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STUDIES ON THE SLOW FLUORESCENCE DECLINE IN ISOLATED CHLOROPLASTS

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SUMMARY

Data presented here indicate that the slow fluorescence decline in osmotically disrupted chloroplasts is not associated with the well known divalent cation effect on fluorescence yield. Thus the two phenomena have markedly different magnesium concentration requirements, magnesium addition after the fluorescence decline did not stimulate the dark reversal, and the characteristics of the fluorescence induction kinetics of the two processes are not similar.

At pH 7.6 the slow fluorescence decline was stimulated by several uncouplers demonstrated to greatly reduce proton pumping, and at pH 9.2 it was stimulated by all uncouplers tested. Acid-base transition was strongly inhibitory, and this inhibition was relieved by uncoupler. Thus the pH gradient seems to inhibit the process. The involvement of coupling factor is suggested by experiments in which phosphorylation substrates were inhibitory, and this inhibition was prevented by uncoupler. These data are explained in terms of coupling factor structural changes which in an unknown manner influence Photosystem II fluorescence emission.

Fluorescence induction curves indicate that the slow quenching decreased only the variable fluorescence. The half rise time was decreased along with the sigmoidicity of the rise curve. These data can be accomodated in terms of a model recently proposed by Butler and Kitajima (Biochim. Biophys Acta (1975) 376, 116–125), involving the transfer of energy from the excited, but closed, reaction centres II to the light harvesting chlorophyll system. The slow fluorescence decline is suggested to represent a decrease of this process.

INTRODUCTION

Krause [1] first reported that isolated intact spinach chloroplasts display a slow fluorescence decline of large magnitude, upon illumination, which is not due to Q re-oxidation. Eperiments with the electron acceptor methyl viologen [1], and the electron transport inhibitor 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) [2] indi-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

cated the involvement of electron transport in the process. Subsequent work demonstrated that the fluorescence decline phenomenon could be reconstituted in broken chloroplasts upon magnesium addition [2], and this lead to the speculation that the fluorescence decline may represent magnesium movement from the thylakoid with the consequent reversal of the well known magnesium stimulation of fluorescence yield [3]. This conclusion was supported by the results of Barber et al. [4] using the Mg^{2+} carrier A23187. Treatment with several uncouplers was also shown to inhibit the fluorescence decline in both intact and disrupted chloroplasts [1, 2], or stimulate its reversal [5], and this was interpreted to indicate that the fluorescence decline is driven by the proton gradient across the thylakoid membrane. By extension it was suggested that magnesium was pumped from the thylakoid space as a counter ion to protons. The nature of the fluorescence decline was not investigated by these workers, though as indicated above it was associated with the divalent cation effect on fluorescence, studied initially by Murata [3], an effect which is itself in dispute [3, 6–9].

In this paper we present data pertinent to these three general areas, i.e. the involvement of Mg^{2+}, the relationship to the proton gradient, and the nature of the fluorescence quenching phenomenon itself. These data do not support the idea that this quenching is due to a reversal of the divalent cation stimulation of fluorescence, and furthermore demonstrate that the proton gradient is not the motive force. Coupling factor involvement with the development of the phenomenon is suggested by experiments with phosphorylation substrates, and the quenching itself may be explained in terms of a decrease in the probability of the back transfer of energy from Photosystem II reaction centres to the fluorescent "light harvesting" chlorophyll system.

MATERIALS AND METHODS

Intact spinach chloroplasts were prepared using a modification of the procedure of Jensen and Bassham [10]. Leaves were briefly homogenised in a solution (medium A) containing 2-(*N*-morpholino)-ethane sulphonic acid (50 mM, pH 6.1), $MgCl_2$ (5 mM), sorbitol (0.33 M). Chloroplasts were centrifuged down at $1000 \times g$ for 1 min, resuspended and stored in a solution (medium B) containing *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) [50 mM, pH 7.6), ethylenediamino tetraacetic acid (tetra sodium salt, 2 mM), $MgCl_2$ (1 mM), $MnCl_2$ (1 mM), sorbitol (0.33 M). All procedures were performed at approx 0 °C. Osmotic disruption of intact chloroplasts was routinely performed by suspending chloroplasts in medium B, modified by the removal of sorbitol, for 2 min.

