Biochimica et Biophysica Acta, 440 (1976) 205–214

BBA 47117

THE I-D FLUORESCENCE TRANSIENT

AN INDICATOR OF RAPID ENERGY DISTRIBUTION CHANGES IN PHOTOSYNTHESIS

ULRICH SCHREIBER* and WILLIAM VIDAVER

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia V5A 1S6 (Canada)

(Received December 17th, 1975)

SUMMARY

The I-D transient in the chlorophyll fluorescence induction curve (Kautsky effect) is investigated in the view of recently discovered rapid changes in energy distribution between the two photosystems (Schreiber, U. and Vidaver, W., FEBS Lett., in the press). Fluorescence induction curves differ appreciably depending on whether measured at $\lambda < 690$ nm, originating in pigment system II, or at $\lambda > 715$ nm, which is in part from pigment system I. The differences occur as well in the rapid part of the induction curve (O-I-D-P) as in the slower P-S decay. Most significant changes in energy distribution are indicated in the region of the I-D dip, being induced by appropriate preillumination. The effect is studied by (a) comparing the individual fluorescence time courses at $\lambda < 690$ nm and $\lambda > 715$, (b) plotting $F < 690$ vs. $F > 715$ and (c) recording time courses of $F < 690/F > 715$ ratios. In (a) the I and D characteristics are delayed at $F > 715$ relative to $F < 690$, which is accompanied by periods close to I and D, where the two emissions follow inverse courses. In (b) the I-D dip corresponds to a loop. And in (c) it is shown that a rapid ratio decay, reflecting increasing excitation of System I pigments, is initiated before the I-D dip. These data indicate that the I-D transient is caused by a rapid switch of energy distribution in favor of System I and the resulting stimulation of Q reoxidation via the electron transport chain. It is suggested that as with the slow fluorescence transients the rapid also can be understood as a composite of two different changes, (1) direct changes resulting from a switch in energy distribution, which are inverse for $F < 690$ and $F > 715$, and (2) indirect changes due to stimulated Q reduction or Q oxidation, which are parallel for both emissions. The rapid ratio decay, correlated to I-D, persists and is even stimulated in the presence of electron transport inhibitors. This and the speed of the phenomenon make it improbable that the rapid energy distribution changes are affected by an ion flux-induced mechanism. It is proposed that the electrical field across the thylakoid membrane is involved in the energy switch mechanism.

Abbreviations: Q, primary electron acceptor of Photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

* Present address: Carnegie Institution of Washington, Department of Plant Biology, Stanford, Calif. 94305, U.S.A.

INTRODUCTION

Chlorophyll fluorescence yield in a living plant is an indicator for the state of the photosynthetic apparatus (for reviews see refs. 1 and 2). Most fluorescence changes can be explained by changes in the redox state of Q, the primary System-II electron acceptor [3, 4]. But in recent years much evidence has been collected showing that certain slow fluorescence transients result from ion-flux induced changes in the thylakoid membrane conformation (see reviews in refs. 1, 2 and 5). Studies of low-temperature fluorescence spectra have revealed that the membrane can exist in two states which differ in the distribution of excitation energy to pigment Systems I and II [6].

It has long been assumed that variable fluorescence yield changes at room temperature are independent of measuring wavelength. But recently it was discovered in Butler's laboratory that 690 and 730 nm fluorescence kinetics display differences which can be correlated with changes in energy transfer rates (Strasser, R. J., personal communication). These differences were seen in the time region, where slow fluorescence transients independent of Q had already been observed [4, 6–12]. By use of a fast method of automatically plotting $F685/F735$ ratios, we recently discovered that energy distribution changes can occur much faster than expected [13].

