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FLASH-INDUCED 519 nm ABSORPTION CHANGE IN GREEN ALGAE

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SUMMARY

1. The 515 nm absorption change induced by isolated flashes was studied in *Chlorella* and *Chlamydomonas*, using a new spectrophotometric method. A strong actinic flash is followed by a weak monochromatic detecting flash (duration 3 μs) which samples the absorption level.

2. The time course of the absorption change after one actinic flash in dark-adapted algae shows four phases: Phase a : a large absorption increase (2/3 of the maximum) occurs in less than 1 μs. 60–80 % of this absorption increase is linked to Photosystem I activity, and 20–40 % to Photosystem II. Phase b: a slow absorption increase occurs in the time range of 1–50 ms. Phase b is derived only from Photosystem I. Phase c: a fast absorption decrease is observed between 50 and 500 ms. Phase c is absent in *Chlamydomonas* mutant (F54) which is blocked at a terminal stage of phosphorylation. Phase d: a slow absorption decrease is observed between 500 ms and 10 s.

3. The turnover times of the reactions driving the absorption change were studied. The turnover times of both photosystems can be distinguished; a fast turnover time ($t_{\frac{1}{2}} = 100$ μs) is observed for Photosystem I.

4. The experimental results are discussed in terms of the chemiosmotic theory of Mitchell (Mitchell, P. (1961) Nature 191, 144–148) and the electrochromic hypothesis of Witt et al. Our results suggest that phosphorylation in *Chlorella* can be driven by an electrical potential only. This is in agreement with formal results obtained in spinach chloroplasts (Witt, H. T. (1971) Q. Rev. Biophys. 4, 365–477).

5. In agreement with Junge et al., we interpret the biphasicity of the absorption decay by the existence of a critical potential for phosphorylation; but we observe that the value of the critical potential depends on physiological conditions, and can be close to zero.

INTRODUCTION

Light-induced absorption changes with positive and negative maxima at 515 and 478 nm, were first observed by Duysens [1] in *Chlorella* and other green plants, using a slow spectrophotometric method. This spectral change is the largest which has been observed either in vivo or in isolated chloroplasts.

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

Beginning in 1955 [2], Witt et al. studied the 515-nm absorption change in detail using flash spectrophotometry, which permits a considerably faster time resolution. In 1968, Junge and Witt [3] ascribed this absorption change to an electrochromic effect on the photosynthetic pigments, induced by an electrical field generated across the thylakoid membrane.

In the following studies with *Chlorella* and *Chlamydomonas*, we further characterized the 515–520 nm absorption change, using a non-repetitive flash spectrophotometric method.

METHODS

Optical device (Fig. 1)

A new spectrophotometric method was developed in which the detecting light is a short monochromatic flash of about 3 μs duration. This duration determines the time of resolution of the method. The xenon flash (General Radio Stroboslave) illuminates the entrance slit of a Bausch and Lomb grating monochromator. A beam splitter divides the pulsed beam into a measuring and a reference beam. The measuring beam illuminates the cuvette. and the transmitted light is collected on the photocathode of a photomultiplier EMI 8558. The reference beam is directed to the photocathode of a second multiplier identical to the first. The cuvette is illuminated on both sides by two actinic flashes (General Radio Stroboslave, duration 3 μs, energy 0.5 J), perpendicular to the axis of the measuring beam. The cuvette measures 8 mm along the axis of the measuring beam, and 3 mm along the axis of the actinic beam. This arrangement allows a homogeneous illumination of the biological sample.

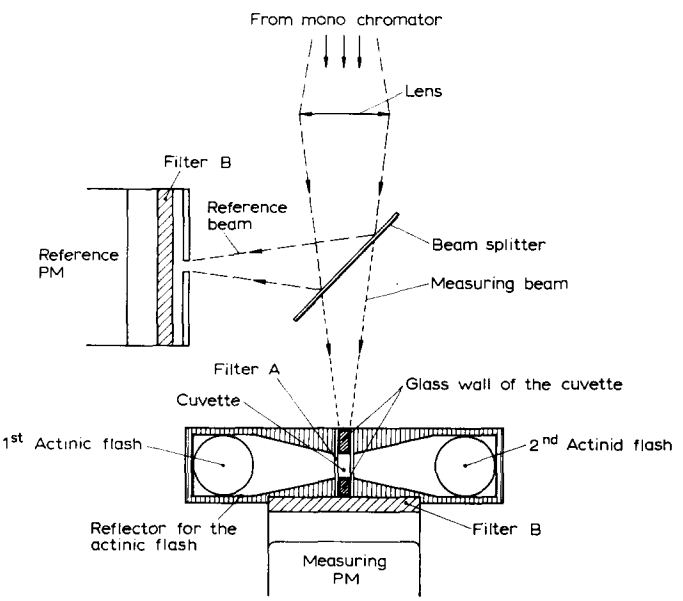

Fig. 1. Optical device (see text). Filter A (red): Wratten 24; Filter B (blue-green): Wratten 44 + Schott BG38.

Two red filters (Wratten No. 24) are interposed between the actinic flashes and the cuvette. A complementary set of blue-green filters (Wratten No. 44 and Schott BG 38) are placed in front of the measuring photomultiplier to eliminate the scattered actinic light.

Electronic device

To avoid saturation of the photomultipliers during the detecting flash, only three dynodes are used for the amplification of the current. The fourth dynode is used as anode. The current of the photocathode is limited to 5 μA, and the anode current to 1 mA. The anode current is integrated by an RC circuit with a time constant of 30 μs. Thus, the signal is proportional to the integral of the light distributed on the photocathode during the detecting flash. The two integrated signals from both photomultipliers are applied to the differential amplifier of a digital averager (Northern NS-560).

In the absence of actinic light, the signals derived from the two photomultipliers are adjusted to the same value by first varying the light intensity transmitted to the reference photomultiplier, and secondly by varying slightly the high voltage of the reference photomultiplier.

