Kinetic Relationship between Energy-Dependent Fluorescence Quenching, Light Scattering, Chlorophyll Luminescence and Proton Pumping in Intact Leaves

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A measuring system was designed for simultaneous recording of modulated chlorophyll fluorescence and light scattering changes. The kinetic relationship was investigated between light-induced changes in non-photochemical fluorescence quenching, as determined by the saturation pulse method, and in light scattering, as measured *via* the apparent absorbance change at 543 nm. Very similar, but not identical kinetics were observed, reflecting a close non-linear relationship between these two indicators of thylakoid membrane energization. Fluorescence was found more sensitive at low levels of energization, while scattering continued indicating further increases in energization when quenching already was saturated. A general relationship between quenching and scattering is demonstrated which holds irrespective of whether energization is varied during induction or *via* changes in light intensity or CO_2 concentration. In the light-off responses, only part of fluorescence quenching was found to relax with the same kinetics as scattering. It is suggested that at high levels of energization slowly reversible membrane changes may be induced which have the potential of non-photochemical quenching at a low level of energization, and which are not accompanied by scattering changes. Neither quenching nor scattering changes displayed kinetics sufficiently fast to be taken as a direct expression of internal thylakoid acidification in intact leaves. This conclusion is drawn from comparative measurements of proton-uptake, as reflected by CO_2-solubilization upon light-induced stroma alkalization, and of chlorophyll luminescence. Both, the initial CO_2-gulp and the pH-dependent luminescence rise were found to clearly precede the development of energy-dependent quenching.

Introduction

Photosynthetic electron transport is coupled to vectorial proton translocation which leads to internal acidification of the thylakoids. The resulting trans-membrane proton gradient (ΔpH) provides energy for ATP synthesis [1]. It energizes the thylakoid membranes, leading to quenching of chlorophyll fluorescence [2] (for recent reviews see ref. [3, 4]). With isolated chloroplasts the extent of this fluorescence quenching is linearly correlated to the proton concentration in the thylakoid interior [5]. Consequently, fluorescence quenching may serve as a non-intrusive indicator of thylakoid membrane energization in intact leaves [6, 7]. However, quenching is also controlled by the redox state of the primary acceptor of PS II, Q_A [8]. Assessment of energy-dependent quenching has therefore been possible only under special conditions [6]. Recently, a technique was introduced by which photochemical quenching (q_P) and non-photochemical quenching (q_{NP}) can be discriminated [9–11]. The so-called saturation pulse method [12] makes continuous quenching analysis possible under a wide variety of conditions. Under physiological conditions, energy-dependent quenching, q_E, is the major component of non-photochemical quenching. In isolated chloroplasts q_E, as determined by the saturation pulse method, was shown to be proportional to 9-aminoacridine quenching [13]. In intact leaves, q_E was abolished by uncouplers and enhanced by energy transfer inhibitors [12], suggesting that also *in vivo* it can serve as an energization indicator.

Kinetically related to fluorescence quenching is the scattering of green light by thylakoid membranes. Scattering is conveniently measured with a

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Abbreviations: PS, photosystem; Q_A, primary stable acceptor of PS II; ΔpH, proton gradient across the thylakoid membrane; q_P, coefficient of photochemical quenching; q_{NP}, coefficient of non-photochemical quenching; q_E, coefficient of energy-dependent quenching.

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measuring beam of about 545 nm by the associated decrease in transmission. It has been used for a long time as a qualitative indicator of changes in thylakoid membrane energization *in vivo* [14–17]. Light scattering is sensitive to changes in the refractive index and the geometrical arrangement of thylakoids. It responds also to changes in the water status of leaves [18].

While there are many indications that fluorescence quenching and light scattering may respond in a similar way to changes in membrane energization, it is not yet clear how well they are correlated. A direct comparison requires simultaneous measurements of these two signals, when changes of energization are induced, *e.g.* by dark-light-dark transitions and by changing CO_2-concentration. We have designed a system for such measurements on the basis of a recently introduced modulation fluorometer [11]. It will be shown that changes in energy-dependent quenching and light scattering are closely correlated under most, but not all conditions. Both signals display a relatively slow response to changes in the acidification of the thylakoid internal space. This conclusion will be derived from the considerably faster responses of chlorophyll luminescence (stimulated by light-driven internal acidification) and CO_2-uptake (linked to external alkalization). Also, differences in the relaxation kinetics of light scattering and energy-dependent fluorescence quenching will be discussed.

Materials and Methods

Most of the experiments were conducted with detached leaves of 6–8 weeks old spinach plants (*Spinacia oleracea* L., cv. hybrid 102, Yates). The spinach was grown in a greenhouse with additional illumination (about 250 µmol m^{-2} s^{-1}) in a 10 h light and 14 h dark cycle. Sunflowers were grown in a growth cabinet. For other experiments, fully developed leaves of potted *Arbutus unedo* L. plants were used which were kept in a shaded place in the Würzburg Botanical Garden. Directly after cutting the petiole, the leaves were placed in a water glass and kept for at least 2 h in darkness before the measurements.