In most plants fluorescence rises rapidly after a period of darkness from an initial level O through an intermediate level I to a peak P from which it slowly decays to a low stationary yield. Under certain conditions there is a transient drop of fluorescence after I, giving rise to the D level and the I-D dip. This I-D dip has been extensively studied in the work of Kautsky and coworkers [3, 14, 15] and more recently by Munday and Govindjee [16] and ourselves [10, 17–22]. It was proposed that besides an "indirect quenching" of pigment system II fluorescence by System I activity via the electron transport chain, there can be "direct quenching" via energy transfer from pigment system II to pigment system I [17]. In order for such a mechanism to cause the I-D dip there has to be an energy switch control, as, for example, proposed by Clayton [23]. While our earlier conclusions were rather intuitive, we are now in a position to support them experimentally, by comparing short and long wavelength fluorescence induction kinetics. The interpretation of our data will be based on a model derived by Butler and Kitajima [24] from fluorescence measurements at −196 °C in isolated spinach chloroplasts. In this model, Photosystem I and II pigment complexes are separated by a light-harvesting pigment complex, which can transfer excitation energy to either of the two photosystems.

MATERIALS AND METHODS

Phaseolus vulgaris was grown in chambers with 20 °C day temperatures, 15 °C night temperatures and an 11 h photoperiod. Measurements were on whole attached leaves if not stated otherwise. All experiments were at room temperature (22 °C). Illumination and collection of surface fluorescence was achieved by trifurcated fiber-optics [20]. Fluorescence excitation was by a broad blue band from a movie projector lamp (Sylvania, Type DLG) together with 10 mm Corning 9782 filter and two layers of Balzers K6 interference filter with the cut-off filter removed. Photomultipliers (EMI 9658 R) were screened by 3 mm Corning 2600 filter for collection of long wavelength fluorescence ($F > 715$ nm) and 3 mm Corning 2030 filter and Balzers K6 interference filter for collection of short wavelength fluorescence ($F < 690$ nm). With these filter combinations, stray light artifacts as estimated by replacing a leaf by green paper, were less than 1 %. Photomultiplier anode currents were fed into operational amplifiers (RCA CA 3130); dark currents were compensated. By applying an appropriate stabilised voltage to the amplifier non-inverting input, the constant fraction of the System I $F > 715$ nm signal ("α-fraction") was compensated as well. Signals were recorded in three different ways: (a) as time courses of $F < 690$ nm and $F > 715$ nm; (b) as x-y plots of $F > 715$ nm vs. $F < 690$ nm; (c) as time courses of the ratio $F < 690$ nm/$F > 715$ nm. All recordings were on a dual beam storage oscilloscope (Tektronic 5103 N) and photographed from there. Ratios were recorded electronically by use of an analog divider (Burr and Brown, model 4290). Response time was limited by RC values at the amplifier and was approx. 500 μs. Time resolution for recordings of individual fluorescence curves was approx. 1 ms as determined by the shutter opening time (Compur-electronic-m). This did not affect rise times of ratio curves, as ratios were measured with 1 % accuracy at fluorescence levels only 1/100 of the initial value (approx. 0.5 V). The electronic shutter was controlled by a pulse generator (Simon Fraser University Electronic Workshop).

RESULTS AND DISCUSSION

A pronounced I-D dip can be readily induced by appropriate preillumination in higher plant leaves [3], but has been observed in green algae as well [17]. Recently it was also demonstrated in isolated spinach chloroplasts [25]. Fig. 1 compares fluorescence curves in bean leaves at $\lambda < 690$ nm and $\lambda > 715$ nm with and without preillumination. While without preillumination the kinetics of the two emissions seem very similar, more distinct differences are displayed after preillumination in the

