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CHANGES IN CHLOROPHYLL FLUORESCENCE IN RELATION TO LIGHT-DEPENDENT CATION TRANSFER ACROSS THYLAKOID MEMBRANES

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SUMMARY

Based on cation effects on chlorophyll *a* fluorescence and the light scattering behaviour of chloroplasts, a new interpretation of energy-dependent fluorescence quenching in intact leaves and isolated spinach chloroplasts is given. This type of fluorescence quenching is suggested to reflect movement of Mg^{2+} and other cations from the thylakoids to the stroma compartment. Cation efflux processes are associated with light-dependent proton uptake by thylakoids. Since cations strongly increase the fluorescence yield, their efflux leads to fluorescence lowering, apparently by means of structural changes of the membrane system. Similarly, the light-induced increase of apparent absorbance at 535 nm (caused by increased light scattering), which parallels fluorescence quenching, may reflect structural changes due to cation efflux from the thylakoids. In the dark these processes are reversed. The following results support this view:

1. After the envelopes of intact chloroplasts had been ruptured by osmotic shock in a medium of low cation content, the fluorescence yield was drastically lowered, and the long-term fluorescence quenching, as well as the light-dependent absorbance increase were missing. This is understood as being caused by loss of cations, which had been retained within the envelope.

2. Addition of certain cations to these thylakoid preparations largely restored the fluorescence signal characteristic of intact chloroplasts.

3. A dark period of 2–3 min in the presence of cations was required to produce the maximum fluorescence response.

4. When chloroplasts were ruptured in the presence of 5 mM $MgCl_2$, both the signals of fluorescence and apparent absorbance at 535 nm remained very similar to those of the intact chloroplasts.

5. The described cation-dependent phenomena are sensitive to FCCP and closely correlated with light-induced proton uptake into the thylakoids, thus showing a relation to the energy conserving mechanism of photosynthesis.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; HEPES, *N*-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; PMS, phenazine methosulfate.

INTRODUCTION

Recently, several workers have shown that the yield of chlorophyll *a* fluorescence of chloroplasts or algae is strongly influenced by the energy state [1–3], or light-induced structural changes [4–7] of the thylakoid system. Energy-dependent fluorescence quenching has been discussed in terms of increase in spillover of excitation energy [8–12] from the strongly fluorescent Photosystem II to the pigments of Photosystem I, which emit much less fluorescence. Light-induced structural changes of the thylakoid membranes related to the energization of the photosynthetic apparatus may be thought to stimulate the rate of spillover or, alternatively, the dissipation into heat of excitation energy and, therefore, quench fluorescence. It has been postulated that the rate of spillover from Photosystem II to Photosystem I is controlled by ions of magnesium and other metals [8, 13–16]. As has first been observed by Homann [17], these cations drastically increase fluorescence of broken chloroplasts, presumably due to structural changes of the thylakoids [16,17].

Furthermore, structural changes due to the energization of the thylakoid membranes are thought to be responsible for the light-induced increase of apparent absorbance or light scattering at 535 nm (chloroplast shrinkage) [18, 19]. In a preceding study [20] a close inverse relationship between long-term changes in fluorescence and in apparent absorbance at 535 nm has been found with intact systems such as leaves of higher plants and isolated chloroplasts. These slow changes are kinetically closely related to the light-induced proton uptake into the thylakoids [20] which, however, because of the permeation barrier of the chloroplast envelope to protons [21], can only be measured in envelope-free chloroplasts. It has been postulated that the light-induced proton uptake into the thylakoids leads to structural changes of the membrane system which affect both fluorescence and apparent absorbance [20].

However, several observations indicate that the relationship between chlorophyll *a* fluorescence, light scattering, and light-dependent proton transfer is a complex one. Mohanty and Govindjee [22] found an energy-dependent fluorescence increase and absorbance decrease (540 nm) with *Anacystis* cells. The two changes did not show the same kinetics, indicating that different types of structural alterations are involved. Broken chloroplasts, i.e. chloroplasts having lost their envelope and water soluble stroma content after osmotic rupture, showed little or no fluorescence and absorbance changes accompanying light-dependent proton uptake [20]. Thus, the proton pumping process does not seem to be directly responsible for the phenomena observed in intact systems. This is in agreement with a recent work of Mohanty et al. [8], who found that proton uptake in glutaraldehyde-fixed thylakoids may proceed without concomitant conformational changes. The experimental evidence presented in this communication suggests that the light-induced structural membrane alterations that underlie the changes of fluorescence and apparent absorbance seen with intact systems strongly depend on metal ion transport processes. The latter have, in turn, been found to be dependent on active proton uptake [23]. As the cations are thought to regulate the decay of excited chlorophyll a_2 via structural alterations of the thylakoid system [8, 9], their energized movement would explain those long-term fluorescence phenomena which are known to be independent of the redox state of the quenching substance of Duysens and Sweers [24].

