystem II. The main conclusion from this result is that the formation of triplet of carotenoid that we detect in the 6 to 40 μs range is linearly dependent only upon system II excitation.

Consequently, one would expect that the absorption change linked to the formation of the carotenoid triplet is independent of the state of Photosystem I centers. Thus, the contribution of the carotenoid triplet can be eliminated from Curves 2A and 2B by subtracting Curves 3A and 3B respectively. For Curves 1A and 1B, one can assume, according to Wolff and Witt [9], that the contribution of the carotenoid triplet is negligible, because the actinic flash is not saturating and the reaction centers of Photosystem II are active.

The following conclusions on the absorption changes induced by both photoreactions can be drawn:

(1) The 515 nm absorption increase induced by photoreaction I is biphasic: a first phase completed in less than 6 μs is followed by a slower phase with a half-rise time of 15–20 μs. We checked that contrary to "phase b" (1–20 ms), the "slow" phase (6–100 μs) induced by the actinic flash is still observed when algae are illuminated for longer than 3 min. with a weak continuous background (5 photons/s per reaction center).

(2) The absorption increase induced by photoreaction II is monophasic, since the difference between curves 1A and 2'A or 1B and 2'B does not vary significantly as a function of time after 6 μs.

These results are similar to those reported by Cox and Delosme at 480 nm.

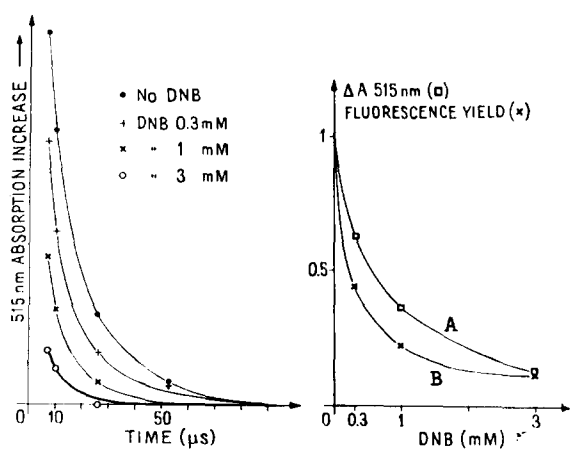


Fig. 3. Effect of dinitrobenzene on the absorption change due to the carotenoid triplet. Algae are submitted to a strong background of red light (Wratten No. 24) in the presence of 20 μM DCMU + 500 μM hydroxylamine.

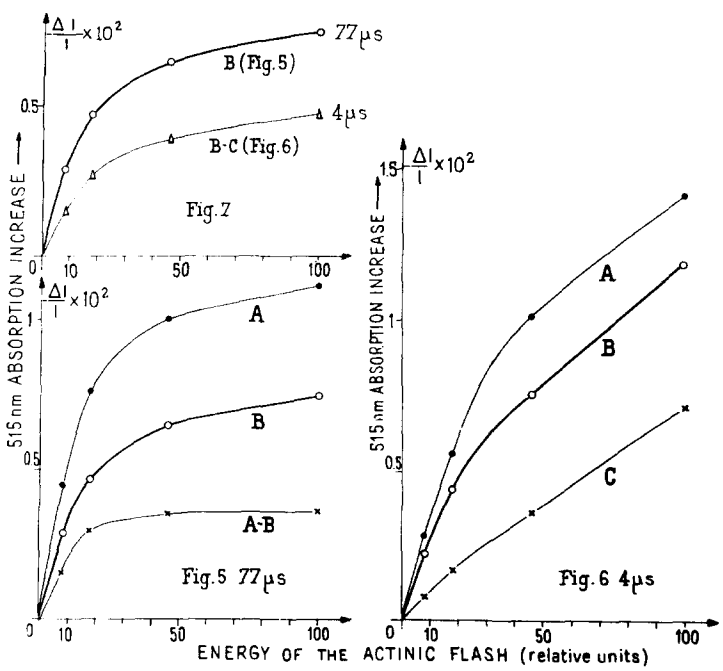
Fig. 4. Effect of increasing concentrations of dinitrobenzene on: (A), the 515 nm absorption change at 7 μs after the actinic flash (same conditions as in Fig. 3); (B), the maximum fluorescence yield in continuous light, in the presence of 20 μM DCMU + 500 μM hydroxylamine.

However, the amplitude of the slow phase compared to the total absorption change induced by photoreaction I is significantly smaller at 515 nm than at 480 nm.