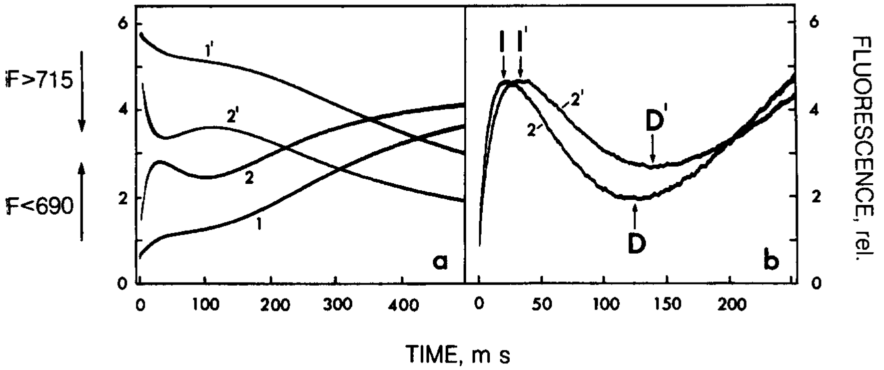

Fig. 1. Differences in the I–D dip after preillumination depending on emission wavelength (a) $F < 690$ and $F > 715$ recorded inversely. A constant amount, corresponding to 50 % of the O level is subtracted from $F > 715$; true base at approx. −0.5 unit. Traces 1 and 1′ without preillumination; traces 2 and 2′ after 1.5 s light and subsequent 10 s dark. Light intensity, 2 mW/cm^2. (b) Same conditions as a. Traces 2 and 2′ displayed enlarged and on the same upward going scale. Curves shifted relative to each other to match I levels for better comparison. Both curves are triggered simultaneously at time 0. Note the delay of I′ and D′ in trace 2′ relative to I and D in trace 2.

relative size of the I-D dip and the times at which I and D occur. The latter aspect becomes more evident with higher magnification of the fluorescence changes and matching of the I levels by appropriate shifts of the base lines (Fig. 1b). I′ and D′, measured at $\lambda > 715$ nm, are clearly delayed relative to I and D, measured at $\lambda < 690$ nm. A similar delay is observed at P and in later curve characteristics (not shown in the figures). Most important for the interpretation of this finding is the fact that there are periods where the two emissions display inverse changes. To explain this unique effect, we propose that with illumination from O to I a change in energy distribution in favor of System I is induced, which causes a quenching of pigment system II fluorescence paralleled by an increase in pigment system I fluorescence. A similar change, but in favor of System II, is induced with illumination from I to D, and then again in favor of System I with illumination from D to P. From this point of view the $F > 715$ nm as well as the $F < 690$ nm induction curves are composites of one curve representing the redox state of Q, which is kinetically identical for both emissions, and another curve representing the energy distribution parameter, which is inverse for the two emissions. In order to study the latter parameter it is necessary to eliminate the first parameter as fas as possible.

With the basic assumption that Butler and Kitajima's model [24] is valid at room temperature, one should expect $F > 715$ nm to be excited (a) within pigment system II, (b) within pigment system I by energy transfer from pigment system II and (c) within pigment system I by direct absorbance. While (a) and (b) display an initial and a variable part with illumination, (c) remains constant. Separate *x*-*y* plots of (a), (b) and (c) vs. $F < 690$ nm should yield a straight line for (a), a line with changing slope reflecting energy transfer changes for (b), and a line parallel to the $F < 690$ nm axis for (c). The fraction (c) is equivalent to Butler and Kitajima's α-fraction. At room temperature the assumption that α is constant will only hold for the early part of the induction, where ion flux effects are still negligible. For both the straight line in (a) and the initial slope in (b) increments in $F < 690$ nm and $F > 715$ nm are in the same ratios as the corresponding initial values, which means that both

