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THE RELATIONSHIP BETWEEN DELAYED FLUORESCENCE AND THE H^+ GRADIENT IN CHLOROPLASTS

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SUMMARY

1. The induction kinetics of delayed fluorescence have been studied in isolated chloroplasts and compared with the kinetics of H^+ uptake. The slow phase of the delayed fluorescence rise, after replotting on a logarithmic scale, had the same half-rise time as H^+ uptake.

2. The kinetics of decay of the "state" filled during the slow phase of delayed fluorescence induction have been investigated by following the reappearance of the slow phase with increasing dark time after a prior period of illumination.

3. The decay of the "state" filled during the slow phase was found to parallel the decay of H^+ uptake under a variety of conditions in which the ionic environment was varied, or in the presence of ionophores or uncoupling agents.

4. It is suggested that the slow phase of the delayed fluorescence induction occurs as a pH gradient develops across the thylakoid membrane, and that the pH gradient is equivalent to the "state", the decay of which gave rise to a reappearance of the slow phase.

INTRODUCTION

The relationship between delayed fluorescence[1] and the high energy state in chloroplasts was first investigated by Mayne[2,3] who concluded that the "high-energy phosphorylation intermediate" was necessary for delayed fluorescence in spinach chloroplasts. Further investigation by Fleischmann and Clayton[4] with chromatophores from *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* showed that ionophores such as valinomycin, in the presence of K^+, NH_4^+ or Cs^+ had a marked effect on delayed fluorescence. Further observations led Fleischmann and Crofts (see ref. 5) to propose a mechanism by which the electrical component of the electrochemical H^+ gradient could be coupled to delayed fluorescence in bacterial chromatophores.

Wraight and Crofts[6] investigated the kinetics of the onset of 1 ms delayed fluorescence in spinach chloroplasts and interpreted their results in terms of a mechanism dependent on both electrical and chemical components of a H^+ gradient of the type proposed by Mitchell[7,8]. The kinetics of induction of 1 ms delayed fluorescence were resolved into two phases: a fast phase complete in less than 0.2 s, and a slower phase which was half complete in 0.3–0.5 s. They suggested that the slow

phase was dependent on the development of a pH gradient across the chloroplast membrane.

This relationship has been further investigated in the present paper, in which we have compared the kinetics of onset and decay of the slow phase of delayed fluorescence induction with those of light induced H^+ uptake. Some of these results have been briefly reported elsewhere[9].

METHODS

Preparation of chloroplasts

Chloroplasts were prepared from leaves freshly picked from spinach grown either outside or in a greenhouse. Finely chopped leaves were blended in 400 mM sucrose, 30 mM Tricine, 5 mM $MgCl_2$ and 2 mM sodium ascorbate (pH 7.6) at top speed for 6 s in an Atomix homogeniser (M.S.E.).

The homogenate was filtered through 8 layers of cheese cloth and chloroplasts were precipitated by centrifugation at 4500 × *g* for 5 min. The pellet was resuspended in a tenth dilution of the grinding medium and recentrifuged as above. The washed, broken chloroplasts were resuspended in 200 mM sucrose, 30 mM Tricine and 5 mM $MgCl_2$ (pH 7.6). Total chlorophyll was assayed by the method of Arnon[10].

Measurement of delayed fluorescence

For studies of delayed fluorescence, the stock suspension of chloroplasts was diluted to give 5 μg chlorophyll/ml final concentration in the suspending medium indicated. All experiments were done at pH 7.6 and 20 °C. Benzyl viologen (0.16 mM) was used in all experiments as electron acceptor. Delayed fluorescence was measured with a rotating sector phosphoroscope of conventional design as previously described[6].

Measurement of initial kinetics of H^+ uptake

Rapid H^+ changes were measured by following the colour change of phenol red. The stock suspension was used at a concentration of 5 μg chlorophyll/ml, diluted in 0.1 M KCl adjusted to pH 7.6 with tetramethyl ammonium chloride.