This type of differential measurements is necessary to cancel the small energy fluctuations of the detecting flash (1 $^{0}/_{0}$).

Actinic and detecting flashes can be programmed in a very flexible manner using an electronic device constructed by Mr Daniel Beal. The delay between actinic flashes, or between actinic and detecting flashes, can be varied over a large time range (from 1 μs to 30 s). An automatically programmed remote-control valve is used to renew the biological sample.

Experimental protocol

In the simplest type of experiments, the absorption of the dark-adapted material is first sampled by a series of two–four detecting flashes separated by 0.1–1 s. The biological sample is then illuminated by actinic flashes, each followed by a detecting flash. The time course of the absorption change induced by an actinic flash can be measured in two ways: (1) in the time range from 3 μs–50 ms, only one detecting flash is fired per actinic flash, thus only one point of the time curve is measured per experiment; (2) in the time range from 50 ms–30 s, the detecting flash can be used in a repetitive mode, with a dark time from 30–500 ms between the detecting flashes.

We checked that the detecting flashes had no appreciable actinic effect. With the highest flash energy available, less than one reaction center out of 1000 was hit during a detecting flash at 519 nm. However, for repetitive detecting flashes separated by less than 100 ms, it it necessary to decrease the flash energy to avoid actinic effects of the detecting beam.

The chlorophyll concentration is about 60–70 μg per ml, which in the large optical path of the detecting beam represents an absorbance of 1–1.2. This high absorbance explains why we observe a larger $\Delta I/I$ ratio than is normally reported in the literature. Despite this chlorophyll concentration, the actinic light in the algal suspension remains homogeneous.

The stability of the base-line is improved by the addition of ficoll to a final concentration of 5 %, which increases the viscosity of the medium. Under these conditions, the variation of the base-line is not a limiting factor, compared to the amplitude

of the measured signal. We checked that this introduction of ficoll had no effect on the biological properties reported in this paper.

If necessary, the signal-to-noise ratio can be improved by averaging a series of measurements.

The temperature is 20 °C.

RESULTS

Time course of the 515 nm absorption change after one isolated flash

In Fig. 2 is shown the relaxation of the 515 nm absorption change following one short saturating flash in dark-adapted *Chlorella*. The kinetics of the absorption change are extremely complex, and at least four phases can be distinguished. Phase a: a large increase of absorption is observed in a time shorter than the time resolution of the method. We checked carefully that no significant absorption change occurs in the time range of 3–200 μs. Phase b: the absorption increases slowly to a maximum in 20–40 ms, depending upon the physiological state of the algae. This time can be considered as infinitely long relative to the duration of the flash. Phase c: a relatively fast decrease of the absorption is observed in the range of 50–400 ms. Phase d: the absorption slowly returns to the dark-adapted level in about 5–10 s.

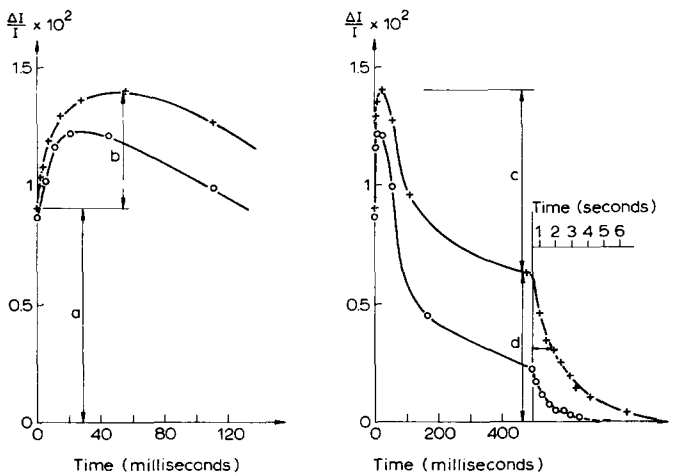

Fig. 2. Time course of the 515 nm absorption change after one saturating flash. +–+, *Chlorella* dark-adapted for 1 h; ○–○, *Chlorella* dark-adapted for 5 min.

Phases a, b and c were described by Witt and Moraw [18]. However, the amplitude of Phase b can be significantly higher than reported by these authors (55 % of Phase a in our Fig. 2, upper curve).

The relative amplitude of Phase d compared to Phase c depends upon the physiological state of the algae. After a weak illumination followed by 0–10 min in the dark, the amplitude of the slow Phase d is small or even negligible. This amplitude increases with the dark time, without significant change in the amplitude of Phase a.

Phase d is maximal for a dark time of about 1 h. For longer dark times, a decrease of the amplitude of Phase d is generally observed.

Anaerobic conditions, or chemical treatment with DCMU or NH_2OH, also affect the relative amplitudes of Phases c and d. For example, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 10 μM, suppresses Phase c after one isolated flash. Further study is required to determine the factors responsible for the level of the break between Phases c and d.

Spectrum of the absorption change (Fig. 3)

We have checked if only one type of absorption change was involved during the different phases enumerated above. The absorption was measured at different times after one saturating flash, for several wave-lengths of the detecting light. Within experimental error, no significant change was observed in the shape of the spectrum.

In Fig. 3, the amplitude of the negative peak is small compared to the positive one, in agreement with the spectra published by Witt et al. [27], Rumberg [22] and De Kouchkovsky [24]. These spectra of *Chlorella* are significantly different from those observed in isolated chloroplasts by Emrich et al. [25].