MATERIALS AND METHODS

Intact chloroplasts (Type A, see [25]) were isolated from freshly harvested leaves of *Spinacia oleracea* L. according to the method of Jensen and Bassham [26], modified by additional washing and centrifugation steps and slight variation of the isolation medium [27, 28]. On the average, 80% of the chloroplasts were intact as shown by their rate of $Fe(CN)_6^{3-}$ reduction [28]. The assay medium (Soln C) contained 0.33 M sorbitol, 0.5 mM KH_2PO_4, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 10 mM NaCl, 2 mM EDTA and 40 mM *N*-hydroxyethylpiperazine *N*-2-ethanesulfonic acid (HEPES) buffer (pH 7.6). No artificial electron acceptors were used. For osmotic rupture of chloroplasts, which results in the loss of envelope and stroma, Type A chloroplasts were suspended in water or, alternatively, in a solution of 5 mM $MgCl_2$, and a few seconds later were brought back to isosmotic conditions by adding an equal volume of double strength Soln C. Breakage was checked with a phase contrast microscope.

Simultaneous measurements of proton uptake, chlorophyll *a* fluorescence and apparent absorbance at 535 nm were carried out at room temperature (20–23 °C) as described previously [20]. A broad band of red light (half band width from approx. 630–680 nm) was used for excitation, and fluorescence was recorded in the far-red band at 740 nm [20]. Additional experiments with blue light excitation (Filters 4303 and 5562 from Corning, New York, and 1 mm infrared absorbing filter Calflex C from Balzers, Liechtenstein) and fluorescence recording at 683 nm (interference filter 683 nm, and 1 mm Calflex C, Balzers, Liechtenstein; Filter 2403, Corning, New York; cutoff filter RG 665 and RG 630, Schott and Gen., Mainz; 8 mm heat filter from a Leitz projector) gave essentially the same fluorescence signals as the former method.

RESULTS

Upon prolonged illumination intact isolated chloroplasts exhibit slow fluorescence quenching to a low level parallel to the slow increase of apparent absorbance at 535 nm (Fig. 1A), which reflects increased light scattering. Both phenomena have been discussed as being indicators of the high-energy state of the thylakoid system built up in the light [20]. However, after the chloroplasts have been exposed to osmotic shock (see Materials and Methods), the fluorescence yield remains low initially and within the following light period is decreased only slightly (Fig. 1B). Furthermore, the slow light-dependent absorbance change is largely or totally absent. The reason for these drastic differences between intact and broken chloroplasts suspended in the same assay medium may lie in the removal of certain substances by osmotic shock and (or) in structural alterations of the thylakoids. Since the broken chloroplasts can carry out photosynthetic reactions at high rates as, e.g. phenazine methosulfate (PMS) catalyzed photophosphorylation and electron transport to $Fe(CN)_6^{3-}$ or methylviologen, the osmotic shock does not seem to impair basic functions of the thylakoid membranes. Therefore, it seems likely that substances are retained within the envelope of intact chloroplasts, which in some way are responsible for the observed energy-dependent fluorescence and absorbance changes.

Anions of weak organic acids are known to cause light-dependent shrinkage of broken chloroplasts, as expressed by the increase of light scattering and, thus of