Phenol red absorbance changes were measured using a dual wavelength spectrophotometer[11] with 560 and 540 nm as measuring and reference wavelengths. For measurement of rapid changes the time constant of the decoding electronics was set to approx. 5 ms. A Wratten 70 filter was used to cover the actinic light, and the measuring photomultiplier was screened by 1 cm of saturated copper sulphate. Slower H^+ changes were measured using a glass electrode and recording pH meter[11].

Characterisation of phenol red as a pH indicator in suspensions of chloroplasts

Phenol red has not previously been used to study pH changes in chloroplast suspensions. The experiments below showed that phenol red was acting as an indicator of changes of pH in the external phase of the suspension.

(i) A difference spectrum of the change between 540 and 575 nm, using 540 nm as reference wavelength showed that the changes observed were due to phenol red, and that at 560 nm no other species contributed significantly to the changes observed.

(ii) The titration curve of phenol red with and without chloroplasts showed that the pK of the indicator was unchanged by the presence of chloroplasts. No binding of the indicator was detected at the chloroplast concentration used in these experiments.

(iii) A comparison of the light induced pH change in a chloroplast suspension as monitored by a pH electrode and by the phenol red absorbance change showed that the two light induced changes correspond to the same pH change. Both changes were abolished when the buffering power of the suspending medium was increased.

The light intensity dependence of the phenol red change was measured. At the intensity used the extent of the pH change was maximal, although the initial rate was not saturated.

Measurement of 9-aminoacridine fluorescence

This was measured as previously described[12] except that an additional actinic light covered by a Wratten 70 filter was used. The reaction mixture was 0.1 M KCl, 10 mM potassium phosphate (adjusted to pH 7.6 with KOH). Benzyl viologen (0.16 mM) was used as an electron acceptor and 4 μM 9-aminoacridine was present.

Materials

Simple organic and inorganic reagents were of Analar grade where obtainable, and otherwise of the highest grade commercially available.

Benzyl viologen and phenol red were obtained from British Drug Houses.

Valinomycin was obtained from Sigma (London) Chemical Co.

Nigericin was a generous gift of Dr R. L. Harned (Commercial Solvents Corp., Ind., U.S.A.).

RESULTS

Kinetics of induction of delayed fluorescence

Fig. 1 shows the kinetics of the rise in delayed fluorescence as measured 0.8–1 ms after flashes of light in the phosphoroscope. In Fig. 1a the intensity of the delayed fluorescence was measured directly while in Fig. 1b the same signal is shown after passing through a logarithmic amplifying circuit suitably offset to display the slow portion of the change on a similar scale. The logarithmic display was used in most experiments because of the exponential relationship expected between delayed fluorescence and the high energy state[6], and because the traces were obtained more conveniently than by replotting (see, for instance, Fig. 2).

Comparison of the H^+ change and the slow phase of delayed fluorescence

In Fig. 2 a trace of the rise in intensity of delayed fluorescence is shown together with a replot of the slow plase on a logarithmic scale. The second trace shows the H^+ change, as monitored by phenol red on a similar time scale. The slow phase of delayed fluorescence on a logarithmic scale, and the phenol red change, are plotted to test for first-order kinetics in the fourth part of the figure. The two processes clearly have a very similar time course.

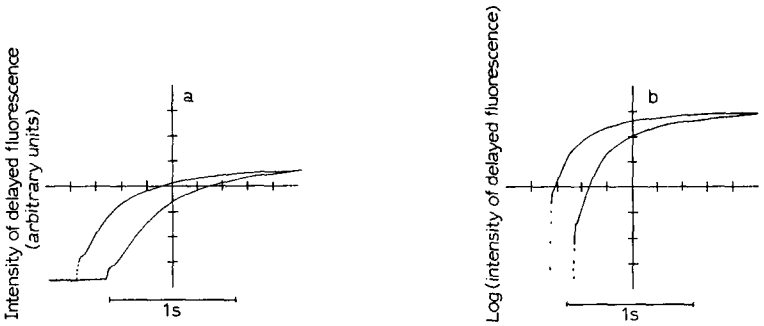

Fig. 1. Induction kinetics of delayed fluorescence. (a) Intensity of delayed fluorescence recorded directly from the photomultiplier output after decoding and smoothing. (b) The same signal after passing through a logarithmic amplifying circuit. Chloroplasts (5 μg chlorophyll/ml) were suspended in 100 mM KCl, 20 mM Tricine and 0.16 mM benzyl viologen.

