

THE EFFECT OF TEMPERATURE ON ENERGY-LINKED FUNCTIONS IN CHLOROPLASTS

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1. Introduction

The effect of temperature on the "energized state" (high-energy compound [1] or conformation [2] or electrochemical membrane potential [3]) is of interest from different points of view. In the first place, the chemical reactions involved presumably proceed at a decreased rate at lower temperatures thus being more accessible to kinetic analysis. In the second place, the reactions that generate or dissipate the energized state may show a *different* temperature-dependence so that they can be more readily resolved. In the third place, it is appropriate to study the *in vitro* reactions over a temperature range that includes the variations occurring *in situ*.

Various extrinsic fluorescent probes have provided a dynamic read-out of energy-linked phenomena in several types of biological membranes. The application of such probes has been discussed in detail [4] and excellently reviewed [5]. We have recently found that the fluorescent uncoupler atebirin provides an interesting energy-probe in chloroplasts [6] and coupled membrane particles of beef-heart mitochondria [7] and *Azotobacter vinelandii* [8].

Energization of these organelles leads to a quenching of the atebirin fluorescence, the extent of which is related to the actual *rate* of energy generation. Moreover, the fluorescence quenching occurs concomitantly with a proportional binding of atebirin to the organelles. More recently we have extended these studies to other types of fluorescent probes and compared the responses with other energy-linked changes in chloroplasts [9].

In this paper we compare several energy-linked functions in chloroplasts at different temperatures between 0–30°. It was found that the light-induced fluorescence lowering of both atebirin and chlorophyll *a*, cyclic photophosphorylation and the formation of the Δ pH across the membrane are not affected much by lowering the temperature to 0–5°. In contrast, the light-induced pH rise and light-scattering increase are already largely absent at 10–15°. At lower temperatures the dark-restoration of the fluorescence of both atebirin and chlorophyll *a* is much slower and ATP synthesis continues for some time in the dark. The dark-decay of the energized state as read out by the atebirin fluorescence shows a sharp break in the Arrhenius plot at 19.5°, being more temperature-sensitive above this break.

It is concluded that at lower temperatures chloroplasts contain a much higher steady-state level of energy because of the much lower activity of energy-dissipating reactions.

Some of these results have been presented at the Second International Congress on Photosynthesis Research, Stresa, Italy, 1971.

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Abbreviations:

- DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea
S-6 : 5-chloro,3-(*p*-chlorophenyl),4'-chloro-salicylanilide
MES : 2-(*N*-morpholino)ethanesulphonic acid
TES : *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid
Tricine: *N*-tris(hydroxymethyl)methyl-glycine

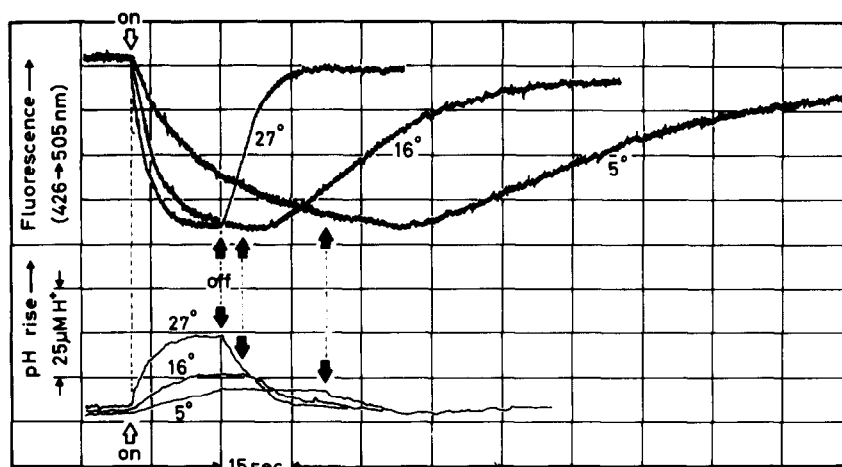


Fig. 1. Simultaneous recording of light-induced quenching of atebtrin fluorescence and pH rise at different temperatures. The standard medium contained 250 mM sucrose, 2 mM MES buffer (pH 6.8), 3 mM $MgCl_2$, 10 mM NaCl, 5 μ M pyocyanine and in addition 4 μ M atebtrin and chloroplasts (chlorophyll content 45 μ g/ml).

