# ENERGY TRANSDUCTION IN PHOTOPHOSPHORYLATION

M. AVRON
*Department of Biochemistry, Weizmann Institute of Science Rehovot, Israel*

Received 6 September 1978

## 1. Proton uptake and proton gradients

Chloroplast membranes isolated from higher plants or algae show rapid and reversible electron transport-dependent proton uptake from the medium into their innervesicular space (see [15]). The number of protons taken up is a function of the natural internal buffering power, and can therefore be increased considerably by the addition of components which act as internal buffers [1,17]. In order to evaluate the possible contribution of the proton concentration gradients created during such proton uptake to the energy conserving system several methods were developed to measure the size of such gradients. Most of these methods depend on the equilibration of amines across the chloroplast membrane in response to the pH gradient (fig.1).

The non-protonated form of the amines ($RNH_2$) is in all cases assumed to freely permeate the membrane and therefore its concentration inside equals its concentration outside. Amines with a p$K$ more than 1 pH unit above that of the reaction medium will be mostly in their protonated form in the medium. When a pH gradient exists across the vesicle due to proton uptake, the free amine inside will be protonated by the incoming protons pulling more free amine in, until at steady state the following relation will hold [30]:

$$\frac{(H^+)_{in}}{(H^+)_{out}} = \frac{(RNH_3^+)_{in}}{(RNH_3^+)_{out}} \qquad (1)$$

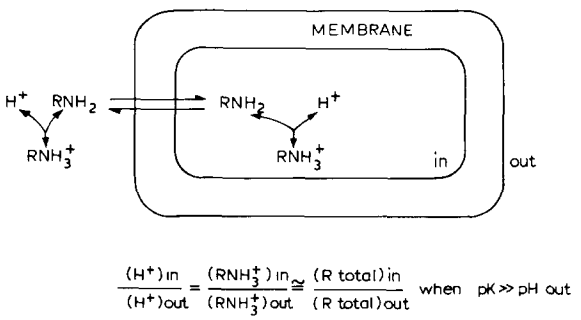


Fig.1. Equilibration of amines across membrane vesicles.

The first method devised for measuring the transmembrane pH gradient utilized $^{14}C$-labelled methylamine (p$K$ 10.6), where under all experimental conditions employed, the total amine concentration is essentially equal to the protonated amine concentration. Chloroplasts were illuminated in the presence of trace (10 $\mu$M) amounts of labelled methylamine, centrifuged in the light, and the $^{14}C$ content of pellet and supernatant determined. Independently the osmotic volume of the same chloroplast preparation was determined using $^{14}C$-labelled sorbitol which does not enter the chloroplast vesicle and therefore labels only the nonosmotic compartment. From these values the methylamine concentration inside and outside the vesicle can be determined and hence (eq. (1)) the $\Delta$pH. Values of $\Delta$pH approaching 3 were determined with this method [30]. Similar techniques using other amines have been employed [25].

The main disadvantage of such methods is the requirement for rather high chloroplast concentrations and for centrifugation to separate chloroplasts from supernatant, both of which result in under-saturation with light, and a consequent underestimation of the pH gradient.

This difficulty does not arise in the following methods, which monitor the pH gradient continously in the reaction mixture. Rottenberg and Grunwald [29] added low concentrations of ammonium and

followed the ammonium concentration in the medium directly with an ammonium-sensitive electrode. This is an excellent method but its usefulness is limited to media which contain no other ions to which the electrode responds.

Schuldiner et al. [33] followed the fluorescence of a fluorescent amine, 9-aminoacridine, since it was demonstrated [16] that the fluorescence of such amines is totally quenched when taken up by chloroplast vesicles. From the light-induced change in fluorescence and the osmotic volume of the vesicles the light-induced ΔpH can be accurately and continuously followed. This is probably the most widely used method and with proper precautions [11,12] its reliability has been adequately established in a variety of biological and model systems [3,7–10,14,18].