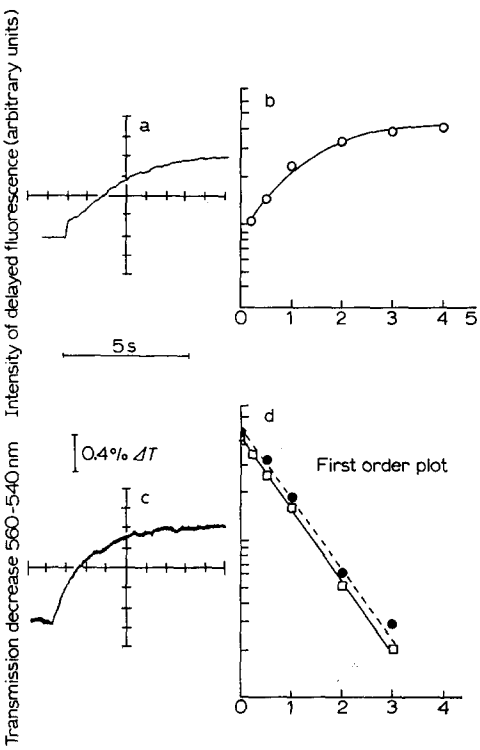

Fig. 2. Initial kinetics of delayed fluorescence and H+ uptake. Chloroplasts (5 μg chlorophyll/ml) were suspended in 100 mM KCl and 0.16 mM benzyl viologen with further additions as indicated. (a) Delayed fluorescence intensity at 1 ms, 20 mM Tricine present. (b) The trace from (a) replotted with intensity on a logarithmic scale. (c) H+ uptake indicated by phenol red (5.6 μM). (d) First-order plot of the curves from (b) and (c).

Variation of rise kinetics with dark time

When the rise kinetics, recorded as in Fig. 1b were measured after a preceding phase of illumination, it was found that the extent of the slow phase varied with the length of the intervening dark period (Fig. 3a), and that the decreased slow phase was associated with an increase in the extent of the initial fast phase of the rise kinetics. In Fig. 3b a plot of the height of the slow phase (on an inverted scale) against the dark time is shown. This plot shows the reappearance of the slow phase (or decay of the extra fast phase) with increasing dark incubation. Since the total extent did not vary, the change can be interpreted as the dark decay of a state induced

during illumination. The half-time of this decay varied from 7 to 25 s, depending on the age of spinach leaves and the conditions under which they had been grown.

Fig. 4a shows a similar effect, but demonstrated on a segment of spinach leaf suspended in distilled water. Again Fig. 4b shows a plot of the extent of the slow phase (on inverted scale) against dark time.

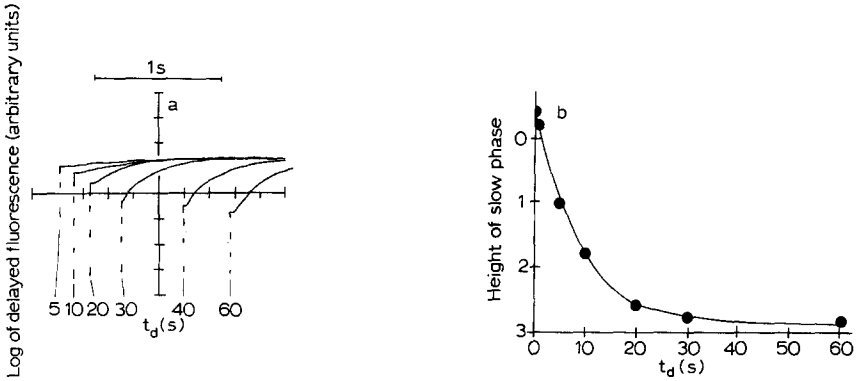

Fig. 3. Dark decay of the state generated during the slow phase of the induction. (a) Logarithm of intensity of delayed fluorescence rise kinetics after various dark times, t_d, following actinic illumination for 2 s. (b) Height of slow phase, from experiments similar to those shown in (a) plotted against t_d. Conditions as in Fig. 1.