Measurement of slow fluorescence changes were routinely performed in a Perkin-Elmer MPF-3 spectrofluorimeter with 440 nm exciting light of varying intensities. The emission wavelength was 686 nm. Fast fluorsecence induction experiments were performed in an instrument to which an oscilloscope equipped with memory storage was attached. The photomultiplier was situated at 90 ° to the exciting beam. The exciting beam was filtered through a broad band Corning 4-96 filter, and fluorescence was measured through a Balzers 691 nm interference filter. No scattered light reached the photomultiplier in either insturment. Chlorophyll was 3–4 μg/ml for all fluorescence measurements.

Proton pump measurements were made in an unbuffered solution containin $MgCl_2$ (5 mM) and methyl viologen (0.5 mM) at pH 7.0. The chloroplasts wei added as a 40-μl sample in medium B and the final volume was 2 ml. Chlorophy was 45 μg/ml. Chlorophyll was measured by the technique of Arnon [11].

RESULTS

Involvement of magnesium

As reported by Krause [2], when intact chloroplasts are osmotically disrupte In a medium deficient in divalent cations, the fluorescence decline was extreme iow (Fig. 1). However, addition of magnesium both raised the initial maxim

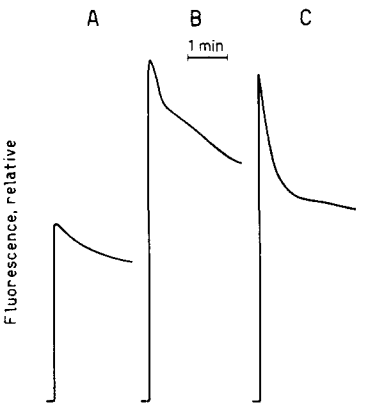

Fig. 1. Effect of various magnesium concentrations on the slow fluorescence decline. Chloroplas were disrupted osmotically in medium B (see Materials and Methods) minus sorbitol and illuminatio was with light of 100 000 ergs · cm^{-2} · s^{-1} of 440 nm. A, no further additions; B, 5 mM MgC added 15 s before illumination; C, 25 mM $MgCl_2$ added 15 s before illumination.

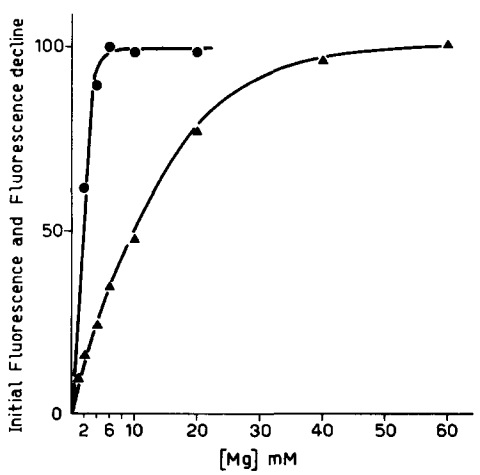

Fig. 2. Titration with $MgCl_2$ of the stimulation of initial fluorescence and the fluorescence declin phenomenon. Conditions were as described for Fig. 1, except the magnesium was added 1.5 mi before illumination and the data, along with those for the initial fluorescence have been standardise to the maximal value. ● — ●, initial fluorescence; ▲ — ▲, fluorescence decline after 15 s.

fluorescence (F_{max}) and greatly stimulated the fluorescence decline, but whereas about 5 mM was sufficient to saturate the stimulation of F_{max}, it required around 40 mM magnesium to saturate the stimulation of the fluorescence decline (Fig. 2). The magnesium stimulation of F_{max} is clearly associated with the well known divalent cation fluorescence stimulation, and the concentration dependency is similar to that normally reported [3, 8], whereas the stimulated fluorescence decline seems to represent another process.

