Biochimica et Biophysica Acta, 292 (1973) 715–728

BBA 46495

THE HIGH-ENERGY STATE OF THE THYLAKOID SYSTEM AS INDICATED BY CHLOROPHYLL FLUORESCENCE AND CHLOROPLAST SHRINKAGE

G. H. KRAUSE

Institute of Botany, University of Düsseldorf, 4 Düsseldorf (Germany)

(Received September 4th, 1972)

SUMMARY

Certain long-term fluorescence phenomena observed in intact leaves of higher plants and in isolated chloroplasts show a reverse relationship to light-induced absorbance changes at 535 nm ("chloroplast shrinkage").

1. In isolated chloroplasts with intact envelopes strong fluorescence quenching upon prolonged illumination with red light is accompanied by an absorbance increase. Both effects are reversed by uncoupling with cyclohexylammonium chloride.

2. The fluorescence quenching is reversed in the dark with kinetics very similar to those of the dark decay of chloroplast shrinkage.

3. In intact leaves under strong illumination with red light in CO_2-free air a low level of variable fluorescence and a strong shrinkage response are observed. Carbon dioxide was found to increase fluorescence and to inhibit shrinkage.

4. Under nitrogen, CO_2 caused fluorescence quenching and shrinkage increase at low concentrations. At higher CO_2 levels fluorescence was increased and shrinkage decreased.

5. In the presence of CO_2, the steady-state yield of fluorescence was lower under nitrogen than under air, whereas chloroplast shrinkage was stimulated in nitrogen and suppressed in air.

6. These results demonstrate that the fluorescence yield does not only depend on the redox state of the quencher Q, but to a large degree also on the high-energy state of the thylakoid system associated with photophosphorylation.

INTRODUCTION

Light-induced "chloroplast shrinkage" as measured by slow light scattering or absorbance changes at 535 nm in intact green leaves has been interpreted as an indication of electron transport coupled to photophosphorylation[1]. The light-generated H^+ gradient across the thylakoid membrane is thought to cause shrinkage[1,2] as far as this gradient is not dissipated by phosphorylation. Thus, if the rates of proton pumping and photophosphorylation exceed the rates of energy-consuming processes in the illuminated green cell, *e.g.* in case of CO_2 starvation or at high light intensities,

Abbreviation: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

the ADP supply would limit further phosphorylation. The shrunken state of the chloroplasts would then indicate a high phosphorylation potential, or the high-energy state of the system. Evidence has been put forward by Gimmler[3] that in cells of *Dunaliella parva* the ATP level indeed correlates with the light-induced absorbance change at 535 nm. As has been discussed by Dilley[4], the slow apparent absorbance changes at 535 nm presumably reflect conformational changes of the thylakoid membranes due to protonation processes, in addition to shrinkage of the thylakoid system caused by osmotic movement of weak organic acids and water.

The steady-state yield of chlorophyll *a* fluorescence can, according to the hypothesis of Duysens and Sweers[5], partly be viewed as an indicator of the redox state of the quencher "Q", *i.e.* of the balance between reduction of Q *via* Photoreaction II and reoxidation *via* Photoreaction I. But the fluorescence yield does not seem to be exclusively determined by the redox state of Q. Long-term fluorescence changes observed with algae have been interpreted as being related to conformational changes of the thylakoid membranes[6]. On the basis of salt and pH effects on chlorophyll fluorescence emission of *Chlorella* cells De Kouchkovsky[7] proposed that the fluorescence lowering from the initial peak to the steady-state level corresponds to structural membrane changes caused by ion movement. Furthermore, a number of workers[8-10] have emphasized that the steady-state fluorescence yield depends, to a considerable extent, on the energy state of the thylakoid system. In those studies chloroplasts or thylakoids isolated from spinach leaves were poisoned with 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) preventing reoxidation of the fluorescence quencher by Photoreaction I. In this case, coupled electron flow mediated by Photosystem I caused fluorescence quenching, which was reversed by uncouplers. It has been suggested that this quenching process reflects the high-energy state associated with active proton transport.

Energy-dependent fluorescence quenching should also be expected to reflect the high-energy state of the thylakoid membranes in photosynthetic organisms *in vivo*. However, in the absence of DCMU we have to deal with a complex system, in which both the redox state of Q and the energy state of the membrane system contribute to the fluorescence yield. Reduction of Q should raise the fluorescence level and a high phosphorylation potential should lower it. In the present study attempts are made to distinguish between these two effects on fluorescence *in vivo* and in intact isolated chloroplasts. It is demonstrated that under certain conditions the steady-state fluorescence yield is determined mainly by the energy state of the membranes rather than by the redox state of Q.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea*) was grown in the greenhouse or in a growth chamber during winter months. Otherwise, field-grown plants were used.

Isolated chloroplasts from spinach leaves ("Type A" or "complete" chloroplasts according to the nomenclature suggested by Hall[11]) were obtained using a modification[12,13] of Jensen and Bassham's method[14]. Pyrophosphate and nitrate were omitted from the buffer solutions. Osmotically shocked (Type C), envelope-free chloroplasts were prepared by suspending Type A chloroplasts in water. An equal volume of double strength solution C (without isoascorbate) was immediately added. To

record the proton uptake with a pH electrode the buffer was omitted from the latter solution. Chloroplasts equivalent to 200 μg of chlorophyll were added to 4.5 ml of reaction medium. To the reaction cuvette a pH electrode and a Clark type oxygen electrode were attached. Chloroplast shrinkage and fluorescence were measured as described below.

