Biochimica et Biophysica Acta, 681 (1982) 405–411
Elsevier Biomedical Press

BBA 41131

ADENOSINE TRIPHOSPHATE-GENERATED TRANSMEMBRANE ELECTRIC POTENTIAL IN CHLOROPLASTS

ARIE ADMON, YOSEPHA SHAHAK and MORDHAY AVRON*

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

(Received January 26th, 1982)
(Revised manuscript received May 21st, 1982)

Key words: Photosynthesis; Membrane potential; ATPase; Oxonol VI; (Lettuce chloroplast)

Utilizing oxonol VI as a transmembrane electric potential indicating dye, chloroplasts are shown to develop rapid transient light-induced and ATP-induced potentials. Following the large transient signal smaller steady-state potentials are maintained with either driving system. The ATP-induced potential in the dark depends upon preactivation of the light-triggered ATPase of the chloroplasts, and is inhibited by uncouplers, ionophores such as valinomycin, and energy-transfer inhibitors such as tentoxin, Dio-9 or DCCD. Nigericin increased the signal of both the light- and the ATP-induced reactions. The fact that relatively large transient membrane potentials are induced by either a dark-to-light transition or ATP in the dark provides an explanation for previously observed phenomena such as early kinetics of photophosphorylation and the ATP-induced luminescence.

Introduction

Following proper activation, the membrane-bound ATPase of isolated chloroplasts has been shown to catalyse an ATP-dependent pumping of H^+ into the inner thylakoid space [1], an ATP-dependent reverse electron flow leading to the oxidation of cytochrome *f* and the reduction of Q (the primary electron acceptor of Photosystem II) [2], and an ATP-dependent reverse electron flow leading to luminescence emitted from Photosystem II [3].

The ATP-induced H^+ uptake was shown to result in the maintenance of a large H^+ concentration gradient ($\Delta pH > 3$), similar in magnitude to that maintained by illuminated chloroplasts [4]. This large transmembrane H^+ concentration gradient has been generally assumed to serve as the major driving force for the other ATP-driven reactions.

Several recent observations [5,6] have indicated that following dark-to-light transition, isolated chloroplasts develop a significant transient transmembrane electrical potential which is utilized as a driving force for ATP synthesis in the first few seconds of illumination. The development and the decay of this potential in chloroplasts were recently shown [7] to be probed by the potential-sensitive dye, oxonol VI, which is thus far the only extrinsic probe shown to respond to membrane potentials in chloroplasts.

The possible existence of ATP-generated transmembrane electrical potentials in the dark was previously suggested on the basis of analysis of the ATP-induced luminescence reaction [3]. In this communication, oxonol VI was used to show the

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; SF 6847, 3,5-di(*tert*-butyl)-4-hydroxybenzylidenemalononitrile; Tricine, *N*-tris(hydroxymethyl)methylglycine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll.

* To whom correspondence should be addressed.

existence of ATP-induced transmembrane electrical potentials in chloroplasts in the dark. Such potentials are shown also to be transient in nature and fully dependent upon the operation of the membrane-bound ATPase.

Methods

Chloroplasts were prepared from lettuce leaves essentially as previously described [8] except for the final washing which was with a solution containing: 200 mM sucrose, 10 mM KCl, 5 mM $MgCl_2$, 10 mM Na^+-Tricine, pH 7.8. Chlorophyll was determined as described previously [9].

Absorbance changes of oxonol VI (603–590 nm) were measured in an Aminco DW-2 dual-wavelength spectrophotometer, fitted with a magnetic stirrer to enable fast mixing of added solutions to the sample during measurement. Actinic light was provided by a 250 W projector through 10 cm of water and a Schott RG665 filter. Light intensity in the cuvette was about 10^6 erg · cm^{-2} · s^{-1}. The photomultiplier was protected from the actinic light by 1 cm of half-saturated $CuSO_4$ solution. The reaction mixture contained 200 mM sucrose, 10 mM KCl, 5 mM $MgCl_2$, 10 mM Na^+-Tricine, 2 μM phenazine methosulfate, 2 μM oxonol VI, and chloroplasts containing 20 μg Chl/ml, pH 7.8. Oxonol VI and tentoxin were kindly provided by Dr. Bashford of the Johnson Foundation, Philadelphia, PA, and Dr. Selman of the Department of Biochemistry, University of Wisconsin, Madison, WI, respectively.