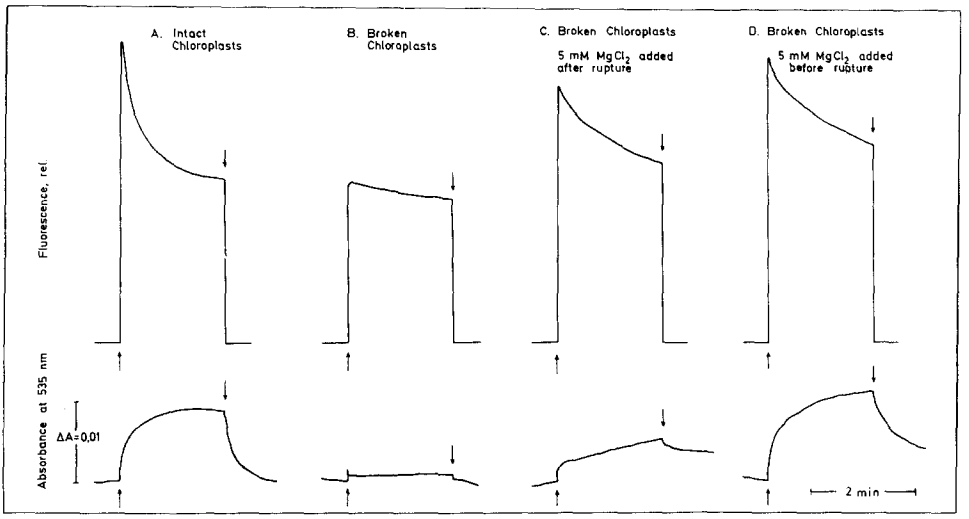

Fig. 1. Fluorescence and light-induced changes in apparent absorbance of isolated spinach chloroplasts. Chloroplasts were suspended in Solution C (see Materials and Methods), pH 7.6 (0.1 mg chlorophyll per 1.4 ml). Half band width of exciting light from 627 to 679 nm. Intensity: 38 kergs · $cm^{-2} \cdot s^{-1}$. Light on, upward arrows; light off, downward arrows; light period 2 min; 3-min dark periods before start of illumination. Fluorescence was measured at 742 nm, absorbance at 535 nm; light path 5 mm. A, intact chloroplasts. B–D, broken, osmotically shocked chloroplasts; B, suspended in the same medium as in A; C, 5 mM $MgCl_2$ added after rupture; D, with 5 mM $MgCl_2$ added before osmotic shock.

apparent absorbance [29]. It has been suggested [30] that these anions act similarly in vivo. Fig. 2 demonstrates that sodium glycolate (30 mM) added to a suspension of broken chloroplasts produced, in particular at low pH, large light-induced absorbance changes, but scarcely changed fluorescence. Consequently, weak organic acid anions, though common in chloroplasts in vivo, are not involved in long-term fluorescence changes.

Effects of Mg^{2+} and other cations on fluorescence and light-induced absorbance changes

Fig. 1 C shows that the fluorescence signal characteristic of intact chloroplasts, i.e. a high fluorescence yield at the maximum followed by quenching to a low level is largely restored if $MgCl_2$ (5 mM) is added after osmotic shock. The high yield at the maximum represents the well documented [13–17] fluorescence increase observed on cation addition to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-poisoned thylakoids. As will be discussed below, the following fluorescence quenching obviously reflects energized movement of Mg^{2+} out of the thylakoids.

As in the experiments of Murata et al. [13–16] the effect of Mg^{2+} is fully saturated at a concentration of 5 mM. Similar effects are exerted by Ca^{2+} and Mn^{2+}, whereas K^+ and Na^+ are effective only at much higher concentrations. In order to achieve an effect on fluorescence comparable in size to that of 5 mM $MgCl_2$ (Figs 1C and 1D) 150–200 mM KCl or NaCl were required.

As seen from Figs 1B and 1C, the light-dependent absorbance signal of broken chloroplasts is increased only slightly by addition of $MgCl_2$. In contrast to intact

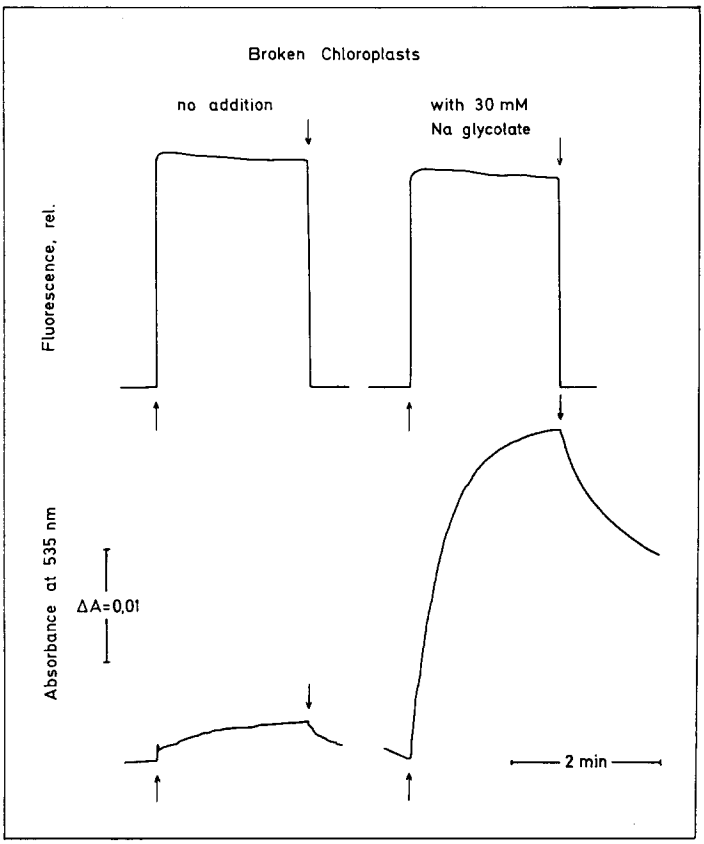

Fig. 2. Fluorescence and light-induced changes in apparent absorbance of broken spinach chloroplasts before and after addition of sodium glycolate (30 mM). Chloroplasts were suspended in water. The pH was approximately 6.5. Other conditions as for Fig. 1. Light on, upward arrows; light off, downward arrows.