Fig. 3 represents the results of an experiment in which the effect of magnesium addition on the dark recovery of fluorescence yield was examined after the decline in osmotically disrupted chloroplasts. In Figs 3A and 3B chloroplasts were first disrupted in 5 mM magnesium and then diluted to 1 mM magnesium immediately before illumination. This treatment permitted a satisfactory rate of fluorescence decline while maintaining a high F_{max} at a low concentration of magnesium in the medium. As can be seen magnesium addition did not stimulate the dark recovery of fluorescence yield. On the other hand when the chloroplasts were initially disrupted at the low magnesium concentration and subsequently subjected to an identical treatment (Figs 3C and 3D), magnesium addition in the dark greatly stimulated the fluorescence. This clearly separates the fluorescence decline phenomenon from the magnesium effect on fluorescence yield.

The fluorescence induction parameters are compared in Table I for the magnesium stimulation of fluorescence and the slow fluorescence decline. As previously reported [8] magnesium stimulated only the variable fluorescence and it can

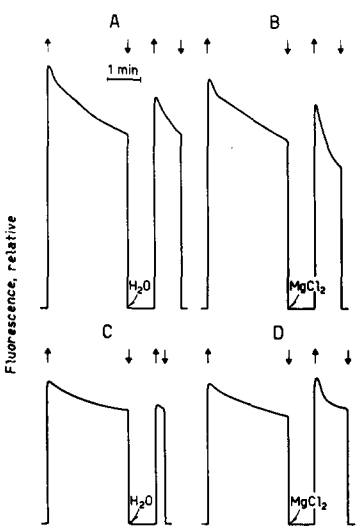

Fig. 3. Effect of magnesium addition on the dark fluorescence recovery. In A and B, chloroplasts were osmotically disrupted in a medium containing 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and HEPES (50 mM, pH 7.6). The $MgCl_2$ concentration was adjusted to 1 mM 5 s before illumination, with all other constituents remaining at the same level. In C and D, disruption was in a similar medium except that $MgCl_2$ was 1 mM from the beginning. $MgCl_2$ (20 mM) was added after the fluorescence decline and during the dark, where indicated. Upward arrows (↑) indicate the onset of illumination with 100 000 ergs · cm^{-2} · s^{-1} of 440 nm light, and downward arrows (↓) indicate its termination.

TABLE I

FLUORESCENCE INDUCTION PARAMETERS IN THE PRESENCE AND ABSENCE OI MAGNESIUM, AND BEFORE AND AFTER THE SLOW FLUORESCENCE DECLINE

Fluorescence induction was measured in the presence of 16 μM DCMU and with 6 500 ergs · cm^{-2} s^{-1} of Corning 4-96 filtered light.(A) Magnesium experiment. Osmotic disruption was with eithe 1 or 5 mM $MgCl_2$ in the presence of 3 mM EDTA, 1mM $MnCl_2$ and HEPES (50 mM, pH 7.6) Results are the mean of 10 reactions. (B) Fluorescence decline experiment. Both samples were dis rupted with 5 mM $MgCl_2$, 2 mM EDTA, 1 mM $MnCl_2$ and HEPES (50 mM, pH 7.6). Just befor illumination of one of the samples, to bring about the fluorescence decline, 20 mM $MgCl_2$ and μM desaspidin were added to each sample. Desaspidin was added as it prevents the dark reversal o the fluorescence decline (see below). The light intensity was about 500 000 ergs · cm^{-2} · s^{-1} o $CuNO_3$-filtered light and the exposure time was 2 min, after which there was a 20-s exposure to weal 722 nm light in order to reoxidise Q. Both illuminated and non-illuminated samples were then placec in ice for 5 min before fluorescence induction measurements was commenced. Results are the mear of 15 reactions.

