Effect of Electron Transfer Inhibitors and Uncoupling Agents on the Chlorophyll Fluorescence Lifetime during Slow Fluorescence Decline in Bean Leaves and Intact Chloroplasts

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Fluorescence measurements were performed with bean leaves and intact chloroplasts, which are characterized by a slow fluorescence decline. The fluorescence lifetime (τ) and the fluorescence relative quantum yield (φ) of intact chloroplasts, measured with the phase fluorometer, showed nearly proportional light-induced decrease with a halftime of ~30 s, which was removed by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), by 4,5,6,7-tetrachloro-2′-trifluoromethylbenzimidazole (TTFB), by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at a low concentration and by gramicidin D. Simultaneously the slow light-induced increase in absorbance at 518 nm (ΔA_{518}), reflecting thylakoid membrane energization, was eliminated. The data on τ of intact chloroplasts are original, the other results agree with literature data. In leaves, the slow light-induced fluorescence decline, with τ dropping from ~2 to ~0.6 ns, was abolished by FCCP at a concentration of 5 μM. However, while τ was stabilized at a level close to the initial (maximum) one or somewhat higher, φ became close to the minimum value. Besides, the amplitude of ΔA_{518} was lowered about three times. These effects seemed to be due to multiple action of FCCP as a protonophoric uncoupler and an electron transfer inhibitor. In the presence of another uncoupling agent, TTFB, which, besides, is a diuron-like inhibitor of the electron transfer in chloroplasts, we observed the light-dependent, but hardly linked to changes in membrane potential, great increase in τ of a leaf from ~1.9 to ~4.3 ns, with φ decreasing slightly. Addition of DCMU together with FCCP to the incubation medium or infiltration of a leaf with DCMU alone stimulated the rise in τ only to ~3 ns. The increase in τ of a leaf observed in the presence of FCCP and DCMU, and especially with TTFB, may be associated with protein conformation changes which (i) alter the lifetime of nanosecond recombination luminescence of the photosystem II and/or (ii) disturb excitation energy transfer from the light-harvesting chlorophyll *a*/*b* complex to other pigment-protein complexes.

Introduction

At the onset of illumination intact chloroplasts retaining envelopes, cells of green algae and whole leaves exhibit a rather fast increase in fluorescence to a maximum level P, which is followed by a slow (with a halftime of 30–60 s) decline to a steady-state level S. Algae and leaves can show additional S → M → T fluorescence transition [1, 2]. The process is reversed in the dark. The slow light-induced fluorescence decline has a complex nature. Firstly, under certain conditions it may be associated with reoxidation of the so-called "primary" electron acceptor of PSII, Q, which is in the reduced state (Q^-) when fluorescence reaches the P level [3, 4]. Secondly, it may be linked to energization of a thylakoid membrane, in particular to magnesium ion release from the intrathylakoid space to the stroma in response to the light-driven uptake of protons by thylakoids [4–9]. Thirdly, a slow light-dependent quenching of chloroplast fluorescence may be caused by ATP-dependent phosphorylation of the light-harvesting chlorophyll *a*/*b* complex (LHC) catalyzed by kinase, with the activity being controlled by the redox state of plastoquinone [10, 11 and refs. therein]. In a leaf, the most complex intact system, probably, each of the three mecha-

Abbreviations: RC, reaction center; LHC, the light-harvesting chlorophyll *a*/*b* complex; P680, the primary electron donor of PSII; Q, the primary nonporphyrine electron acceptor of PSII; B, the secondary electron acceptor of PSII; Ph, pheophytin, the intermediary electron acceptor of PSII; DCMU, diuron, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; TTFB, 4,5,6,7-tetrachloro-2′-trifluoromethylbenzimidazole; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, N-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonic acid; Mes, morpholinoethane sulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Tricine, N-tris(hydroxymethyl) methylglycine; τ, fluorescence lifetime; φ, relative fluorescence quantum yield; ΔA, absorbance change; $\Delta\psi$, transmembrane electric potential difference.

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nisms makes a certain contribution to the P → T fluorescence transition [3, 12, 13].