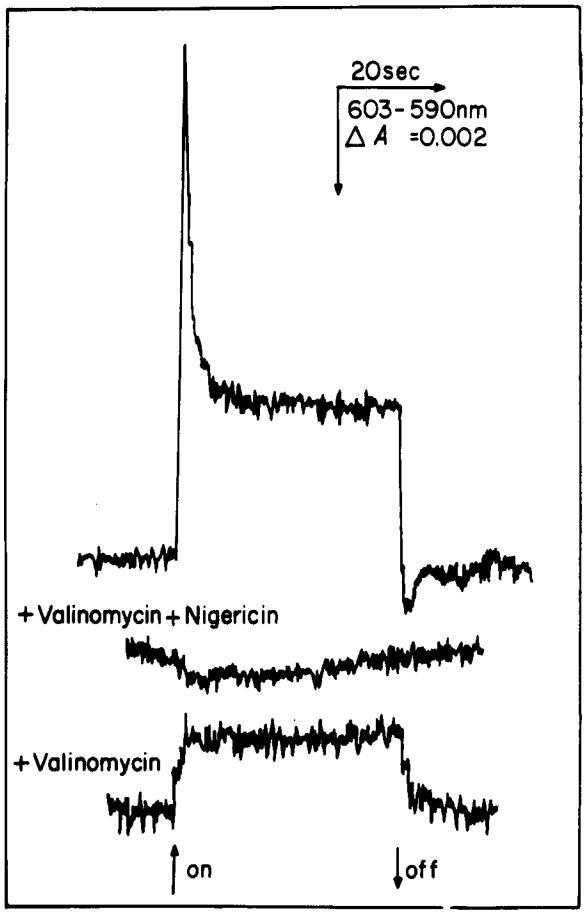

Fig. 1. Light-induced changes of oxonol VI in chloroplasts. Upward and downward pointing arrows indicate light on and off, respectively. The ionophores were added approx. 1 min before the illumination. Final concentrations: nigericin, 0.4 μM; valinomycin, 1 μM. Other reaction conditions as described in Methods.

Results

Fig. 1 shows the time course of the absorbance changes of oxonol VI in a chloroplast suspension during one illumination cycle. The change in absorbance is clearly biphasic with an initial rapid increase, within the response time of the instrument, followed by a slow decay to a steady-state level. Terminating the illumination returns the absorbance change to zero with a small undershoot. Valinomycin added in the presence of KCl eliminated the rapid signal and markedly reduced the steady-state level. Valinomycin and nigericin together abolished it completely (Fig. 1). Nigericin by itself, which catalyzes an H^+-K^+ exchange, would be expected to increase the magnitude of the light-generated transmembrane electrical gradient. Indeed, addition of nigericin increased both the initial peak and the steady-state levels (Fig. 2). These data confirm and extend the report of Schuurmans et al. [7] that the oxonol VI signals correspond to the transmembrane electrical potential across the thylakoid membrane.

The effect of adenine nucleotides on the light-induced signal is shown in Fig. 3. In the absence of phosphate, ATP or ADP increased the signal. This is in agreement with the previously described effect of these nucleotides, decreasing

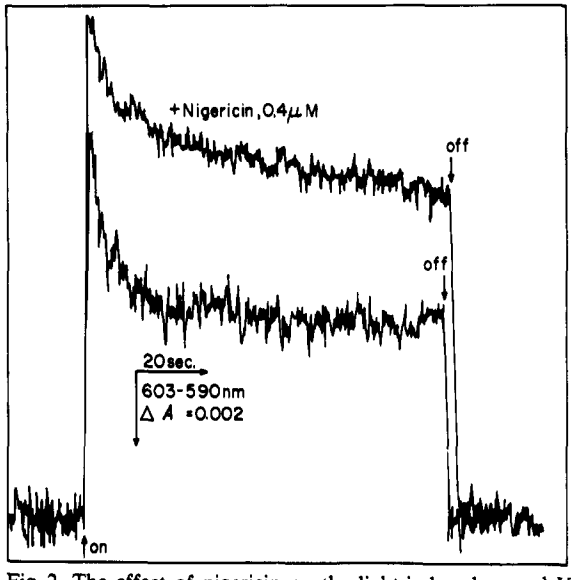

Fig. 2. The effect of nigericin on the light-induced oxonol VI signal. In the upper trace, 0.4 μM nigericin was added before illumination.