chloroplasts, the absorbance increase usually was very slow, did not correspond to the fluorescence quenching and was only partially reversed in the dark. Only when Mg^{2+} was present during osmotic shock (Fig. 1D), did the broken chloroplasts exhibit changes of absorbance as well as of fluorescence very similar to those of the intact system. From this it appears that also the light-induced absorbance change is a function of metal ion concentration, but in this case presence of cations prior to osmotic rupture is essential for preserving the normal size and time course of the signal. Presumably, osmotic shock in the absence of high cation concentrations results in structural alterations of the membrane system, which are not totally reversed by subsequent Mg^{2+} addition. Presence of Mg^{2+} in the medium during osmotic shock largely seems to prevent these alterations.

In contrast to broken chloroplasts, the fluorescence signal and the light-induced apparent absorbance changes of intact chloroplasts are not or are only slightly influenced by addition of 5 mM $MgCl_2$. This is not surprising, since high concentrations of monovalent and divalent cations reportedly have been found in

chloroplasts [31, 32]. Apparently, the chloroplast envelope is capable of retaining saturating amounts of cations within the stroma compartment. It should be noted, however, that the suspension medium (Soln C, see Materials and Methods) is not free of cations. The divalent cations present (1 mM $MgCl_2$ and 1 mM $MnCl_2$) are largely bound by EDTA (2 mM) and thus should not affect fluorescence [13]. Omission of the cations from the medium does not, in fact, influence the signals of fluorescence and absorbance of broken chloroplasts, but in this case, the signals of intact chloroplasts, as depicted in Fig. 1A, are considerably distorted. This suggests damage of the chloroplast envelope at very low cation concentrations.

Relation of the Mg^{2+}-dependent changes to the energy conserving mechanism of photophosphorylation

As in the case of intact chloroplasts [20] uncouplers of photophosphorylation inhibit light-dependent long-term fluorescence quenching and absorbance increase of chloroplasts ruptured in the presence of Mg^{2+}. This is shown in Fig. 3 with 10^{-6} M carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP). The high-energy state

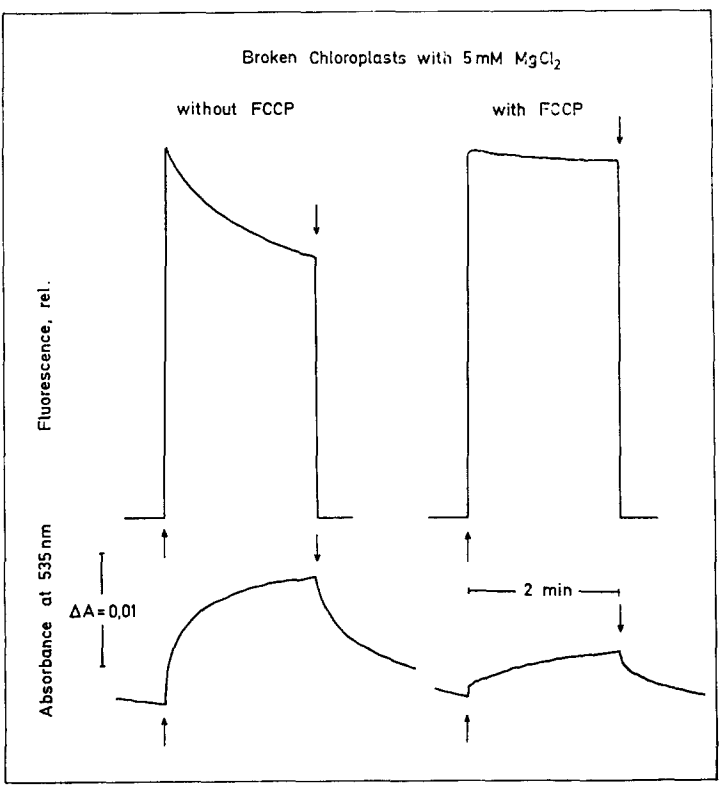

Fig. 3. Fluorescence and light-induced changes in apparent absorbance of broken chloroplasts; effect of uncoupling. Conditions as for Fig. 1. Left: broken chloroplasts with 5 mM $MgCl_2$, added before osmotic rupture; right: same chloroplast sample after addition of 10^{-6} M FCCP. Light on, upward arrows; light off, downward arrows.