During *in vivo* measurements the leaf was still attached to the plant. In a translucent cuvette (volume 29 ml) a round segment of the leaf (diameter 4 cm) was exposed to the exciting and measuring beams. A moistened gas stream of known composition was passed through the cuvette at a flow rate of 20 l/h. After drying the gas stream was passed through an infrared analyzer (Beckman model 215A) recording the CO_2 level. The oxygen content of the gas stream was measured with a Clark type electrode. Fluorescence, photo-induced chloroplast shrinkage and, *in vivo*, CO_2 uptake or, *in vitro*, proton uptake were excited by short-wavelength red light and measured simultaneously. The exciting light was provided by a 240 W–24 V iodine lamp and was filtered through a 6-cm water layer, a RG 610 cutoff filter (3 mm, Schott and Gen., Mainz), an interference filter Filtraflex K 6, an infrared absorbing filter Calflex C(1 mm), both from Balzers, Liechtenstein, and a heat filter (8 mm) from a Leitz projector. The resulting beam had a half band width from 627 to 679 nm. At an intensity of 50–60 k ergs$\cdot$cm$^{-2}\cdot$s^{-1} and a CO_2 concentration of 500 ppm in the gas phase it almost saturated the CO_2 uptake by intact leaves. Light intensities were measured with a silicon photodiode, which was calibrated with a thermopile.

The photomultiplier used for fluorescence detection was placed at a 45° angle to the exciting beam. It was protected by a 742-nm interference filter (Balzers, Liechenstein) in combination with two 5030 filters (4 mm each, Corning, New York), a RG 715 cutoff filter (3 mm, Schott and Gen., Mainz) and a 1-mm Calflex C filter. By means of this filter set mainly 740 nm fluorescence was recorded. Separate experiments had established that the ratio of variable to fast fluorescence at 742 nm was not significantly different from that at 684 nm.

The slow changes in apparent absorbance at 535 nm (attributed to conformational changes of the chloroplast lamellae[1]) induced by the exciting light were recorded with a weak measuring beam of 535 nm from a Bausch and Lomb monochromator. The measuring beam passed through the leaf to a second photomultiplier placed under the cuvette. This multiplier was protected from exciting red light by blue and green filters: BG 18 (2 mm, Schott and Gen., Mainz), 9782 (8 mm), and 9780 (4 mm, Corning, New York). The signals from the photomultipliers were usually recorded with a two-channel Kompensograph III (Siemens) recorder. The sensitivity was increased by zero suppression. Exciting light was admitted to the leaf or chloroplast samples by an electric shutter (Compur Electronic) with an opening time of about 4 ms. At very low intensities of exciting light, fast oscillographic recording of fluorescence induction with a Tektronix oscilloscope, type 564, was used to discriminate between fast ("constant") and variable parts of the total fluorescence yield. At higher intensities the rise in variable fluorescence was too fast to be clearly separated from the fluorescence rise during the opening of the shutter. Therefore, the fast (constant) part of the total fluorescence at high light intensities was calculated from that measured at low light intensities on the basis of the known[15,16] linearity of fast fluorescence with light intensity. The linearity of the experimental set-up was tested with a chlorophyll solution.

Chlorophyll in chloroplasts and leaf samples was determined according to Arnon[17].

RESULTS

The recordings of absorbance at 535 nm usually show fast changes on illumination (seen as spikes, *e.g.* in the lower part of Fig. 1), followed by a slow increase in apparent absorbance. The latter can be viewed as a measure of light-induced conformational changes of the thylakoid system[2], conveniently denoted as chloroplast shrinkage.

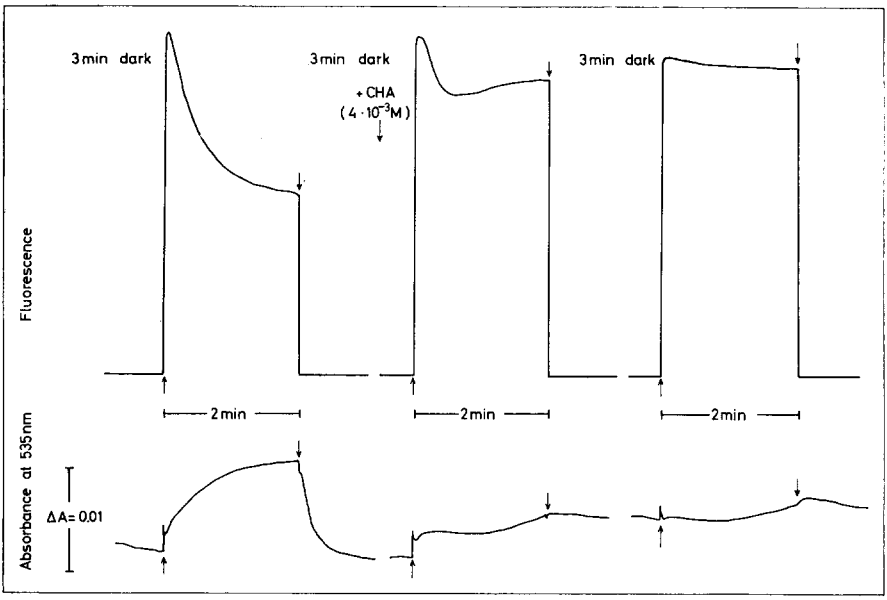

Fig. 1. Fluorescence and light-induced changes in apparent absorbance of intact isolated spinach chloroplasts; effects of uncoupling. Chloroplasts were suspended in buffer Solution C (see ref. 14), without pyrophosphate, nitrate and isoascorbate, pH 7.6 (45 μg chlorophyll per ml). Half band width of exciting light from 627 to 679 nm. Intensity, 58 k ergs·cm^{-2}·s^{-1}. Fluorescence was measured at 742 nm, absorbance at 535 nm. Slow absorbance increase denotes shrinkage. Cyclohexylammonium chloride (CHA) was added as indicated. Light on, upward arrows; light off, downward arrows. The effect of cyclohexylammonium chloride is fully visible in the second light period after addition.