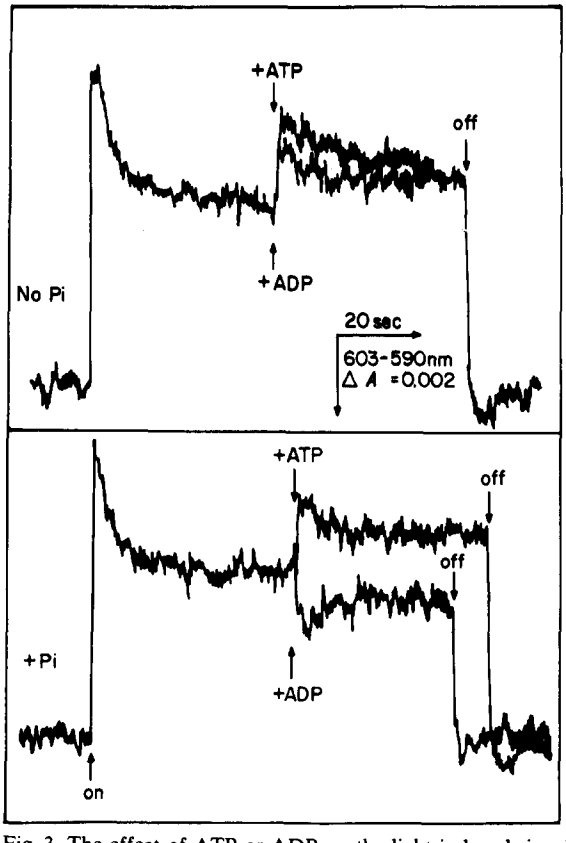

Fig. 3. The effect of ATP or ADP on the light-induced signals of oxonol VI. ATP, ADP and phosphate were added to a final concentration of 1, 1 and 2 mM, respectively.

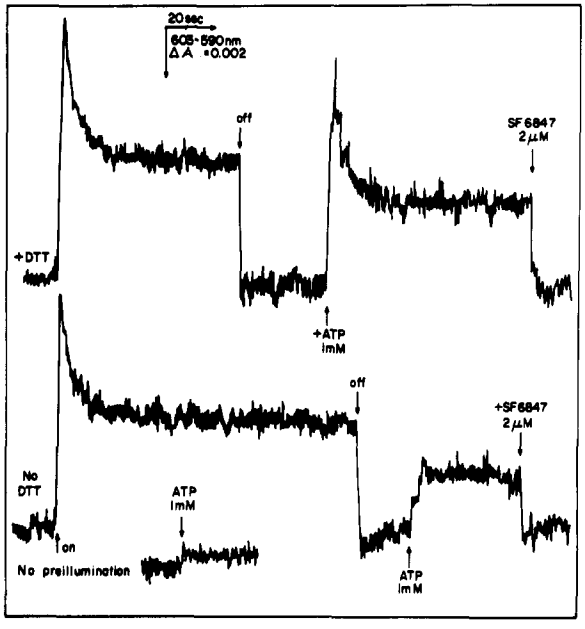

Fig. 4. ATP-induced transmembrane electrical potential in chloroplasts in the dark. In the control (upper trace) ATP was added following preactivation of the ATPase by illumination in the presence of 5 mM dithiothreitol (DTT).

the H^+ leakage through the coupling factor [10,11]. In the presence of phosphate, ATP still increased the signal but, addition of ADP, which initiated phosphorylation, decreased the observed transmembrane potential.