(A)	$MgCl_2$ (1 mM)	$MgCl_2$ (5 mM)
Non-variable	26.7	26.4
Variable	33.7	97.2
Half rise time	26.8 ms	20.7 ms
(B)	Before fluorescence decline	After fluorescence decline
Non-variable	29.4	30.0
Variable	57.7	14.2
Half rise time	29.5 ms	25.8 ms

be seen that the slow fluorescence decline was also only due to the variable fluores· cence. Low magnesium, however, lead to a somewhat longer half time of the fluo· rescence rise, whereas the post fluorescence decline chloroplasts displayed a shorter half time. Thus the two processes are not identical kinetically.

Relationship to the proton gradient and coupling factor

In Fig. 4 the effects of four uncouplers on the fluorescence decline are indicated. While gramicidin and ammonium inhibited the decline at pH 7.6, desaspidin and

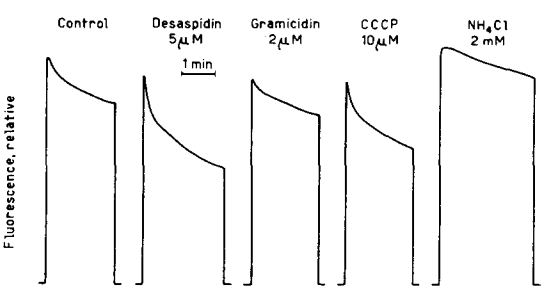

Fig. 4. Effect of uncouplers on the slow fluorescence decline. Chloroplasts were osmotically disrupted in a medium containing 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and HEPES (50 mM, pH 7.6). The uncoupler was added 30 s before the commencement of illumination, which was with 100 000 ergs · cm^{-2} · s^{-1} of 440 nm light.

TABLE II

EFFECT OF UNCOUPLERS ON THE SLOW FLUORESCENCE DECLINE AT pH 7.6 AND pH 9.2

Chloroplasts were disrupted at the pH indicated in a medium containing 50 mM HEPES, 5 mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM EDTA. Just before illumination 40 mM $MgCl_2$ was added. The light intensity was 50 000 ergs · cm^{-2} · s^{-1}. Results are given as the percentage decline of fluorescence in 2.5 min with the transient initial value taken as 100 %.

	pH 7.6	pH 9.2
Control	33	16
Desaspidin (5 μM)	45	70
CCCP (10 μM)	33	73
Gramicidin (2 μM)	13	70
NH_4Cl (2 mM)	16	22

usually carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were stimulatory. At pH 9.2, all uncouplers were stimulatory, with NH_4Cl being the least effective (Table II). All uncouplers prevented the recovery of fluorescence yield in the dark. All four uncouplers were checked for their effect on proton pumping, and all were shown to be strongly inhibitory at these concentration with desaspidin being the most effective (Table III).

As the above experiments indicated that the pH gradient may inhibit the slow fluorescence decline with uncouplers, under certain conditions, removing this inhibition, acid-base transition experiments were performed to further investigate this aspect. As reported initially by Jagendorf and Uribe [12] this treatment generates a proton gradient across the thylakoid membrane, and in the pH 4.2 to pH 7.6 transition experiment we observed a dramatic inhibition of the fluorescence decline (Table IV). This inhibition was relieved by the uncoupler desaspidin.

These data implicated the transmembrane proton gradient in the slow fluorescence decline which prompted investigation into the possible involvement of the coupling factor. To this end we tested the effect of phosphorylating conditions on the fluorescence decline and from Fig. 5 it can be seen that while phosphate and ADP when added separately had little effect, a pronounced inhibition was observed when the two compounds were added together. A similar inhibition, of even some-

TABLE III

EFFECT OF UNCOUPLERS ON PROTON UPTAKE

Chloroplasts were disrupted osmotically in 2 ml of a medium containing 5 mM $MgCl_2$, and 0.5 mM methyl viologen. With the chloroplast addition the constituents of medium B (see Materials and Methods) were also added, but these were diluted by a factor of 50. The final pH was between 6.9 and 7.0 Chlorophyll was 40 μg/ml, and the data are given as nano-equivalents of H^+ per mg chlorophyll per reaction mixture.