Experiments with intact spinach chloroplasts

In preparations of complete (Type A[11]) chloroplasts the electron transport chain is expected to be in a highly reduced state during illumination, if CO_2 is absent from the medium. However, as shown in Fig. 1, the fluorescence yield, following a transient peak during induction, declined to a low level parallel to an increase in absorbance at 535 nm. Uncoupling with cyclohexylammonium chloride inhibited shrinkage and led to a high fluorescence yield.

Because of its sensitivity to uncoupling the low steady-state fluorescence level seen before addition of uncoupler can be regarded as a manifestation of the high phosphorylation potential, which is also indicated by shrinkage. Obviously, the type

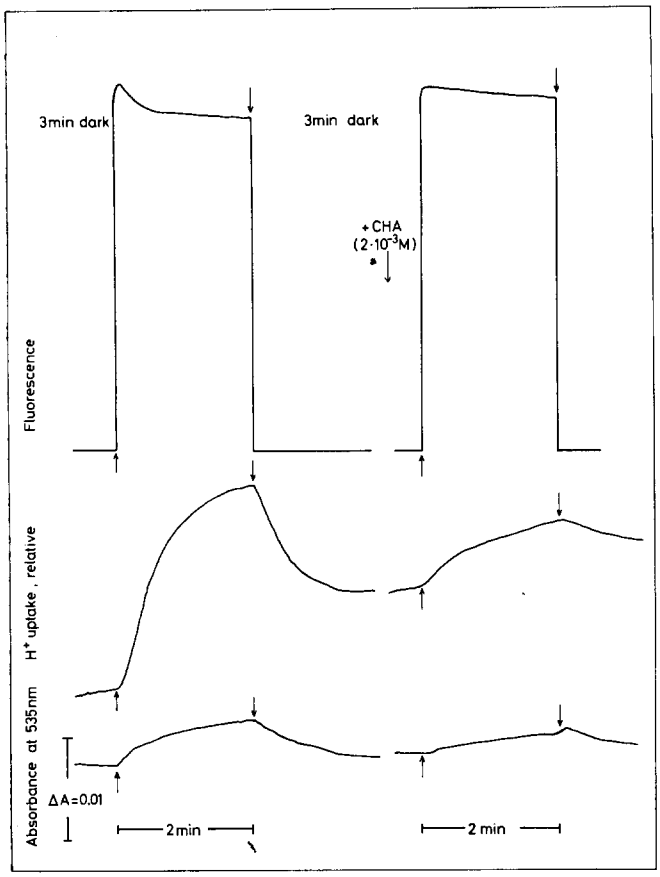

Fig. 2. Fluorescence, light-induced proton uptake and absorbance changes of ruptured, envelope-free spinach chloroplasts; effects of uncoupling. Osmotically shocked chloroplasts were suspended in unbuffered Solution C (see Methods), containing $3 \cdot 10^{-2}$ M sodium glycolate. The pH of the reaction mixture was 6.7. Proton uptake was recorded with a glass electrode and is indicated by upward deflection of the trace. Other conditions as for Fig. 1. Light on, upward arrows; light off, downward arrows.

of quenching depicted in Fig. 1 depends on the integrity of the chloroplast membrane system, as has also been noticed by Wraight and Crofts[9]. In osmotically shocked, envelope-free chloroplasts (Fig. 2) considerably less energy-dependent quenching is observed. Fig. 2 depicts fluorescence, shrinkage, and proton uptake of envelope-free chloroplasts. The fluorescence yield remained high, and measurable light-induced changes in apparent absorbance were only obtained in the presence of weak organic acid ions. Cyclohexylammonium chloride raised the fluorescence level slightly and inhibited both shrinkage and proton uptake. At pH 6.7 (Fig. 2) the steady-state fluorescence yield was 6% lower in the coupled than in the uncoupled system. Not more than 14–18% quenching was found in the pH range from 7–8. On the other hand, with chloroplasts that had retained intact envelopes (Fig. 1) we found a 40–55% lower fluorescence yield in the coupled system.

Fig. 3 shows the relation between the dark restoration of the transient fluorescence peak and the dark decay of photo-induced shrinkage in intact chloroplasts. After prolonged illumination, maximum shrinkage and minimal fluorescence are attained in the steady state (*cf.* Fig. 1, first part). On turning off the light, the high-energy state of the membrane system will be dissipated. Within 2–3 min the chloroplasts gradually return to the dark state as testified by the reversal of shrinkage. If fluorescence and shrinkage indeed reflect the energy state of the system, the fluorescence peaks observed on illumination after different dark times should correspond to the extent of shrinkage reversal reached at the end of the dark periods. In fact, the peak of fluorescence as seen on illumination during the dark decay of the high-energy state (Fig. 3, upper part) follows closely the reversal of shrinkage in intact isolated chloroplasts (Fig. 3, lower part).