## 2. Proton gradients and ATP formation

The relation of the measured ΔpH values to ATP formation in chloroplasts was investigated using several approaches. As expected from the chemiosmotic hypothesis, which postulates that ATP formation is driven by utilizing the transmembrane electrochemical potential of protons, the gradient observed during phosphorylation was smaller by about 0.5 pH units than that observed in the absence of phosphorylation [21,25]. When very low light intensities were used, the kinetics of the development of the pH gradient and of ATP synthesis could be simultaneously observed. As can be seen in fig.2 [22] the gradient developed immediately upon turning the light on, but a distinct lag in ATP formation was observed. On plotting the rate of ATP synthesis versus the magnitude of ΔpH at any time point (fig.2), it is clear that no ATP formation could be observed before a threshold of ΔpH (~2.3 units) was built up. Beyond that point the rate of ATP formation was sharply and linearly dependent upon further increase in ΔpH. Clearly, ATP formation and ΔpH are linked but their relation is not linear.

To quantitate the relation of proton movement across the membrane vesicle to ATP formation chloroplasts were permitted to synthesize ATP at low light intensities until a steady state was achieved where no further ATP could be synthesized. At this point both the magnitude of the proton concentration gradient and of the phosphate potential were measured. In fig.3 [2] the results of such an experi-

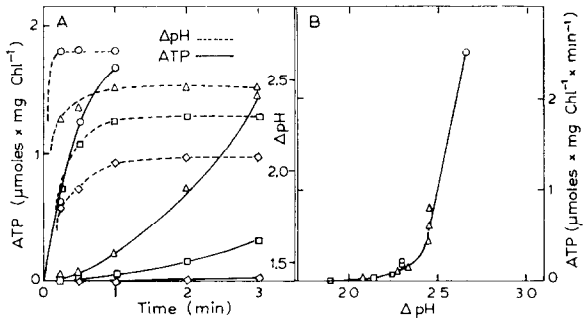


Fig.2. Time course of development of ΔpH and ATP formation at low intensities. The reaction mixture contained in 3.0 ml: tricine–maleate, 30 mM (pH 7.2); sorbitol, 30 mM; $MgCl_2$, 1 mM; ADP, 1 mM; $P_i$, 1 mM (containing $3 \times 10^7$ cpm $^{32}P$). ATP, 100 μM, pyocyanine, 25 μM; 9-aminoacridine, 0.5 μM; and chloroplasts containing 75 μg chlorophyll. In (A) light intensities were in ergs × $cm^{-2}$ × $s^{-1}$: (○- - -○) $4.5 \times 10^5$; (△- - -△) $6 \times 10^4$; (□- - -□) $4 \times 10^4$; (◇- - -◇) $1.5 \times 10^4$ [22].

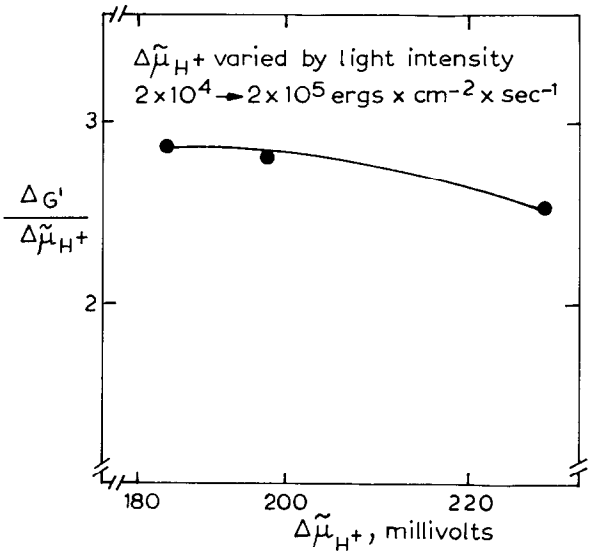


Fig.3. Analysis of ΔpH as a driving force for ATP formation in the steady state. Reaction mixture in 3 ml: KCl, 20 mM; phosphate, 2 mM (pH 8.0); $MgCl_2$, 4 mM; ADP, 0.2 mM; phenazine methosulfate, 10 μM; 9-aminoacridine, 1 μM; and chloroplasts containing 54 μg chlorophyll. It was illuminated for 10–20 min until a steady state phosphate potential was attained. Phosphate potential values were calculated from ADP content determination with pyruvate kinase and phosphoenolpyruvate. $\Delta G^{o'}$ of 7.8 kcal × $mol^{-1}$, at pH 8.0, 10 mM $Mg^{2+}$ 25°C, 0.1 ionic strength was used. $\Delta\mu_{H^+}$ was assumed to be composed of ΔpH only [28].

ment are plotted. The value of $\Delta F'/\Delta\mu_{H^+}$ represents the minimal number of protons which must traverse the membrane for the synthesis of an ATP molecule under the given conditions. It is evident that at least 3 protons must traverse the membrane per ATP molecule synthesized under all the measured conditions. Membrane potential values were not considered in the calculation, since it has been amply demonstrated [9,30,32] that no significant membrane potentials exist in the steady state in chloroplasts.