We stated above that the formation of carotenoid triplet depends only on Photosystem II excitation. If chlorophyll excitation energy not trapped by System II reaction centers is converted to triplet excitation of carotenoid, one would expect that any quencher of the chlorophyll fluorescence would prevent the formation of the carotenoid triplet. Dinitrobenzene is such a quencher [12], and its effect on the formation of the carotenoid triplet is shown in Fig. 3. One observes a large decrease of the magnitude of the triplet absorption change at 515 nm. The half-lifetime is slightly shortened from 7 μs to 5 μs with 3 mM dinitrobenzene. In Fig. 4, we compared the effect of increasing concentrations of dinitrobenzene on the 515 nm triplet absorption change and the maximum fluorescence yield of chlorophyll.

Saturation curves of the different absorption changes at 515 nm

Figs. 5 to 7 show the absorption increase at 515 nm as a function of the energy of the actinic flash, at 77 and 4 μs following the flash. At 77 μs (Fig. 5) the metastable state of carotenoid has completely disappeared, and the absorption changes only arise from the two photoreactions. This experiment measures the saturation curve for



Figs. 5, 6 and 7. 515 nm absorption changes as a function of the energy of the actinic flash (red light, Wratten No. 24). The energy has been varied with neutral density grids. Fig. 5: the absorption change has been measured 77 μs after the actinic flash. A, control; B, algae preilluminated in the presence of 20 μM DCMU + 400 μM hydroxylamine. Fig. 6: absorption change measured 4 μs after the actinic flash. A and B as in Fig. 5; in C, the algae have been illuminated with a red continuous light during the measurement. Fig. 7: absorption change induced by photoreaction I, measured 4 μs and 77 μs after the actinic flash (see text).

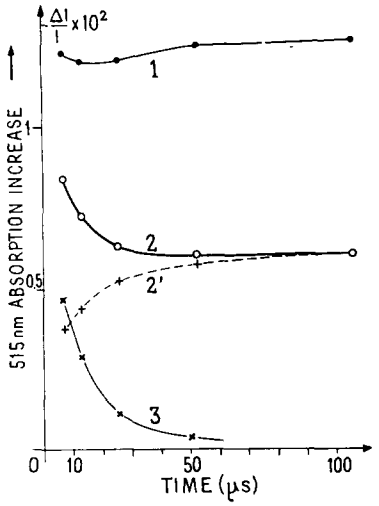


Fig. 8. 515 nm absorption change induced by a saturating flash. 1: Control; 2: algae preilluminated in the presence of 20 μM DCMU+100 mM hydroxylamine. 2': difference between curves 2 and 3; 3: 20 μM DCMU+100 mM hydroxylamine (the algae are illuminated by a continuous background during the measurement).

each photosystem: curve B is the saturation curve of photoreaction I; the saturation curve of photoreaction II is calculated from the difference between curve A (control) and curve B (DCMU plus hydroxylamine).

The saturation curves of the two photoreactions differ by the slightly positive slope observed at high flash energy for photoreaction I. This positive slope is likely due to double hits occurring during the tail of the Xenon flash, the turnover of Photosystem I being faster ($t_{\frac{1}{2}} \cong 80$ μs, [6]) than the turnover of Photosystem II ($t_{\frac{1}{2}} \cong 500$ μs in *Chlorella* after a single actinic flash [13]).

At 4 μs following the actinic flash (Fig. 6), the contribution of the metastable state of carotenoid increases with the intensity of the flash. The intensity curve of this effect (curve C) exhibits no saturation in the intensity range where photosynthesis is saturated. This result is in agreement with those of Wolff and Witt [9] and Mathis [14, 15].

In Fig. 7 is plotted the absorption change induced by photoreaction I at 77 μs (same curve as B, Fig. 5) and 4 μs. The 4 μs curve has been computed by subtracting curve C from curve B in Fig. 6, according to the method stated above. The proportionality between the two curves of Fig. 7 indicates that the ratio of the amplitudes of the fast and slow rises does not depend on the energy of the flash.

Absorption changes at 515 nm induced by a saturating flash

In the experiment shown in Fig. 8, we studied the absorption changes at 515 nm induced by a saturating flash (i.e. during which all reaction centers undergo a photoreaction). A large contribution of carotenoid triplet is observed, even when both photosystems are active. As in Fig. 2, the absorption change induced by photoreaction I at 515 nm is obtained by subtracting curve 3 (both photoreactions blocked) from Curve 2 (photoreaction II blocked).

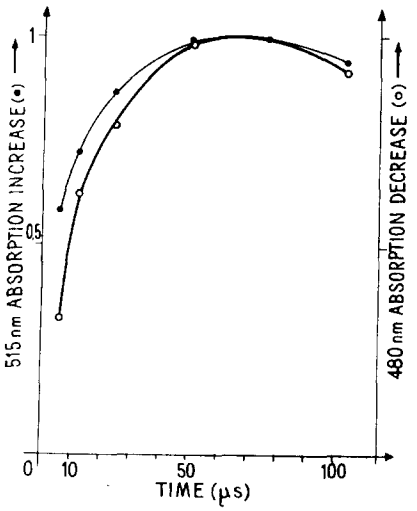


Fig. 9. 515 nm absorption increase and 480 nm absorption decrease induced by a non-saturating far red flash. The two curves have been normalized to the same total amplitude.