2. Materials and methods

Intact spinach chloroplasts were prepared as described previously [10]. Total chlorophyll was determined by the method of Whatley and Arnon [11].

Spectrofluorimetric experiments were performed with a Perkin-Elmer MPF-2A spectrofluorimeter that was modified so as to accommodate a thermostated cylindrical plexiglass cuvette (volume 1 ml), suited with a quartz window. Fluorescence emission was measured "front-face". Illumination with red light was provided by a 200 W quartz-iodide bulb using a Corning CS1-69 heat filter and a Schott RG 665 cut-off filter (controls were done with an RG 610 filter).

Chlorophyll *a* fluorescence (at 683 nm, 20 nm band width) was measured by "single-beam excitation" [12] (at 467 nm, 12 nm band width).

Simultaneous recordings of pH changes and either fluorescence or light-scattering changes were displayed using an apparatus described previously [10].

The determination of the Δ pH across the chloroplast membrane was made from the distribution of 14 C-methylamine as described by Rottenberg et al. [13].

Phosphorylation experiments were performed in a thermostated plexiglass syringe (volume 8 ml) from which samples were acidified at the desired

time intervals. Incorporation of $^{32}P_i$ into ATP was measured after extraction of P_i according to Nielsen and Lehninger [14].

3. Results

When we tested the atebtrin fluorescence responses in chloroplasts at lower temperatures a surprising result was obtained. As shown by the upper traces in fig. 1 the light-induced quenching of atebtrin fluorescence reached its full extent (100% quenching). At pH 8.0–8.5 the rate of this process was also hardly temperature-dependent. It was found (not shown) that at lower temperatures the maximal atebtrin concentration where a 100% quenching is still observable is much higher than at 25°C (cf. [6], fig. 4) and — more important — that more atebtrin is actually bound to the chloroplasts as determined after centrifugation [7, 9]. Binding saturation was obtained at 130 nmole/mg chlorophyll at 26°, 268 at 15° and 495 at 4.5°. Moreover, much higher concentrations of other uncouplers were required to inhibit the atebtrin responses at lower temperatures. These observations suggest that the energy content of the chloroplasts is much higher at lower temperatures.

The experiment in fig. 1 further presents the light-

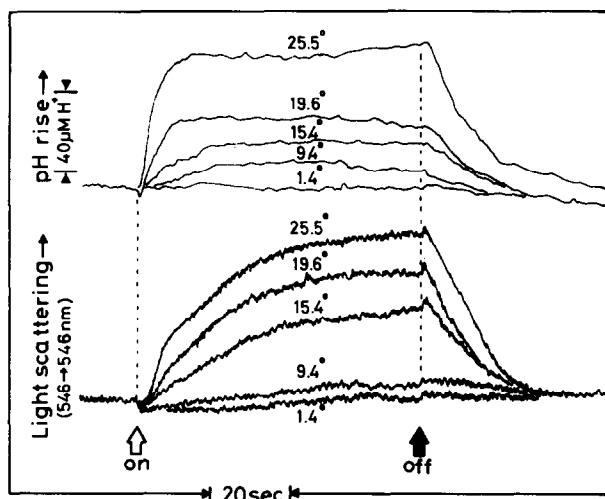


Fig. 2. Simultaneous recording of the light-induced pH rise and light-scattering increase at different temperatures. Conditions as in fig. 1, except that no atebirin was present. The pH was 6.4, the chlorophyll content 32 $\mu\text{g/ml}$.

induced pH rise, recorded simultaneously with the atebirin fluorescence, at 27°, 16° and 5°. Both the rate and the extent of the proton uptake are markedly inhibited at lower temperatures, whereas the rate of the fluorescence lowering of atebirin is much less affected and its extent remains the same. The kinetic profile of the "off" responses of the two processes is quite different as well. The restoration of the atebirin fluorescence in the dark is considerably delayed at lower temperatures, whereas the pH drops immediately.

