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FACTORS REGULATING THE SLOW ELECTROGENIC PHASE IN GREEN ALGAE AND HIGHER PLANTS

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Summary

The electrochromic rise in the millisecond range, corrected for the subsequent decay, has been studied in *Chlorella* cells and spinach chloroplasts.

The half-time of the electrochromic rise in the millisecond range is independent of the redox states of the components involved in the electrogenic loop. It is also independent of pH in the absence of a pH gradient, but it is dependent upon the transmembrane electric field and the pH gradient. The limiting step of the overall process is thus the electrogenic reaction itself.

The amplitude of the electrochromic rise in the millisecond range is controlled by two classes of factors: the redox state of its reactants and the free energy available in the overall process including the electrogenic reaction. This free energy depends upon ΔpH and transmembrane electric field.

A fast reduction of cytochrome f^+ (and thus probably of the Rieske protein) by Photosystem II is still possible when the electrogenic reaction is impeded for energetic reasons.

The kinetic behavior of the electrogenic reaction could be interpreted by the following reaction:

$$UH_{2int} + FeS^+ + X_2 + H^+_{ext} \leftrightharpoons U + 2H^+_{int} + FeS + X_2H$$

where FeS is the Rieske protein; X_2 would be a new protein (observed in Bouges-Bocquet, B. (1980) FEBS Lett. 117, 54—58); UH_2 which is likely a special

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Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; FeS protein, Rieske iron-sulfur protein; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

quinone, would release its two protons inside the thylakoids and X_2 would use protons from the external medium.

Introduction

According to the theory of Mitchell [1], the components of the electron transfer chain are located in the membrane in such a way that photoreactions generate a transmembrane electric field. The increase of the absorption at 515 nm, which is a linear measure of the transmembrane electric field [2], takes place less than 20 ns after the photochemical reactions in chloroplasts [3], due to charge separation. An additional increase of the absorption at 515 nm has been observed in the millisecond range after flashes, with *Chlorella* cells [4,5] and with chloroplasts [6—8]. According to the terminology in Ref. 5, the fast absorption increase at 515 nm due to charge separation is referred to as phase a, the slow one in the millisecond time range as phase b.

The 515 nm absorption increase due to phase b cannot be distinguished from the 515 nm absorption increase due to phase a, i.e. both originate from an electrochromic effect which reflects a transmembrane electric field [5,10,16]. For example, both are slowed down when phosphorylation is inhibited [5]. One must conclude that phase b as well as phase a originate from charges crossing the membrane.

What is the reaction generating phase b? It could be either the end of the delocalisation of the charges separated by the photoreactions or additional charges crossing the membrane. Three arguments favor the second hypothesis:

1. The amplitude of phase b at its maximum is identical to the amplitude of the phase a generated by Photosystem I [5,11].

2. It is possible to obtain electron transfer from system II to system I having similar charge separation and similar phase a, but either with or without phase b (see below Fig. 7).

3. The compounds involved in the reaction giving rise to phase b [11,39] are very similar to the compounds involved in the reaction giving rise to the slow electrochromic rise (phase III) in bacteria. This latter rise has been demonstrated to be due to an additional charge crossing the membrane (for review see Ref. 40).

In this paper, I thus consider phase b to be an additional charge crossing the membrane in the millisecond time range after photoreactions.

The slow electrochromic rise was not observed after a few flashes in Chlorella cells [5,11]. However, no precise analysis of the kinetics of the electrochromic effect, taking into account the following decay, has been performed. In contrast, Velthuys [7] reported, for chloroplasts under stationary conditions, an H^+/e ratio of 2.4 which would indicate that, at least in a fraction of the chains, an extra proton-pumping loop and its associated electrogenic reaction takes place under stationary conditions. It is important to known in which conditions this electrogenic phase occurs, to understand its role under physiological conditions, its eventual contribution to the proton gradient and, thus, to phosphorylation.

Materials and Methods

Chlorella pyrenoidosa was grown on Knopp medium [12] to which were added Arnon's trace elements A_5 and B_6 [13]; the culture was illuminated by white fluorescent light. Before use, cells were resuspended at a chlorophyll concentration of 25 μg/ml in 0.1 M phosphate buffer containing 7% Ficoll.

Chloroplasts were isolated according to a method developed by Diner (Diner, B., unpublished results). Leaves of market spinach were cut into 1 cm^2 pieces and kept in distilled water, under white fluorescent light, during 1 or 2 h. They were ground in Tricine buffer (0.02 M, pH 7.4) with 0.4 M sorbitol and 0.01 M ascorbate, for 5 s at high speed and 10 s at low speed. The suspension was then filtered through 16 layers of cheese cloth and centrifuged for 10 min at 1000 $\times g$. The pellet was resuspended at a chlorophyll concentration of 60 μg/ml in a 0.02 M buffer with 0.4 M sorbitol, 0.01 M ascorbate and 7% Ficoll. The buffers were: Tricine above pH 7.4, Mops between pH 7.4 and pH 6.6; Mes below pH 6.6.