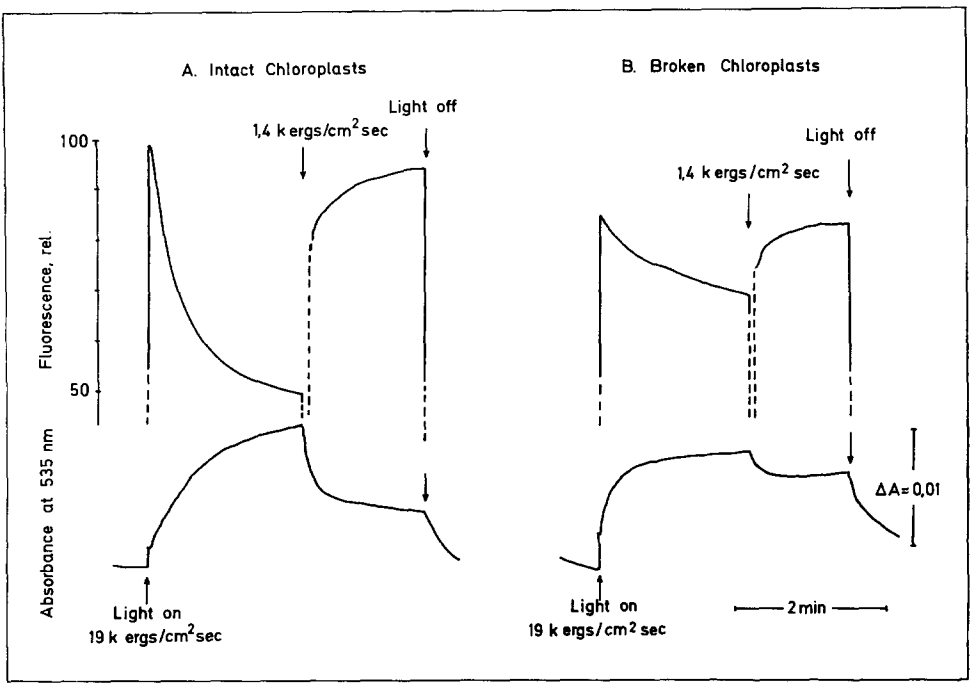

Fig. 4. Fluorescence and light-induced changes in apparent absorbance of isolated chloroplasts; effect of lowering the exciting light intensity. After 2 min illumination at 19 kergs · cm^{-2} · s^{-1} the intensity was lowered to 1.4 kergs · cm^{-2} · s^{-1}. The photomultiplier used for fluorescence detection was then switched to higher amplification in order to compensate the decline in absolute fluorescence emission. A, intact chloroplasts; B, broken chloroplasts with 5 mM $MgCl_2$, added before osmotic shock. Other conditions as for Fig. 1.

related to photophosphorylation should be partly dissipated when the light intensity is lowered below the saturation level. The reduction of light intensity then should be followed by a fluorescence increase and absorbance decrease. This is, in fact, the case as shown in Fig. 4 for intact chloroplasts and chloroplasts broken in the presence of 5 mM $MgCl_2$.

The light-dependent proton uptake into the intrathylakoid space is thought to be closely related to photophosphorylation. Fig. 5 demonstrates that the proton uptake in the absence of Mg^{2+} occurs without concomitant fluorescence and absorbance changes. However, if Mg^{2+} is present prior to osmotic shock (Fig. 5), the changes of fluorescence and absorbance show kinetics very similar to the light-induced pH change. The light saturation characteristic of absorbance increase corresponds closely to that of proton uptake. Both phenomena showed half saturation at about 1 kerg · cm^{-2} · s^{-1} (Fig. 6). Intact chloroplasts needed a higher exciting intensity for saturation of absorbance increase (half saturation at approx. 3.5 kerg · cm^2 · s^{-1}; see also Fig. 4).

The results of Figs 3–6 demonstrate that the cation-dependent light-induced changes in fluorescence and absorbance can be regarded as indicators of the high-energy state associated with photophosphorylation.