### 3. Proton gradient and reverse electron flow

We have previously shown that, under appropriate conditions, the coupled reaction between electron transport and ATP formation in chloroplasts is reversible [27]. Thus addition of ATP caused the reduction of 'Q' and oxidation of cytochrome *f* (fig.4,5). According to the chemiosmotic hypothesis proton gradients should be obligatory energy transducing intermediates in this process. Indeed, transmembrane proton gradients were shown to be created during the action of the ATPase (see [5]).

As obligatory intermediates, the build up of proton gradients would be expected to be kinetically at least as fast as the induction of reverse electron flow under all experimental conditions. Thus, treatments which slow down the development of the transmembrane proton gradient would be expected to induce a corresponding slow down in the development of the ATP-

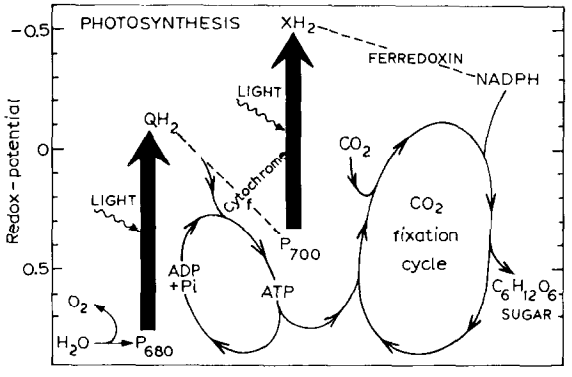


Fig.4. An oversimplified scheme of photosynthesis indicating a site of coupling between electron transport and ATP formation.

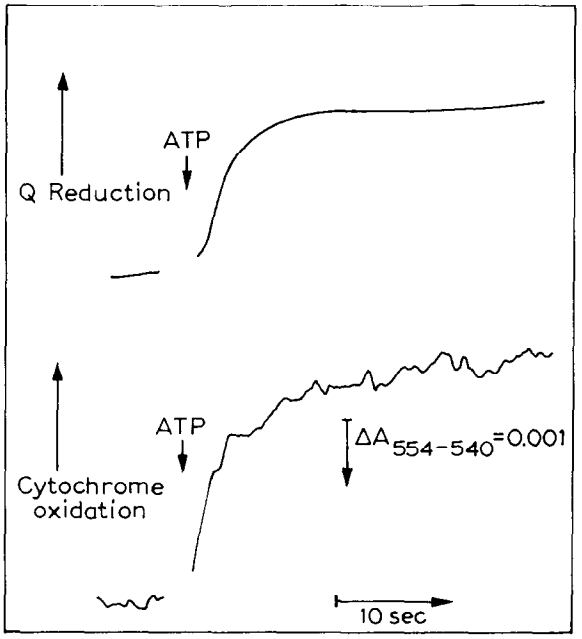


Fig.5. ATP-driven reduction of Q and oxidation of cytochrome *f*. Q reduction was followed by the increase in chlorophyll fluorescence yield which accompanies it. Cytochrome *f* oxidation in a dual wavelength spectrophotometer at 554–540 nm [27].

driven reverse electron flow. We constructed an apparatus which enabled us to simultaneously monitor the ATP-driven development of ΔpH and reduction of Q under a variety of conditions, and thus test the above prediction [4].

Figure 6 illustrates the simultaneous recording of 9-aminoacridine and chlorophyll fluorescence, and the effect of adding increasing concentrations of three internal buffers on the ATP-induced ΔpH build up and reverse electron flow. All three caused a decrease in rate and extent of not only the ATP-induced ΔpH, as expected, but also of the ATP-induced reverse electron flow. These results are essentially in agreement with the predictions of the chemiosmotic hypothesis. Thus, slowing the build up of ΔpH by internal buffers brought about a corresponding change in the rate and extent of the observed reduction of Q by reverse electron flow.