Light scattering and chlorophyll fluorescence yield were measured simultaneously using a fiber optics system as depicted in Fig. 1. A leaf was enclosed in a black perspex cuvette with 2 cm^3 volume. To minimize changes in water status, water was fed through the petiole and a moistened air stream was passed over the leaf. When needed, CO_2 was removed by bubbling the air through a bottle filled

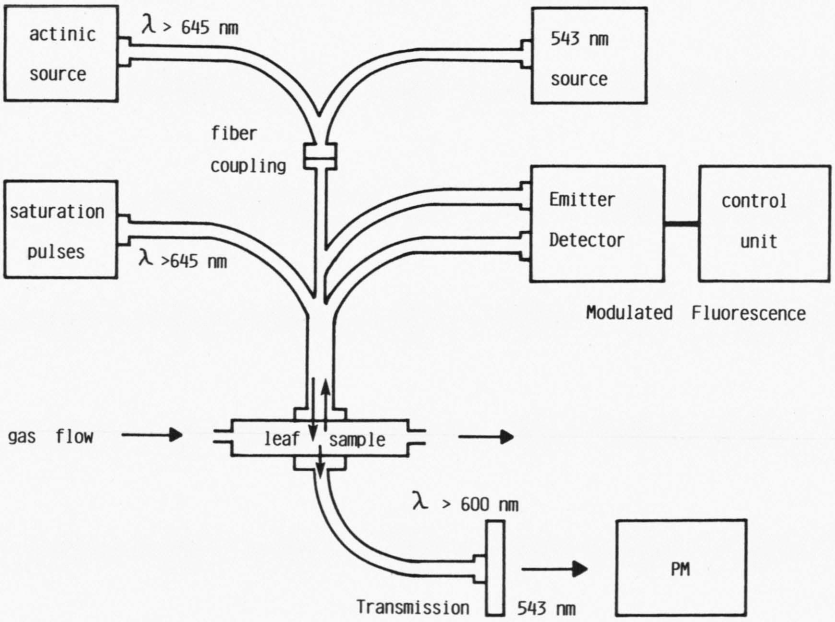

Fig. 1. Block diagram of the fiber optic system used for simultaneous measurements of modulated chlorophyll fluorescence and light scattering changes. The system allowed application of saturation pulses to suppress photochemical quenching and to determine non-photochemical quenching.

with saturated KOH solution. The optical system was based on the fiber optics of the PAM Fluorometer (Walz, Effeltrich, F.R.G.) which were slightly modified by dividing one of the fiber bundles to accomodate the measuring beam of the light scattering arrangement. Measuring light for light scattering was obtained from a halogen lamp with stabilized power supply, and filtered through a 543 nm interference filter (Balzers B40, 8 nm half band width). Transmitted measuring light was collected by an additional fiber bundle below the leaf disc and guided to a photomultiplier (EMI 9558) which was protected by 8 mm BG 39 (Schott, Mainz, Germany) and 8 mm CS 4-96 (Corning, Corning, N.Y., U.S.A.) filters. The signal of the photomultiplier was amplified and compensated by a laboratory-built amplifier. Before onset of actinic illumination, the signal was zeroed by changing the voltage at the photomultiplier power supply (Knott NSHM BN600). The signals were recorded on chart recorder. When the water status of the leaf changed during the experiments, there was a slow baseline shift of the light scattering signal. Curves were discarded when no unambiguous baseline was apparent.

Fluorescence yield was measured with a PAM Chlorophyll Fluorometer (Walz, Effeltrich, F.R.G.) as previously described [11]. The integrated photosynthetic photon flux density (PPFD) of the modulated measuring beam was 0.04 µmol m^{-2} s^{-1}. Red actinic light of varying PPFD was provided by a fiber illuminator (KL 1500, Schott, Mainz), filtered through a combination of RG630 and 645 (Schott, Mainz) and DT Cyan (Balzers, Liechtenstein). The same filter combination was used for saturating light pulses from a second fiber illuminator (FL 103, Walz). The PPFD of the saturation pulses was 2400 µmol m^{-2} s^{-1}. PPFD was determined by means of a LICOR 185 B Quantummeter (Lincoln, Nebraska).

Chlorophyll luminescence was measured with a laboratory built phosphoroscope based on fiber optics. Actinic light was chopped by a rotating disk at a frequency of 625 Hz with dark and light periods of equal duration. Luminescence intensity was measured between 240 µs and 560 µs after light-off by means of a photomultiplier (Hamamatsu R 928), protected by a red plastic filter (Roscolene 821, Edmunds Scientific). The photomultiplier signal was processed by a laboratory built lock-in amplifier and subsequently recorded on a storage oscilloscope (Nicolet Explorer III). Actinic light was provided by a halogen lamp (Osram Xenophot XLX) connected to a stabilized power supply (Rohde and Schwarz NGRE 30/15). The actinic beam passed two blue-green filters (Schott BG 39) and an electromagnetic shutter (Compur electronic-m). The integrated (photosynthetic photon flux density) PPFD was 80 µmol m^{-2} s^{-1}. During the light phases, chlorophyll fluorescence was measured by means of a photodiode (UDT 500) at wave-lengths above 695 nm (RG 695, Schott). Measurements were carried out with leaf discs (∅ 13 mm) enclosed in a cuvette described elsewhere [19]. The temperature of the leaf discs was controlled by a flow of thermostatized water.