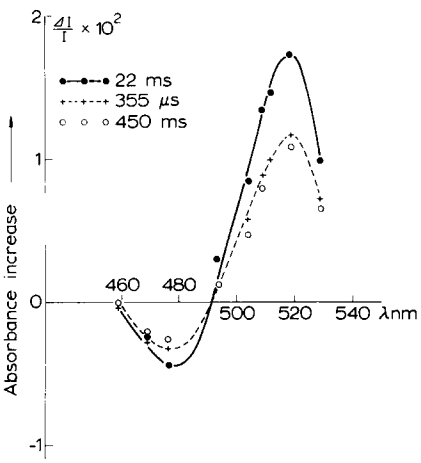

Fig. 3. Spectrum of the absorption change, measured at three different times after one saturating flash. *Chlorella* dark-adapted for 1 h.

Effect of pre-illumination

In the experiment of Fig. 4, the algae were first dark-adapted during 1 h. The upper curve represents the time course of the absorption change after one isolated flash. In a second experiment, the dark-adapted algae were illuminated by a long series of flashes (more than 60) separated by 450 ms: the lower curve represents the dark relaxation after the last flash of the series. We observe little change of the amplitude of Phase a, but a large modification of the time course of the absorption change: Phase b is suppressed, Phase c is accelerated and its amplitude increased, and the amplitude of Phase d is considerably decreased. Similar results were reported by Witt and Moraw [18], who observed after pre-illumination disappearance of Phase b and acceleration of Phase c.

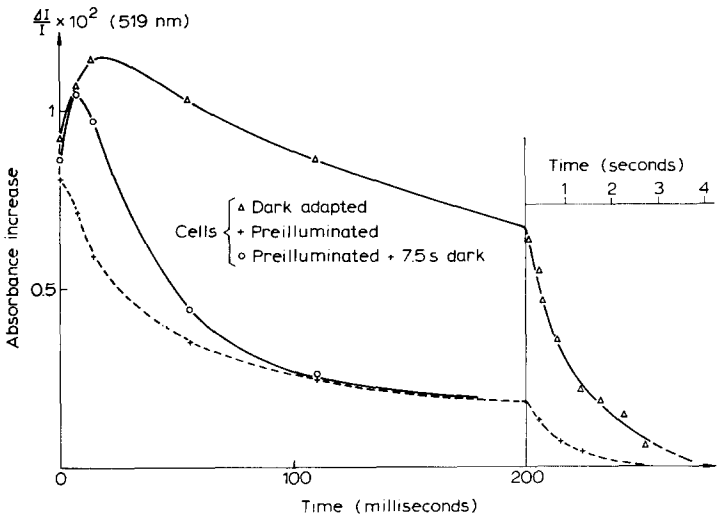

Fig. 4. Time course of the 519 nm absorption change, measured: △–△, after 1 h dark; +–+, 450 ms after a long series of flashes (more than 60 flashes separated by 450 ms); ○–○, 7.5 s after a long series of flashes.

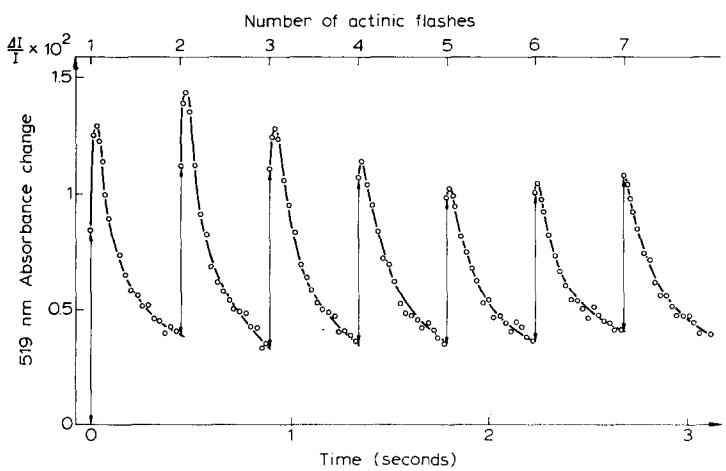

Fig. 5. Time course of the 519 nm absorption change induced by a series of saturating flashes. *Chlorella* dark-adapted for 10 min. The arrows indicate the amplitude of Phase a.

An intermediate situation is observed when the pre-illumination by a series of flashes is followed by a 7.5-s dark period (middle curve): the amplitude of Phase d remains small, but Phase b can be clearly observed.

In Fig. 5 is shown the effect of a series of seven actinic flashes separated by 450 ms. We observe a slight variation of Phase a, and a decrease of Phase b. Phase b has practically disappeared by the seventh flash.

We have never observed, during such flash sequences at room temperature, a periodicity of 4 which could be related to the charge accumulation in the oxygen-evolving system. It is interesting to point out that in isolated chloroplasts at −40 °C, Amesz et al. [28] observed a dependence of the 518 nm absorption change on the number of charges accumulated on the donor side of Photosystem II.

Flash-induced 519 nm change in a Chlamydomonas mutant (F54)

The mutant F54 isolated by Bennoun [4] includes a functional electron transfer chain, but is not able to perform photophosphorylation. Sato et al. [5] demonstrated that this mutant is blocked at a terminal stage of the mechanism of phosphorylation, probably at the level of the ATPase.

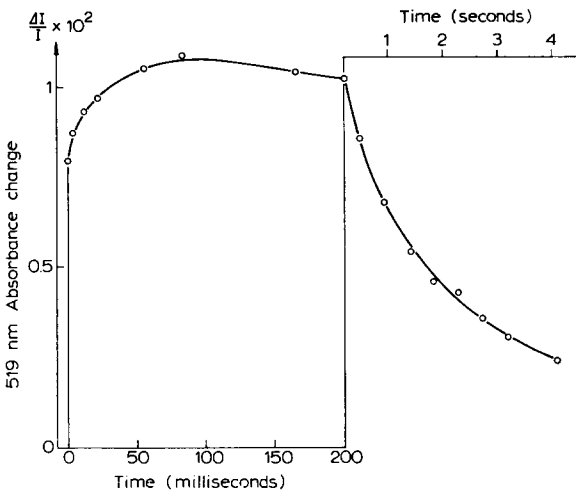

Fig. 6. Time course of the 519 nm absorption change after one saturating flash in dark-adapted *Chlamydomonas* mutant F54.