Control	130
Desaspidin (5 μM)	14
CCP (10 μM)	40
NH_4Cl (2 mM)	52
Gramicidin (2 μM)	53

TABLE IV

EFFECT OF ACID-BASE TRANSITION ON THE SLOW FLUORESCENCE DECLINE

Chloroplasts were disrupted at the pH indicated in a medium containing 50 mM HEPES, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 3 mM sodium succinate. After 2 min 30 mM $MgCl_2$ was added and the pH adjusted to the indicated value with an identical medium of the requisite pH. Illumination with light of 50 000 ergs · cm^{-2} · s^{-1}, was commenced 2 s after the pH adjustment. Results are given as the percentage fluorescence decline in 2 min with the transient initial fluorescense value taken as 100 %. At the pH 4.2 this value was 0.35 that at 7.6.

pH transition	Fluorescence decline
7.6 → 7.6	20
4.2 → 4.2	30
4.2 → 7.6	3
4.2 → 7.6 (+5 μM desaspidin)	30

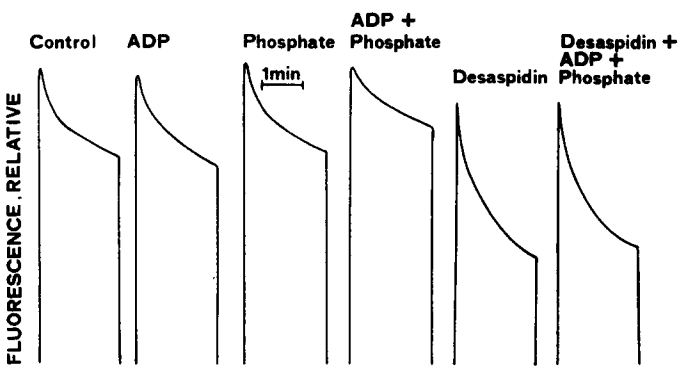

Fig. 5. Effect of phosphorylation substrates on the slow fluorescence decline. Conditions as in Fig. 1 1 mM ADP, 2 mM phosphate and 5 μM desaspidin were added 1 min before illumination where indicated.

what greater magnitude, was also observed with methyl viologen present as electron acceptor [13] and under these conditions we noted the usual 2–3 fold stimulation of electron transport on addition of the phosphorylation substrates. We have also observed that GDP functions almost as well as ADP in inhibiting the fluorescence decline in the presence of phosphate, while CDP and ATP were without effect in the presence or absence of phosphate. In the presence of the uncoupler desaspidin ADP and phosphate were without effect (Fig. 5).

The fluorescence quenching phenomenon

In order to examine the fluorescence induction parameters after the slow quenching, osmotically disrupted chloroplasts were illuminated in the presence of desaspidin in order to prevent the subsequent dark recovery of fluorescence yield. After reoxidation of Q with far red light, fluorescence induction was measured on samples stored in ice, and compared with similarly treated chloroplasts which had not been exposed to the fluorescence quenching-inducing illumination. Results of a typical experiment are shown in Fig. 6, and data from a large number of such experiments are summarised in Table 1B. It can be seen that only the variable compo

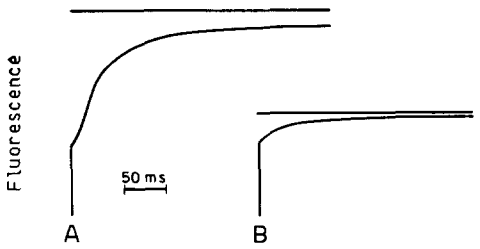

Fig. 6. Fluorescence induction before (A) and after (B) the slow fluorescence decline. Experimental conditions were as described in Table 1B.

nent of the fluorescence was quenched. The half time of the fluorescence rise was faster after the slow quenching phenomenon, and this is correlated with the decrease or loss of the sigmoid nature of the induction curve.