Comparison between the absorption changes at 480 nm and 515 nm

Fig. 9 shows the absorption changes in the same batch of algae at 480 and 515 nm, induced by a far red non-saturating flash exciting Photosystem I preferentially. In these conditions, the contribution of the carotenoid triplet is negligible at both wavelengths. We observe that the slow change between 6 and 100 μs represents a larger fraction (approx. 70 %) at 480 nm than at 515 nm (approx. 40 %) of the total absorption change. The same conclusion is reached by comparing the results of Cox and Delosme obtained at 480 nm with that of Fig. 8 at 515 nm.

No significant absorption change in the 0–1 ms time range has been observed at 490 nm, which thus represents an isobestic point for all the absorption changes we studied here. A detailed study of the spectrum of the absorption changes has been undertaken.

DISCUSSION

Absorption changes linked to the carotenoid triplet

The absorption change we observed at 515 nm when both photoreactions were blocked has the same characteristics as the carotenoid effect described by Mathis [7, 14, 15], and Wolff and Witt [9] in isolated chloroplasts. The difference spectrum (cf. ref. 10) and the dependence on the light intensity (Fig. 6) are very similar. The longer life-time we observed is partially due to the longer duration of the actinic flash we used. Comparison between isolated chloroplasts and algae (data not shown) suggests the existence of a component of longer life time in algae, the amplitude of which varies from one culture to another.

Our experiments prove that the 515 nm absorption change in the microsecond time range due to the formation of the carotenoid triplet, is only induced by Photosystem II excitation. This conclusion is in agreement with the fact that a quenching

of Photosystem II excitation energy by artificial traps such as dinitrobenzene, inhibits the formation of the carotenoid triplet. In the same time range, Duysens et al. [16, 17] and Zankel [18] observed a photoinduced quenching of the chlorophyll fluorescence. High concentrations of oxygen decrease both the life time of this quenching state and of the carotenoid triplet. These authors assumed that the two phenomena were related, thus associating the formation of the carotenoid triplet with Photosystem II excitation.

Wolff and Witt [9] affirmed that the formation of the carotenoid triplet occurs only when the excitation energy is not trapped by photoactive centers. From our results, we can conclude that the trapping by active Photosystem II centers, and not that of Photosystem I, can compete with the formation of the carotenoid triplet. Favoring this hypothesis is the effect of artificial traps (e.g. dinitrobenzene molecules) which decrease the amplitude of the signal due to the carotenoid triplet. A second argument arises from the comparison between 480 nm and 515 nm experiments: at 480 nm where there is no contribution of the carotenoid triplet, Cox and Delosme (confirmed by P. Joliot and A. Joliot [24]) do not observe any absorption change linked to the Photosystem II reaction in the 5–200 μs time range. At 515 nm, the close parallelism of curves 1A and 2'A (Fig. 2) obtained with non-saturating flashes suggests that the contribution of the carotenoid triplet to curve 1A is rather small.

Absorption changes due to photoreaction I

We observed an absorption increase at 515 nm linked to photoreaction I in the 6–100 μs time range. The time course of this absorption increase is similar to the absorption decrease at 480 nm reported by Cox and Delosme [10] and confirmed in this paper. The time course of this increase at 515 nm and its amplitude compared to the total absorption change due to photoreaction I are independent of the energy of the flash (Figs. 2, 7 and 8). No absorption changes were detectable at 490 nm. Thus the difference spectrum is similar to that of the electrochromic effect. Nevertheless, some difficulties arise when one compares at 480 and 515 nm the magnitude of the different phases of the absorption change: at 480 nm, the slow phase (6–100 μs) is 70 % of the total absorption change induced by a far red flash. At 515 nm, in the same algae, we observed only 30–40 % for the same phase. Thus, the difference spectra of both phases of absorption change are not exactly the same, as would be expected if both changes were exclusively due to variations of the electrical field.

Different hypotheses can be proposed to explain our experimental results:

(1) If we assume, in agreement with Wolff et al. [4], that the electric field induced by Photosystem I rises to its maximum value in less than 100 ns, a time much shorter than the time resolution of our apparatus, we must ascribe the slow phase to the relaxation of an additional spectral change induced by the flash. This additional change would have opposite signs at 480 nm and 515 nm and an isobestic point around 490 nm. In this hypothesis, this spectral change could be ascribed to a change in the redox state of either the primary donor or the primary acceptor of Photosystem I. As the time course (20 μs) is faster than the reduction of P^+-700 measured on *Chlorella* in similar conditions [6], it is more likely that a reaction on the acceptor side is involved.