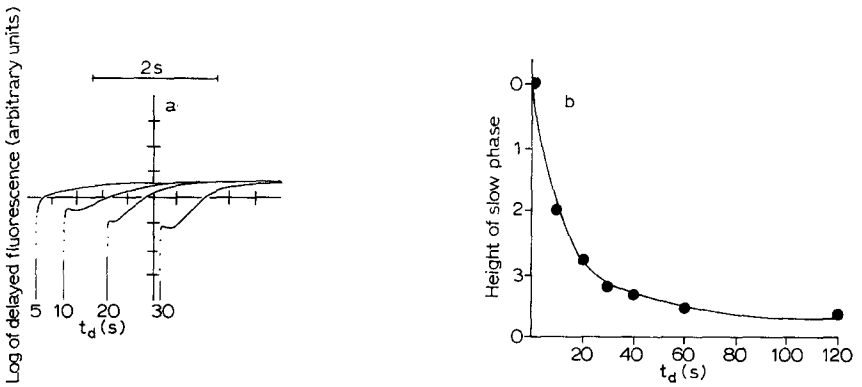

Fig. 4. Delayed fluorescence from spinach leaf segments. (a) Logarithm of intensity of delayed fluorescence measured as in Fig. 1. (b) Height of slow phase plotted as in Fig. 2b. Strips of freshly cut spinach leaves about 3 cm × 1 cm were suspended in distilled water.

Effect of ionophores on the decay of the state generated in the slow phase

In Fig. 5 the effect of valinomycin (4 nM) on the dark decay is shown. At this low concentration, valinomycin increased the rate of the decay from a half-time of 25 s to one of 12 s. The total extent of the change was the same, but the slow phase contributed a greater proportion in the presence of valinomycin so that the extent of the decay appeared to be stimulated. The effect of nigericin at two different concentrations is shown in Fig. 6. At a low concentration (4 nM) the rate of the decay was increased but the extent of the decay was unchanged. At higher concentrations (40 nM) the decay was no longer observed, and the slow phase of the rise kinetics was abolished, while the height of the fast phase was unaffected.

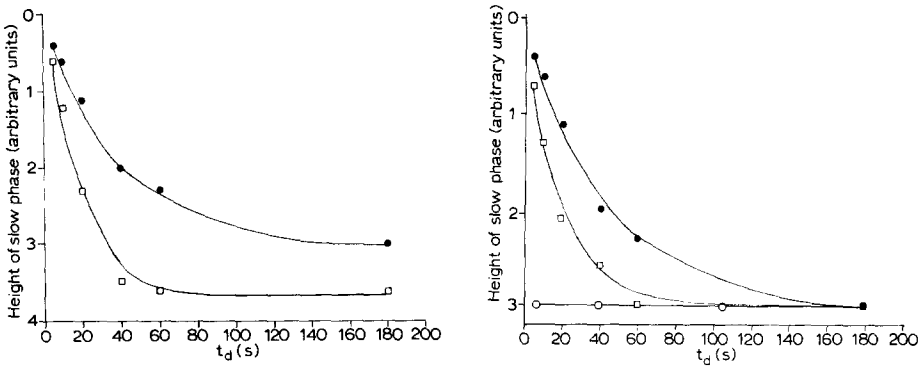

Fig. 5. Effect of valinomycin on the dark decay of the state generated during the slow phase induction of delayed fluorescence. ●—●, chloroplasts (5 μg chlorophyll/ml) suspended in 100 mM KCl, 20 mM Tricine and 0.16 mM benzyl viologen; □—□, the same but with 4 nM valinomycin.