On the other hand, Nam Hai Chua and Levine [6] observed that this mutant displays an abnormally large 520 nm absorption change under continuous illumination. We observe (Fig. 6) that Phases a and b of the 519 nm absorption change induced by an isolated flash are normal compared to the wild type of both *Chlamydomonas* and *Chlorella*. But the maximal absorbance is reached later (100 ms) than in the wild type, Phase c is suppressed and only the slow decay (Phase d) is observed. Pre-illumination does not modify this situation. Fig. 7 presents the absorption level measured 50 μs after each flash of a series of seven actinic flashes separated by 450 ms. We observe a gradual increase of the absorption level; the effects of the first flashes are additive. After seven actinic flashes, the absorption decrease is sampled by a series of detecting flashes separated by 450 ms. The absorption returns slowly to the dark-adapted level, with a half-time close to that observed for Phase d in the wild type.

This experiment is consistent with the results of Rumberg and Siggel [11], who observed that the time course of the 515 nm absorption change is a function of the efficiency of phosphorylation.

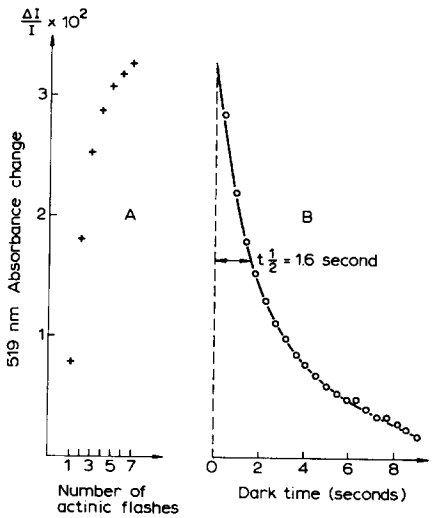

Fig. 7. 519 nm absorption change induced by a series of seven saturating flashes in *Chlamydomonas* mutant F54. (A) Absorption level sampled 50 μs after each actinic flash. (B) Time course of the absorption change after the last flash of the series.

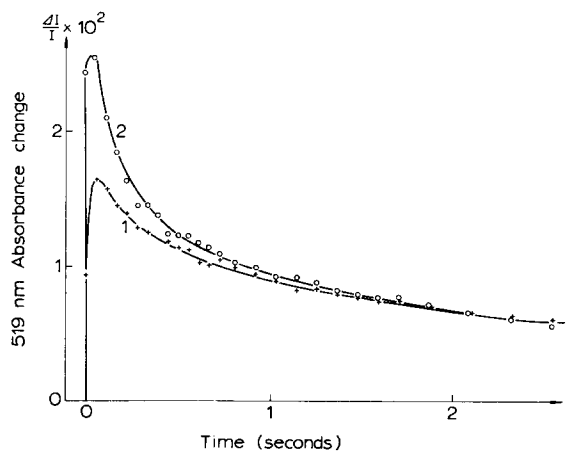

Fig. 8. Time course of the 519 nm absorption change. Curve 1, after one saturating flash. Curve 2, after two saturating flashes separated by 22 ms.

Biphasicity of the absorption decrease at 519 nm

In Fig. 8, the algae were pre-illuminated firstly by one saturating flash (Curve 1) and secondly by a pair of saturating flashes separated by 22 ms (Curve 2). The double flash induced a much higher absorption increase than the single saturating flash. Phase c was correspondingly increased, but Phase d remained unmodified. Thus the absorption level at the break between Phases c and d is independent of the absorption level immediately following the last flash.

We conclude from this experiment the existence of a threshold absorption level, which depends on the state of the algae. Above this absorption level, a fast reaction rapidly decreases the absorption, while below this level only a slow reaction occurs.

Junge et al. [7, 8] obtained similar results with isolated chloroplasts. We will discuss later the differences between their results and our own.

Photoreactions driving the 519 nm absorption change

We studied the effect of inhibition of System II on Phases a and b.

1. Phase a. In the experiment reported in Table I, the amplitude of Phase a was measured after one saturating flash, for two different batches of algae, under three experimental conditions: 1, uninhibited, dark-adapted cells, 2, DCMU-treated, dark-adapted cells and 3, DCMU plus NH_2OH-treated cells, pre-illumination followed by 10 s dark. When algae are pre-illuminated in the presence of both inhibitors, the electron acceptor Q is irreversibly blocked in the reduced form [9, 10], i.e. the System II reaction center is not photoactive.

TABLE 1

EFFECT OF INHIBITION OF PHOTOSYSTEM II ON THE AMPLITUDE OF PHASE a AT 519 nm IN *CHLORELLA*

Condition 1, Control (uninhibited). Condition 2, 10 μM DCMU. Condition 3, 10 μM DCMU + 100 μM NH_2OH; pre-illumination with continuous light, followed by 10 s dark. Units: the control value is taken as reference (100).

	Dark-adapted		Pre-illuminated
	(1) Control	(2) DCMU	(3) DCMU+NH_2OH
Batch 1	100	93	81
Batch 2	100	97	61

As compared to the control (Condition 1), Condition 2 induces only a small decrease of Phase a; but Condition 3 induces a decrease of Phase a by 20–40 %. We checked that in Condition 3 the value of Phase a was not modified when the dark period between pre-illumination and the flash was varied from a few seconds to 20 min. This latter result is in agreement with Bennoun's observation of an irreversible blockage of System II reaction centers.

We conclude from these experiments that Phase a depends on both System I and System II reaction centers. This is in agreement with the results of Schliephake et al. from isolated chloroplasts [13]. However, in our experiments, the contribution of System I is 1.5–4 times higher than that of System II.