The absorption changes were measured on the single beam kinetic spectrophotometer described in Ref. 14. Flash excitation was provided by a red-filtered Xenon flash of 6 μs pulse width which induced 1.9 photochemical reac-

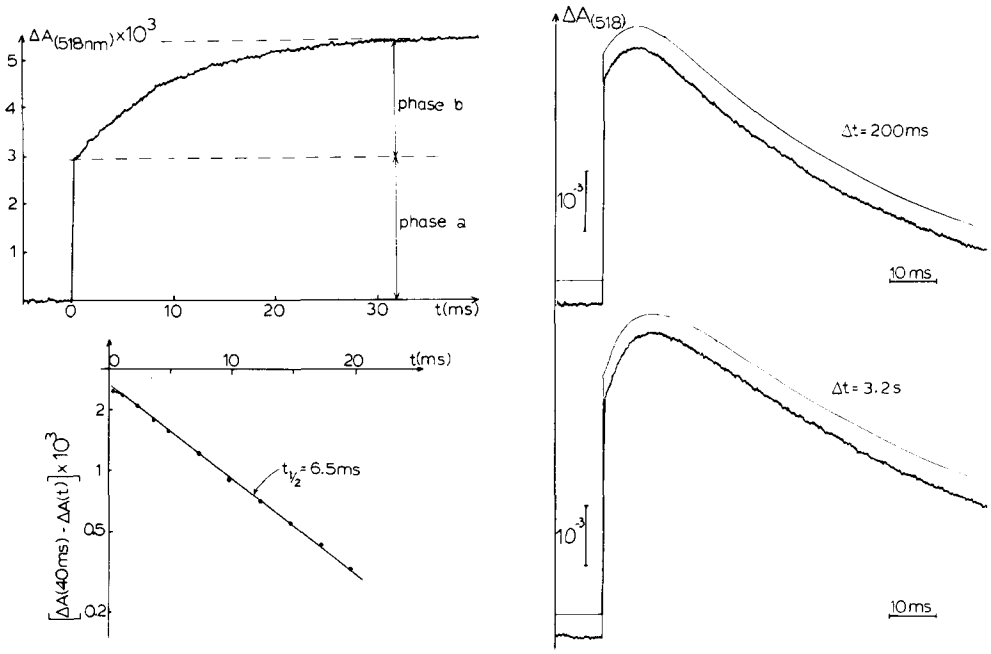

Fig. 1. Top. Absorption changes at 518 nm, induced by a flash in *Chlorella* cells with $2 \cdot 10^{-4}$ M dithionite. Average of 64 flashes fired on the same sample every 6.4 s. Bottom. Kinetics of the slow phase (phase b) in semilogarithmic plot.

Fig. 2. Absorption changes at 518 nm, induced by a flash in *Chlorella* cells. Average of 64 flashes fired on the same sample every 200 ms (top trace) and every 3.2 s (bottom trace). The upper lines are computed curves (see text). The parameters used are $a = 3.8 \cdot 10^{-3}$, $b = 1.8 \cdot 10^{-3}$, half-time of field-decay = 30 ms, half-time of phase b = 5.6 ms (top trace); $a = 4 \cdot 10^{-3}$, $b = 2.1 \cdot 10^{-3}$, half-time of field-decay = 54 ms, half-time of phase b = 4.9 ms (bottom trace).

tions per system I center in *Chlorella* cells [15] and around 1 reaction per center in chloroplasts. For Fig. 4, a short Xenon flash of 1 μs pulse width (Verrerie Scientific type CA 972), which induced 0.85 photochemical reaction per system I center in *Chlorella* cells, was used.

The most precise kinetics of the slow electrochromic rise (Fig. 1, top curve) were obtained in *Chlorella* cells which had slow electric field decay, in the presence of a small amount of reductant. In effect, as shown in Ref. 11, the existence of the slow electrochromic rise (phase b according to the terminology of Joliot et al. [5]) depends upon the presence of a compound in the reduced state, and addition of a small amount of reductant increases the amplitude of phase b.

The semi-logarithmic plot of phase b (Fig. 1, bottom curve) indicates that the kinetics of phase b follow exponential kinetics. A spectral analysis of the small lag at the beginning of the curve shows that it is due to an additional absorption change due to the formation of some photoreduced ferredoxin in the presence of reductant (Bouges-Bocquet, B., unpublished results).