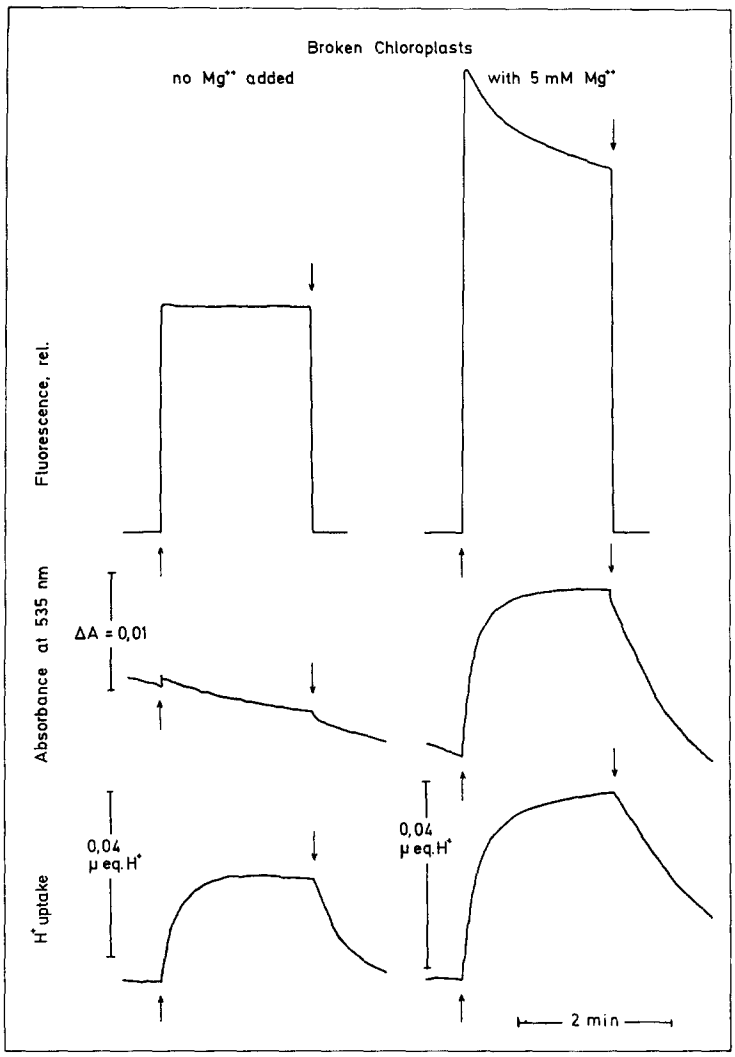

Fig. 5. Fluorescence, light-induced changes in apparent absorbance, and proton uptake of broken chloroplasts. In a cylindrical translucent cuvette (diameter 2 cm) equipped with a glass electrode chloroplasts equivalent to 0.1 mg chlorophyll were suspended in 3.6 ml buffer-free Solution C (see Materials and Methods). The pH was 7.1. Light on, upward arrows, light off, downward arrows. Left: broken chloroplasts, no $MgCl_2$ added; right: broken chloroplasts with 5 mM $MgCl_2$, added before osmotic shock. Other conditions as for Fig. 1.

Requirement of a dark interval for the Mg^{2+}-dependent fluorescence increase

In order to exert its full effect on fluorescence, Mg^{2+} has to be added in the dark, 2–3 min before illumination. Fig. 7 shows that addition of Mg^{2+} in the light to broken chloroplasts does not result in a significant fluorescence increase (at lower light intensities a somewhat larger increase was seen). However, the fluorescence yield is high after a dark period of 3 min. The fluorescence peak recorded after intermittent darkening was a function of the dark time (not shown). Apparently, Mg^{2+}

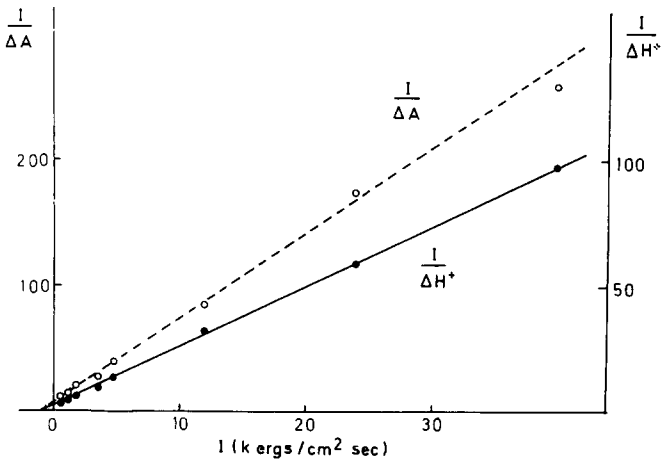

Fig. 6. Light saturation characteristics of the apparent absorbance increase (ΔA) and proton uptake (ΔH^+) of broken chloroplasts (with 5 mM $MgCl_2$, added before osmotic rupture). $I/\Delta A$ and $I/\Delta H^+$ are a linear function of the light intensity, I (kergs · cm^{-2} · s^{-1}). ΔA is defined as difference between apparent absorbance after 2 min illumination and that in the preceding dark state, ΔH^+ the number of μequiv H^+/mg chlorophyll taken up within the same light period. The plot shows half saturation for both phenomena at 1 kerg · cm^{-2} · s^{-1}. Experimental conditions as for Fig. 5.