The small decrease of Phase a induced by DCMU alone can be related to the fact that, independent of the block of the electron transfer, DCMU reduces appreciably the number of System II reaction centers [12].

2. Phase b. The amplitude of Phase b was compared in the presence or absence of DCMU plus NH_2OH for algal Batch 2 (see Table I), which displays the larger

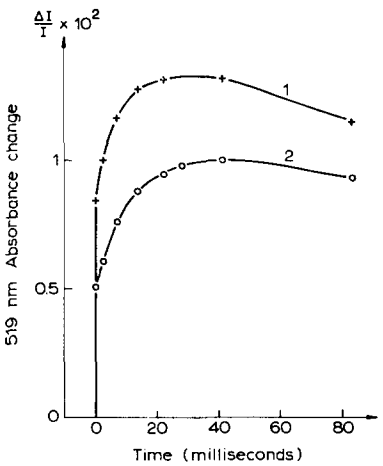

Fig. 9. Time course of the 519 nm absorption change after one saturating flash. Curve 1, control (dark-adapted). Curve 2, 10 μM DCMU + 100 μM NH_2OH; pre-illumination by several flashes, 10 s before the experiment.

519 nm absorption change linked to System II (Fig. 9). While the amplitude of Phase a is decreased about 40 % as pointed out previously, the amplitude of Phase b is not modified. This result suggests that Phase b is associated only with System I activity.

From these experiments we conclude that the contribution of Photosystem I to the 519 nm absorption change is 60–80 % if we consider only Phase a, and 70–85 % if we consider the total absorption increase (Phases a+b). These values are higher than those reported by Witt et al. (see refs 13 and 23).

Dependence of the absorption change on the energy and the duration of the actinic flash

In Fig. 10, we plotted the fast 519 nm absorption change (Phase a) as a function of the energy of the actinic flashes. In the first experiment, the two actinic flashes, placed on either side of the cuvette, are synchronized (Curve 1); in a second experiment, the two actinic flashes are separated by 700 μs dark (Curve 2). The experiments are performed on pre-illuminated algae in the presence of DCMU plus NH_2OH, so that only the 519 nm absorption change linked to System I activity is observed.

When the total energy distributed by the two flashes is low, the absorption change is independent of the time distribution of the energy, which means that the probability of hitting the same photocenter twice is low. At higher energy, the absorption change induced by the two flashes separated by 700 μs is much higher than when the two flashes are synchronized. We conclude that Photosystem I reaction centers have a turnover time longer than the flash duration, but faster than 700 μs.

In the case of the synchronized flashes, the energy available is sufficient to attain saturation. This energy is not fully saturating when the two flashes are separated by 700 μs dark, which is understandable because in this case more than one photoreaction occurs per reaction center.

We observed similar saturation curves (not shown) in the absence of DCMU and NH_2OH. This result suggests that there is little difference between the optical cross-sections of System I and System II reaction centers.

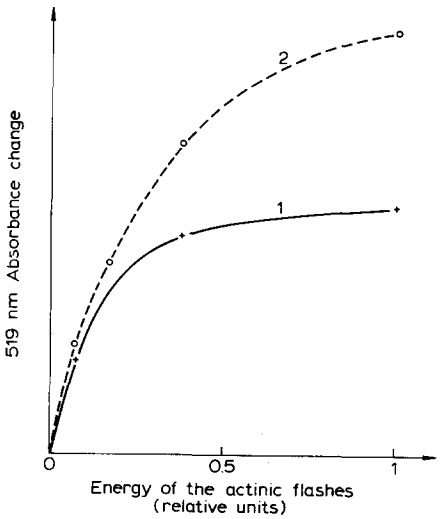

Fig. 10. Dependence of the fast 519 nm absorption increase (Phase a) on the energy of the actinic flashes. Curve 1, after two synchronized actinic flashes; Curve 2, after two actinic flashes separated by 700 μs. *Chlorella*+ 10 μM DCMU+100 μM NH_2OH, pre-illuminated. The absorption level is sampled 13 μs after the last flash.

Turnover of the photoreactions driving the 519 nm absorption change

The 519 nm absorption change induced by the second of a series of two actinic flashes was measured as a function of the time interval Δt between the two flashes, in 1 h dark-adapted *Chlorella* (Fig. 11). Two measurements are required for this purpose: in a first experiment, two actinic flashes are given with a dark interval Δt, and the absorption is sampled by a detecting flash 13 μs after the second actinic flash; in a

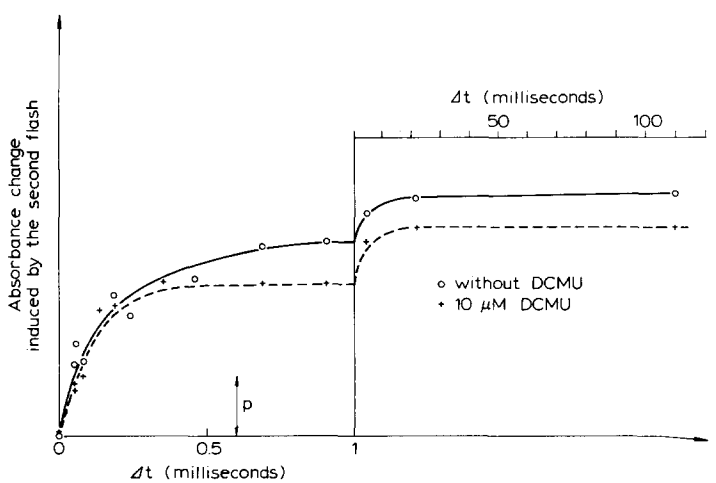

Fig. 11. Fast 519 nm absorption increase (Phase a) induced by the second actinic flash of a series of two (see text). p = absorption increase due to the second actinic flash for $\Delta t = 0$. p was substracted from each measurement. *Chlorella* dark-adapted for 1 h.

second experiment, the second actinic flash is suppressed, and the detecting flash is given at the time Δt after the first actinic flash. The difference between the two measurements gives the absorption increase induced in 13 μs by the second flash (Fig. 11). A control experiment was performed for $\Delta t = 0$: a difference p was observed between the absorption measured respectively after two flashes and after one flash. This difference p is due to the fact that the energy of a single flash is not fully saturating (see Fig. 10), and perhaps also to the intervention of double hits during the tail of the xenon flash. In Fig. 11 the value of p was subtracted from each measurement to consider only the effect of Δt.