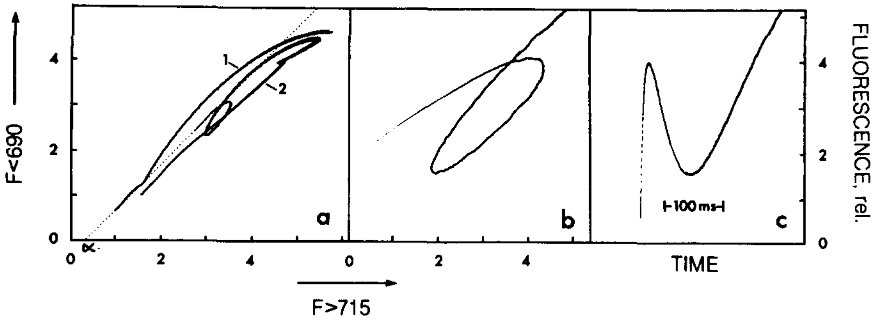

Fig. 2. The loop in the *x*-*y* plot corresponding to the I-D dip. Same light intensity and preillumination conditions as in Fig. 1. (a) Determination of α as the intercept of the initial slope at the $F > 715$ axis. The dotted straight line represents the initial "energy distribution line"; similar straight lines can be drawn for any point on the curve, all originating at α on the $F > 715$ axis. The slope of these lines is taken as a measure for energy distribution towards Photosystem II. (b) Loop enlarged by five-times increased sensitivity on both scales; signals compensated for parts not shown. (c) I-D dip in $F < 690$ recorded under the conditions of b, with the $F > 715$ base replaced by the time base.

cut the x-axis at the origin. As the sum function of the straight line in (a) and the initial slope in (b) will also go through the origin, the α-fraction can be determined from an experimental x-y plot, which comprises (a), (b) and (c), as the intercept of the initial slope with the $F > 715$ nm axis. This is indicated in Fig. 2a. The figure shows the x-y changes corresponding to preillumination to P (trace 1) and subsequent illumination following 10 s dark time (trace 2). With the assumptions outlined above, any changes in slope of the x-y curves reflect changes in energy transfer efficiency from pigment system II to I. The slope of the straight line connecting any point on the curve with the origin corrected for α is a measure for the energy distribution between the two photosystems as affected by energy transfer efficiencies. In this view the x-y curve travels through a multitude of "energy distribution lines", and "spill-over" of excitation energy from pigment system II to I is the stronger the flatter the line cut. Both with and without preillumination there is an energy distribution change in favor of System I corresponding to the I level. The effect is more spectacular after preillumination where a pronounced loop in the x-y plot corresponds to the I-D dip. In Fig. 2b this loop is enlarged by increasing sensitivities at the oscilloscope and suitable compensation. Fig. 2c shows the corresponding spike in the $F < 690$ nm time course. As with comparison of the direct traces in Fig. 1 the x-y plot displays regions where $F < 690$ nm and $F > 715$ nm show opposite changes.

While in our experiments with bean leaves the I-D dip and the x-y loop was induced by preillumination, rapid energy distribution changes as such do not require preillumination (see e.g. trace 1 in Fig. 2). In red algae a pronounced I-D dip occurs in totally dark-adapted samples and is also there accompanied by a x-y loop (Schreiber, U., unpublished data).

The slope of the energy distribution lines (see Fig. 2) is identical to the ratio of $F < 690$ nm and $F > 715$ nm corrected for the α-fraction. Once the α-fraction has been estimated from x-y plot, this ratio can be determined electronically for any point in time and recorded continuously [13]. This method has the advantage of displaying the time dependency of the energy distribution changes, which is lost in the x-y representation. Furthermore, the ratio method is more sensitive than the x-y method, as small ratio changes are more readily distinguished than small slope changes. Hence the weakest point in the ratio method is the limited accuracy with which the α-fraction is determined from the initial slope of the x-y curve. Inaccurate correction for the α-fraction may lead to distortion of the ratio curves, as will be realised from the following: assuming there are no energy transfer changes the x-y plot will show a straight line and the intercept at the x-axis gives the α-fraction. With correct compensation for α, the ratio will keep constant. But with α being underestimated the ratio will consistently be too small, and the more so, the smaller the corresponding $F < 690$ nm value. This will essentially lead to a transient ratio rise in the O-I region. For the same reasons, overestimation of α will result essentially in a transient ratio decay in the O-I region. These ratio transients, originating from possible errors in the determination of α, thus have to be taken into consideration as possibly overlapping the ratio transients due to energy distribution changes. To demonstrate further the importance of correct determination of α, in Fig. 3 a set of ratio curves is displayed, which is derived from the same individual traces at $F < 690$ nm and $F > 715$ nm with the assumption of different values of α. It is evident that with too low compensation all rise phases are overstressed while all decay phases are suppressed and vice