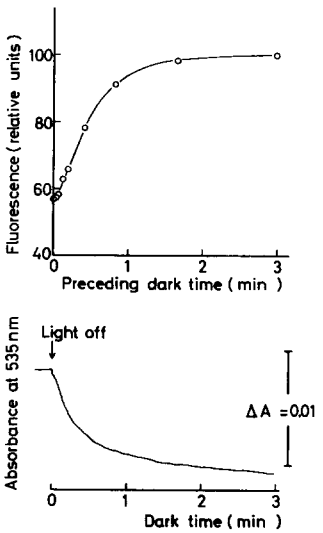

Fig. 3. Dark restoration of the transient peak of fluorescence and dark decay of shrinkage in intact isolated chloroplasts. In the upper part the fluorescence yield in the transient peak is plotted *versus* the length of the preceding dark period. Lower part, absorbance decrease recorded upon darkening. Experimental conditions as for Fig. 1. Time 0 represents the steady state in the light.

Methyl viologen, which transmits electrons from Photosystem I to oxygen, caused fluorescence quenching and increased shrinkage (Fig. 4), obviously due to an accelerated electron transport. A similar effect of methyl viologen on fluorescence of *Chlorella*, the disappearance of the peak ("P"), was observed by Munday and Govindjee[19]. The low steady-state fluorescence level in the presence of methyl viologen, as depicted in Fig. 4, may result partly from oxidation of Q and it may partly indicate a higher energy state. Accordingly, uncoupling with cyclohexylammonium chloride in the presence of methyl viologen increased the fluorescence level considerably, but not as much as in the absence of methyl viologen (*cf.* Fig. 1). The fluorescence increase did not result from oxygen depletion in the medium, though in the light oxygen was taken up, if methyl viologen was added. The oxygen uptake was stimulated by uncoupling. The transient peak of fluorescence induction (Fig. 4)

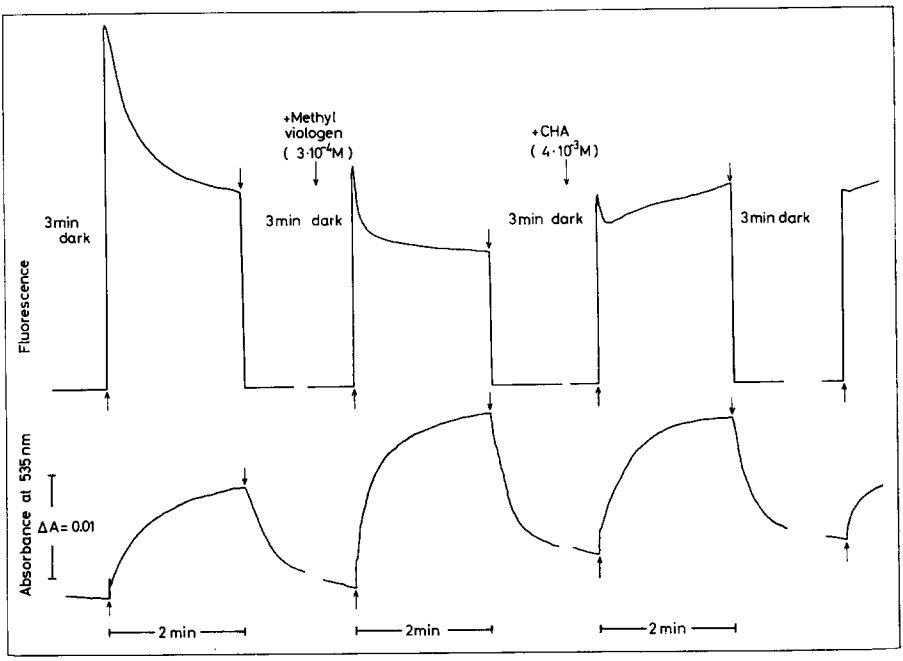

Fig. 4. Effect of methyl viologen on fluorescence and light-induced shrinkage of intact isolated chloroplasts. Conditions as for Fig. 1. Light on, upward arrows; light off, downward arrows. Methyl viologen and, additionally, cyclohexylammonium chloride (CHA) added as indicated.

was lowered further by uncoupling, possibly owing to a more rapid initial oxidation of the quencher.

Cyclohexylammonium chloride ($4 \cdot 10^{-3}$ M) inhibited shrinkage only partially in this and other experiments. The degree of inhibition varied with the leaf batches used for chloroplast isolation. Apparently, organic acid ions present in the chloroplast preparations may compete with the uncoupler for protons pumped into the intra-thylakoid space. This would explain the finding that addition of more cyclohexylammonium chloride decreased shrinkage further, but did not significantly change the fluorescence level.

Experiments with intact spinach leaves

Intact leaves illuminated with a high intensity of red light exhibited a high fluorescence yield under nitrogen. Admission of oxygen or CO_2 to the gas phase passing over the leaf surface effectively quenched fluorescence. In CO_2-free air only a few percent of the variable fluorescence was left in the steady state, while in the presence of CO_2 in air a considerably higher yield of variable fluorescence was obtained (Fig. 5). This is difficult to explain by the quencher theory, which demands a lowering of fluorescence in the presence of the effective electron acceptor CO_2. In the presence of CO_2 the variable fluorescence was lower in nitrogen than in air. Fig. 5 also shows the chloroplast shrinkage attained in the steady state. Under nitrogen shrinkage is more or less suppressed at high light intensities. This effect has been explained as inhibition of electron transport due to overreduction[1]. Oxygen, which acts as an electron acceptor