CO_2 gas exchange was measured by infrared absorption in a system specially adapted to record rapid transients [20, 21].

Results and Discussion

Fig. 2 shows simultaneous recordings of chlorophyll fluorescence and absorbance changes around 543 nm during illumination of a leaf of *Arbutus unedo* with short wave-length red light. The lower trace shows the fluorescence yield, as monitored by a modulated measuring beam, which is sufficiently weak to leave the redox state of Q_A unaffected. The signal in the presence of the measuring beam alone indicates the dark-level yield, F_o, which is indicative of practically completely oxidized Q_A. Under these conditions, photochemical quenching of chlorophyll fluorescence is at its maximum. With onset of the actinic light the continuous trace displays the fluorescence induction kinetics known as Kautsky effect [22], with a fast rise to a peak level, P, followed by a slower decline. Once in the preceding dark period and then every 20 s during actinic illumination, pulses of saturating light intensity (2400 µmol m^{-2} s^{-1}) were applied. This strong light reduces all primary PS II acceptors, and hence eliminates photochemical quenching. The saturated fluorescence yield seen in the presence of actinic illumination during each pulse is termed F_s. In relation to the maximal possible yield, F_m, observed with a dark-adapted leaf, the lowering of fluorescence yield in a saturation pulse is a measure of non-photochemical quenching. During the first minute of actinic illumination, F_s dropped considerably. Subsequently, it slowly increased again.

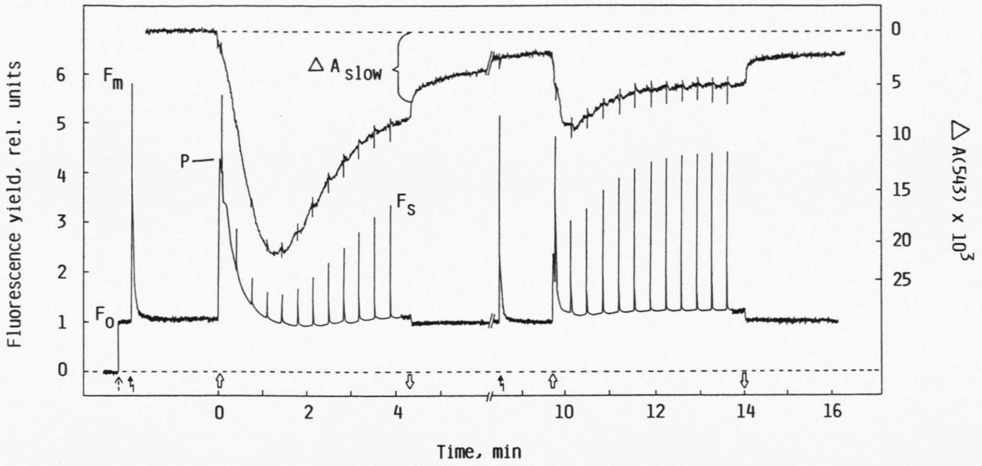

Fig. 2. Original recordings of fluorescence yield (lower trace) and light scattering changes (upper trace) during two periods of actinic illumination of a leaf of *Arbutus unedo*. The arrows below the zero line of fluorescence yield denote switching-on of the weak measuring beam, ↑, application of a short pulse of saturation light (PPFD 2400 μmol $m^{-2}s^{-1}$), ↿, and switching on (⇧) or off (⇩) of the actinic light (PPFD 160 μmol $m^{-2}s^{-1}$). Characteristic fluorescence levels are indicated: F_o, dark-level fluorescence; F_m, maximal fluorescence yield after dark adaptation; F_s, fluorescence yield during a saturating pulse applied during actinic illumination; P, peak fluorescence level of the Kautsky transient. At the light scattering trace, the evaluation of the slow ΔA transient after light off is depicted. Note the break in time scale between the two subsequent illumination periods. Before the first illumination the leaf was dark-adapted for 2 h. During measurement, the leaf was enclosed in a cuvette and exposed to a stream of moistened air (20 l/h).

The upper trace of Fig. 2 shows the corresponding changes of apparent absorbance at 543 nm. Difference spectra have revealed that these reflect light-induced changes of light scattering [14]. The scattering signal exhibited kinetics which were very similar to those of F_s. This was true for the first as well as for a second illumination, when fluorescence and scattering changes were considerably speeded up, due to light-activation and to stomatal opening. When entrance of CO_2 is permitted and photochemical energy can be used for its reduction, the extent of light-induced energization is lowered.