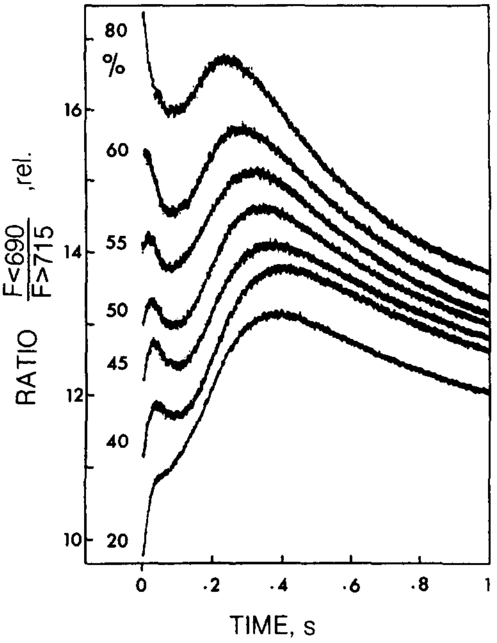

Fig. 3. Ratio curves in dependence of assumed values for the α-fraction. Numbers at beginning of each curve indicate compensation in percent of initial fluorescence of $F > 715$. All curves recorded from different areas of the same dark-adapted leaf. Light intensity, 2 mW/cm^2. Note: the traces are shifted relative to each other for clearer presentation. The ordinate scale gives for 50 % compensation the recorded ratio as voltage output from the amplifier and bears no numerical significance for the actual emission ratios.

versa with compensation being too high. The present accuracy of an α-determination suggests the true ratio curve to be between 40 and 60 % compensation in the given example of Fig. 3. The important issue is that in any case there are rapid ratio changes, reflecting some rapid energy distribution switch mechanism. An initial ratio rise is not observed in the upper region of the possible α-compensation values. The initial part of the x-y curve often shows, although not always, a slightly sigmoid shape, which argues for the existence of a small initial ratio rise.

Figs. 4a–4c show ratio curves measured under the same conditions as Figs. 1 and 2. 40, 50 and 60 % α-compensation have been assumed. While the first traces differ substantially for the three assumed α-values, the second traces are almost identical. This is due to the fact that the distortion by a certain error in α-compensation decays with the amplitude of $\lambda < 690$ nm; and after preillumination the initial fluorescence is approximately at the same level as I in the curve without preillumination. The predominant feature in all traces after preillumination is a pronounced ratio decay beginning after approx. 5 ms and terminated after approx. 60 ms under the given experimental conditions. In a corresponding $F < 690$ nm trace, I occurs after approx. 20 ms and D is reached after approx. 100 ms (see Fig. 2c). Obviously the ratio decay precedes the I-D dip. Thus it appears reasonable to consider the I-D dip a consequence of the ratio decay. The O-I rise reflects the rapid exhaustion of System II centers and it appears to be even speeded up by a short transient stimulation of

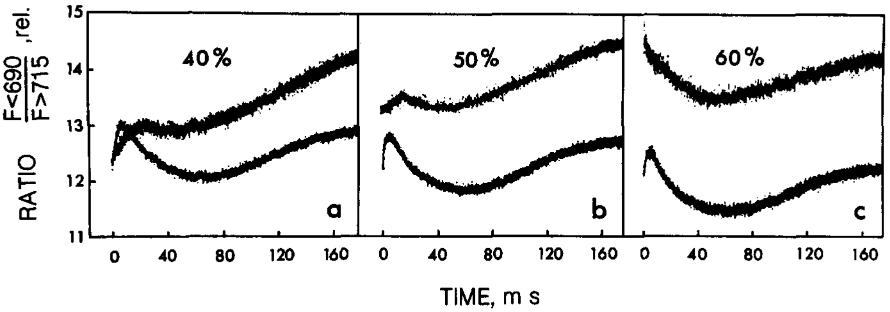

Fig. 4. Ratio transients corresponding to the stimulation of the I-D dip by preillumination. Same conditions as in Figs. 1 and 2. Three different compensation values were assumed for a, b and c. For ordinate see Fig. 3. Upper curves without preillumination.