The ability of the light-triggered ATPase to produce a transmembrane potential in the dark is illustrated in Fig. 4. Following activation of the latent ATPase by light and dithiothreitol, addition of ATP caused a rapid increase in signal with biphasic kinetics similar in shape and magnitude to those of the light-induced response. The ATP-induced signal was eliminated by uncouplers such as SF 6847 and ionophores like valinomycin (Fig. 4 and Table I). In order to obtain an ATP-induced signal, the latent ATPase had to be preactivated by light in the presence of dithiothreitol. When dithiothreitol was omitted the ATP-induced signal was decreased, and without preillumination it was essentially absent (Fig. 4). As would be expected from the known properties of the light-triggered ATPase [1], an ATP-induced signal was observed when ATP was added after several minutes in the dark following light preactivation; this signal was

TABLE I

EFFECT OF INHIBITORS

Reaction conditions as described in Fig. 4. The effects on the steady-state signals are recorded.

Additions	Concentration	Oxonol VI signal (% of control)	
		Light induced [a]	ATP induced [b]
None	–	(100)	(100)
Valinomycin	1 μM	20	0
Nigericin	0.4 μM	170	210
SF6847	2 μM	0	0
Gramicidin	4 μM	0	0
Tentoxin	2 μM	95	0 [a]
DCCD	40 μM	66	12 [a]
Dio-9	4 μg/ml	80	0 [a]
Dio-9	4 μg/ml	–	0
Dio-9(after ATP)	4 μg/ml	–	0
DCMU	10 μM	109	88
No PMS	–	100	80
No PMS+DCMU	10 μM	0	82

[a] Inhibitor added about 1 min before light.
[b] Inhibitor added after light before ATP.

maintained better when phosphate was present, and decayed fully when ADP was present during the dark period before ATP addition (Fig. 5).

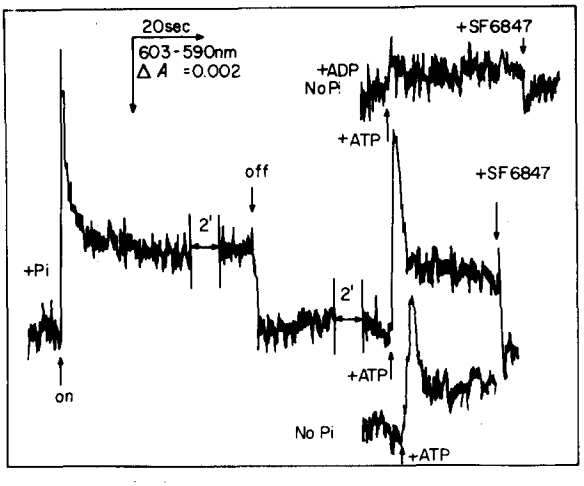

Fig. 5. The effect of dark decay of the ATPase on the ATP-induced oxonol VI signal. As was previously shown [1], the ATPase remains active during preincubation for several minutes in the dark in the presence of phosphate (2 mM) but is rapidly inactivated when ADP (1 mM) replaces phosphate. In the lower trace the ATPase was partially inactivated by preincubation in the dark in the absence of any addition. ATP and SF6847 were added to final concentrations of 1 mM and 2 μM, respectively.

In Table I the effects of a variety of inhibitors on the light- and ATP-induced signals are summarized. As was already indicated, protonophores like SF 6847 or gramicidin and ionophores such as valinomycin abolished both the light-induced and ATP-induced signals. Nigericin increased both (see also Fig. 6 below). As may be expected, energy transfer inhibitors like tentoxin, DCCD or Dio-9 inhibited the ATP-induced signal but did not affect the light signal. In agreement with the known slow reaction of tentoxin and DCCD with the coupling factor, the inhibition of the ATP-induced signal by these inhibitors was seen only when the inhibitors were added before the light-activation step. However, Dio-9 caused immediate inhibition of the ATP-induced signal also when given with or following ATP addition (Table I).

Nigericin, which uncouples photophosphorylation in chloroplasts, prevents the activation of the ATPase if added before the light-activation step. However, when added following activation or ATP addition, it enhances the signal, by promoting H^+-K^+ exchange, as it does with the light-induced signal (Fig. 2). Fig. 6 indicates that all these effects of nigericin are indeed observed.