We have also noticed in chloroplasts not treated with desaspidin that the fluorescence quenching phenomenon was also only due to the variable fluorescence component, and this was accompanied by a decrease of sigmoidicity. However, we were unable to quantitate these data owing to the rapid dark recovery of the fluorescence yield, which necessitated fluorescence induction experiments being made before Q could be fully reoxidised.

DISCUSSION

It is well established [14, 15] that the velocity of Photosystem II photochemistry may be expressed by

$$v = \frac{Q}{1-(1-Q)\alpha} \tag{1}$$

which comes from the series

$$v = Q+Q(1-Q)\,\alpha+Q(1-Q)^2\alpha^2 \cdots \tag{2}$$

Q is the probability of a Photosystem II trap being open and α is the probability of energy transfer between Photosystem II units.

Recently Butler and Kitajima [16] have suggested a model for Photosystem II in which reaction centres where Q is already reduced, may become excited and would then either transfer energy back to the light harvesting chlorophyll system with a probability constant k_t, or would dissipate this by non-radiative decay processes. This concept easily includes the possibility that reaction centres may not be able to transfer back to the light harvesting chlorophylls, in which case excitation of a closed trap would lead only to non-radiative dissipation and k_t would then be the probability constant for a closed reaction centre not trapping. Including this constant k_t in Eq 1 and 2 gives the velocity of Photosystem II photochemistry as

$$v = \frac{Q}{1-(1-Q)\,\alpha \cdot k_t} \tag{3}$$

This equation is consistent with the experimental observations of Joliot et al. [14] and Marsho and Kok [15] but with inclusion of k_t there is the possibility that α has previoulsy been somewhat underestimated.

Using the same approach an equation for variable fluorescence yield, ΦF may be represented by the series:

$$\Phi F = (1-Q)k_t(1-\alpha)k_F+(1-Q)^2 \cdot k^2{}_t\alpha(1-\alpha)k_F+(1-Q)^3k^3{}_t \cdot \alpha^2(1-\alpha)k_F \ldots \quad (4)$$

where k_F is the probability constant for fluorescence emission from the light harvesting chlorophylls of Photosystem II. This yields

$$\Phi F = \frac{(1-Q)k_t(1-\alpha) \cdot k_F}{1-(1-Q)\,\alpha \cdot k_t} \quad (5)$$

Integrating Eq. 3 with respect to time, t, gives

$$t = k_t \cdot \alpha \cdot (1-Q)-(k_t \cdot \alpha-1) \ln Q \quad (6)$$

From Eq 5 and 6 theoretical fluorescence rise curves may be calculated (Fig. 7). The sigmoid nature of the rise curve at high k_t is a consequence of energy transfer between Photosystem II units [17, 18], and resembles the experimental situation before the slow fluorescence decline (Fig. 6). Interestingly, decreasing k_t not only decreased the variable fluorescence, as has been shown by Butler and Kitajima [16], but also decreased both the half time of the fluorescence rise and the sigmoidicity. This situation resembles the fluorescence rise curves measured before and after the slow fluorescence decline (Table 1B, Fig. 6). Moreover, the decrease in half rise time, such as the situation in Fig. 7 where the variable fluorescence was calculated to be the same as that for the fluorescence decline experiment of Table 1B (ratio of high variable/low variable = 4.08), is very similar to the measured half rise time changes. The possibility that the fluorescence decline may represent changes favouring an increased spillover of energy from Photosystem II to Photosystem I does not seem likely, as it can be seen from Eq 5 that sigmoidicity is not eliminated by increasing spillover (decreasing k_F). Furthermore increased spillover leads to

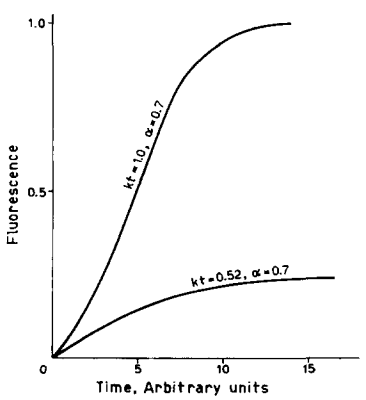

Fig. 7. Theoretical fluorescence induction curves plotted according to Eqns 5 and 6 for k_t = 1 and 0.52, α constant at 0.7, and k_F constant at 1.

large increases in the half time of the fluorescence rise. Thus we believe that the slow fluorescence decline probably represents a situation where k_t is progressively decreased during the first few minutes of illumination.