For a more detailed comparison of the kinetics, the fluorescence and scattering responses of Fig. 2

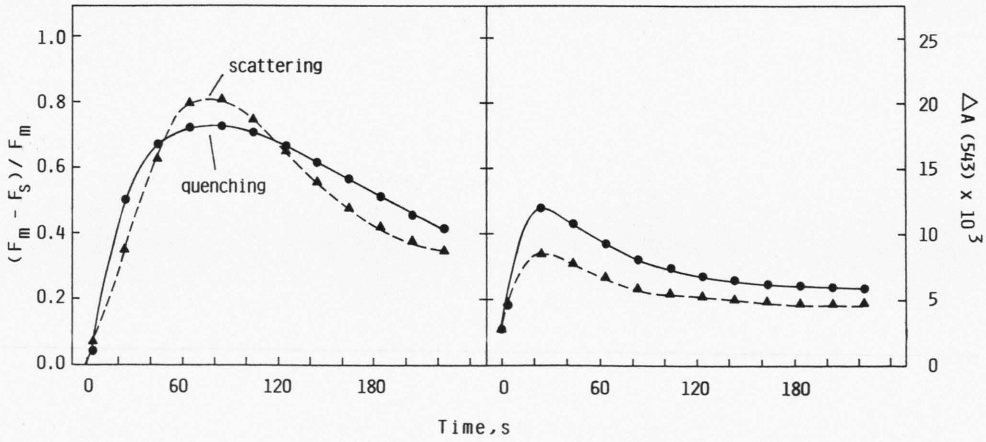

Fig. 3. Time course of non-photochemical fluorescence quenching (●), expressed as $(F_m\text{-}F_s)/F_m$, and light scattering (▲), evaluated from the traces shown in Fig. 2. The left hand panel shows the first, the right hand panel the second illumination period. For other conditions, see Fig. 2.

were replotted (Fig. 3). The term $(F_m-F_s)/F_m$ was chosen as a measure of non-photochemical fluorescence quenching instead of q_{NP} (*i.e.* $[(F_m-F_o)-(F_s-F_o)]/(F_m-F_o)$, representing changes in the variable part of chlorophyll fluorescence only). This was necessary, as under the given conditions F_o-quenching was apparent, without a simple way of correcting for this type of quenching. Determination of F_o by switching off the actinic light [12] would have disturbed the course of the induction kinetics.

The plotted values of $\Delta A(543)$ were sampled briefly before saturation pulses were applied. Fluorescence quenching and scattering increased relatively slowly, reaching their maximum after about 80 s during the first illumination cycle. The second illumination caused both light scattering and fluorescence quenching to develop faster, with a sharp maximum already after 24 s. Although the time courses of $(F_m-F_s)/F_m$ and $\Delta A(543)$ were quite similar, they were not identical. In particular, it may be noticed that during the first illumination the fluorescence quenching built up somewhat faster than the scattering response, whereas its relaxation was somewhat slower than that of scattering.

In Fig. 4 plots of simultaneously measured non-photochemical fluorescence quenching, $(F_m-F_s)/F_m$, and light scattering changes, $\Delta A(543)$, are presented. The data were derived from experiments similar to those of Fig. 2 and 3 with varying light intensities and using four different leaves of *Arbutus*. A general relationship between non-photochemical quenching and $\Delta A(543)$ is apparent, which is independent of light intensity and preillumination state. The relationship is non-linear, with the sensitivity of fluorescence quenching being higher than that of light scattering at low energization levels, and the scattering response becoming more sensitive to energization than fluorescence quenching at high energization levels.

Still, there are some small systematic differences in response at the different conditions, suggesting that both fluorescence and light scattering to some extent

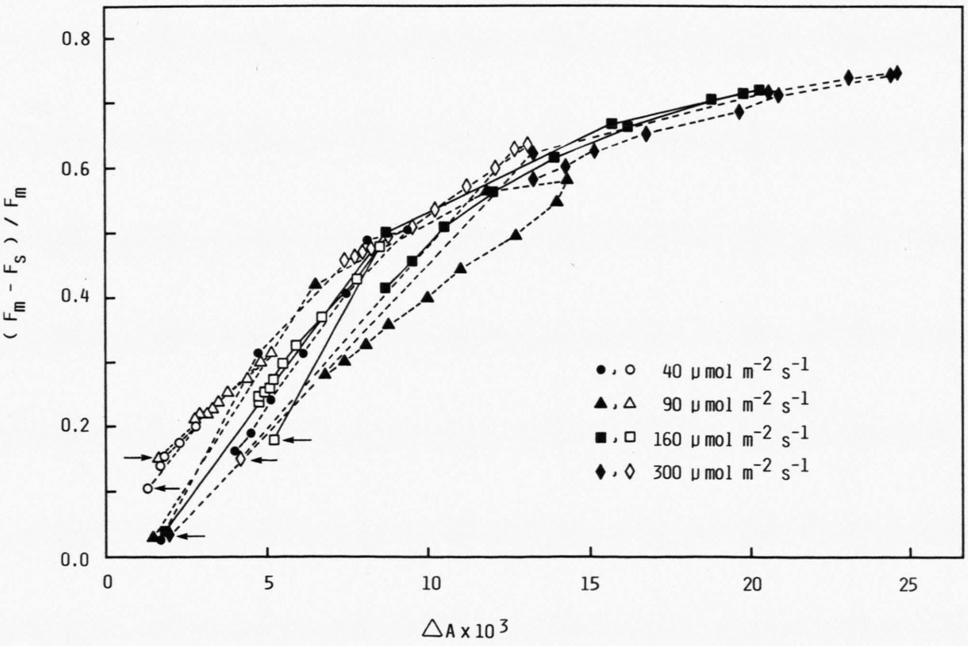

Fig. 4. Relationship between non-photochemical fluorescence quenching and the full extent of light scattering measured simultaneously during actinic illumination. The data are derived from traces identical (■, □) or similar to those shown in Fig. 2. Different symbols represent different leaves of *Arbutus unedo* and different light intensities, as indicated in the figure. Closed symbols denote values from the first illumination period, open ones from the second period. Data points are joint by continuous (■, □) or dashed lines, following the time course of the respective induction curves. The first values of the inductions are marked by arrows.