If proton gradients serve as intermediate energy pools between electron transport and ATP formation,

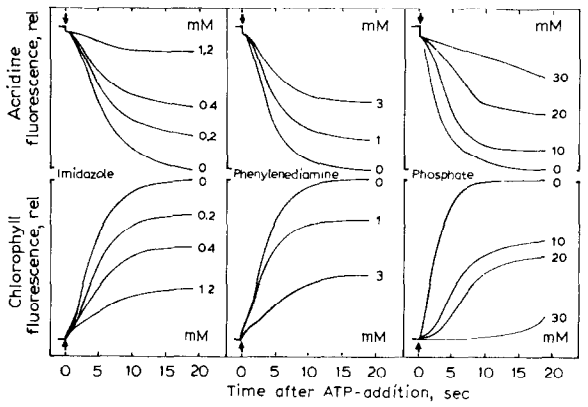


Fig.6. The effect of internal buffers on the kinetics of ATP-driven build up of ΔpH and reduction of Q. Buffers pre-adjusted to pH 8.0 were added to the indicated final concentration before activation [4].

it should be possible to demonstrate also proton gradient-dependent reverse electron flow. Indeed [34,35], such a reaction was found in our laboratory (fig.7). Rapid addition of Tris to chloroplast suspensions pre-equilibrated at pH 5.3 created a momentary pH gradient with the inner vesicular space at pH 5.3

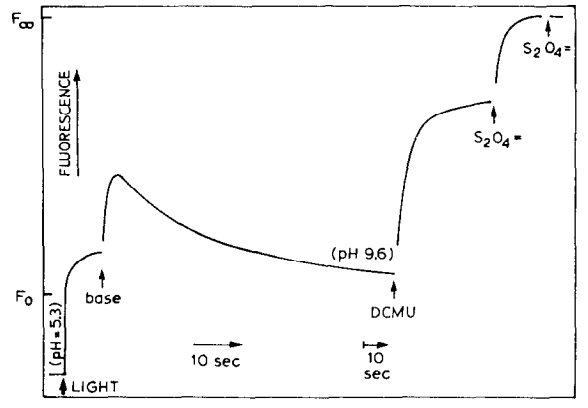


Fig.7. Proton gradient-driven reduction of Q. $F_0$ level indicates fully oxidized Q and $F_\infty$ level (observed on addition of DCMU and/or dithionite) fully reduced Q. Base refers to an injection of 0.2 ml 1 M Tris, at a predetermined pH to give final pH 9.6, into a reaction mixture which contains in 2.0 ml: maleic acid, 3 mM (pH 5.3); $MgCl_2$, 10 mM; KCl, 30 mM, and chloroplasts concerning 40 μg chlorophyll. Where indicated 1.5 μM DCMU and a few grains of dithionite were added [34].

and the medium at pH 9.6. This momentary gradient served as a driving force for reverse electron flow as indicated by the transient reduction of Q (fig.7). Reverse electron flow is clearly occurring since oxidizing the reduced electron carriers between the two photosystems by pre-illumination with far-red light which excites only the photoreaction sensitizing the P-700 to X electron transfer (see fig.4), severely inhibits the acid–base-induced reduction of Q, and a following pre-illumination with green light (which excites also the second photoreaction, thus rereducing the electron carriers) fully restores the reaction (fig.8).

Recently, conditions were found under which ATP or proton gradient-driven reverse electron flow were extended to include the photosystem [31,36]. Thus, ATP or acid–base transition induced the emission of