In Fig. 11, we can distinguish at least three dark reactions, with three different turnover times. A first reaction is complete in about 300 μs. This reaction is not modified by addition of DCMU, and is attributed to the turnover of Photosystem I reaction centers.

A second reaction, in the range of 1 ms ($t_{\frac{1}{2}}$ approx. 0.5–1 ms), is DCMU-dependent, and is thus attributed to System II reaction centers. The half-time of this reaction is consistent with the turnover time of Photosystem II in *Chlorella* as determined by oxygen measurements [14, 29–31].

A third reaction, in the range of 10–20 ms, is DCMU-insensitive, and is attributed to System I.

In the presence of DCMU, the fraction of System II reaction centers regenerated in 50 ms is negligible [10]. Nevertheless, DCMU induces only a small decrease of the absorption change caused by the second flash. Thus, we conclude (as from the experiment of Fig. 9) that for this algal batch most of the 519 nm absorption increase is linked to Photosystem I activity.

519 nm absorption change after saturating and non-saturating actinic flashes

To determine if the kinetics of the 519 nm absorption change depend on the number of reaction centers excited, we studied these kinetics in saturating and non-saturating flashes.

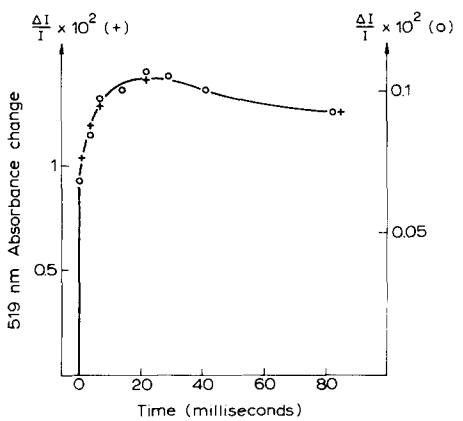

Fig. 12. Time course of the 519 nm absorption increase induced by saturating and non-saturating flashes. +–+, after one saturating flash; ○–○, after one non-saturating flash. *Chlorella* dark-adapted for 1 h. The two curves were normalized to the same amplitude of Phase a.

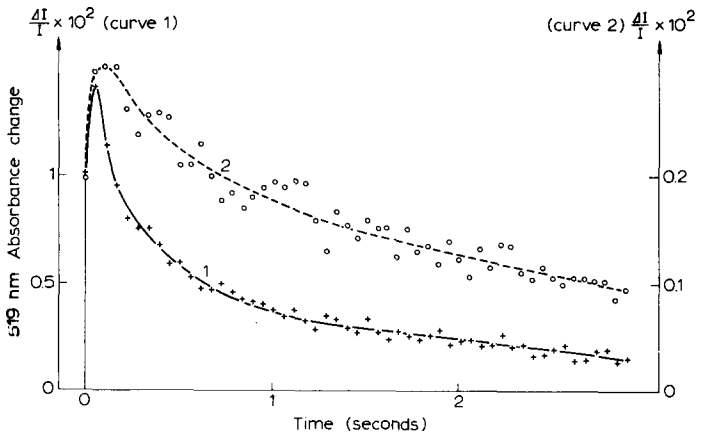

Fig. 13. Time course of the 519 nm absorption decrease induced by saturating and non-saturating flashes. +–+ (Curve 1), after one saturating flash. ○–○ (Curve 2), after one non-saturating flash. *Chlorella* dark-adapted for 10 min. The two curves were normalized to the same amplitude of Phase a. In Curve 1, Phase b is slightly shortened by the large Phase c.

1. Phases a and b (Fig. 12): to observe the increasing Phase b under the best conditions, we used *Chlorella* cells dark-adapted for 1 h, so that the fast decreasing Phase c was small. If we admit that the amplitude of Phase a is proportional to the number of reaction centers hit, then about 1/14 of the reaction centers were hit during the weak flash. After normalization to the same amplitude of Phase a (Fig. 12), the relative amplitude and the time course of Phase b appear to be perfectly independent of the number of reaction centers hit during the flash.

2. Phases c and d (Fig. 13): in this experiment, we used *Chlorella* cells dark-adapted for 10 min, which show a large Phase c and a small Phase d after one saturating flash. We compared the time course of the 519 nm absorption change after a saturating flash (Curve 1) and after a weak flash (1/5 of reaction centers hit: Curve 2). The two curves were normalized to the same amplitude of Phase a. We observe a longer lifetime after the weak flash than after the saturating flash: thus, contrary to Phase b, the time course of the absorption decrease depends on the number of reaction centers hit.

Dependence of Phases a and b upon the wave-length of a non-saturating actinic flash (Fig. 14)

When algae are illuminated by weak actinic flashes far below saturation, the proportion of System I and System II reaction centers hit during the flash depends on the wave-length. In the case of Fig. 14, two different actinic wave-lengths were used.

1. Curve 1: the algae are illuminated through a Wratten filter 24, which transmits all the red light. In these conditions, we can estimate that the two photosystems are excited approximately to the same extent (all the experiments reported above were performed under these conditions).

2. Curve 2: the algae are illuminated through two Wratten filters (97 and 24) which transmit only the light of wave-lengths larger than 700 nm. Under these conditions, the excitation of System I is about 10 times larger than that of System II [15].