energy distribution to System II, as expressed by the transient ratio rise. While this rise is still proceeding, some regulatory feedback mechanism appears to have already switched to more favorable System I excitation. As a result Q reoxidation is speeded up, fluorescence decays and the feedback mechanism again switches to direct more energy to System II, which is first reflected by a ratio rise and only after a delay by a significant fluorescence rise. In addition there is the delay between $F > 715$ nm and $F < 690$ nm curves, which was discussed above, and which is due to the antiparallel course of that part of the fluorescence changes which is directly caused by the ratio changes.

The fact that the ratio decay precedes I-D suggests that the feedback mechanism affecting the rapid energy distribution changes is not controlled by electron transport and the coupled ion flux changes through the thylakoid membrane. This would be even more true for the rapid initial ratio rise, for which, though, the present evidence is less certain. Definite proof that electron transport is not involved in the energy switch comes from studies with inhibitors, which are known to block electron transport. As we already demonstrated before [13], DCMU does not inhibit the rapid ratio changes but rather stimulates them. Figs. 5a and 5b show ratio curves in the

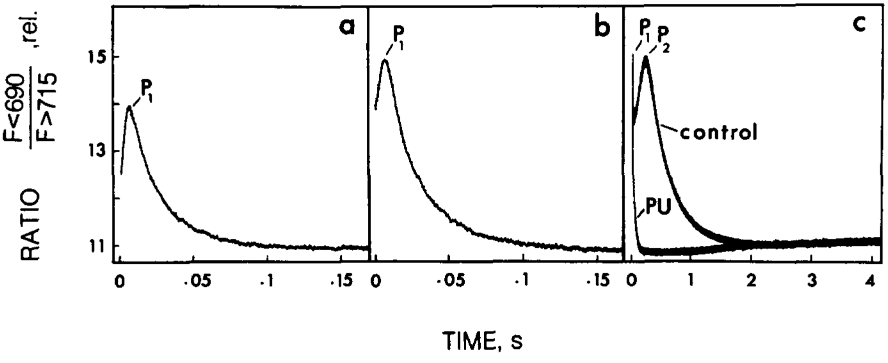

Fig. 5. Isolation of a rapid ratio decay in the presence of electron-transport inhibitors. a, $5 \cdot 10^{-5}$ M DCMU; b, $5 \cdot 10^{-3}$ M phenylurethan; c, comparison of transients with and without phenylurethan at slower speed of recording. All curves at 50 % compensation for α-fraction. Light intensity, 1 mW/cm^{-2}. Experiments after 3 h incubation of excised leaf discs in inhibitor solutions.

presence of DCMU and phenylurethan. Comparison with a control (see Figs. 5c and 4) suggests that the rapid ratio changes persisting in the presence of the inhibitors correspond to the transient rise and decay in the control, which have been correlated above with the O-I-D characteristics. Inhibitor treatment appears to stimulate P_1, the first peak in the ratio curve, and to eliminate P_2, the second peak. This and a stimulation of the decay following P_1 makes this decay the predominant feature of the ratio curve in the presence of electron-transport inhibitors. The fact that this leads to a strong shift of energy distribution towards System I is advantageous, as System II is totally blocked, while System I may still make use of the energy in cyclic photophosphorylation. An important conclusion is that the process which initiates the I-D dip in a control sample, still takes place and is even stimulated when electron transport between the two photosystems is blocked. Why then does the fluorescence curve in presence of DCMU or phenylurethan not show a pronounced I-D dip? Fig. 6 shows simultaneously measured fluorescence curves of $F < 690$ nm and $F > 715$ nm in the presence of DCMU and phenylurethan. There is no I-D dip, but even visual comparison indicates that the short wavelength fluorescence rise is flattened in its upper part relative to the long wavelength one. No dip occurs as there is a concomitant and stronger rise of fluorescence due to Q reduction. From this it may be concluded that the I-D dip after preillumination is primarily due to reoxidation of Q, while the effect of the energy distribution change is mostly on the last part of the O-I rise. But it is the energy distribution change which induces the speeded up reoxidation of Q and thus the I-D dip. There has been confusion in the past about the kinetics of the fluorescence rise in presence of DCMU [26–29]. From our findings it is apparent that the kinetics differ appreciably depending on the wavelength where fluorescence is measured. What Doschek and Kok [28] refer to as "tail" of the rise curve appears to correspond to the part of the curve which is distorted by the energy distribution change in favor of System I (see Figs. 5 and 6). This is only one example where realisation of rapid energy distribution changes requires a reconsideration of previous data collected without taking this phenomenon into consideration. This is not only true for most fluorescence data but also for practically any other study of photosynthesis where