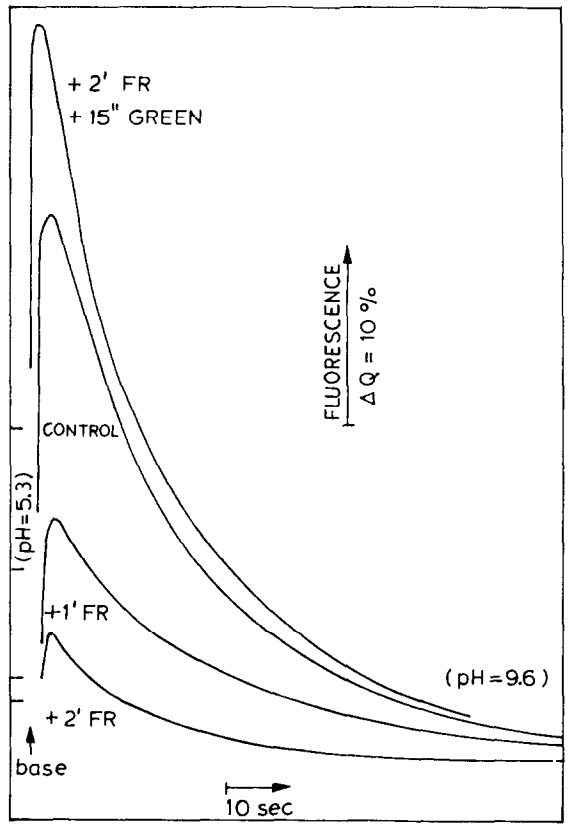


Fig.8. Effect of far-red (FR) and green pre-illumination on the proton gradient-driven reduction of Q. Reaction conditions were as described in fig.7 [34].

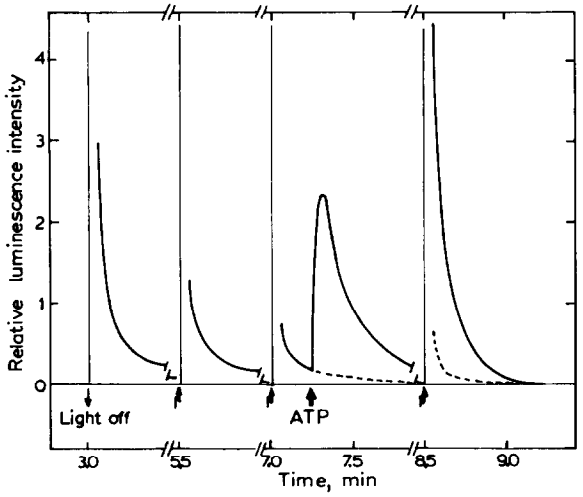


Fig.9. ATP-induced stimulation of luminescence. The light off arrow indicated the end of the 3 min light-activation period. The application of flash pairs is indicated by zig–zag arrows. The broken lines represent the decay of flash-induced luminescence when no ATP was added. During illumination or flashing the photomultiplier was turned off; monitoring of post-illumination luminescence was initiated about 5 s after pre-illumination. Temperature, 10°C [31].

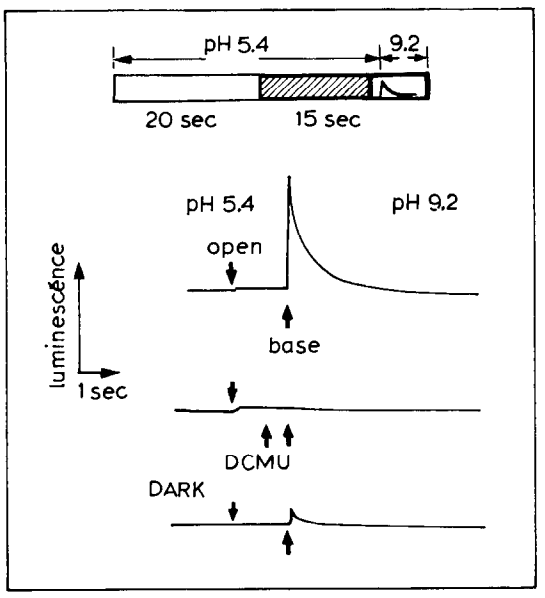


Fig.10. Proton gradient-driven reverse electron-flow luminescence. Reaction mixture included in 2 ml: succinate, 3 mM; KCl, 30 mM; $MgCl_2$, 10 mM and chloroplasts containing 40 μg chlorophyll. Final pH 5.4. Where indicated 0.15 ml, 0.5 M Tris and 0.1 ml 0.1 mM DCMU in 10% methanol were injected. Pre-illumination was with green light: $8 \times 10^4$ ergs $\times$ $cm^{-2} \times s^{-1}$ [36].

light from the photosystem. The experimental procedure and results are illustrated in fig.9,10.

The chloroplast suspension was first illuminated with strong, heat-filtered white light for 3 min to activate the latent ATPase. From ~5 s after the activating light was turned off, the decay of post-illumination luminescence was monitored for 90 s, by which time it approached zero. Two saturating flashes were given and the flash-induced luminescence was recorded for 90 s. Two more flashes were then given and ATP was injected before the flash-induced luminescence had delayed completely. ATP induced a marked burst of luminescence which decayed with a half-time of about 12 s.