are also influenced by events which are unrelated to the formation of the transthylakoid proton gradient. Besides energy-dependent quenching, other forms of non-photochemical deexcitation are known such as energy transfer from PS II to PS I, increasing with a so-called state I-state II shift [23, 24]. In addition to light scattering the ΔA(543) signal also contains an electrochromic component peaking around 515 nm [25]. This so-called electrochromic shift does not find expression in chlorophyll fluorescence. It is a component of membrane energization caused by light-dependent formation of a membrane potential, which together with the proton gradient constitutes the proton motive force driving ATP-synthesis.

It is possible to estimate the contribution of the 515 nm shift to the overall ΔA(543), as the membrane potential relaxes considerably more rapidly on darkening than light scattering (see Fig. 2). We have determined the slowly relaxing component of ΔA(543) (ΔA_{slow}) after approx. 4 min illumination at a variety of different light intensities in the presence and absence of CO_2 in air. Removal of CO_2 stimulated non-photochemical quenching and light scattering by increasing the proton gradient. In Fig. 5 the resulting plot of $(F_m-F_s)/F_m$ *versus* ΔA_{slow} is shown. Essentially, the relationship is similar to the one shown in Fig. 4. As expected, elimination of the P515 component from the ΔA(543) signal caused a small shift of the response curve to the left, accentuating the earlier response of fluorescence quenching to energization. It is important to note that the general relationship between fluorescence quenching and scattering holds, irrespective of whether membrane energization was increased by increasing the light intensity at constant CO_2 or by decreasing CO_2 at constant light. Hence, at least during the first minutes of illumination, the correlation between quenching and scattering may be considered rather tight.

Some important differences between these two signals were, however, revealed when the dark relaxation kinetics were investigated. As shown in Fig. 6, only part of energy-dependent quenching relaxed within the time period of complete relaxation of a light scattering change. Both signals initially decayed with identical kinetics, displaying a lag phase of about 7 s, followed by a quasi-exponential phase (t 1/2 about 45 s). However, whereas the scattering signal almost completely relaxed within 5 min, restoration of the original F_m-level took about 45 min (not shown) (see also ref. [12]).

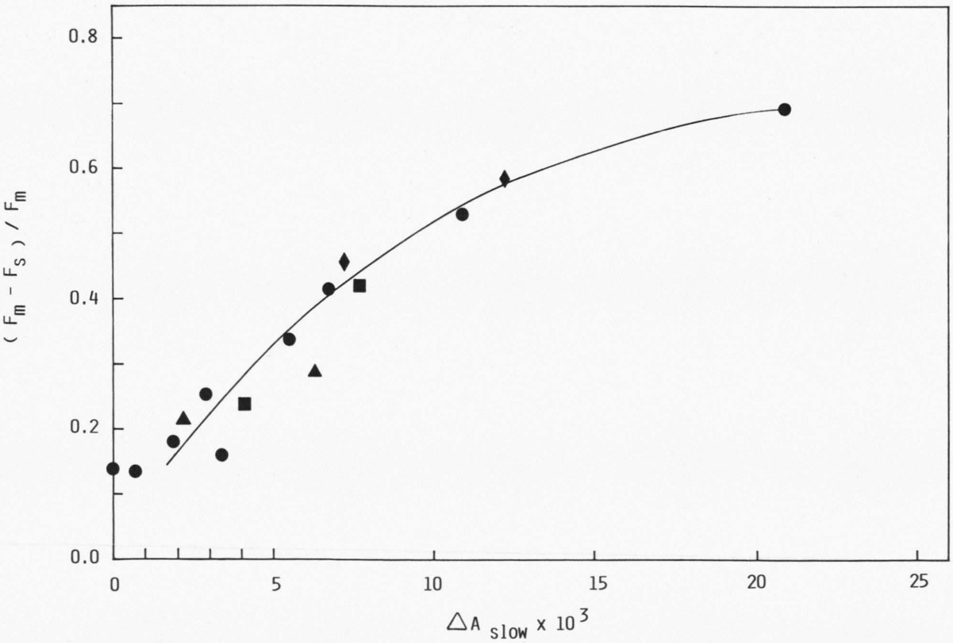

Fig. 5. Relationship between non-photochemical quenching and the slow light-off signal of light scattering (compare Fig. 2) in leaves of *Arbutus unedo*. The data are measured after 4 min illumination at varying PPFD. Different symbols denote different leaves. The circles are obtained by variation of actinic light intensity in the presence and absence of CO_2.