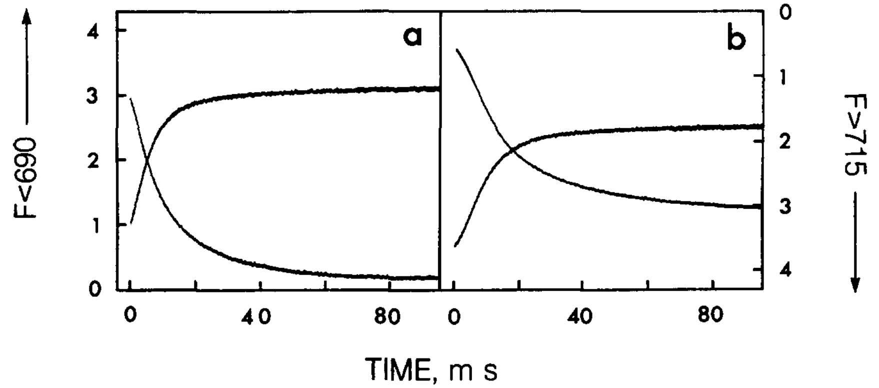

Fig. 6. Individual traces of $F < 690$ and $F > 715$ in presence of a, $5 \cdot 10^{-5}$ M DCMU and b, $5 \cdot 10^{-3}$ M phenylurethan. Curves recorded simultaneously and displayed on inverse scales for better comparison. True bases for $F > 715$ are at −0.5 unit (see also Fig. 1). Other conditions as in Fig. 5.

conclusions have been drawn from a quantitative analysis of kinetic data. We foresee a great impact of our findings on the interpretation of certain oscillatory phenomena observed following brief saturating flashes [30–32]. Undoubtedly the energy distribution changes display some oscillatory pattern. One may expect alternating switching from preferential System I to System II excitation in a series of saturating flashes. If the flashes were truly saturating, this should not necessarily lead to any oscillations in the rate of the induced reactions; but it may be difficult to saturate System I, and a flash which was found saturating in one state may not be saturating in another.

At present we have no clue as to what mechanism controls the rapid energy distribution changes. With the maximum light intensities of the present set-up we were not able to saturate the rate of the ratio changes, and maximum rise times with DCMU were in the order of 500 μs and the corresponding decays around 5 ms [13]. Up to our maximum intensity, rise and decay rates were proportional to light intensity. One may conclude that unless at still higher intensities saturation is reached, the rate of the ratio change is limited by the rate of quanta absorption. Possibly charge separation at System I and System II reaction centers and the electrical field thereby generated across the thylakoid membrane [33] induces the energy distribution changes. In the view of Seely's computer model of the photosynthetic unit [34] it appears possible that the field affects the orientation of some pigment molecules in a strategic position for energy transfer between the two photosystems.

ACKNOWLEDGEMENTS

We gratefully acknowledge support from the Simon Fraser University Faculty of Science and the Department of Biological Sciences, which made it possible for one of us (U.S.) to continue this work while being in Canada. We are also grateful for support by National Research Council of Canada operating grant A2908.

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