ATP addition stimulated luminescence by about 10-fold, as compared with the post-illumination luminescence during the same time period (dashed line in fig.9). At 90 s after the second flash pair, a third flash pair was given and the luminescence monitored. The dashed lines in fig.9 illustrate the decays of flash-induced luminescence when no ATP was added.

This sequence displays the ATP-induced luminescence in two ways:

(i) The burst of luminescence upon injection of ATP.

(ii) The much enhanced luminescence following a flash couple, when ATP is present and hydrolysed by the ATPase (compare the dashed and solid curves following the last flash couple).

These experiments demonstrate that after proper activation, the ATPase system can affect the electron-transport system all the way to the photosystem II reaction center. A similar procedure was followed to observe an acid–base-induced reverse electron flow luminescence. Following a pre-illumination period, the chloroplasts are placed in the dark during which their native luminescence decays essentially completely, the shutter in front of the photomultiplier is then opened to permit observation, Tris is injected and immediately a transient light emission becomes apparent (fig.10). DCMU, which blocks electron flow between the site of coupling and Q, fully inhibits the reaction.

## 4. Initial kinetics of photophosphorylation

If transmembrane proton concentration gradients are the only intermediary energy transducing devices in chloroplasts, it could be expected that on a dark–light transition photophosphorylation should always proceed with a measurable lag period, as demonstrated in the low light experiments of fig.2. However, several laboratories reported recently that such lags (the length of which can be roughly calculated by considering the rate of proton pumping and the internal buffer content) are not observed [6,19,20].

We decided, therefore, to compare photophosphorylation with a model reaction where one can be fairly certain that ATP formation is driven exclusively by a transmembrane proton concentration gradient. Luckily, such a reaction has been described in detail as post-illumination phosphorylation (see [13]). In this reaction chloroplasts are pre-illuminated, in the absence of ADP and phosphate, and the latter are added after the light has been turned off. The ATP formed in the dark was convincingly shown to be due only to the transmembrane proton concentration gradient built up during the pre-illumination period. Figure 11 shows a comparison of the early kinetics of photophosphorylation and post-illumination phosphorylation. In agreement with the previous reports we find no detectable lag in the photophosphorylation reaction, but a clear lag of about 500 ms in the post-illumination reaction [37]. Thus, a driving force of ATP formation seems to exist during the first 1 s of illumination, which is not a transmembrane proton concentration gradient. An alternative possibility appeared to be that the initial formation of ATP is driven by a transmembrane electric potential. Ample evidence exists for the transient formation of a transmembrane electric potential following illumination, although during steady state photophosphorylation little or no such potential is present (see [3]).

To test this possibility we utilized the ion transporting agents valinomycin and nigericin. In the presence of $K^+$, valinomycin promotes the transport of that ion across membranes, anihilating a transmembrane electric potential where it exists without affecting the proton concentration gradient. Nigericin, on the other hand, promotes a $H^+$–$K^+$ exchange, thus anihilating a pH gradient, without affecting the transmembrane electric potential. As can be seen in fig.11,