The ideas of the above mechanisms are based to a considerable extent on data concerning action of different agents on fluorescence intensity. The reversible light-induced quenching of fluorescence of intact chloroplasts is removed by a number of uncoupling agents, e.g., the ionophorous antibiotic gramicidin D [6] and the protonophoric uncoupler FCCP [5, 7, 8]. FCCP and its analogue CCCP produce the similar effect on the fluorescence decline in green algae [14, 15]. However, addition of FCCP at concentrations exceeding 1 μM leads to irreversible light-dependent quenching of chloroplast fluorescence [7, 8]. Probably, this is related to the discovered in earlier studies [16–19] light-dependent action of CCCP and FCCP on electron transfer on the donor side of PSII.

The slow fluorescence decline is abolished also by diuron-like inhibitors in intact chloroplasts [4–6], green algae [20, 14] and leaves [12]. It is well-known that diuron-like inhibitors block electron transfer on the acceptor side of PSII between Q and the next electron carrier, plastoquinone B [21], but the mechanism of their inhibitory action remains unclear. It was considered to be based on reduction of the midpoint potential of the couple B/B^- [22]. Now it is suggested [23, 24] that an inhibitor molecule can displace B from its binding site on the special protein with a molecular weight of 32 kDa regulating electron transfer between Q and B (see [25, 26] and refs. therein). Another possibility is discussed also: binding of the inhibitor with the 32-kDa protein may induce structural rearrangements in it, which disturb electron transfer between Q and B [21, 26–29].

A study of the effect of diuron-like inhibitors on fluorescence induction still remains urgent to date, as in the presence of these agents exciting light provides accumulation of PSII reaction centers (RCs) in the state $P680\ Q^-$. (The same occurs in the dark upon addition of the reductant-dithionite). According to recent data (see [30]), this state of the RCs is not actually the "closed" one. Between P680 and Q, there is an intermediary electron acceptor, pheophytin (Ph), which operates similarly to bacteriopheophytin in purple bacteria [31–33]. In this connection, it has been suggested that the increase in the chlorophyll fluorescence yield and the emergence of a fluorescence component with a lifetime of 2–4 ns in chloroplasts [34, 35] and 4.3 ns in pigment-protein complexes of PSII [33], observed upon reduction of Q, are due to nanosecond luminescence, resulting from charge recombination in the ion-radical pair $P680^+Ph^-$ [34]. The following arguments are in favour of this hypothesis: (i) the nanosecond luminescence stimulated by dithionite vanishes, when RCs turn into the state $P680Ph^-Q^-$ [33, 34]; (ii) the fluorescence yield in the presence of dithionite is affected by magnetic field (see [36] and refs. therein). However, its effect in chloroplasts and algal cells increases (but not drops) upon lowering the temperature from 200 to 100 K, in contrast to that in purple bacteria (see [36] and refs. therein). One of the arguments against the recombination nature of the nanosecond luminescence is the absence of the long-lived (nanosecond) component in fluorescence of mutant barley chloroplasts lacking LHC [37]. Besides, it should not be neglected that, according to the data in [38], the lifetime of the PSII recombination luminescence is of the order of 150 ns.

In purple bacteria, the important fact that has lent support to the idea of recombination nature of the nanosecond fluorescence is the high sensitivity of the latter to the transmembrane electric potential difference ($\Delta\psi$) [39–41]. Addition of uncoupling agents to energized chromatophores leads to the increase in the fluorescence lifetime (τ) and the decrease in the relative fluorescence quantum yield (φ) which have been attributed to the change in the activation energy of the nanosecond recombination luminescence. The effect of an uncoupler on fluorescence has been recently used to study the nature of nanosecond emission in green algae [15].

It is noteworthy that in the early phase fluorometric studies [42, 20] of the induction phenomena in fluorescence of green algae and higher plant leaves, τ and φ were measured simultaneously and the ratio of these parameters (τ/φ) was shown to remain constant in the course of their slow parallel decline. Formally, this is indicative of the homogeneous character of the fluorescence. Nevertheless, the computer analysis of the fluorescence decay after a weak laser pulse applied to green algae and higher plant chloroplasts has revealed that it is well simulated by a sum of three exponential components [35, 43, 44].

In view of elucidation of the nature of nanosecond emission of chlorophyll in higher plants, it

seemed interesting to us to perform measurements of τ in intact objects (intact chloroplasts, leaves) and to study sensitivity of τ and φ to electron transfer inhibitors and uncoupling agents under the conditions of the slow fluorescence decline.