Fig. 6. Effect of nigericin on the dark decay. ●—●, conditions as for Fig. 5; □—□, the same but with 4 nM nigericin; ○—○, the same but with 40 nM nigericin.

Effect of varying the suspending medium on the decay

The life-time of the state associated with the slow phase varied markedly with the ionic composition of the medium.

Fig. 7 shows the effect of substituting acetate for chloride as the anion of the reaction mixture. With increasing proportions of acetate the rate of decay was increased, and at 100 mM acetate the maximal extent of the slow phase was decreased.

The effect of substituting sulphate for chloride in the reaction medium is

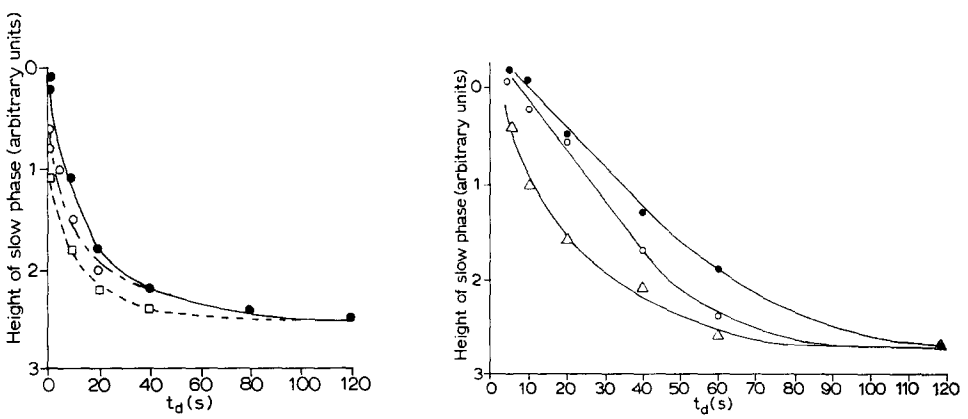

Fig. 7. Effect of substituting acetate for chloride in the suspending medium. ●—●, conditions as for Fig. 5; ○—○, chloroplasts (5 μg chlorophyll/ml) in 10 mM potassium acetate, 90 mM KCl, 20 mM Tricine and 0.16 mM benzyl viologen at pH 7.6; □—□, chloroplasts (5 μg chlorophyll/ml) in 50 mM potassium acetate, 50 mM KCl, 20 mM Tricine and 0.16 mM benzyl viologen.

Fig. 8. Effect of substituting sulphate for chloride in the suspending medium on the dark decay. △—△, chloroplasts (5 μg chlorophyll/ml) in 100 mM KCl, 20 mM Tricine and 0.16 mM benzyl viologen at pH 7.6; ○—○, chloroplasts (5 μg chlorophyll/ml) in 14 mM K_2SO_4, 80 mM KCl, 20 mM Tricine and 0.16 mM benzyl viologen; ●—●, chloroplasts (5 μg chlorophyll/ml) in 35 mM K_2SO_4, 50 mM KCl, 20 mM Tricine and 0.16 mM benzyl viologen.

shown in Fig. 8. The rate of the dark decay was decreased with increasing sulphate concentrations, but the total extent was unaffected.

Figs 9a and 9b show a comparison of the half-times of the decay of the light induced pH change in chloroplasts as monitored by phenol red, and the dark decay of the delayed fluorescence when acetate or sulphate replaced chloride in the medium.

It can be seen that the half times of the decay of the delayed fluorescence state, and of the phenol red change decreased in parallel with increasing acetate concentration, and increased with increasing sulphate concentration.