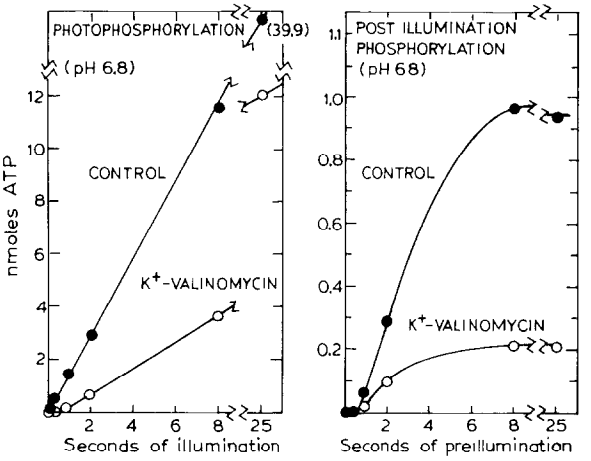


Fig.11. Time course of ATP formation in photophosphorylation and post-illumination phosphorylation in the presence and absence of $K^+$ and valinomycin. Reaction mixture included in 1.0 ml: Na–Hepes, 33 mM (pH 6.8); $MgCl_2$, 5 mM; KCl, 100 mM; EDTA, 0.1 mM; methyl viologen, 1 mM; and chloroplast membranes containing 73 μg chlorophyll/ml. Where indicated 4 μl 0.5 mM ethanolic solution of valinomycin was added. A reaction mix containing the same constituents with 2 mM ADP and 4 mM $^{32}P_i$ ($6 \times 10^6$ cpm/μmol) but no chloroplasts or valinomycin was rapidly injected before light exposure (photophosphorylation) or immediately following light exposure (post-illumination phosphorylation) for the indicated length of time [37].

addition of valinomycin and $K^+$ induced a lag in the photophosphorylation reaction of a similar length to that observed in its absence in the post-illumination reaction. Figure 12 shows similar experiments in the

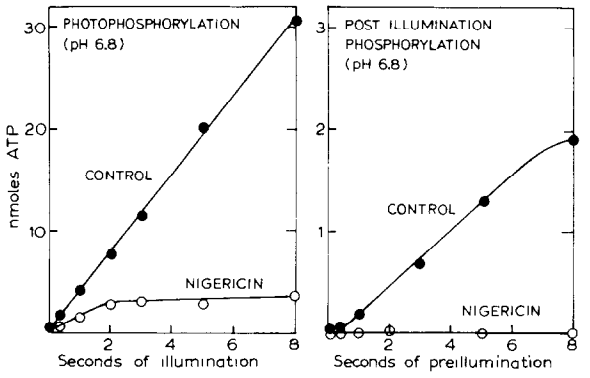


Fig.12. The effect of nigericin on photophosphorylation and post-illumination phosphorylation. Reaction conditions as under fig.11 except that 20 μl 10 μM nigericin replaced valinomycin [37].

presence and absence of nigericin and $K^+$. Nigericin completely abolished post-illumination photophosphorylation and steady state photophosphorylation, as expected, but did permit significant ATP formation to proceed during the first 2 s of photophosphorylation. Both results support the suggestion that a transmembrane electric potential is the major initial driving force during the first 1 s or 2 s after a dark–light transition, changing into a proton concentration gradient as the major driving force of ATP formation during steady state phosphorylation.

## 5. Conclusion

I have tried to show data from experiments with isolated chloroplasts which demonstrate the intimate qualitative and quantitative relation of proton gradients as an intermediary energy pool between electron transport and the ATP synthesizing apparatus. The system can be experimentally manipulated to show light-induced electron transport, light-induced proton gradient, proton gradient-induced ATP formation, and in reverse, ATP-induced proton gradient formation, ATP-induced reverse electron transport, ATP-induced luminescence, proton gradient-induced reverse electron transport and proton gradient-induced reverse electron-transport luminescence.

About 3 $H^+$ seem to be necessary to drive the synthesis of an ATP molecule in the steady state. Even though the chloroplast system uses the transmembrane proton concentration gradient as the major energy storage device in the steady state, transmembrane electric potentials serve as an important energy transducing intermediate during the first 1 s or 2 s following a dark–light transition in strong actinic light. All this, and much more information which space and time do not permit me to include here, provide us with much insight into the overall outline of coupled ATP synthesis in the chloroplast. However, the mechanism through which this feat is achieved still eludes us, and remains as a challenge for the future.