When the electric field decay is more rapid, the kinetics of the electrochromic effect can be fitted with two exponentials (Fig. 2):

$$\Delta A = a\, e^{-k't} + b \frac{k}{k - k'} (e^{-k't} - e^{-kt}) \quad (1)$$

In this paper, amplitude and half-time of phase b will refer to the parameters, b and k, which allow this equation to fit with the 518 nm absorption changes and thus will correspond to the amplitude and half-time of phase b corrected for the electric field decay.

Results

Fig. 3 presents the dependence of the half-time and amplitude of phase b on the pH in chloroplasts. Clearly the half-time of phase b does not depend on pH. On the other hand, the relative amplitude of phase b, which can be equal to that of phase a in *Chlorella* when system II is inhibited, is strongly decreased when the pH is decreased and becomes almost negligible below pH 6.

Another factor which could induce a change in the half-time of phase b is the redox state of the compounds involved in the electrogenic reaction. The amplitude of phase b can be decreased either by action of an oxidant [11] or by action of a strong reductant (Table I). However, both treatments, which vary the redox state of the compounds involved in the electrogenic reaction, leave the half-time of phase b unchanged (Table I). In contrast, Table II shows that factors changing the electric field decay modify the half-time of phase b: freshly harvested cells exhibit a fast field decay and a fast phase b; when in these cells the field decay was slowed down by exposure to dim light [16], phase b was also showed down; after addition of dicyclohexyl-18-crown-6, a compound which accelerates the field decay (Diner, B., personal communication), phase b was accelerated.

In addition, in cells in which the electric field decayed slowly and in which, during a series of flashes, the electric field generated by the electron transfer chain was not collapsed between two flashes, the half-time of phase b increases

TABLE I

AMPLITUDE AND HALF-TIME OF PHASE b IN *CHLORELLA* CELLS IN DIFFERENT REDOX CONDITIONS

Average of 64 flashes fired on the same sample every 3.2 s.

	Amplitude of phase b (%)	Half-time of phase b (ms)
Ferricyanide, $2 \cdot 10^{-4}$ M + DAD, $5 \cdot 10^{-5}$ M	25	3
Dithionite, $2 \cdot 10^{-4}$ M	100	3
Dithionite, $2 \cdot 10^{-2}$ M	30	3

TABLE II

HALF-TIME OF PHASE b IN *CHLORELLA* CELLS UNDER DIFFERENT CONDITIONS OF ELECTRIC FIELD DECAY

Average of 64 flashes fired on the same sample every 6.4 s.

	Half-time of the electric field decay (ms)	Half-time of phase b (ms)
Freshly harvested cells	40	2
Same batch kept in dim light	330	10
After further addition of dicyclohexyl-18-crown-6, $5 \cdot 10^{-5}$ M	56	6

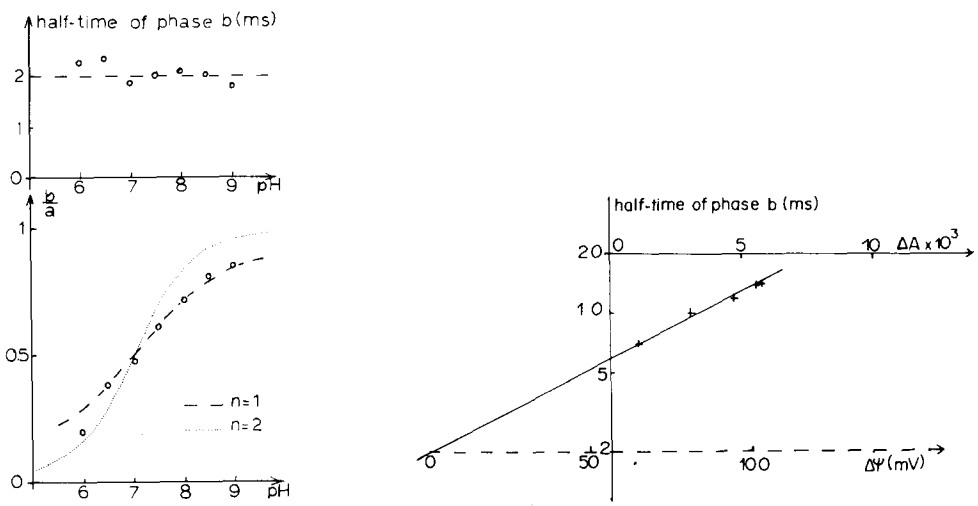

Fig. 3. Half-time (top trace) and relative amplitude (bottom trace) of phase b in spinach chloroplasts as a function of pH. Dithionite concentration was adjusted at each pH value to get the maximal amplitude of phase b. Average of 64 flashes fired on the same sample every 6.4 s. The dotted lines are computed curves (see Discussion).