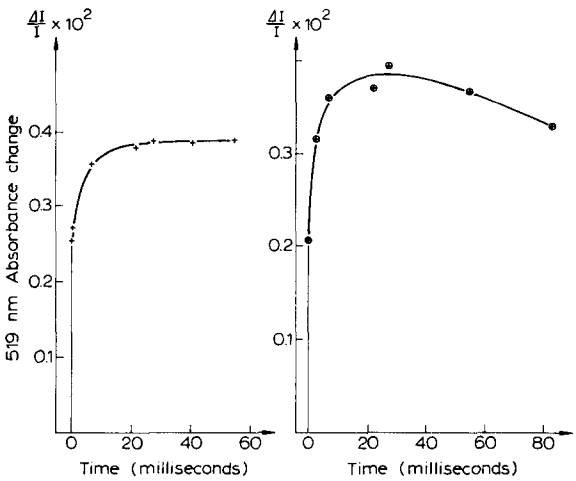

Fig. 14. Dependence of the 519 nm absorption increase on the wave-length of a non-saturating actinic flash. Curve 1, Filter A (Wratten 24), transmitting red light ($\lambda > 585$ nm). Curve 2, Filter A (Wratten 24+97) transmitting only far red light ($\lambda > 695$ nm). The two curves were normalized to the same amplitude of Phase a. The absorption change $\Delta I/I$ corresponding to Phase a was $2.5 \cdot 10^{-3}$ for Curve 1, and $2.0 \cdot 10^{-3}$ for Curve 2. A saturating actinic flash induced an absorption change $\Delta I/I \simeq 8 \cdot 10^{-3}$.

We observe (Fig. 14) that the relative amplitude of Phase b (compared to Phase a) is much larger in the case of the far-red excitation. We conclude (as from Fig. 9) that Phase b is associated only with System I activity.

DISCUSSION

The 520 nm absorption change and the electrochromic hypothesis

The only coherent interpretation of the 520-nm absorption change was proposed by Junge and Witt [3]. These authors suppose that the photochemical charge separation creates an electrical field across the membrane of the thylakoid. This field induces a small absorption shift which concerns all the pigments included in the membrane.

The experiments described in this paper, while not proving this hypothesis, are coherent with it.

1. Although the fast 520 nm absorption increase (Phase a) follows almost instantaneously the photoreaction ($t_{\frac{1}{2}} \leqslant 20$ ns according to Wolff et al. [16]), there is no correlation between the relaxation curve and the turnover of the reaction centers. Thus, the absorption change is no direct or indirect indicator of the redox state of the reaction centers. On the other hand, we were not able to establish a correlation between the time course of the absorption change and the kinetics of any one of the reactions involved in the electron-transfer chain. The field effect is a phenomenon which "memorizes" the photoact a long time (a few seconds) relative to the recovery of the photochemical activity (a few milliseconds).

2. The decreasing phase (c and d) of the absorption change appears as a cooperative phenomenon, since the lifetime is shorter when all the reaction centers are

hit than when a small fraction of them are hit (Fig. 13). On the other hand, one cannot distinguish in Phases c and d the absorption change generated by System I from that generated by System II. This result suggests that the mechanism for the decay is independent of the photoreaction producing the original absorption change (cf. ref. 13). In this respect also, the electrochromic hypothesis developed by Junge and Witt [3] is coherent with the experimental facts. First, these authors assume that the functional unit involved in the absorption change is the whole thylakoid membrane, including a great number of reaction centers: this takes into account the co-operation observed during Phases c and d. Secondly, they suppose that both types of reaction centers are included in the same membrane and co-operate to create the field and the proton gradient [13]. While it is not possible to discuss here the numerous arguments developed particularly by Witt et al., and also by Jackson and Crofts [17], we consider the electrochromic interpretation as sufficiently coherent to choose it as a working hypothesis.

Dependence of the 520 nm absorption change upon the two photoreactions

Our results confirm anew the fact that the 520 nm absorption change depends upon both photoreactions. Nevertheless, we find that the contribution of System I is in large excess compared to that of System II (Fig. 11). This result does not seem valid for isolated chloroplasts, according to Schliephake et al. [13]. As our experiments are performed with saturating actinic flashes (see Fig. 10), and if we consider with Witt et al. [13] that both systems are included in the same membrane and co-operate to create the field, we conclude that System I reaction centers in *Chlorella* are in excess compared to System II reaction centers. On the other hand, the saturating flash experiments performed here do not permit a conclusion about the relative sizes of the collecting antennae associated with each of the photoreactions to be drawn.

Interpretation of Phase b

Witt and Moraw [18] observed an absorption increase similar to our Phase b (but of smaller amplitude) after one flash in *Chlorella*. This experimental point was not discussed in recent papers or reviews by these authors. The experiments of Figs 9 and 14 demonstrate clearly that Phase b is only related to Photosystem I activity. Furthermore, the fact that the time course of Phase b is independent of the number of reaction centers hit (Fig. 12) proves that Phase b is a non-co-operative phenomenon, independent of interactions which might exist between reaction centers.

It seems unlikely that this phase can be attributed to a spectral change different from the fast-induced 520-nm change (Phase a): first, the spectra are very similar (Fig. 3); secondly, it is not possible during the subsequent absorption decrease to distinguish a component linked to Phase a from a component linked to Phase b.

Two types of interpretation can be proposed for Phase b:

1. In a first hypothesis, we postulate that the photochemical charge separation does not occur between the inner face and the outer face of the membrane, but that the photochemical complex is embedded in the membrane itself. Thus a complete charge separation from one side to the other side of the membrane requires some dark steps, e.g. redox reactions in the electron transfer chain.