The steady-state light-induced signal of oxonol VI was increased by lowering the temperature of

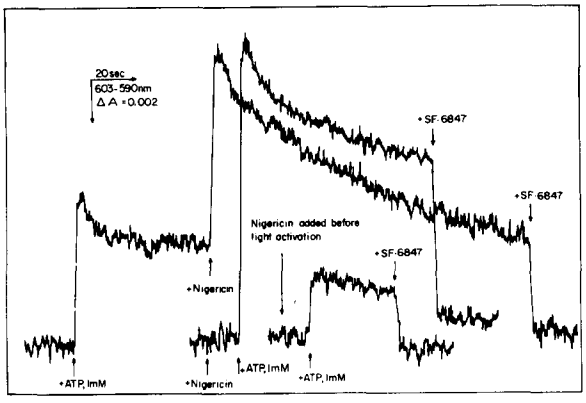

Fig. 6. The effect of nigericin on the ATP-induced transmembrane electric potential. Nigericin (0.4 μM) was added either after the light preactivation of the ATPase, before or after the ATP, or before preillumination.

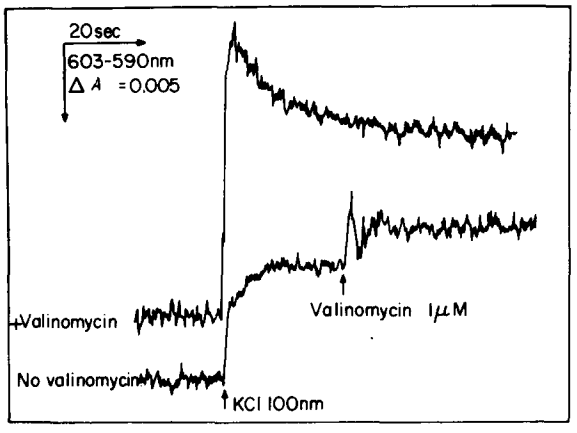

Fig. 8. Valinomycin- and KCl-induced oxonol VI signals.

the reaction medium. The signals at 3 and 28°C are shown in Fig. 7. This may be attributed to a lower permeability of the thylakoid membrane at the lower temperature, which slows the rate of counterion movement more than that of the pumped H^+, leading to a larger transmembrane potenial.

Several attempts were made to calibrate the observed signal in terms of a transmembrane electrical gradient. Fig. 8 shows the response of oxonol VI to a K^+- and valinomycin-induced transmembrane diffusion potential. As can be seen, rather large ionophore-insensitive salt-induced changes in absorbance made it impractical to use this approach for calibrating purposes.

We also attempted to calibrate the signal by following the diffusion potential induced by a rapid injection of a strong acid containing a relatively impermeable anion. Since H^+ permeates the thylakoids more rapidly than such anions, this procedure should result in a transient diffusion potential. Indeed a rapid acid-induced increase in

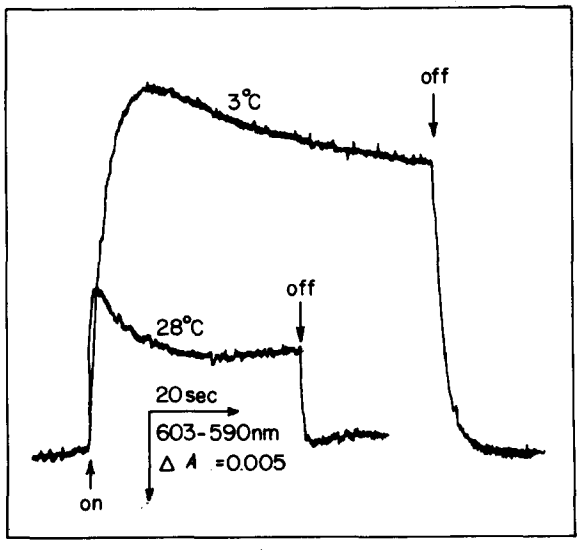

Fig. 7. The effect of temperature on the light-induced transmembrane electric potential. Temperatures were determined in the cuvettes.