## References

[1] Avron, M. (1972) in: Proc. 2nd Int. Cong. Photosynthesis (Forti, G. et al. eds) pp. 861–871, N. V. Junk, The Hague.
[2] Avron, M. (1976) in: The Structural Basis of Membrane Function (Hatefi, Y. and Djavani-Ohaniance, L. eds) pp. 227–238, Academic Press, New York.
[3] Avron, M. (1977) Ann. Rev. Biochem. 46, 143–155.
[4] Avron, M. and Schreiber, U. (1977) FEBS Lett. 77, 1–6.
[5] Bakker-Grunwald, T. (1977) in: Encycl. Plant Physiol. (Trebst, A. and Avron, M. eds) vol. 5, pp. 369–373, Springer-Verlag, Heidelberg.
[6] Beyeler, W. and Bachofen, R. (1978) Eur. J. Biochem. 88, 61–67.
[7] Casadio, R., Baccarini-Melandri, A. and Melandri, B. A. (1974) Eur. J. Biochem. 47, 121–128.
[8] Casadio, R. and Melandri, B. A. (1977) J. Bioenerg. Biomembr. 9, 17–30.
[9] Chow, W. S. and Hope, A. B. (1976) Aust. J. Plant Physiol. 3, 141–152.
[10] Deamer, D. W., Price, R. C. and Crofts, A. R. (1972) Biochim. Biophys. Acta 274, 323–335.
[11] Fiolet, J. W. T., Bakker, E. P. and Van Dam, K. (1974) Biochim. Biophys. Acta 368, 432–445.
[12] Fiolet, J. W. T., Haar, L. V. D., Kraayenhof, R. and Van Dam, K. (1975) Biochim. Biophys. Acta 387, 320–334.
[13] Galmiche, J. M. (1977) in: Encycl. Plant Physiol. (Trebst, A. and Avron, M. eds) vol. 5, pp. 374–392, Springer-Verlag, Heidelberg.
[14] Graber, P. and Witt, H. T. (1976) Biochim. Biophys. Acta 423, 141–162.
[15] Jagendorf, A. T. (1975) in: Bio-energetics of Photosynthesis (Govindjee, ed.) pp. 423–492, Academic Press, New York.
[16] Kraayenhof, R. (1970) FEBS Lett. 6, 161–165.
[17] Nelson, N., Nelson, H., Naim, V. and Neumann, J. (1971) Arch. Biochem. Biophys. 145, 263–267.
[18] Nichols, J. W. and Deamer, D. W. (1978) in: Abstracts, Frontiers of Biological Energetics no. 143, Johnson Foundation, University of Pennsylvania.
[19] Ort, D. R. and Dilley, R. A. (1976) Biochim. Biophys. Acta 449, 95–107.
[20] Ort, D. R., Dilley R. A. and Good, N. E. (1976) Biochim. Biophys. Acta 449, 108–124.
[21] Pick, U., Rottenberg, H. and Avron, M. (1973) FEBS Lett. 32, 91–94.
[22] Pick, U., Rottenberg, H. and Avron, M. (1974) in: Proc. 3rd Int. Cong. Photosynthesis (Avron, M. ed) pp. 967–974, Elsevier, Amsterdam.
[23] Pick, U., Rottenberg, H. and Avron, M. (1974) FEBS Lett. 48, 32–36.
[24] Pick, U. and Avron, M. (1976) FEBS Lett. 65, 348–353.
[25] Portis, A. R. and McCarty, R. E. (1973) Arch. Biochem. Biophys. 156, 621–625.
[26] Portis, A. R. and McCarty, R. E. (1974) J. Biol. Chem. 249, 6250–6254.
[27] Rienits, K. G., Hardt, H. and Avron, M. (1974) Eur. J. Biochem. 43, 291–298.
[28] Rosing, J. and Slater, E. C. (1972) Biochim. Biophys. Acta 267, 275–290.

[29] Rottenberg, H. and Grunwald, T. (1972) Eur. J. Biochem. 25, 71–74.
[30] Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54–63.
[31] Schreiber, U. and Avron, M. (1977) FEBS Lett. 82, 159–162.
[32] Schroder, H., Muhle, H. and Rumberg, B. (1971) in: Proc. 2nd Int. Cong. Photosynthesis (Forti, G. et al., eds) pp. 919–930. N. V. Junk, The Hague.
[33] Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.
[34] Shahak, Y., Hardt, H. and Avron, M. (1975) FEBS Lett. 54, 151–154.
[35] Shahak, Y., Pick, U. and Avron, M. (1976) in: Proc. 10th FEBS Meet. (Desnuell, P. and Michelson, A. M. eds) vol. 40, pp. 305–314, Elsevier, Amsterdam.
[36] Shahak, Y., Siderer, Y. and Avron, M. (1977) Plant Cell Physiol. spec. iss. no. 3. 'Photosynthetic Organelles, Structure and Function' (Miyachi, S., Katoh, S., Fujita, Y. and Shibata, K. eds) pp. 115–127, Jap. Soc. Plant Physiol, Japan.
[37] Vinkler, C., Avron, M. and Boyer, P. D. (1978) FEBS Lett. 96, 129–134.