In this hypothesis, Phase b is attributed to the change from an initial field

induced by the dipoles resulting from the charge separation, to that observed when the charges are delocalized as free ions in the inner and outer phases. Several arguments can be made against this hypothesis: (a) Phase b was not observed in isolated chloroplasts; (b) Phase b in *Chlorella* disappears after illumination by several flashes (about seven); (c) The amplitude of Phase b in *Chlorella* decreases when the temperature is lowered from 20 °C to 5 °C [18]. Thus it seems difficult to associate Phase b to a redox reaction of the main electron-transfer chain.

2. A second type of hypothesis is to suppose that, in dark-adapted algae, the photochemical charge separation starts a charge transfer through the membrane by a channel other than that of the photochemical complex. A support for this hypothesis is that when only the System I photoreaction occurs, the amplitudes of Phases a and b appear equal (Figs 9 and 14). Thus, in the frame of this hypothesis, we can admit that the photochemical transfer of one charge is followed by the active transfer of a second charge via an unknown channel. The disappearance of Phase b after six or seven flashes (whatever the dark time between flashes) suggests that this active transfer involves a substance present in limited concentration.

Jackson and Dutton [26] recently reported the observation of a slow increasing phase in the carotenoid band shift induced by a single flash in *Rhodopseudomonas spheroides.*

Turnover time of System I

Whatever the correct interpretation of the 520 nm absorption change, the measurement of Phase a provides a simple and efficient method to follow the photochemical activity, especially for Photosystem I. An example is the measurement of the turnover time of Photosystem I in *Chlorella* (Fig. 11).

It is interesting to compare our data with the results recently reported by Haehnel and Witt [19] in isolated chloroplasts. In our experiments, we observe that the fast phase (0–300 μs) represents a larger component of the turnover reaction than in Haehnel's experiments. The difference between our experiment and those of Haehnel and Witt might be due to the fact that we work with dark-adapted algae while Haehnel and Witt use chloroplasts subjected to repetitive flashes. We would like to pursue this study further to determine if, in *Chlorella*, a simple series scheme involving a fast step (probably Cyt *f* to P_{700} reaction) and a slow step (plastoquinone to Cyt *f* reaction) is sufficient to explain our kinetic data. In chloroplasts, the data published by Marsho and Kok [20] and Haehnel and Witt suggest a more complex scheme.

520 nm absorption change and phosphorylation

One basic assumption of the theory of Mitchell [21] is that the synthesis of ATP requires the transfer of hydrogen ions from the inside to the outside of a closed membrane (probably the thylakoid). The transfer of electrons through the chain of electron carriers of photosynthesis pumps hydrogen ions towards the inside of the thylakoid. The energy necessary for ATP synthesis comes from the electrical potential and the proton gradient developed across the membrane. A correlation between the life time of the 520 nm absorption change and the efficiency of phosphorylation was reported by Rumberg and Siggel [11] and Junge et al. [7]. These authors observed a considerable acceleration of the decay of the absorption change when ADP was

added to isolated chloroplasts. They interpreted this observation in terms of a breakdown of the field during phosphorylation associated with a flux of protons from the inside to the outside of the thylakoid. Similar conclusions can be obtained from the comparison of the 519 nm relaxation curves for *Chlorella* or *Chlamydomonas* (wild type) and for the mutant F54 blocked at a terminal stage of phosphorylation (Figs 6 and 7): we observe that the decay of the 519 nm absorption change is always very slow in the mutant F54, even after pre-illumination by several flashes (Fig. 7). The lifetime of the 519 nm absorption change in mutant F54 ($t_{\frac{1}{2}}$ approx. 1.5 s) is considerably longer than the lifetime in isolated chloroplasts in the absence of phosphorylating reagent (200–250 ms after Rumberg and Siggel, and Junge et al.). This result suggests that the permeability of membranes in vivo is lower than in isolated chloroplasts.

Significance of the break between the fast and the slow phase (Phases c and d) during the decay of the absorption change

We pointed out that the decay of the absorption change induced by a single flash is clearly biphasic, the time course of the slow phase being very similar to the one measured in mutant F54. Thus it is reasonable to suppose that phosphorylation occurs only during Phase c and not during Phase d. Our results are very similar to those obtained by Junge et al.: on isolated chloroplasts, they also observed a biphasicity in the decay of the 520 nm absorption change. In both cases, it can be demonstrated that the biphasicity of the decay does not depend upon heterogeneity of the biological material. For instance, if we suppose that the chloroplasts include two types of thylakoids, one associated to active phosphorylation enzyme and the other not, we predict for the double-flash experiment (Fig. 8) a doubling of both Phases c and d. On the contrary, the experiment shows a large increase of Phase c without change in Phase d. Similar experiments have already been performed by Junge et al., and we concur with these authors as to the existence of a critical potential for phosphorylation. The main difference between our experiment and that of Junge et al. is that we observe on average a much lower value for the critical potential, which varies widely depending upon the state of the algae. In our experiments, the value of the critical potential is always lower than the value of the potential reached after one isolated flash. On the contrary, Junge et al. observed the fast Phase c only when the chloroplasts were illuminated by a series of flashes, which generate a much larger potential.

A few minutes after a weak pre-illumination, the critical potential is small compared to the potential generated by a single flash. For this reason, our results are not in contradiction with experiments by Boeck and Witt, who observed that a critical potential is not essential for phosphorylation [23].

Two main conclusions can be drawn from our experiments, if we admit the fast decay (Phase c) is associated with phosphorylation.

1. A single flash is able to drive phosphorylation. After a single flash, a large electrical potential is produced, but the proton gradient remains negligible. Thus we must concede that phosphorylation can occur when the proton-motive force is exclusively the electrical potential, as observed in chloroplasts by Boeck and Witt [23].

2. After a weak pre-illumination, no critical potential is required for phosphorylation. We must recall that such an absence of critical potential can also be observed after very long dark adaptation (several hours). Under these conditions, it seems that the ATPase can work in a completely reversible manner. It is not the case under other

physiological conditions (i.e. 1 h after a weak pre-illumination), where a critical potential is observed.

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