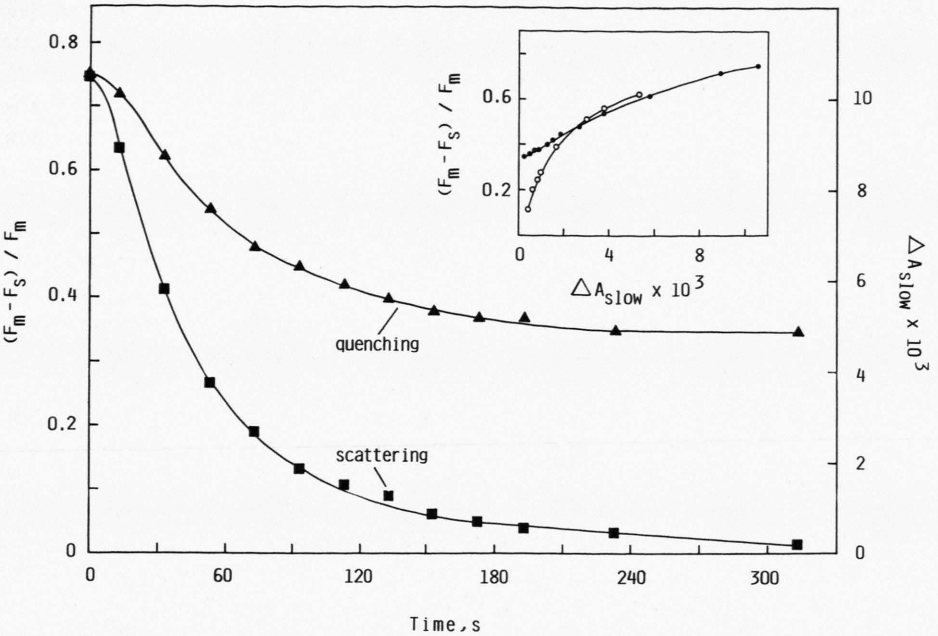

Fig. 6. Time course of non-photochemical fluorescence quenching and light scattering in a spinach leaf after turning off the actinic light (PPFD 500 µmol $m^{-2}s^{-1}$, absence of CO_2). Fluorescence quenching was assessed by applying saturating light pulses every 20 s. The relationship between both signals is shown by the closed symbols in the inset. The open symbols show steady state values during actinic illumination obtained with a different spinach leaf by changing PPFD in the presence and absence of CO_2.

In the insert of Fig. 6 the relationship between $(F_m-F_s)/F_m$ and $\Delta A(543)$ is compared for steady state conditions at different levels of illumination (open circles), and during dark relaxation (closed circles). In the first case, the non-linear relationship extrapolates to the origin, whereas in the second case the relationship is almost linear and extrapolates for zero scattering to a considerable residual fluorescence change. The relative extent of slowly reversing non-photochemical quenching was found to be variable depending on the plant material. It increased with light intensity (not shown). Apparently, secondary events altered the nature of the fluorescence response during illumination. This alteration was not accompanied by light scattering changes.

By comparison with previously published data is appears that even the relaxation kinetics of light scattering are slower than the relaxation kinetics of the transthylakoid proton gradient. In isolated spinach chloroplasts, the relaxation of 9-aminoacridine fluorescence quenching can be used to obtain information on the rate of H^+ efflux from the intrathylakoid space on darkening [26]. Obtained values are maximum figures, as relaxation may be determined by diffusion of 9-aminoacridine rather than by H^+ efflux. When CO_2 assimilation in the light was inhibited by nitrite (no or little contribution of ATP synthesis to H^+ efflux in the dark), the half relaxation time of 9-aminoacridine fluorescence quenching was about 20 s [27], and when CO_2 had been assimilated in the light, it was about 10 sec [28]. Illuminated sunflower leaves acidified the chloroplast stroma on darkening with a half time of about 20 sec, when CO_2 was absent, and with little more than 5 sec, when it was present [21]. Considering that the light scattering measurements of the present study were made in the presence of CO_2, it may be concluded that the decay of the proton gradient is considerably faster ($t\ 1/2=5-10$ s) than the relaxation of light scattering ($t\ 1/2=45$ s, plus 7 s lag phase).

In Fig. 7 information is presented which allows a comparison between the rate of proton pumping and the rate with which fluorescence quenching is established upon illumination of a leaf. A sunflower leaf was predarkened to deactivate Calvin cycle enzymes. CO_2 exchange and chlorophyll fluorescence were simultaneously measured as the light was turned on. Initial CO_2 uptake was rapid. It was proportional to CO_2 concentration and could not be saturated by CO_2. This rapid phase was caused by the light-dependent alkalization of the chloroplast stroma which results in CO_2 uptake according to the reversible reaction

$$CO_2 + OH^- \rightleftharpoons HCO_3^- \quad (1)$$

which is catalyzed by carbonic anhydrase. Evidence for such interpretation of the rapid CO_2-uptake phase has been presented elsewhere [29].

A second slow phase of CO_2 uptake could be saturated by CO_2. It reflects CO_2 assimilation which is slowly activated in the light.