Fig. 4. Half-time of phase b as a function of the electrochromic effect before the flash in slow field decaying *Chlorella* cells with dithionite $2 \cdot 10^{-4}$ M. These results were obtained from a series of five flashes spaced by 100 ms. Average of 256 series fired on the same sample once every 6.4 s. The bottom scale is a computed scale (see Discussion).

when the flash number increases. This effect is not observed in cells where the electric field collapses between two flashes. Fig. 4 presents, in semi-logarithmic plot, the half-time of phase b during a series of flashes, in cells with slow electric field decay, as a function of the electrochromic change before the flashes (top axis). On the top axis, the zero corresponds to the state of the electrochromic effect at the beginning of the series of flashes, and does not necessarily correspond to the zero of the transmembrane electric field. This figure shows that the bigger the transmembrane electric field, the slower the kinetics of phase b.

Fig. 5 presents the relative amplitude and half-time of phase b as a function of flash frequency with and without NH_4Cl in chloroplasts. Dithionite $2 \cdot 10^{-4}$ M had been added to maintain phase b at its maximal value. In this experiment, I verified that all 518 nm absorption change reflecting the transmembrane electric field had decayed between two flashes to its value after a long dark incubation. The factor of the transfer chain linked to the electrogenic reaction, which is varied when the time between flashes is decreased from 3.2 s to 400 ms in the absence of ammonium chloride, is the proton gradient. The proton gradient was difficult to estimate in these chloroplasts. However, the decrease of the relative amplitude of phase b, when the flash frequency is increased, is, according to Fig. 3, consistent with the acidification of the inside of the thylakoids. The bottom curve of Fig. 5 thus indicates that the rise-time of phase b is an increasing function of the proton gradient.

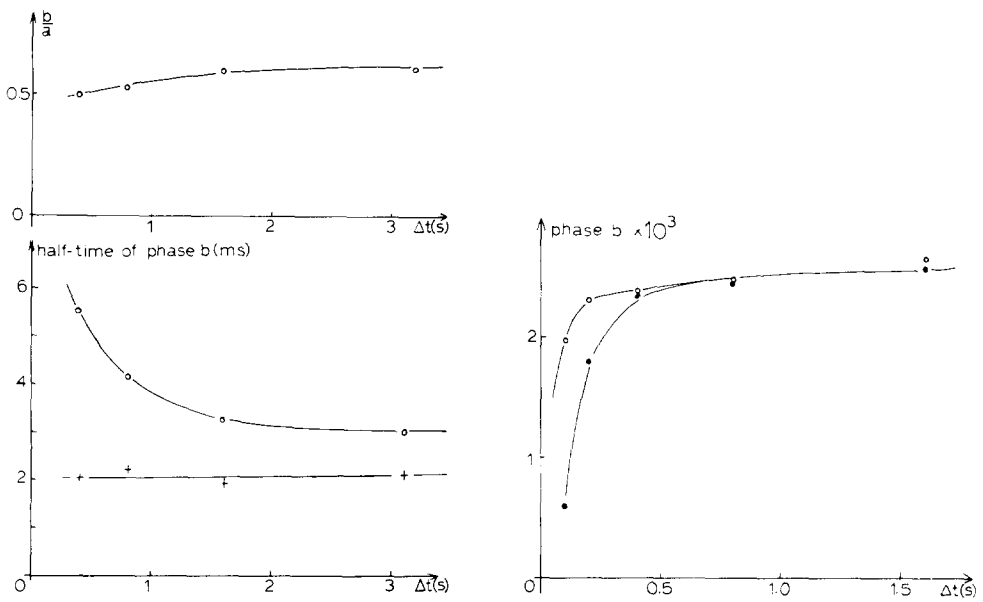

Fig. 5. Relative amplitude (top trace) and half-time (bottom trace) of phase b in spinach chloroplasts at pH 7.5, for an average of 64 flashes fired on the same sample as a function of the time Δt between two consecutive flashes. ○———○, $2 \cdot 10^{-4}$ M dithionite; +———+, $2 \cdot 10^{-4}$ M dithionite + 10^{-3} M NH_4Cl.

Fig. 6. Amplitude of phase b for an average of 64 flashes fired on the same sample as a function of the time Δt between two consecutive flashes, in *Chlorella* cells with dithionite $2 \cdot 10^{-4}$ M. ○———○, cells with fast electric field decay; ●———●, cells with slow electric field decay.