Fig. 2 shows the effect of temperature on the light-induced pH rise together with the light-scattering increase that is thought to reflect an energy-dependent shrinkage of the organelle [15]. It is clear from this picture that the influence of temperature on these processes is most pronounced between 25° and 10°.

The pH changes as recorded by the electrode are no measure for the actual pH gradient created upon illumination. Therefore, the ΔpH was measured by ^{14}C -methylamine distribution experiments [13]. Fig. 3 pictures the dark-decay of the ΔpH at 25° and 5°. The half times of the decay are about 10 and 25 sec, respectively.

The apparent discontinuity in the temperature

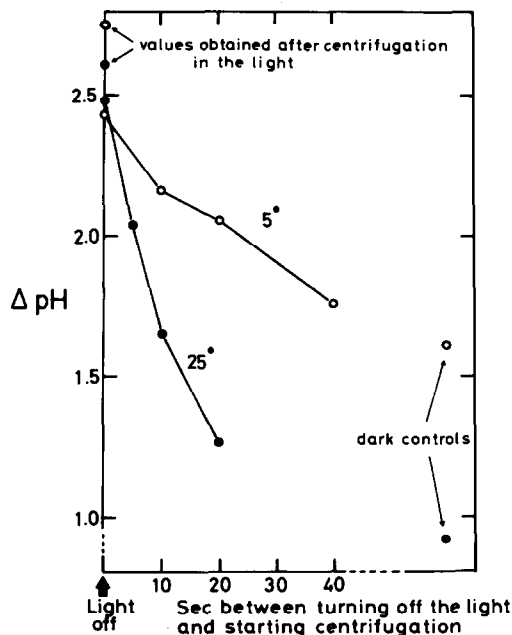


Fig. 3. The effect of temperature on the dark-decay of the ΔpH in chloroplasts. The medium contained 100 mM sucrose, 20 mM glycylglycine (pH 8.5), 5 mM MgCl_2 , 20 mM KCl, 5 μM ^{14}C -methylamine (specific activity 32 $\mu\text{Ci}/\mu\text{mole}$), $^3\text{H}_2\text{O}$ (specific activity 0.2 $\mu\text{Ci/ml}$), 60 mg dextran per ml, 10 μM pyocyanine and chloroplasts with a chlorophyll content of 48 $\mu\text{g/ml}$. The experiment was performed as described [13], except that the chloroplasts were centrifuged through a layer of silicone oil. For the calculation of the ΔpH an osmotic space of 10% of the total pellet water content (as determined by ^{14}C -sucrose binding [13]) was used.

effect noticed in the experiments shown in figs. 1 and 2 led to a careful study of this effect on the atebirin responses. The results are illustrated by the traces in fig. 4 and the Arrhenius plot in fig. 5. At the lower temperatures the quenching of atebirin fluorescence brought about by previous illumination of the chloroplasts is maintained in the dark for a long period. As shown in fig. 4, addition of the potent uncoupler S-6 during the quenched state induces a more rapid restoration of the atebirin fluorescence. The initial rates and overall half times of the "on" reactions are hardly influenced by