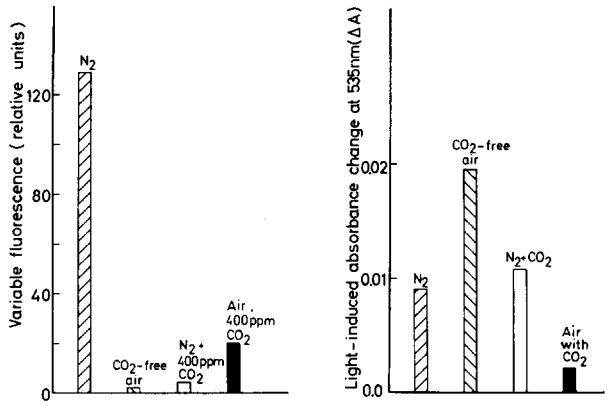

Fig. 5. Steady-state values of variable fluorescence and of light-induced absorbance changes of an intact spinach leaf. The values were obtained after prolonged illumination with red light; half band width from 627 to 679 nm; intensity at the leaf surface, 53 k ergs · $cm^{-2} \cdot s^{-1}$. The variable part of total fluorescence at 742 nm was calculated as described in Methods. Gas phase composition as given in the figure.

releases the inhibition and causes a strong shrinkage response. Shrinkage in air is largely suppressed by CO_2. Changing the atmosphere in the presence of CO_2 from air to nitrogen stimulates shrinkage. Except for the stituation in CO_2-free nitrogen, where reduction of Q appears to dominate fluorescence, there exists a clear reverse relationship between shrinkage and fluorescence. This is shown in detail in Figs 6 and 7. Gradual admission of CO_2 to a nitrogen atmosphere shows that the fluorescence quenching achieved at low concentrations of CO_2 (up to about 200 ppm) is reversed to a large extent at higher concentrations (Fig. 6). Shrinkage was intermediate in the absence of CO_2, increased with CO_2 up to a concentration of 100 ppm (by relieving the inhibition of electron transport[1]), and then declined with increasing CO_2 levels. Similarly, the shrinkage in air was gradually suppressed and the fluorescence level increased with CO_2 concentration (Fig. 7).

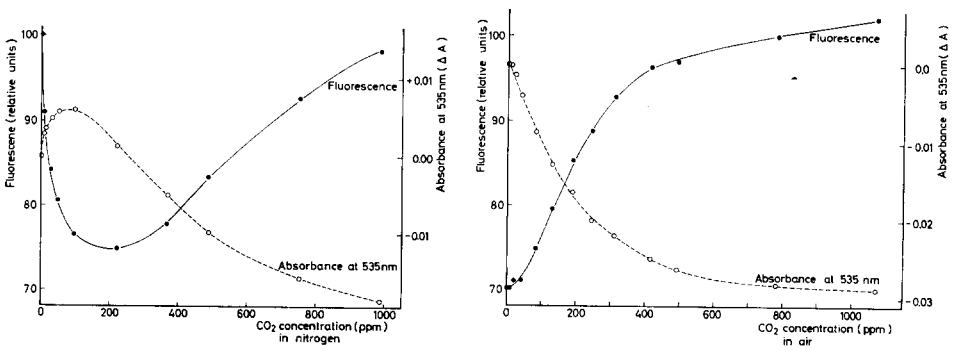

Fig. 6. Effects of CO_2 in nitrogen on steady-state fluorescence and absorbance of a spinach leaf under continuous illumination. Half band width of exciting light from 627 to 679 nm. Intensity at the leaf surface, 42 k ergs · $cm^{-2} \cdot s^{-1}$. The total fluorescence yield was measured at 742 nm.

Fig. 7. Effects of CO_2 in air on steady-state fluorescence and absorbance of an illuminated spinach leaf. Conditions as for Fig. 6. The fluorescence yield is given in the same scale as in Fig. 6.

As can be concluded from Fig. 6, CO_2 exerts a 2-fold effect. As it is fixed and reduced *via* the carbon reduction cycle, it acts as an effective electron acceptor. At low concentrations under nitrogen it causes changes in fluorescence and shrinkage which are very similar to the effects of oxygen. Fluorescence is quenched approximately to the level found in CO_2-free air. (The system is not oxygen-free in this case because of photosynthetic O_2 evolution.) Higher CO_2 levels largely dissipate the high-energy state in chloroplasts due to ATP consumption, as indicated by the gradual inhibition of shrinkage under nitrogen (Fig. 6) as well as in air (Fig. 7). The fluorescence phenomena, when evaluated in relation to the accompanying changes in shrinkage (Figs 6 and 7), can be viewed as an expression of changes in the energy state. Only in N_2, in the presence of very low levels of CO_2 or in its absence, can the high fluorescence level be attributed to the reduced state of the quencher Q. In agreement with the findings of other authors[8-10] a low steady-state fluorescence level indicates, under the conditions of the *in vivo* experiment, a high-energy state of the chloroplast system. A high fluorescence level, as seen in the presence of sufficient CO_2, indicates a low energy state.

The situation in the absence of CO_2 is demonstrated in Fig. 8. It has been mentioned that high intensities of red light overreduce the electron transport chain under nitrogen. In fact, on changing the gas phase from CO_2-free air to nitrogen a depression of shrinkage, *i.e.* swelling, is observed, which is relieved on readmission of oxygen. With the onset of swelling, fluorescence rises to a high level and is subsequently quenched by addition of oxygen. The effect of nitrogen and oxygen on fluorescence at high-intensity illumination can be understood both in terms of the quencher theory and of the theory relating a low fluorescence level to a high-energy state of the membranes. Notably, opposite effects are seen at low intensities of exciting