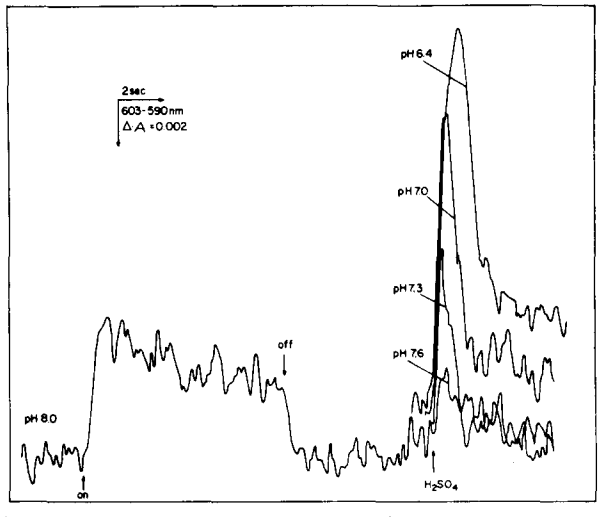

Fig. 9. Acid-induced oxonol VI signals. Initial pH was 8.0. The final pH values measured inside the cuvette, following the rapid injection of aliquots of H_2SO_4, are indicated on the figure. Note the faster time scale relative to the other figures.

signal which decayed almost completely within a few seconds was observed within reasonable pH limits. The signal increased linearly with the magnitude of the change in pH (Fig. 9). A similar transient signal was induced by a variety of strong inorganic acids such as HCl, HNO_3, H_2SO_4, or H_3PO_4, but not by weaker organic acids like succinate or benzoate (not shown). The acid-induced signal was not abolished by uncouplers or by valinomycin with KCl, but was abolished by addition of a detergent like Triton X-100 at 0.1–0.01%. Injection of a base, resulting in alkalinization of a sample previously kept at low pH (from pH 6 to 8), did not cause any change in absorbance (not shown).

Calibration of oxonol signals by acid injection was attempted also in asolectin liposomes (not shown). In this case, the acid-induced signals developed rapidly but decayed slowly ($t_{1/2} > 10$ min). Addition of valinomycin brought about an immediate decay of the signal.

Discussion

The most widely employed method for following transmembrane electric potentials in chloroplasts has utilized the electrochromic shift in the absorbance of endogenous carotenoids [12,13]. This method has proved less valuable for measurement of potentials under continuous illumination because of interfering absorption changes which occur in the same wavelength region [12]. The use of oxonol VI for measurements of membrane potential in chloroplasts seems to provide a reasonable alternative. Although not as rapid in response, it permits observation of both rapid transients and steady-state potentials. The kinetics of the oxonol VI signal (Fig. 1) are in agreement with the results obtained using the electrochromic shift, and confirm the previous observations by Schuurmans et al. [7]. The effects of ionophores and inhibitors are in agreement with the conclusion that the response is mostly reflecting the magnitude of the existing transmembrane electrical potential (Figs 1 and 2, and Table I).

The data presented here indicate that by utilizing the oxonol VI response, we could demonstrate that ATP added in the dark to the light-triggered ATPase of chloroplasts generated a transmembrane electrical potential (in addition to the transmembrane pH gradient previously described). An ATP-induced oxonol VI signal in broken intact chloroplasts has been demonstrated recently [14,15]. An ATP- and pyrophosphate-induced transmembrane electrical potential was measured in chromatophores by following the carotenoid shift [16] or the oxonol VI signal [17].

The elimination of the ATP-induced signal in the dark by valinomycin or by protonophores like SF 6847 and the enhancement of this signal by nigericin confirm the conclusion that it corresponds to the transmembrane electrical potential. Inactivation of the ATPase by ommission of light or dithiothreitol, the rapid decay of the ATP effect on addition of ADP and the inhibition by energy-transfer inhibitors such as Dio-9, tentoxin or DCCD indicate that the ATP-induced signal is produced by the light-triggered ATPase.

A major difficulty in the interpretation of oxonol VI signals as indicating a transmembrane electrical potential is the lack of a simple and reliable calibration method. K^+- and valinomycin-induced diffusion potentials do not provide satisfactory results because of interfering absorption changes which are not related to transmembrane potentials. Similar observations were made by previous investigators who tried this technique to calibrate the oxonol VI or the carotenoid shift signal (see Refs. 18 and 19).