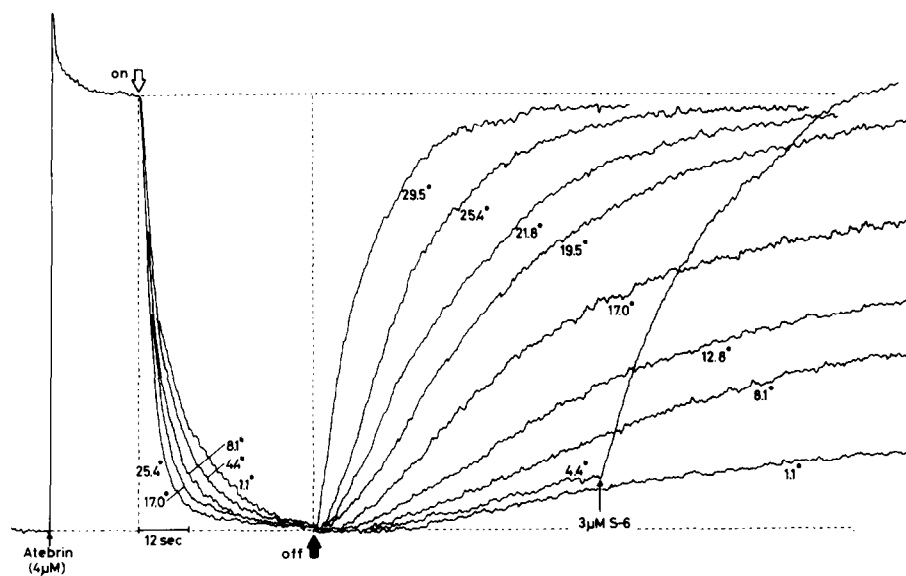


Fig. 4. The effect of temperature on the light-induced quenching of atebtrin fluorescence. Reaction conditions as in fig. 1 except that 20 mM TES buffer at pH 7.9 was used. The chlorophyll content was 32 $\mu\text{g/ml}$.

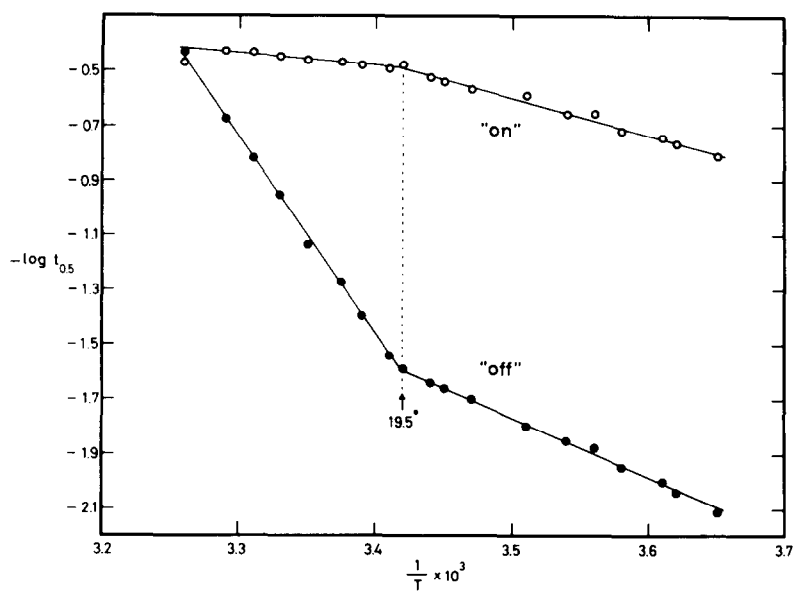


Fig. 5. Arrhenius plot of the overall half times of the "on" and "off" responses of atebtrin fluorescence under the conditions of fig. 4.

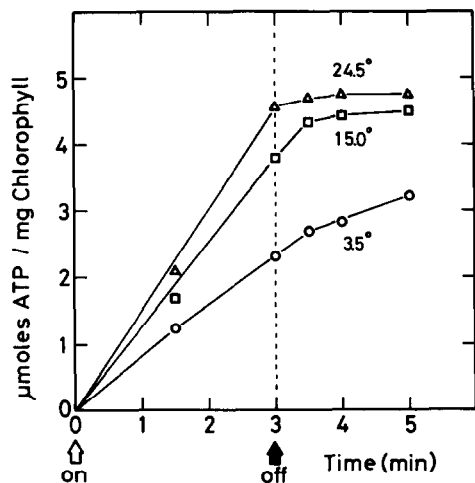


Fig. 6. The effect of temperature on photosynthetic phosphorylation during light and dark stages. The standard medium (fig. 1) contained 20 mM Tricine buffer (pH 7.8), and in addition: 5 mM $^{32}\text{P}_i$ buffer (specific activity 650 cpm/nmole), 20 mM glucose, 4 IU ammonium-free hexokinase, 2 mM ADP and chloroplasts (40 μg chlorophyll per ml).