Unfortunately the mechanism whereby this change in k_t processes is effected is still very unclear. We are not able to agree with the earlier suggestion [2, 4] that the slow fluorescence decline represents a reversal of the well-known magnesium stimulation of fluorescence yield, first reported by Murata [3], though magnesium does seem necessary for the phenomenon, high concentrations being required to saturate it. We therefore presume that the fluorescence increase effected by magnesium addition to uncoupled chloroplasts during illumination by Krause [2] and Barber et al [5] does not represent a reversal of the slow fluorescence decline but simply the magnesium stimulation of fluorescence yield. We have noticed (unpublished observations) that when magnesium of 10–30 mM was added during illuminations to chloroplasts already exposed to 5 mM $MgCl_2$ there was an immediate and rapid stimulation of the fluorescence decline.

Evidence with uncouplers and from acid-base transition experiments strongly suggests that the proton gradient across the thylakoid membrane is inhibitory. This conclusion opposes that of other workers [2, 5] who considered that the proton gradient in fact drove the slow fluorescence decline. Their conclusions were based on experiments with several uncouplers which inhibited these fluorescence changes or stimulated their reversal. We have also presented data that several uncouplers gramicidin and NH_4Cl) under certain conditions of pH are inhibitory, but this seems not to be due to their property of reducing the proton gradient since uncouplers such as desaspidin and CCCP, which reduced the pH gradient substantially, were stimulatory at all pH values tested, and gramicidin and NH_4Cl also stimulated at high pH. We have also noticed that desaspidin and CCCP are stimulatory with intact chloroplasts not subjected to osmotic shock (unpublished data).

The necessity of electron transport is well established by the work of Krause [1, 2] using electron acceptors and inhibitors of electron transport, and from many similar experiments we have performed. Thus, in general terms we may conclude that during electron transport structural changes occur in the membrane which influence k_t processes at the reaction centre of Photosystem II and which are inhibited by the proton gradient.

It would appear that the coupling factor may be involved here as ADP or GDP in the presence of phosphate strongly inhibited the fluorescence decline. Other nucleotides were without effect and the inhibition was completely prevented by the uncoupler desaspidin and substancially overcome by antibody to CF_1 and the energy transfer inhibitor phloridzin [13]. This inhibitory effect cannot be related to altered rates of electron transport as ADP and phosphate increase this, and would therefore be expected to increase the rate of fluorescence decline. We also think it unlikely that the small decrease in transmembrane pH gradient associated with phosphorylation [19] is involved here as the arguments presented above with respect to uncoupler and acid-base transition experiments lead one to expect that decreased pH gradient would be stimulatory and not inhibitory. We therefore suggest that the most likely explanation is that upon binding of ADP and phosphate to the coupling factor at the phosphorylation site a conformational change occurs which in some way interferes with the fluorescence decline phenomenon. Coupling factor

conformational changes associated with ADP plus phosphate addition have previ ously been postulated by Ryrie and Jagendorf [20] on the basis of measurement of ^{3}H-H exchange on the coupling factor. However, these authors indicated that th energy transfer inhibitor phloridzin was without effect on ^{3}H-H exchange, wherea we have shown that phloridzin largely prevented the ADP plus phosphate inhibitio of the fluorescence decline. Thus while we expect those CF_1 structural change measured by Ryrie and Jagendorf [20] to be involved in the effect of ADP plu phosphate on the fluorescence decline, the two phenomena do not appear to b identical.

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