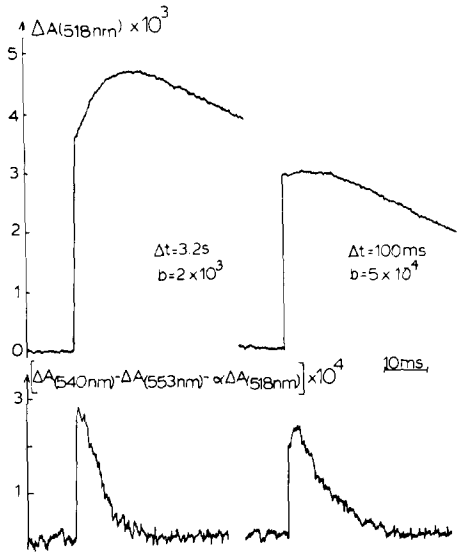

Fig. 7. Absorption changes at 518 nm (top traces) and cytochrome *f* kinetics (bottom traces) after one flash in *Chlorella* cells with 10^{-1} M NH_4Cl. Average of 64 flashes (top traces) and 256 flashes (bottom traces) fired on the same sample every 3.2 s (left curves) or every 100 ms (right curves).

As mentioned above from Ref. 16, it is possible to modify the rate of electric field decay in a batch of cells. When the time between flashes is decreased below 200 ms in *Chlorella* cells of fast electric field decay, and below 400 ms in cells of slow electric field decay, the amplitude of phase b, under stationary conditions, drops rapidly (Fig. 6), even in the presence of dithionite.

In Fig. 7, one can observe that, although the amplitude of phase b is decreased at high flash frequency, the reduction of cytochrome f^+ remains total and rather rapid when Photosystem II is active and ammonium chloride added in *Chlorella* cells.

Discussion

Factors modulating the rate of phase b

The effect of pH on the amplitude of phase b (Fig. 3) indicates that protons are involved in the overall process leading to the electrogenic reaction. However, the absence of variation of the half-time of phase b when the pH is varied (Fig. 3) shows that the reactions involving proton uptake or release are not limiting steps for the electrogenic reaction.

Table I indicates that phase b is a true first-order process, the half-time of which does not depend on the redox state of its reactants. Nevertheless, Fig. 4 clearly shows that the rate of phase b is slowed down by an increase of the transmembrane electric field. This point was further improved by the complementary experiment (data not shown) where no slowing down of phase b was observed during a series of flashes in cells where the electric field collapsed between two flashes. The rate-limiting step of the overall process generating phase b is thus the electrogenic reaction itself.

An increase, $\Delta\psi$, in the electric potential across the membrane decreases by $\Delta\psi$ the free energy of the electrogenic reaction. Using Marcus' semiclassical theory which relates the rate of a reaction to its free energy [17,18], it can be determined (see Appendix) that for an increase $\Delta\psi$ of the electrical potential across the membrane, the slowing down of phase b is determined by the following equation:

$$k^*/k = \exp -F\Delta\psi/2RT \quad (2)$$

where k and k_+ are the rate constants of phase b before and after the electric potential increase.

ΔA_{518nm}, the electrochromic effect, is a linear measure of the membrane electric potential [2]. Eqn. 2 could also be written, using the half-times:

$$\ln t_{1/2+} - \ln t_{1/2} = F\Delta\psi/2RT = c \cdot \Delta A_{518nm} \quad (3)$$

Fig. 4 is consistent with a linear relation between the absorption change at 518 nm before the flash and the logarithm of the half-time of phase b. From this figure, one can observe that:

1. The absorption change at 518 nm due to phase a after one flash is $1.1 \cdot 10^{-3}$.

2. When the absorption change is increased by $2 \cdot 10^{-3}$, the half-time of phase b is slowed down from 7 to 10 ms. Using Eqn. 3, one can thus deduce that the transmembrane electric potential generated by one flash in this experiment was 10 mV.

This experiment was done in the presence of dithionite in order to have better precision on the determination of the half-times of phase b. Due to this reductant, the contribution of Photosystem II was negligible. The xenon flash used in this experiment, calibrated with a saturating laser flash, induced around 0.85 photochemical reactions at the level of Photosystem I in *Chlorella* cells. Thus two electrons crossing the membrane would generate an electric potential of 24 mV.

This value is low compared to the usual estimations which gives values around 50 mV (for review see Ref. 20). However, a recent paper of Symons et al. [21] indicates that the usual estimations of the transmembrane electric field may be overestimated by a factor of 2, and would thus be consistent with the values reported here.

The slowing down of phase b when the flash frequency was increased in chloroplasts (Fig. 5) was interpreted as an effect of the formation of a pH gradient. I showed in the preceding section that the kinetics of phase b reflect the kinetics of the electrogenic step itself. The lack of dependence of the half-time of phase b on the pH in the absence of pH gradient (Fig. 3) indicates that the free energy of the electrogenic step is independent on pH and thus that the electrogenic step involves an equal number of proton releases and uptakes. The slowing down of the electrogenic step by a pH gradient (Fig. 5), in addition, would show that the free energy of this step is decreased by pH gradient. It thus would involve a proton uptake from the outside of the thylakoid and a proton release inside.