(2) A second class of hypotheses is that the electric field induced by photoreaction I rises to its maximum value in two phases: (a) a first possibility is that the

distribution of the charges initially localized at the level of the photoinduced dipoles becomes gradually homogeneous on the membrane surfaces because of ion movements in the aqueous phases inside and outside of the thylakoid. As the slow phase is only associated with photoreaction I, we have to admit that the electrochromic probes are localized closer to photocenters II. The same hypothesis was proposed by Conjeaud [19] to explain that at about −170 °C, the contribution to the electrochromic effect is much smaller for photoreaction I than for photoreaction II [20]. However, experiments by Fowler and Kok [21] and Witt and Zickler [22] would predict a more rapid time for charge delocalization than we observe in our experiments, in view of the much shorter distance between adjacent reaction centers relative to the circumference of a thylakoid. (b) A second possibility is that the primary electron donor or acceptor of photocenter I is not located respectively on the internal and external surfaces of the membrane, but is buried within the membrane. In this case, the slow phase would be due to the migration of the charges from the primary to the secondary donor or acceptor located on the membrane surface. Jackson and Dutton [23] proposed a model of this type to explain the different phases of the electrochromic shift in *Rhodopseudomonas* chromatophores.

The discrepancy mentioned above between the difference spectra of the rapid and the slow phases makes an additional assumption necessary: if all the absorption changes we observed are due to an electrochromic effect, we must admit that several probes are involved, which differ in their spectra and in their positions relative to the reaction centers. Another possibility is that a minor absorption change of a different origin contributes to the measurements either at 480 or at 515 nm.

Absorption changes linked to photoreaction II

We pointed out that no absorption change linked to photoreaction II was observed between 6 and 100 μs following the flash, in agreement with the results of Cox and Delosme at 480 nm. Nevertheless, recent experiments of P. Joliot and A. Joliot [24] suggest that the primary electron donor chlorophyll a_{II} is embedded within the membrane. The charge transfer between this chlorophyll and the secondary donor is too fast (< 1 μs) to be detected by our apparatus as a separate phase in the absorption change. In this hypothesis, the structural arrangement of the System II reaction centers is similar to that proposed above in paragraph 2b for the reaction centers of Photosystem I.

ACKNOWLEDGEMENT

The authors thank Dr. B. Diner for his critical reading of the manuscript. This work has been supported by the Centre National de la Recherche Scientifique (Equipe de Recherche N° 16), the Délégation Générale à la Recherche Scientifique et Technique and the Commissariat à l'Energie Atomique.

REFERENCES

1 Duysens, L. N. M. (1954) Science, 120, 353–354
2 Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 244–254
3 Witt, H. T. (1955) Naturwissenschaften 42, 72–73

4 Wolff, C., Buchwald, H. E., Rüppel, H., Witt, K. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1038–1041
5 Witt, H. T. and Moraw, R. (1959) Z. Physik. Chem. Neue Folge 20, 5/6, 253–282
6 Joliot, P. and Delosme, R. (1974) Biochim. Biophys. Acta 357, 267–284
7 Mathis, P. (1966) C. R. Acad. Sc. Paris 263 D, 1770–1772
8 Mathis, P. and Galmiche, J. M. (1967) C. R. Acad. Sc. Paris 264D, 1903–1906
9 Wolff, C. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1031–1037
10 Cox, R. and Delosme, R. (1976) C. R. Acad. Sc. Paris 282D, 775–778
11 Bennoun, P. (1970) Biochim. Biophys. Acta 216, 357–363
12 Teale, F. W. J. (1960) Biochim. Biophys. Acta 42, 69–75
13 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772–785
14 Mathis, P. (1969) in Progress in Photosynthesis Research (Metzner, H., ed.), Vol. 2, pp. 818–822, Tübingen
15 Mathis, P. (1970) Thèse, Orsay
16 Duysens, L. N. M., Van der Schatte Olivier, T. E. and Den Haan, G. (1972) Abstr. VI. Int. Congr. Photobiol. Bochum, No. 277
17 Den Haan, G. A., Warden, J. T. and Duysens, L. N. M. (1973) Biochim. Biophys. Acta 325, 120–125
18 Zankel, K. L. (1973) Biochim. Biophys. Acta 325, 138–148
19 Conjeaud, H. (1976) Thèse de 3e Cycle, Paris VI
20 Vermeglio, A. (1974) Thèse, Orsay
21 Fowler, C. F. and Kok, B. (1974) Biochim. Biophys. Acta 357, 308–318
22 Witt, H. T. and Zickler, A. (1973) FEBS Lett. 37, 307–310
23 Jackson, J. B. and Dutton, P. L. (1973) Biochim. Biophys. Acta 325, 102–113
24 Joliot, P. and Joliot, A. (1976) C. R. Acad. Sci. Paris 283D, 393–396