Materials and Methods

For infiltration, a freshly cut green bean leaf was plunged into 0.05 Tris-HCl or Tricine-NaOH buffer solution (pH 8), which was subsequently degassed with a water-jet pump during 20 min. Then air was let to come into the vessel with a leaf. As a result of a sharp change in pressure, the buffer solution came into the leaf tissues, and the leaf became transparent. A rectangular piece was cut out of the leaf infiltrated in such a way and placed on a transparent plate along the diagonal plane of a measuring cuvette.

For isolation of intact chloroplasts according to the method described in [45], freshly cut bean leaves were ground by means of a domestic homogenizer at 0 °C for 5 s in the solution "A" [46, 47]. After filtration through 8 layers of a cheese-cloth, the suspension was centrifuged for 40 s at $2000 \times g$. The precipitate was carefully separated from the supernatant and resuspended in a small volume ($\sim$0.5 ml) of the solution "C". Both solutions contained 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$. Besides these components, the solution "A" contained 0.05 M Mes-NaOH (pH 6.1), 10 mM NaCl, 4 mM cysteine, 2 mM ascorbate, 1 mg/ml bovine serum albumin, the solution "C" contained 0.05 M Hepes-NaOH (pH 7.6).

The fluorescence parameters, τ and φ, were measured with the phase fluorometer, as in [48]. Fluorescence was excited by 404 and 436 nm mercury lines, with the intensity of exciting light being $50\ J \cdot m^{-2} \cdot s^{-1}$. Fluorescence spectra were recorded with the spectrofluorimeter Hitachi MPF-4.

The light-induced absorbance changes (ΔA) in the region of 400–600 nm were measured with the differential dual-beam spectrophotometer, as in [40]. The 400 W tungsten lamp was used as a source of the actinic light. The glass filter transmitting the $\lambda > 620$ nm range and the interference filter IF 675 (VEB Carl Zeiss Jena) were placed in front of this lamp. The photomultiplier was protected from scattered actinic light by the glass filter cutting off the $\lambda > 600$ nm range.

Results and Discussion

In preliminary experiments we did not detect any effect of the light-induced membrane potential on the fluorescence of broken chloroplasts. Therefore, we decided to study this effect with systems closer to the *in vivo* state – intact chloroplasts retaining envelopes and whole leaves.

If dark-adapted intact chloroplasts are exposed to strong illumination, their fluorescence parameters, τ and φ, show the gradual nearly proportional decrease down to the steady-state levels, which are reached in 1–2 min (Fig. 1 A). The fluorescence

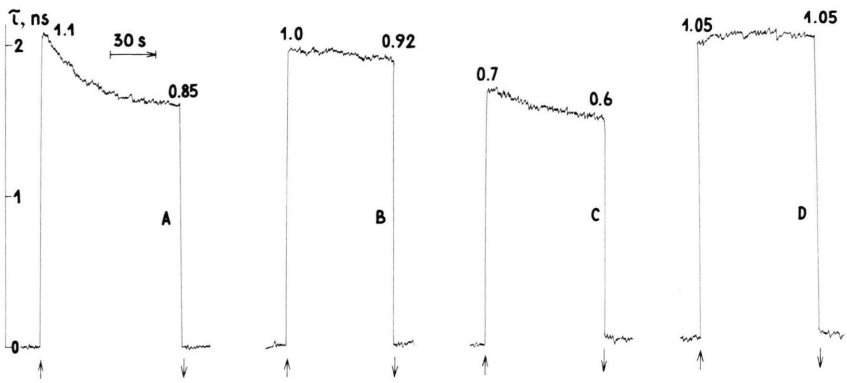

Fig. 1. The fluorescence lifetime (τ) and the fluorescence relative quantum yield (see numbers above the traces of τ) of intact chloroplasts without additions (A), in the presence of 0.5 μM FCCP (B), of 5 μM FCCP (C) and of 30 μM TTFB (D), (↑) and (↓) designate the moments, at which chloroplasts are exposed to the exciting light of the phase fluorometer and then replaced by the reference cuvette with latex.

quenching is reversed in the dark. The slow light-induced decline in both parameters is removed by DCMU, by FCCP at a concentration of 0.5 μM (Fig. 1 B), by TTFB (the diuron-like inhibitor, which is an uncoupler as well [49, 50, 21]) (Fig. 1 D) and by gramicidin D (not shown). The values of τ and φ, which remain constant under prolonged illumination of intact chloroplasts in the presence of these agents, are close to maximum values observed without additions. Raising of FCCP concentration to 5 μM leads to irreversible proportional decrease in τ and φ (Fig. 1 C).