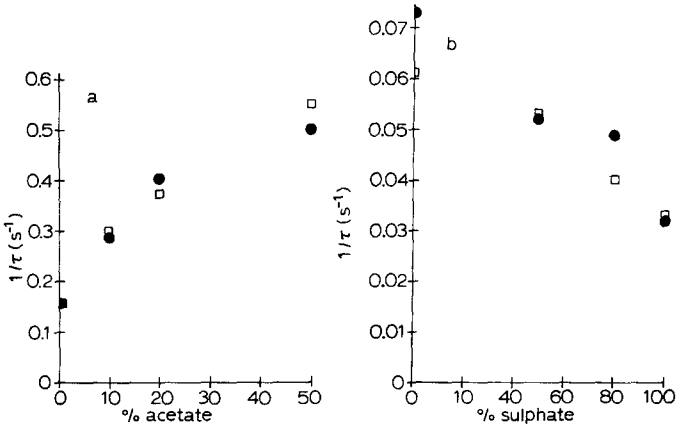

Fig. 9. Effect of substituting sulphate or acetate for chloride on the rate of decay of H^+ uptake and the dark decay of the state generated in the slow phase of delayed fluorescence. (a) Dark decay of H^+ uptake, or of the state generated during the slow phase of delayed fluorescence, plotted against increasing proportions of acetate in the suspension medium. The rate of decay is represented as the reciprocal of the half-time ($\tau_{\frac{1}{2}}$) of the decay under these conditions. □, delayed fluorescence; ●, H^+ uptake decay as monitored by phenol red. In both cases the reaction mixture contained chloroplasts (5 μg chlorophyll/ml) with 0.16 mM benzyl viologen. For delayed fluorescence the suspension medium contained 20 mM Tricine and various proportions of acetate and chloride to maintain total salt concentration at 100 mM. For H^+ uptake, Tricine was not included but 5.6 μM phenol red was. Otherwise the same as for delayed fluorescence. (b) As for (a) except with sulphate instead of acetate. Concentration of K_2SO_4 and KCl were adjusted to maintain constant ionic strength.

Relation between slow phase and Δ pH

Rottenberg *et al.*[13] have introduced a method for estimating the pH gradient across the thylakoid membrane by measuring the quenching of 9-aminoacridine fluorescence. We have attempted to use this method to follow the kinetics of onset and decay of the pH gradient. The quenching of 9-aminoacridine fluorescence on illumination of a chloroplast suspension was considerably slower (half rise time about 5 s) than the H^+ uptake. The time course of reappearance of fluorescence in the dark was similar to the decay of the state discussed above, and to the reversal of H^+ uptake. It seems likely that the quenching associated with uptake of the amine lagged behind the development of the pH gradient (*cf.* ref. 14), and that no great reliance can be placed in the kinetics of onset indicated by these measurements.

DISCUSSION

From the effects of ionophorous antibiotics and amines on the slow phase of the delayed fluorescence induction, Wraight and Crofts[6] suggested that the devel-

opment of the slow phase was dependent on the generation of a pH difference across the thylakoid membrane. Theoretical considerations suggested that the intensity of delayed fluorescence should be exponentially related to the pH gradient; replotting the induction kinetics on a logarithmic scale showed a slow phase the kinetics of which were similar to those reported for H^+ uptake, and which responded in parallel with H^+ uptake to ionophores and uncouplers.

The results reported here have extended these observations. We have also investigated some of the characteristics of the state generated in the light, the decay of which in the dark is associated with a reappearance of the slow phase of the delayed fluorescence induction.

Induction kinetics of delayed fluorescence, and the relation of H^+ uptake

The kinetics of the rise in intensity of delayed fluorescence showed several components. On replotting on a logarithmic scale these were resolved into a rapid phase, followed by a slow phase, the kinetics of which were approximately first order. The kinetics of H^+ uptake measured by the phenol red change under similar conditions were essentially the same as the replotted show phase of the delayed fluorescence induction (Fig. 2). The half-rise time of both changes ($t_{½}$ 0.3–0.7 s), was similar to values reported by others for the rise time of H^+ uptake (ref. 14).