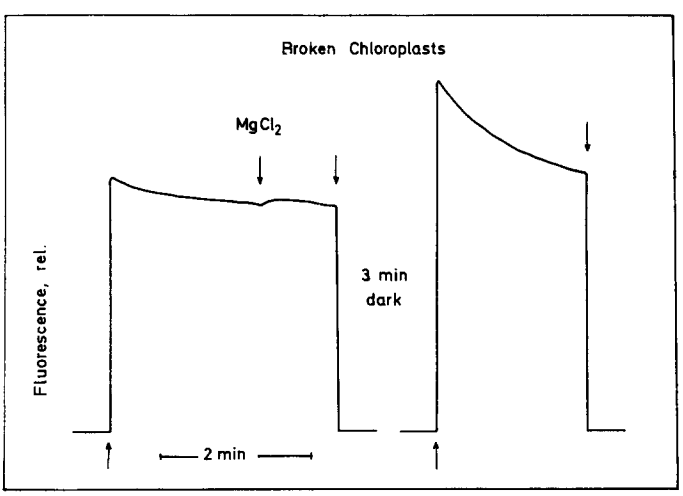

Fig. 7. Effect of Mg^{2+} on fluorescence of broken chloroplasts; requirement of a dark interval. Osmotically ruptured chloroplasts (0.1 mg chlorophyll), suspended in 2.4 ml Solution C (see Materials and Methods), were placed in a cylindrical cuvette (diameter 2 cm) and agitated with a magnetic stirrer during measurement. Exciting light as for Fig. 1. Light on, upward arrows; light off, downward arrows. $MgCl_2$ (5 mM), added in the light as indicated, caused no significant fluorescence change. Disturbance caused by addition has been omitted.

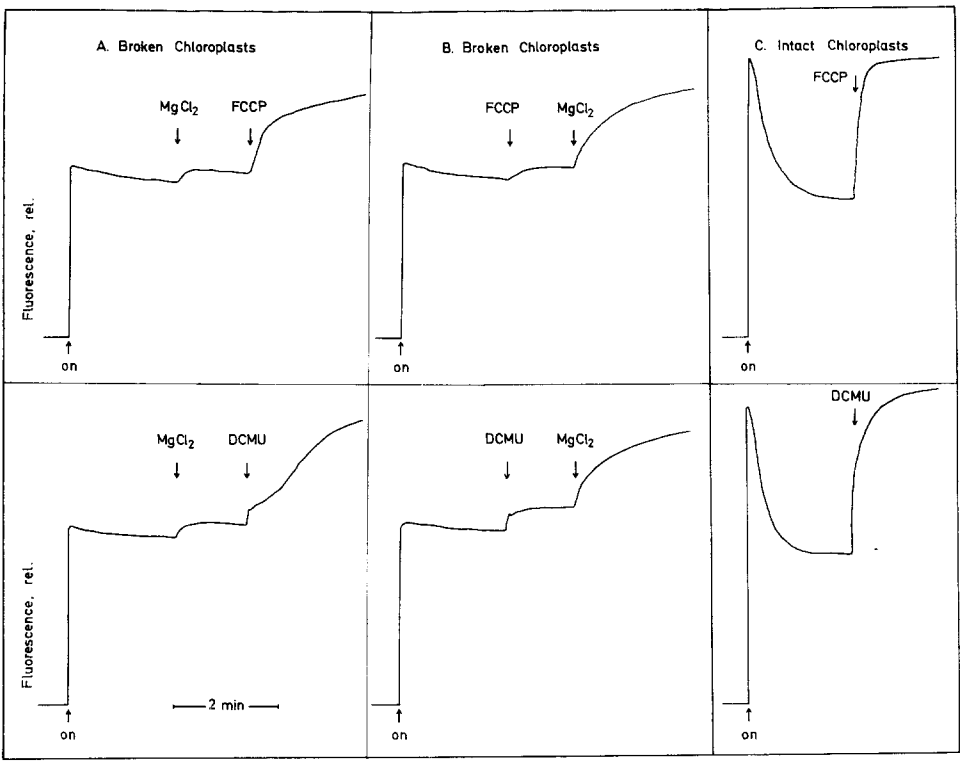

Fig. 8. Effects of FCCP and DCMU, with and without added Mg^{2+}, on the fluorescence of chloroplasts. Light intensity 10 kergs · cm^{-2} · s^{-1}; light on, upward arrows. $MgCl_2$ (5 mM), FCCP (10^{-6} M), and DCMU (10^{-5} M) were added in the light as indicated. A and B, broken chloroplasts; C, intact chloroplasts in Solution C. Other conditions as for Fig. 7. Disturbance caused by additions has been omitted.

can affect the thylakoids only in their dark state or in a state of low energy. The latter may be attained in the light by uncouplers of photophosphorylation or inhibitors of electron transport. This explains (Figs 8A and 8B) why Mg^{2+} added in the light increases fluorescence only if uncoupler (FCCP) or inhibitor (DCMU) is added beforehand. The combination of Mg^{2+} plus uncoupler (or inhibitor) is needed in order to achieve maximum fluorescence under steady illumination. On the other hand, for intact chloroplasts (Fig. 8C), obviously due to their cation content, the uncoupler (or inhibitor) alone is sufficient.