An increase, ΔpH, of the pH gradient would decrease by 2.3 (RT/F) ΔpH the free energy of the electrogenic step. Applying Marcus' theory [17,18] to

this free energy drop (see Appendix) one obtains

$$\ln t_{1/2+} - \ln t_{1/2} = \frac{2.3}{2} \Delta pH \quad (4)$$

where $t_{1/2}$ and $t_{1/2+}$ are the half-times before and after the increase of the pH gradient. From Eqn. 4 and Fig. 5 (bottom curve) one can compute that when the time between flashes varied from 3.2 s to 400 ms, the pH gradient was increased by 0.5 units. This value is consistent with the one obtained from the relative amplitude of phase b (comparison of Fig. 5 top curve and Fig. 3 bottom curve).

The dependence of the half-time of phase b on the transmembrane electric field and on the pH gradient explains why phase b is accelerated by uncouplers. Furthermore, the presence of a transmembrane electric field and/or of a pH gradient in dark-adapted cells could explain the variability of the half-times of phase b under apparently similar conditions (see Table II). Such a proton gradient in the darkness, in *Chlorella* cells, is also proposed on a different experimental basis by Joliot and Joliot [36].

The smallest half-time of phase b which I could observe in fast field decaying *Chlorella* cells or chloroplasts was 2 ms. It seems reasonable to assume that this value corresponds to an absence of any protonmotive force. Applying a combination of Eqns. 3 and 4 to this assumption, one obtains:

$$\Delta\psi + 2.3 \frac{RT}{F} \Delta pH = \frac{2RT}{F} \ln \frac{t_{1/2}}{2 \text{ ms}} \quad (5)$$

where $t_{1/2}$ is the half-time of phase b, $\Delta\psi$ the electric potential and ΔpH the proton gradient at the boundaries of the membrane [22]. From this equation and from Fig. 4, one can compute a protonmotive force of 120 mV under stationary conditions in *Chlorella* cells with a slow electric field decay. This value is similar to those estimated for chloroplasts under similar conditions [23].

Equation 5 would indicate that the half-time of phase b could be used to measure the proton gradient efficient for phosphorylation. Some more work would probably be necessary to improve it definitively. This equation would then be a very useful tool to study phosphorylation in whole organisms.

Factors modulating the amplitude of phase b

It was observed [11] that phase b of the electrochromic effect is suppressed by the addition of an oxidant and restored by the addition of a reductant, and thus is dependent upon the reduced state of a compound which I will here refer to as U. Velthuys [7] showed that phase b of the electrochromic effect could also be restored after photoreduction of the plastoquinone pool and concluded that the reductant necessary for phase b was the plastoquinone pool. However, in *Chlorella* cells, after dark-adaptation, the concentration of reduced plastoquinone estimated from the fluorescence gush [24] is around 12% and phase b (with or without DCMU) reaches 80% of its maximal amplitude. Unless U can be any member of the plastoquinone pool, which is unlikely due to the first-order behavior of phase b (Table I), the amplitude of the phase b developed by one flash would titrate the concentration of reductant U before the flash. At a

given pH, it is possible to determine a pseudo-equilibrium constant between U and the plastoquinone pool PQ. As U is a quinone [39], i.e. a two-electron carrier,

$$K = \frac{[U_{red}][PQ_{ox}]}{[U_{ox}][PQ_{red}]} = \frac{0.8 \cdot 0.88}{0.2 \cdot 0.12} \sim 30$$

The difference of the midpoint potential of (U_{ox}, U_{red}) and (PQ_{ox}, PQ_{red}) would be $(RT/2F) \ln K \sim 40$ mV at 25°C.

Thus U would be able to receive electrons from the plastoquinone pool because of its higher potential.

A similar drop of potential between U and the plastoquinone pool is also likely in chloroplasts, as the maximal amplitude of phase b can be obtained in conditions where the plastoquinone pool is not totally reduced (result not shown).

Phase b can also be inhibited by low potentials (Table I), which thus reduce an oxidant necessary for the electrogenic phase. However, it was not possible to estimate the potential at which this inhibition occurs, as it corresponded to an almost totally reduced plastoquinone pool. There is a large gap between the oxidant and the reductant potentials at which phase b is inhibited, and in this gap, at low flash frequency, phase b reaches its maximal amplitude which is equal to the amplitude of phase a generated by system I in *Chlorella* cells.

Beside this modulation of the amplitude of the electrochromic rise by the redox state of its reactants, Fig. 6 shows evidence for another class of factors which limit the amplitude of phase b. A smaller amplitude of phase b at a given flash frequency is observed in cells of slower field decay, and, thus, the larger the amplitude of the transmembrane electric field at a given flash frequency, the smaller the amplitude of phase b at this frequency.