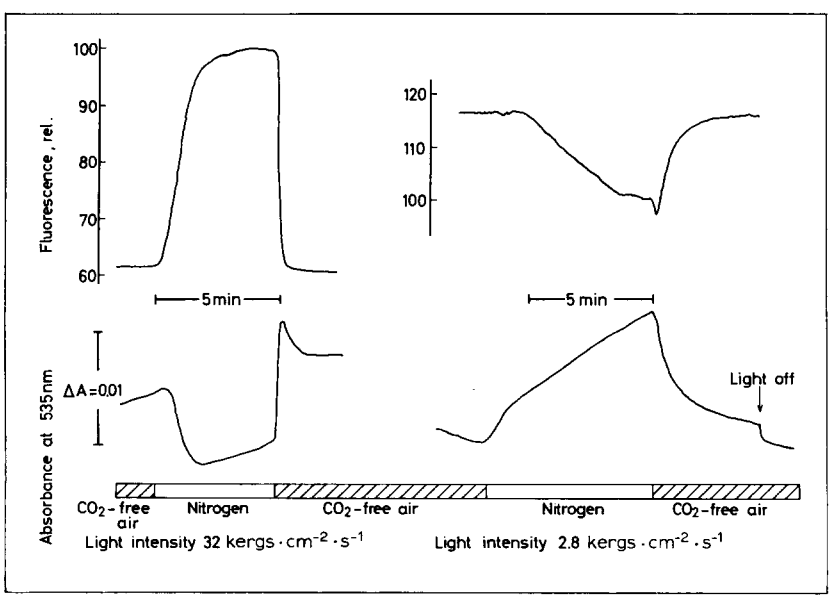

Fig. 8. Effects of oxygen (CO_2-free air) on fluorescence and absorbance of an illuminated spinach leaf. Two different light intensities were applied as indicated. Other conditions as for Fig. 6.

light. According to Heber[1] coupled cyclic electron flow takes place at low intensities of red light under nitrogen. Oxygen interrupts the cyclic flow by draining off electrons Consequently, shrinkage is increased reversibly, when CO_2-free air is replaced by nitrogen. It is significant that in this case the fluorescence is quenched and not stimulated, as should be expected according to the quencher hypothesis. This shows that under controlled conditions the fluorescence level may indicate the energy state of the system rather than the redox state of Q. The latter should be largely reduced under nitrogen even at low light intensities. Readmission of oxygen should quench fluor-

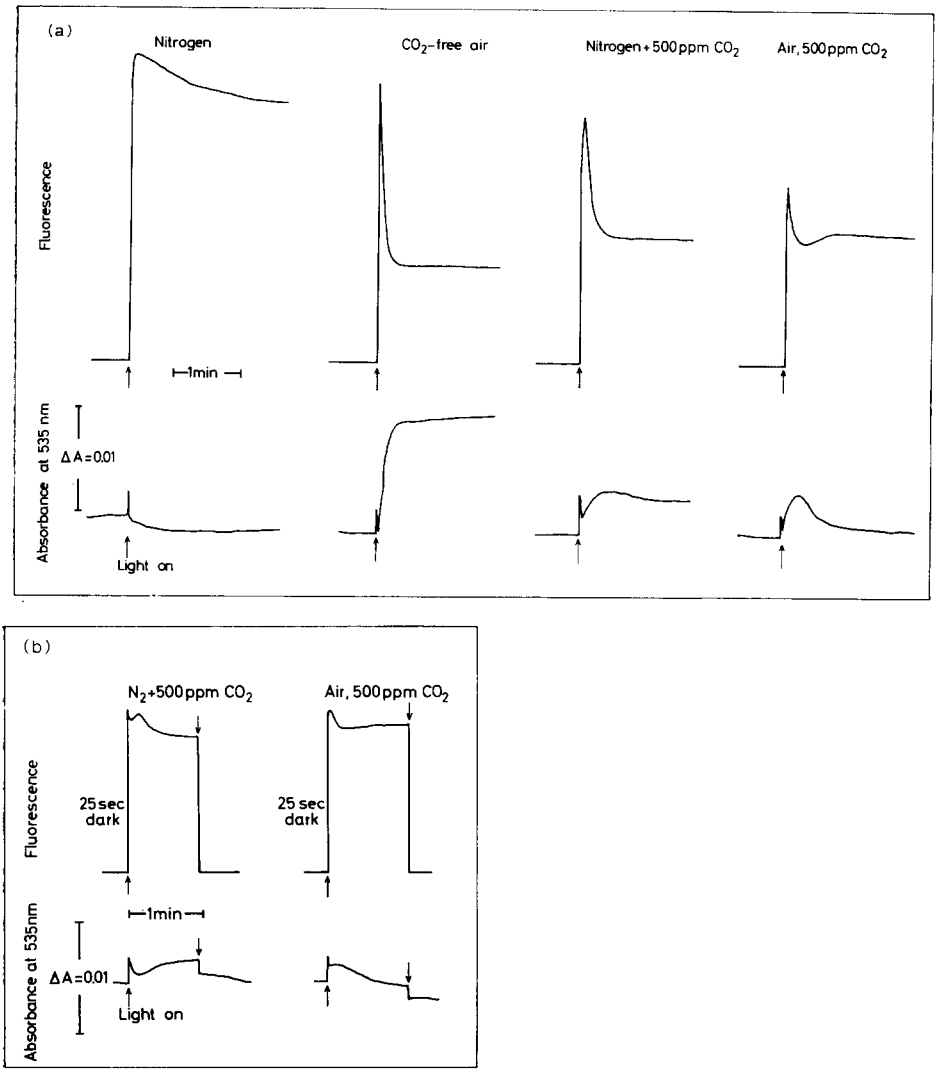

Fig. 9. Fluorescence and light-induced absorbance changes of a spinach leaf. Light intensity, 59 kergs·cm^{-2}·s^{-1}. Other conditions as for Fig. 6. Light on, upward arrows; light off, downward arrows. Gas phase composition as indicated. (a) 3-min dark periods preceding the start of illumination. (b) Preceding dark periods 25 s.

escence *via* oxidation of the quencher. The transient quenching seen in Fig. 8 (right upper trace) on admission of CO_2- free air shows that this effect indeed is present, but is more than compensated by the energy-dependent process.