Injection of acid into a chloroplast suspension produced a transient signal which behaved as expected from a transmembrane electric potential-dependent response in both the chloroplasts (Fig. 9) and in the liposome system. The difference in the decay kinetics in the two systems probably reflects the higher permeability of the chloroplast membrane to counterions. Assuming that this signal is due to an H^+-induced diffusion potential where a pH gradient of 1 unit equals 59 mV, we calculated that the ATP- or light-induced steady-state signal signal amounted to about 15–30 mV. However, the observation that this transient signal was not sensitive to protonophores or ionophores casts some doubt on its suitability as a calibration device for the transmembrane electric potential in chloroplasts. The signal was eliminated only by treating the preparation with a detergent.

The relatively large transient transmembrane

electric potential produced following a dark-to-light transition is in agreement with previous interpretation of the early phosphorylation kinetics [5]. It also supports the previous interpretation of the rapid increase in post-illumination luminescence induced by ATP addition [3]. It was recently shown by Graan et al. [20] that flash-induced ATP synthesis is more effective at lower temperatures. This is in agreement with the observation in this report that the oxonol VI-indicated membrane potential was indeed larger at low temperatures. It may reflect a lower rate of H^+ leakage and/or a slower flow of counterions which dissipate a larger part of the membrane potential at the higher temperatures.

Two reports dealing with the possible relationship between ATPase and the existence of transmembrane electric potentials have recently appeared [21,22].

References

1 Carmeli, C. and Lifschitz, Y. (1972) Biochim. Biophys. Acta 267, 89–95
2 Rienits, K.G., Hardt, H. and Avron, M. (1972) Eur. J. Biochem. 43, 291–298
3 Avron, M. and Schreiber, U. (1979) Biochim. Biophys. Acta 546, 448–454
4 Bakker-Grunwald, T. and Van Dam, K. (1973) Biochim. Biophys. Acta 292, 808–814
5 Vinkler, C., Avron, M. and Boyer, P. (1978) FEBS Lett. 96, 129–134
6 Ort, D.K. and Dilley, R.A. (1976) Biochim. Biophys. Acta 449, 95–107
7 Schuurmans, J.J., Casey, R.P. and Kraayenhof, R. (1978) FEBS Lett. 94, 405–409
8 Avron, M. (1961) Anal. Biochem. 2, 535–543
9 Arnon, D.I. (1949) Plant Physiol. 24, 1–15
10 McCarty, R.E., Fuhrman, J.S. and Tsauchiya, Y. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 2522–2526
11 Karlish, S.J.D. and Avron, M. (1970) Nature 216, 1107–1109
12 Junge, W. (1977) Annu. Rev. Plant Physiol. 28, 503–36
13 Witt, H.T. (1971) Q. Rev. Biophys. 4, 365–477
14 Galmiche, J.M. and Girault, G. (1980) FEBS Lett. 118, 72–76
15 Girault, G. and Galmiche, J.M. (1981) in Photosynthesis II (Akoyunoglou, G., ed.), pp. 965–972, Balaban International Science Service, Philadelphia
16 Baltscheffsky, M. (1969) Arch Biochem. Biophys. 130, 646–652
17 Bashford, C.L., Baltscheffsky, M. and Prince, R.C. (1979) FEBS Lett. 97, 55–60
18 Gross, E.L. and Libbey, J.W. (1972) Arch. Biochem. Biophys. 153, 457–467
19 Strichartz, G.R. and Chance, B. (1972) Biochim. Biophys. Acta 256, 71–84
20 Graan, T., Flores, S. and Ort, D.K. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 25–34, Elsevier/North-Holland, New York
21 Schuurmans, J.J., Peters, A.L.J., Leeuwerik, F.J. and Kraayenhof, R. (1981) in Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F., et al., eds.), pp. 359–369, Elsevier/North-Holland Amsterdam
22 Morita, S., Itoh, S. and Nishimura, N. (1982) Biochim. Biophys. Acta 679, 125–130