This decrease of the amplitude of phase b at high transmembrane electric field could be interpreted as an inhibition for energetics reasons: the free energy available would no longer be sufficient to draw electrons across the membrane.

Fig. 3 (top curve) gives further evidence for limitations of the amplitude of phase b due to energetic reasons. To interpret this figure, I shall use the model proposed in Ref. 37: a two-electron carrier U gives one of its electron to the donors of system I, thus forming a low potential semiquinone; the second electron is transferred in a reaction which allows a charge to cross the membrane. In order to keep the generality of the forthcoming reasoning, let us not specify now the nature of these two compounds but let us call them X_1 and X_2. X_1 would have to be oxidized by Photosystem I before being reduced by reduced U.

The following reasonings could be adapted easily to any other model.

Without specifying the number of protons carried by each reactant, the global reaction including the electrogenic reaction would be written:

$$U_{red} + X_{1ox} + X_{2ox} \rightleftharpoons U_{ox} + X_{1red} + X_{2red} + nH^+ \qquad (R1)$$

The maximal amplitudes of phase b in chloroplasts were obtained in the presence of dithionite which prevented fast reoxidation of reduced X_2. In order to simplify the computation, I have assumed that, just after the flash, the con-

centrations were:

$$[U_{red}] = [X_{1ox}] = [X_{2ox}] = 1 \tag{6}$$

From this state, reaction R1 takes place until equilibrium is reached. Thus at the equilibrium.

$[U_{ox}] = [X_{1red}] = [X_{2red}] = \gamma$ = amplitude of phase b.

and

$$\frac{[U_{ox}][X_{1\,red}][X_{2\,red}]}{[U_{red}][X_{1\,ox}][X_{2\,ox}]} = \gamma^3/(1-\gamma)^3 = K/(H^+)^n = K' \tag{7}$$

Let us call E_1 the midpoint potential of the couple (X_{1ox}, X_{1red}), E_2 that of (X_{2ox}, X_{2red}) and E_U that of (U_{ox}, U_{red})

$$K' = \exp(F(E_1 + E_2 - 2E_U)/RT) \tag{8}$$

E_1, E_2 and E_U shall depend upon pH if, respectively, X_1, X_2, and U are proton carriers. If reaction R1 involves the release of n protons, one can then write

$$E_1 + E_2 - 2E_U = E_1^0 + E_2^0 - 2E_U^0 + 2.3\,\frac{nRT}{F}\,\text{pH} \tag{9}$$

E_1^0, E_2^0 and E_U^0 being independent of pH.

When the pH is decreased, K' is decreased if n is positive, and, consequently, the amplitude of phase b ought to be decreased. This is indeed observed (Fig. 3, top curve). In the presence of dithionite Photosystem II was inhibited, and the ratio of phase b to phase a tended towards 1 at high pH, as predicted by the model. At pH 7, the amplitude of phase b is half the amplitude of phase a. From Eqns. 7 and 8, one obtains

$$\gamma = 0.5 \Rightarrow K' = 1 \Rightarrow E_1 + E_2 = 2E_U \text{ at pH } 7 \tag{10}$$

The dotted lines in Fig. 3 are drawn from Eqns. 7—10 for $n = 1$ and $n = 2$. The experimental data fit the theoretical curve for $n = 1$, thus indicating that reaction R1 is a possible model for the electrogenic path if Eqn. 10 is verified and if $n = 1$.

Several arguments converge to localize the Rieske protein between cytochrome *f* and quinone pool in chloroplasts [26—29]. It thus seems probable that X_1 is the Rieske protein, of potential between +290 mV [30] and +330 mV [38], independent of pH. Thus, if the midpoint potential of U can be extrapolated from *Chlorella* to chloroplasts, the potential of X_2 would be, according to Eqn. 10, in the order of −30 mV at pH 7.

The inhibitory effect of DBMIB, a quinone antagonist, on phase b [11,37] suggest that U is a special quinone. U would be very similar to the bacterial Z [31,32], a two proton carrier. X_1 which is very probably the Rieske protein or eventually cytochrome *f*, is not a proton carrier. Reaction R1 can be a good model for the overall process generating phase b if there is one proton released. In addition, it was proposed that the electrogenic step involves one proton release inside and one proton uptake from the outside of the thylakoid.

All these conditions may be integrated in a simple global reaction if it is

assumed that X_2 is a one-proton carrier. The overall process generating phase b would then be written:

$$UH_{2int} + FeS^{+}_{int} + X_2 + H^{+}_{ext} \rightleftharpoons U + 2H^{+}_{int} + FeS + X_2H \quad (R2)$$

In this reaction, UH_2 would release its two protons inside the thylakoids and X_2 would use a proton from the outside for its protonation.