The alkalization of the chloroplast stroma was complete after about 20 s illumination in the first light cycle and, with Calvin cycle being partially activated, after little more than 10 sec in a second light cycle. As shown by the CO_2 gush observed after darkening which indicates stroma acidification, and which was smaller than the initial CO_2 gulp, the transthylakoid proton gradient had decreased during illumination, most probably owing to photosynthetic energy consumption [30]. This decrease is also indicated by the secondary decrease in light scattering and energy dependent quenching after a maximum was observed on illumination (Fig. 2 and 3).

The kinetics of fluorescence quenching were much slower than those of proton uptake into the intrathylakoid space. The CO_2-gulp corresponded kinetically to the P–S decline which has been shown to selectively reflect the development of photochemical quenching [10, 11]. As in this experiment a high actinic light intensity was used (1500 μmol m^{-2} s^{-1}), the P- and F_m-levels of fluorescence are identical. The M-T decline is governed by the build-up of non-photochemical quenching. This quenching actually increased while the proton gradient decreased again after its initial large increase.

Conclusions on the relationship between fluorescence quenching and proton gradient formation can be also drawn from simultaneous measurements of chlorophyll fluorescence and chlorophyll lumines-

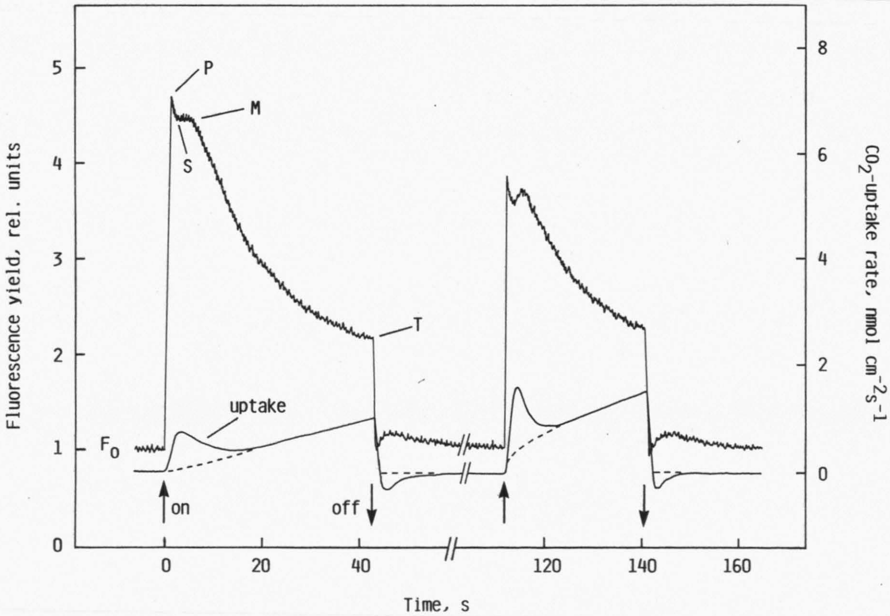

Fig. 7. Simultaneous recordings of modulated chlorophyll fluorescence and CO_2-uptake with a sunflower leaf. The leaf was dark-adapted before the first illumination cycle; 70 s dark time was given between the first and second illumination. The arrows indicate on/off of actinic light (1500 μE $m^{-2}s^{-1}$) which was sufficiently strong to cause almost complete suppression of photochemical quenching (not shown), such that light-induced quenching from M to T is non-photochemical. The characteristic fluorescence levels are indicated. The dotted lines in the CO_2-exchange traces suggest the course of assimilatory CO_2-uptake, as distinguished from H^+-dependent CO_2-solubilization or release. CO_2-concentration, 5000 ppm.

cence (Fig. 8). Luminescence was recorded in repetitive dark cycles and fluorescence measured in the intermittent light cycles. The cycle frequency was 625 Hz. The luminescence signal displayed a two-step rise followed by a slow decline. Whereas the first step to the so-called L_I-level is caused by the rapid formation of a membrane potential, the slower rise to the L_M-level reflects the build-up of the proton gradient [31, 32]. It was slower at 10 °C than at 20 °C. For the given measuring conditions, the dependence of L_M on ΔpH was confirmed by NH_4Cl-infiltration experiments [33]. Luminescence is stimulated by ΔpH, as the activation energy of the recombination reaction is lowered by the internal acidification [34]. The rise of luminescence to L_M, and hence ΔpH formation, coincide kinetically with the P-S decline in fluorescence, at 10 °C as well as at 20 °C. This finding is in agreement with the data of Fig. 7, where the kinetic correspondence of the P-S decline and the CO_2-gulp caused by proton uptake was demonstrated. As has been argued above, the P-S decline clearly precedes the initiation of energy-dependent quenching; and, hence, it may be concluded again that the actual ΔpH formation precedes energy-dependent quenching.

Conclusions

Changes in light scattering and of fluorescence quenching have in the past been interpreted in terms of changes in the energization level of the photosynthetic apparatus as affected by the transthylakoid proton gradient [35, 17, 36]. Still, formation and decay of the proton gradient follows a kinetic pattern which is different from the kinetics of formation and decay of light scattering and q_E. The correlation between the latter two indirect and non-instrusive indicators of membrane energization is close under a variety of conditions, which include light-on induction kinetics and different levels of steady-state energization, varied by light intensity or CO_2-concentration. The correlation is non-linear, with fluorescence quenching responding more sensitively at low levels of energization and light scattering displaying larger changes at high levels of energization. However, when the relaxation of the signals is compared after the light has been turned off, important differences become apparent. Only part of fluorescence quenching relaxes with the same kinetics as scattering, the remaining part showing a very slow recovery.