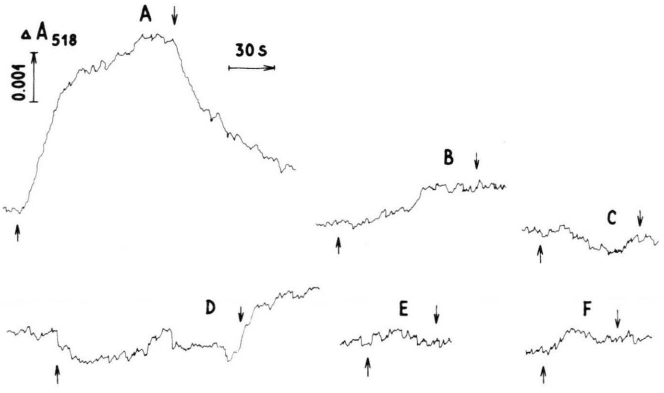

Fig. 2. The light-induced changes in the absorbance at 518 nm (ΔA_{518}) of intact chloroplasts incubated without additions (A), in the presence of 0.5 μM FCCP (B), of 5 μM FCCP (C), of 0.5 μM gramicidin D (D), of 30 μM TTFB (E) and of 20 μM DCMU (F). (↑) and (↓), switching the actinic light on and off, respectively.

The reversible light-induced decline in τ and φ of intact chloroplasts correlates kinetically with the slow light-induced increase in the absorbance at 518 nm (ΔA_{518}) (Fig. 2), which reflects the energy-linked changes in light-scattering by chloroplast membranes [51, 52]. ΔA_{518} disappears upon the addition of any of the above-mentioned agents (Fig. 2).

Thus, if the maximum values of τ and φ measured for chloroplasts under high light conditions (with PS II RCs being in the P680 Ph Q^- state) are believed to be due to the nanosecond recombination luminescence, one may conclude that the data obtained indicate on the absence of the specific (characterized by changes in τ and φ in opposite directions [40]) effect of the light-dependent membrane energization on this luminescence. However, it should be noted, that in the light-induced absorbance changes of intact chloroplasts at ~520 nm there is no fast rise (Fig. 2 A, [8]), which is characteristic of the electrochromic shift of the pigment absorption bands reflecting generation of $\Delta\psi$ across a thylakoid membrane [53].

Figure 3 illustrates fluorescence measurements with an infiltrated leaf. It is seen that the exciting light brings about the gradual decrease in τ (from 2 to 0.6 ns) and in φ (by a factor of 2) of the leaf. The fluorescence decrease is reversible and can be observed again after approx. 10 min of dark adaptation.

Figure 4 shows the light-induced changes in the absorbance of the infiltrated leaf at 518 nm. The