Energy-dependent fluorescence changes are also manifest in transient phenomena following the onset of illumination (Fig. 9). The spikes seen in the fluorescence traces of Fig. 9a represent the well-known Kautsky phenomenon[18] of fluorescence induction after a 3-min dark period. Under nitrogen the fluorescence yield remained high, and no shrinkage response was seen. In CO_2-free air the increase in absorbance at 535 nm, which indicates shrinkage, was paralleled by a fluorescence decline to a low level. In air containing approximately 500 ppm CO_2 the steady-state fluorescence yield was much higher than in the former case, while the absorbance virtually reached the dark state. It should be noticed that the transient shrinkage increase, which indicates a piling up of phosphorylation energy before photosynthesis —following the dark period— becomes fully activated, was reflected by transient fluorescence quenching. The magnitude of both transients effect depended on the length of the dark interval, as seen by comparison of Figs 9a and 9b. In nitrogen containing CO_2 the build-up of a high-energy state appeared delayed as indicated by the comparatively slow kinetics of the absorbance increase. This corresponded to a delay in fluorescence quenching. The effect is more clearly seen after a shorter dark interval (Fig. 9b), when the transient fluorescence peak is less pronounced. In this case, the dark inactivation of photosynthesis presumably has progressed to a smaller degree. The lag phase in the absorbance change is now seen as a minimum and denotes transient swelling, which correlates with a second fluorescence peak.

We would like to emphasize that the effects of gas phase composition on intact spinach leaves with normal rates of photosynthesis (80–150 μmoles CO_2 uptake per mg chlorophyll per h) could in a large number of experiments easily be reproduced in their qualitative aspects. Quantitatively, rather wide variations were found, depending on the plant material used. With all other C_3 plant species investigated (*e.g. Cucurbita pepo*, *Vicia faba*, *Phaseolus vulgaris*, *Nicotiana tabacum*) qualitatively the same responses to changes in the gas phase were observed as in spinach. However, in leaves with low or without measurable rates of CO_2 uptake, the effects of CO_2 on shrinkage and fluorescence were small and often overlapping with effects normally seen in the absence of CO_2, or totally lacking. From this it seems evident that the CO_2 effects are closely related to photosynthetic carbon reduction.

Transient rise during fluorescence induction in vivo

The size of the initial peak, as seen by slow recording of fluorescence induction, depends in a given gas phase on the length of the preceding dark period. From Fig. 9a it is obvious that the height of the transient peak after a 3-min dark time does not correspond to the steady-state yield of fluorescence. In Fig. 10 the values of the peak measured after the light had been switched off for different periods are plotted *versus* the length of the dark interval. Within the period of 3 min, there was an almost linear relation between fluorescence yield in the peak and preceding dark interval. Thus, in intact leaves the restoration of the peak follows kinetics, which are different from the dark decay of the high-energy state as reflected by absorbance decrease in the dark (*cf.* Fig. 3). *In vivo*, the initial peak, therefore, does not seem to be influenced significantly by the energy state. After a prolonged dark time a high peak, though

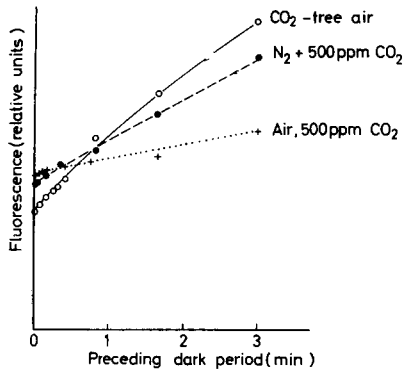

Fig. 10. Dark restoration of the transient peak of fluorescence induction in a spinach leaf. The fluorescence yield in the transient peak is given as a function of the dark interval preceding the onset of illumination. Light intensity 59 k ergs·cm^{-2}·s^{-1}. Other conditions as for Fig. 6.

somewhat lower than that observed in N_2, was obtained in CO_2-free air; the peak was smaller in N_2 containing CO_2, and was quenched further in air with CO_2 (Figs 9a and 10). This result is reasonable, if one assumes that the peak ("P") is influenced substantially by the rates of quencher reduction and reoxidation, as has been proposed by Munday and Govindjee[15,19] and others[20]. CO_2 supposedly is a more effective electron acceptor than oxygen. In air, CO_2 and oxygen together should oxidize the quencher at least as fast, but probably faster than CO_2 in nitrogen.

DISCUSSION

The experimental evidence shown above suggests that energy-dependent fluorescence quenching processes play a role in green plants *in vivo*. The correlation between light-induced chloroplast shrinkage, which has been suggested to reflect the phosphorylation potential[1], and the fluorescence yield points to a strong influence of the high-energy state of the thylakoid membranes on fluorescence. We propose that the light-induced proton translocation may cause certain structural changes of the thylakoid membrane system, which in turn are responsible for both absorbance increase and fluorescence quenching.