DISCUSSION

Homann [17], Murata and co-workers [13–16] and Mohanty et al. [8] have shown that Mg^{2+} and certain other cations strongly increase chlorophyll fluorescence through a mechanism which is independent of the redox state of the fluorescence quencher Q. If the Mg^{2+} concentration determines the fluorescence yield, then the long-term fluorescence quenching, as depicted in Figs 1, 3, 5 and 7 obviously reflects light-induced efflux of Mg^{2+} (or of other metal cations) from the thylakoids. Such

cation movements have been reported by Dilley and Vernon [23] and were partly confirmed by Nobel [33]. The cation movement was found to correspond to active proton transfer across the thylakoid membranes [23]. In intact chloroplasts these cations apparently are retained within the envelope and in the dark may flow back to the thylakoids. When the chloroplast envelope is ruptured, cations are lost. In this case the high fluorescence peak and consecutive quenching will be observed only if Mg^{2+} or other cations are added (Fig. 1). Addition of Mg^{2+} in the light phase, however, is without a strong effect, since in the light the thylakoids are in a state in which they tend to expel rather than to take up cations. A dark interval, therefore, is necessary for cation uptake to proceed (Fig. 7).

If proton uptake is the primary ion transfer process [23], its inhibition by uncouplers or by inhibition of electron transport will also inhibit energy-dependent cation movements. In intact chloroplasts with a high cation level, therefore, the fluorescence yield stays at its maximum during the whole light period, if inhibition is complete (Figs 3 and 8). In broken chloroplasts, at a low cation level, the fluorescence is low but can be raised to the same maximum by cation addition. In this case, addition in the light is as effective as addition during the dark phase. Thus, all fluorescence phenomena reported above may be understood, provided proton uptake causes cation expulsion from the thylakoids and, secondly, the chloroplast envelope is not freely permeable to cations. For both premises there is independent experimental evidence [23, 31].

The results shown, e.g. in Figs 1 and 5, as well as the general correlation between fluorescence and apparent absorbance [20] strongly suggest that the latter is also determined by light-driven cation movements, though cation addition after osmotic rupture of the chloroplast envelope did not restore "normal" light-induced absorbance changes. It is significant that fluorescence quenching and light-dependent absorbance increase can experimentally be separated from each other (Figs 1 and 2). Thus, not exactly the same conformational or configurational changes which conceivably lead to fluorescence changes, may be responsible for changes in apparent absorbance (see also [22], Introduction). In intact chloroplasts both types of structural changes may simultaneously be induced by cation movement. It remains unknown to what extent, additionally, the transfer of weak organic acids contributes to light-dependent absorbance changes observed in intact leaves and preparations of intact chloroplasts.

It has been discussed previously [20] that fluorescence and apparent absorbance (chloroplast shrinkage) may be regarded as indicators of the high-energy state of the thylakoid system. In agreement with other work [8] our present results show that these phenomena are related only indirectly (via cation movement) to the light-driven proton uptake and, thus, to the energy conserving mechanism of photophosphorylation. However, in higher plants, in vivo or under experimental conditions sufficiently close to the in vivo system (intact chloroplasts or chloroplasts ruptured in the presence of high cation strength) this relation, though indirect, seems to be remarkably strict. This is shown by the effects of uncouplers (Fig. 3), reduction of exciting light intensity (Fig. 4) and correlation between fluorescence, absorbance and light-induced pH change (Figs 5 and 6). In terms of hypotheses put forward by the above-mentioned authors [13–17, 23], proton uptake in the light acidifies the thylakoid interior and the resulting high hydrogen ion concentration would neutralize

negative charges on the inner membrane face. Thereby Mg^{2+} and other cations, which at the high pH in the dark are thought to form complexes with the negative groups [34], would be set free. Whether these cations then leave the thylakoid following a concentration gradient, or via obligatory counterexchange with protons remains to be clarified. The exchange of cations against protons at the acidic groups of the interior membrane face would cause various structural changes of the whole membrane system. One type of structural alterations would affect the photosynthetic pigments as manifested in fluorescence quenching. Other changes, possibly modifying the volume of thylakoids or grana stacks, may express themselves in higher light scattering, i.e. increase in apparent absorbance. From this point of view the observed long-term fluorescence and absorbance changes are, indeed, energy-dependent, in other words, indicate the high energy state or the electrochemical potential of the thylakoid membranes. The experimental evidence available fits well into this scheme, though more detailed knowledge on cation transport and binding to the thylakoids is desirable for direct proof.

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