temperature at pH 7.9 (slightly more at lower pH, cf. fig. 1). The Arrhenius analysis of the atebtrin responses in fig. 5 reveals a clear break at 19.5°. The activation energy of the "off" reaction is about -18 kcal/mole above 19.5° and about -6 kcal/mole below 19.5°. This difference is much smaller and in the opposite direction in the case of the "on" reaction.

Recently, Murata and Sugahara [12] described an energy-dependent lowering of chlorophyll *a* fluorescence in chloroplasts. It is of interest to note that the kinetics of the light-induced fluorescence lowering of the intrinsic chlorophyll *a* and extrinsic atebtrin are quite similar. We have confirmed this using the "single beam excitation" method [12] in the presence of 5 μM DCMU and 5 μM pyocyanine. Preliminary experiments (not shown) similar to those described by Wraight and Crofts (cf. [16], fig. 3) indicated that at 25° the chlorophyll *a* fluorescence was rapidly restored in the dark, whereas at 15° and 5° the fluorescence returned only very slowly to its original level.

Finally, we studied the effect of temperature on ATP synthesis. Fig. 6 shows that the rate of cyclic

photophosphorylation at 3.5° is still 50% of that at 24.5°. The experiment is meant to focus attention on the ATP synthesized *after* the light has been turned off. Phosphorylation continues for some time in the dark at 3.5° and also at 15°, but hardly at all at 24.5°.

4. Discussion

From the experiments presented here it is obvious that the steady-state level of energy in chloroplasts is much higher at lower temperatures. The discontinuity in the effect of temperature on the energy-linked atebtrin responses is not particularly surprising. A break in the Arrhenius plot between 17° and 20° has been observed earlier in a wide variety of biological processes [17] including the activity of many purified enzymes [18, 19], the membrane translocation of adenine nucleotides [20] and even the crawling speed of ants [21]. Such breaks are often thought to indicate a conversion between two enzyme conformations with different activities above and below the break. Above 19.5° the energized state is more temperature-dependent while the translocation of protons (and possibly other ions) and the osmotic changes are most active. On lowering the temperature the chloroplast membrane may change to a conformation that does not allow these reactions to proceed to the same extent, so that more energy is available for ATP synthesis and binding of atebtrin.

It may be noted that the light-induced pH rise and chloroplast shrinkage can be easily distinguished from the other energy-linked phenomena in that they have a low pH optimum and slower kinetics [9], and are largely absent at 3–10° — temperatures not unusual for spinach plants *in vivo*.

The restoration of the atebtrin fluorescence in the dark is slower at lower temperatures. That this process is still energy-linked (and not governed by the rate of diffusion of the probe) is evidenced by its sensitivity to uncoupler (fig. 4) and by the prolonged synthesis of ATP in the dark at lower temperatures (fig. 6).

The decay kinetics at lower temperatures of the ΔpH on the one hand and of the 'quenched state' of atebtrin (and chlorophyll *a*) and the ATP synthe-

sis on the other seem to indicate that these processes reflect different 'energized states'. However, if one assumes the quenching of the atebirin fluorescence to be due to a pH-dependent distribution of the probe [22, 23], a linear relationship between the quenching and the Δ pH would not be expected [24]. Obviously, more experiments are necessary to find out what physical mechanisms underlie the energy-linked fluorescence changes of intrinsic membrane constituents like chlorophyll *a* and of extrinsic fluorescent probes. This might enable us to characterize the microenvironmental changes that occur in the membrane upon energization.

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