The experiments with intact isolated chloroplasts support the *in vivo* observations. The dependence of fluorescence on the energy state in isolated chloroplasts is indicated by the strong effect of uncoupling, as well as by the close inverse relation of fluorescence to shrinkage during its induction and dark decay. The latter results do not agree with those of Murata and Sugahara[8] who found with envelope-free chloroplasts that fluorescence and light scattering response at 540 nm did not correspond to each other. However, it should be emphasized that shrinkage cannot be expected to reflect the energy state of the membranes under all circumstances. Without addition of salts of weak organic acids isolated chloroplasts often showed little or no photo-induced shrinkage response after osmotic rupture, though their proton pump appeared intact. If thylakoids are inflated by suspending them in hypotonic media, addition of sodium acetate or another salt of a weak organic acid of appropriate pK causes a strong light-induced absorbance increase which

is not related to fluorescence (Heber, U., personal communication). Probably, it reflects only the change in size of the vesicles as caused by efflux of the undissociated acid and water[2]. Thus, it seems that different processes may lead to the slow absorbance changes at 535 nm, and that conformational changes rather than mere changes in vesicle size may be indicative of the high-energy state. Therefore, light scattering and absorbance at 535 nm appear to follow the energy state of the thylakoid membranes more closely if the latter are tightly packed as in leaves or in isolated chloroplasts with intact envelopes.

It has been shown above that the transient peak of fluorescence induction, as measured during the dark decay of shrinkage apparently did reflect the energy state in isolated chloroplasts (in an essentially CO_2-free, air-saturated medium), but did not so in intact leaves under similar experimental conditions. If the peak is determined mainly by quencher reduction and subsequent reoxidation[15,19,20], the fluorescence decline in leaves under CO_2-free air should largely, be caused by transfer of electrons to oxygen. A Mehler type reaction of the electron transport chain with oxygen *in vivo* has been proposed by Heber[1]. However, in isolated chloroplast preparations this reaction with oxygen seems to be restricted[21]. The fluorescence decline from the peak to the steady-state, therefore may parallel closely the energetic changes occurring upon illumination in isolated chloroplasts.

Several long-term fluorescence phenomena observed *in vivo*, which have been left unexplained by the quencher hypothesis, can easily be interpreted in terms of changes of the energy state. Thus, the extremely low yield of variable fluorescence under strong illumination in CO_2-free air cannot be understood as being caused solely *via* oxidation of the quencher by oxygen. To a considerable extent, this low fluorescence level seems to result from the high potential formed, when ATP cannot be utilized for carbon reduction. Accordingly, CO_2 raises fluorescence, obviously due to energy consumption through operation of the carbon reduction cycle. Furthermore, the lowering of the steady-state fluorescence yield (accompanied by an increase in shrinkage) as observed on exchange of air for nitrogen containing CO_2, may be attributed to energy-dependent quenching. It can be concluded that a higher energy supply than in normal air is available under nitrogen for photosynthetic CO_2 assimilation. This effect will be discussed in more detail in a subsequent paper.

ACKNOWLEDGEMENT

The author wishes to thank Professor U. Heber for the critical review of the manuscript and for his helpful discussions and valuable suggestions during this investigation.

REFERENCES

1 Heber, U. (1969) *Biochim. Biophys. Acta* 180, 302–319
2 Packer, L. and Crofts, A. R. (1967) in *Current Topics in Bioenergetics* (Sanadi, D. R., ed.), Vol. 2, pp. 23–64, Academic Press, New York, London
3 Gimmler, H. (1973) *Z. Pflanzenphysiol.*, 68, 289–307
4 Dilley, R. A. (1971) in *Current Topics in Bioenergetics* (Sanadi, D. R., ed.), Vol. 4, pp. 237–271, Academic Press, New York, London
5 Duysens, L. N. M. and Sweers, H. E. (1963) in *Studies on Microalgae and Photosynthetic Bacteria* (Jap. Soc. of Plant Physiol., ed.), pp. 353–372, The University of Tokyo Press, Tokyo

6 Papageorgiou, G. and Govindjee (1971) *Biochim. Biophys. Acta* 234, 428–432
7 De Kouchkovsky, Y. (1972) in *Proc. 2nd Int. Congr. on Photosynthesis Research, Stresa, 1971*, Vol. 1, pp. 233–245, Dr. W Junk N.V. Publishers, The Hague
8 Murata, N. and Sugahara, K. (1969) *Biochim. Biophys. Acta* 189, 182–192
9 Wraight, C. A. and Crofts, A. R. (1970) *Eur. J. Biochem.* 17, 319–327
10 Cohen, W. S. and Sherman, L. A. (1971) *FEBS Lett.* 16, 319–323
11 Hall, D. O. (1972) *Nat. New Biol.* 235, 125–126
12 Krause, G. H. (1971) *Z. Pflanzenphysiol.* 65, 13–23
13 Heber, U. and Santarius, K. A. (1970) *Z. Naturforsch.* 25b, 718–728
14 Jensen, R. G. and Bassham, J. A. (1966) *Proc. Natl. Acad. Sci. U.S.* 56, 1095–1101
15 Munday, J. C. and Govindjee (1969) *Biophys. J.* 9, 22–35
16 Lavorel, J. (1963) in *La Photosynthèse* (Editions du Centre National de la Recherche Scientifique), No. 119, pp. 161–176, Paris
17 Arnon, D. I. (1949) *Plant Physiol.* 24, 1–15
18 Kautsky, H. and Hirsch A. (1931) *Naturwissenschaften* 19, 964
19 Munday, J. C. and Govindjee (1969) *Biophys. J.* 9, 1–21
20 Schreiber, U., Bauer, R. and Franck, U. F. (1972) in *Proc. Int. Congr. on Photosynthesis Research, Stresa, 1971*, Vol. 1, pp. 169–179, Dr. W. Junk N.V. Publishers, The Hague
21 Krause, G. H. and Heber, U. (1971) in *Proc. 1st Eur. Biophys. Congr., Baden b. Wien, 1971*, Vol. 4, pp. 79–84, Verlag der Wiener Medizin, Akademie, Wien