Reaction R2 could be decomposed into two steps:

$$UH_{2\,int} + FeS^{+}_{int} \rightleftharpoons UH + H^{+}_{int} + FeS \quad (R3)$$

$$UH + X_2 + H^{+}_{ext} \rightleftharpoons U + H^{+}_{int} + X_2H \quad (R4)$$

Reaction R4 is the electrogenic and limiting step. X_2 is very probably the new protein C detected in Ref. 37.

H^+/e ratio, energetics of phase b

The amplitude of the electrochromic rise in the millisecond range is controlled by two classes of factors: the redox state of its reactants (Table I) and the free energy available in the overall process including the electrogenic reaction (Figs. 3 and 6).

Fig. 7 indicates that a fast reduction of cytochrome *f*, though slowed down, is still possible when the electrogenic reaction is impeded for energetic reasons. Thus, electron transfers from Photosystem II to Photosystem I do not necessarily involve the electrogenic reaction.

The mechanism described in this paper starts like a Mitchell's Q-cycle [25]. It would really behave like a Q-cycle if the electron from X_2 came back on U with an associated hydrogen crossing the membrane from the outside towards the inside. In this case, one would expect the H^+/e ratio to be 3 on dark-adapted material or under low light excitation, and, as electron transfer from system II to system I does not necessarily involve the electrogenic reaction, 2 at high pH gradient or at high transmembrane electric field. Values of the H^+/e ratio over 2 have been reported [7,9,41]. Rathenow and Rumberg [41] have even detected an H^+/e of 3 under low light intensity but of 2 at high light intensity, as predicted in this paper. In *Chlorella* cells, the critical value of the light intensity above which the H^+/e ratio drops from 3 to 2 is around one photon per reaction center every 200 ms (Fig. 6).

The reasons why most of the determinations of the H^+/e ratio have reached a value of 2 (for review see Ref. 33) may be numerous. It could be due to use of too intense light, to the presence of oxidants, to imperfect chloroplast preparation. The quality of the chloroplast preparation is an essential factor to observe the slow electrochromic rise. One could suspect that the associated proton pump is even more fragile, and that, in some preparations, the slow electrochromic phase is followed by a back-reaction and not a proton pump.

According to the preceding discussion on the effects of pH and transmembrane electric potential on phase b, the free energy available for reaction R2 would be:

$$\Delta G'_0 = E_1 + E_2 - 2E_U - \Delta\psi$$

If X_2 utilizes protons from the outside of the thylakoid, E_2 is dependent upon

the external pH. On the other hand, the release of protons from U takes place inside the thylakoid and thus E_U should be dependent upon the internal pH. Thus:

$$\Delta G_0' = E_1 + E_2^0 - 2.3(RT/F)\ pH_{ext} - 2E_U^0 + 2.3(RT/F)\ 2pH_{int} - \Delta\psi$$

Using Eqn. 10:

$$\Delta G_0' = 2.3(RT/F)(pH_{ext} - 7) - \Delta\Psi - 2.3(RT/F)\ 2\ \Delta pH \qquad (11)$$

This kinetic study of phase b has provided evidence for a direct contribution of the transmembrane electric potential and pH gradient to the free energy of a reaction.

Appendix

According to Marcus' semi-classical theory [17,18], the activation energy of a reaction is given by Eqn. 12

$$E^{\ddagger} = \frac{(\Delta E - \lambda)^2}{4\lambda} \qquad (12)$$

where ΔE is the free energy of the reaction and λ a coefficient related to the vibration levels. This equation is valid for solid state reactions and it can be applied to the electrogenic reaction which is a true first order process.

When the transmembrane potential is increased by $\Delta\psi$, the free energy of the electrogenic reaction is decreased by $\Delta\psi$. The activation energy after the potential increase will be

$$E_+^{\ddagger} = \frac{(\Delta E - \Delta\psi - \lambda)^2}{4\lambda} = E^{\ddagger} + \left(1 - \frac{\Delta E}{\lambda}\right)\frac{\Delta\psi}{2} + \frac{\Delta\psi^2}{4\lambda}$$

where $E^{\ddagger}$ and $E_+^{\ddagger}$ will be the activation energies before and after the potential increase, ΔE the free energy before the potential increase.

According to DeVault [34], λ is usually on the order of 2 eV for non-tunnelling reactions. Thus ΔE and $\Delta\psi$ are small compared to λ and $E_+^{\ddagger}$ can be approximated by the following equation

$$E_+^{\ddagger} = E^{\ddagger} + \frac{\Delta\psi}{2}$$

From this equation one obtains

$$k_+/k = \exp -F\Delta\psi/2RT$$

where k and k_+ are the rate constants of phase b before and after the electric potential increase.

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