No direct measurements of the kinetics of development of the pH gradient have been reported. Rumberg and Siggel[15] estimated both the kinetics and extent of the pH gradient by comparing the rate of electron flow as a function of the pH of the medium in the presence of uncoupler, and as a function of time of illumination in the absence of uncoupler. Since the former series of measurements showed a marked dependence on pH, they argued that the latter measurements should provide an indication of changes in the pH of the phase which controlled the rate of electron flow. If this phase was taken to be the aqueous phase enclosed by the thylakoid membrane, the measurements of Rumberg and Siggel[15] showed the kinetics of development and the extent of the pH gradient. This elegant line of reason receives some support from our experiments, since the close similarity between the kinetics of the changes we have measured, and the onset of the pH gradient estimated by Rumberg and Siggel[15] are quite consistent with the suggestion[6] that the slow phase of delayed fluorescence reflects the onset of a pH gradient across the thylakoid membrane. Rumberg and Siggel[15] estimated that a difference of 3.5 pH units might exist across the thylakoid membrane at pH 8. Rottenberg, Avron and their co-workers[13,16–18] have more recently arrived at a similar value by measuring the distribution of a variety of amines in equilibrium with the pH gradient. Unfortunately, the kinetics of response of the most promising amine method (using the quenching of 9-aminoacridine fluorescence), are sluggish when compared with H^+ uptake, and so cannot be used to get a true estimate of the rate at which the pH gradient develops.

Decay of the state generated in the slow phase

The decay of the state 'filled' during the slow phase of the delayed fluorescence induction could be followed by varying the dark time after a constant period of light, and measuring the extent of the slow phase on subsequent illumination. By plotting the extent of the slow phase (on an inverted scale) against the dark time, the time

course of the decay was revealed (Figs 3 and 4). Although the half-time for decay was variable, several features of the decay were always present. Thus we have shown that the half-time of the decay and the half-time for reversal of H^+ uptake were the same under a variety of conditions, and that ionophores, uncouplers and the ionic constitution of the suspending medium affected the two changes in parallel. The effects of the ionophores reported above can be easily understood from their mechanisms of action[19,20], and in the light of the extensive literature detailing their effects on H^+ uptake in chloroplasts (*cf.* ref. 21), and on the delayed fluorescence rise[6]. The effects of replacing chloride in the suspending medium by a weak acid anion (acetate) or by a sulphate, which appears to penetrate the thylakoid membrane less readily than chloride[22] merit further comment. Increasing the proportion of acetate in the suspending medium increased the decay of the state generated in the slow phase, and inhibited its extent, and these effects were parallelled by effects on H^+ uptake. The undissociated acid in equilibrium with acetate anion is freely able to cross the chloroplast membrane[21,22]. Movement of the acid would allow a rapid equilibration of the pH gradient across the thylakoid membrane, and the effects of acetate described here on delayed fluorescence can be readily interpreted along these lines. The effect of sulphate in slowing the decay of the delayed fluorescence is discussed above; the parallel effect on the decay of H^+ uptake, can be explained in terms of the relative permeability of the membrane to sulphate and chloride[22]. Bulk movement of H^+ across the thylakoid membrane is dependent on the movement of a co- or counter-ion to preserve a balance of charge. Hence the efflux of accumulated H^+ would be slower in the presence of the less readily penetrating sulphate.

Delayed fluorescence in intact leaves

Kinetics of 1 ms delayed fluorescence similar to those of isolated chloroplasts were demonstrated in spinach leaf slices. Both fast phase and slow phases of the rise kinetics were present and the fast phase showed a similar dark decay to that observed in chloroplasts. This raises the possibility that delayed fluorescence could be used as a direct measurement of the energetic state of intact leaves.

Conclusions

The observations discussed above are consistent with the conclusion that the state generated in the slow phase of delayed fluorescence induction is a pH gradient across the thylakoid membrane, and that the decay of the state reflects the decay of the pH gradient. The results lend further support to the mechanisms relating the high energy state to the intensity of delayed fluorescence proposed by Crofts and co-workers[6,24], and by Kraan[25].

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