An obvious difference between fluorescence

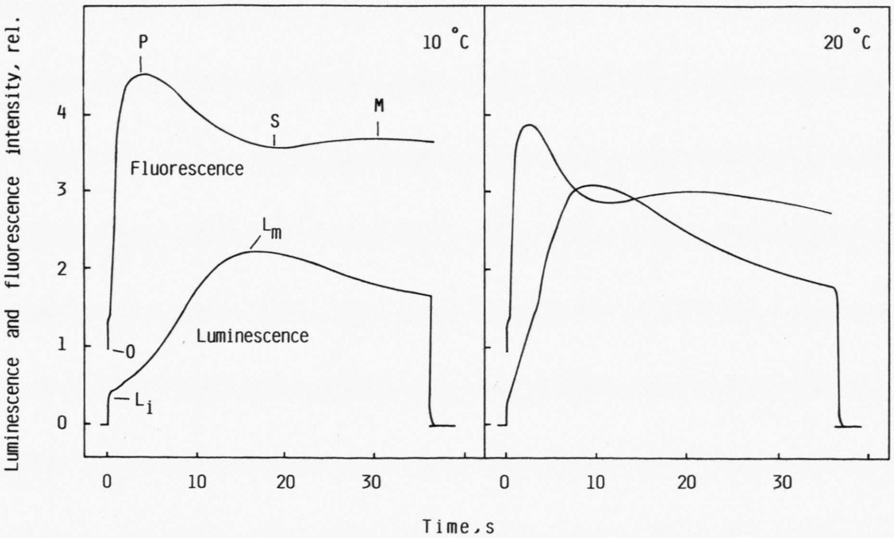

Fig. 8. Fluorescence and simultaneous luminescence transients of predarkened spinach leaf disks upon illumination at different temperatures. The temperature of the leaf disks was controlled by a flow of thermostatized water directly over the surface of the disks. The PPFD of the blue-green actinic light (Schott BG 39) was 80 μmol $m^{-2}s^{-1}$. Characteristic fluorescence and luminescence levels are indicated at the curves. For further details of the measurement, see Materials and Methods.

quenching and light scattering is that there is only a limited amount of fluorescence which can be quenched whereas there is no obvious limits for light scattering. This feature appears likely to cause the non-linearity in the correlation between non-photochemical quenching and light scattering as shown in Fig. 5. When almost all variable fluorescence is already quenched, fluorescence cannot be expected to respond to further changes in membrane energization which at a lower level of quenching would have caused substantial energy-dependent quenching, and which may well further stimulate light scattering. Also, in a state of strong non-photochemical quenching, additional changes at the thylakoid membrane level may occur, which are neither directly expressed in fluorescence quenching (as there is no variable fluorescence left to be quenched) nor expressed in a stimulation of scattering. If such changes cause non-photochemical quenching and if they are slowly reversible, they could explain the results of Fig. 6. A possible candidate for additional, slowly reversible membrane changes occurring in a state of strong energization is the formation of zeaxanthin which has been shown by Demmig and co-workers [37, 38] to be correlated with the build-up of a slowly reversible form of non-photochemical quenching.

The kinetic differences between formation and decay of the transthylakoid proton gradient on one side and development and relaxation of light scattering and q_E on the other side confirm and extend for leaves previous observations of Quick and Horton [10] and Laasch [13] who demonstrated a delayed response of q_E with respect to the build-up of 9-aminoacridine quenching in isolated chloroplasts. Obviously, neither q_E nor scattering respond directly to the internal acidification of the thylakoids. Acidification must be considered a necessary but not sufficient condition for both signals. A relatively slow change in the structure or orientation of membrane components appears to constitute an additional requirement. In isolated chloroplasts, this change can be prevented by brief glutaraldehyde treatment which does not yet affect electron transport and H^+-pumping [39]. It can also be inhibited by antimycin A [40] which also affects the light-induced scattering change (not shown).

Light scattering and q_E are under many conditions reliable non-intrusive indicators of thylakoid energization by ΔpH in the steady state. They complement each other, with fluorescence quenching being more sensitive at low levels of energization and scattering more sensitive at high levels. However, the information they provide must still be interpreted with some caution. Scattering is also affected by changes in the water status of leaves [18] and q_E represents only part of non-photochemical fluorescence quenching. From the experimental point of view, the measurement of fluorescence quenching is more easily performed with small leaves or needles of conifers and less sensitive to signal drift than the measurement of light scattering. Neither signal can provide accurate kinetic information on changes in the transthylakoid proton gradient in view of their sluggish response, although it appears that after proper light activation of the photosynthetic apparatus the time lag between thylakoid acidification and fluorescence quenching or the increase in light scattering becomes small compared to that seen when a leaf is illuminated which had been predarkened long enough to deactivate Calvin cycle enzymes.

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