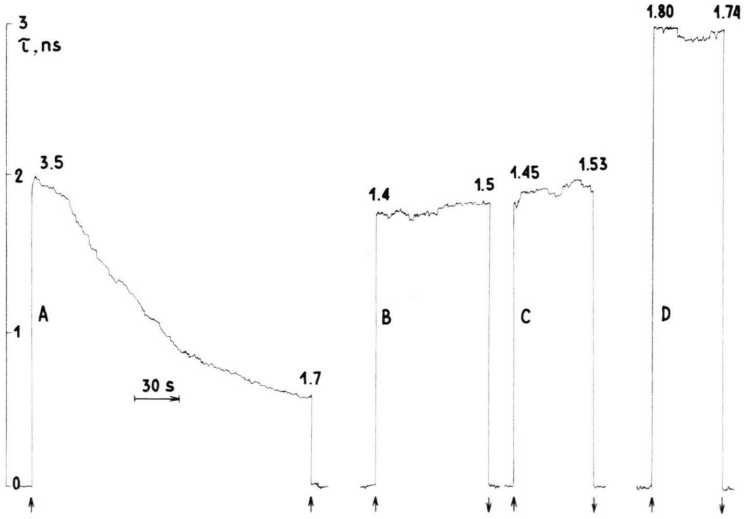

Fig. 3. The fluorescence lifetime (τ) and the fluorescence relative quantum yield (see numbers above the traces of τ) of a bean leaf infiltrated with 0.05 M Tricine-NaOH buffer solution (pH 8): A, without additions; B, after 40-min incubation in the dark with 5 μM FCCP; C, after 80-min incubation with 5 μM FCCP; D, (C) after 30-min incubation in the presence of 100 μM DCMU.

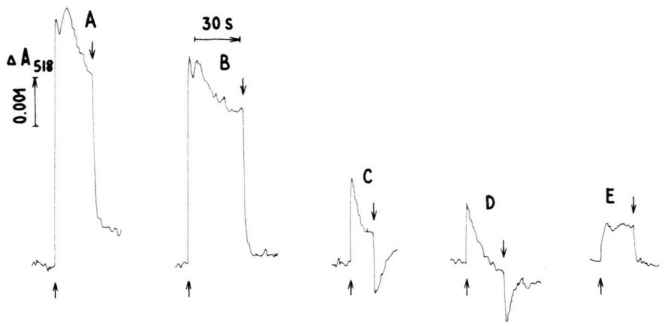

Fig. 4. The light-induced changes in the absorbance at 518 nm (ΔA_{518}) of a bean leaf infiltrated with 0.05 M Tricine-NaOH buffer solution (pH 8): A, without additions just after infiltration; B, without additions after 30-min keeping in the dark; C, after 55-min incubation with 5 μM FCCP; D, after 75-min incubation with 5 μM FCCP; E, (D) after 30-min incubation with 100 μM DCMU.

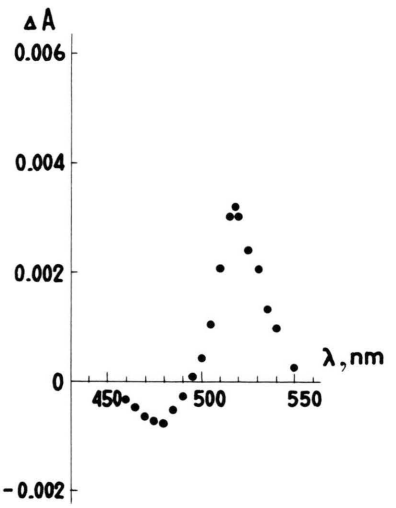

Fig. 5. The spectrum of the fast phase of the light-induced changes in absorbance (ΔA) of an infiltrated bean leaf.

kinetic trace of ΔA_{518} is composed of a fast phase (see its spectrum in Fig. 5) and a slow one. The amplitude of the fast phase, probably, is proportional to $\Delta\psi$ [53], while complex transients of the slow phase are associated with structural rearrangements induced by pH changes within the intrathylakoid space [52, 54]. The kinetics of ΔA_{518} poorly correlates with that of changes in τ and φ of the leaf.

Addition of 5 μM FCCP to the buffer medium, in which the infiltrated leaf is plunged, leads (after keeping for 40 min) to elimination of the light-induced changes in τ and φ (Fig. 3 B, C). The value of τ is maintained close to the maximum, or higher by ~ 15% (in other experiments), but the value of φ – close to the minimum one, observed without additions. The amplitude of the fast ΔA_{518} drops by a factor of approx. 3. Subsequent addition of 100 μM DCMU brings about the increase in τ to 2.9 ns (Fig. 3 D) and a change in the pattern of ΔA_{518} with a slight decrease in its amplitude (Fig. 4 D, E). After keeping of a leaf for 10–15 min in the incubation mixture containing 100–200 μM DCMU, but without FCCP, the light-induced decline in τ and φ is also abolished, with τ and φ values being stabilized near the initial level ($\tau = 2.4$ ns) (data not shown). If a leaf is infiltrated in the presence of DCMU, τ amounts to 2.9 ns.

The absence of correlation in τ and φ variations induced by FCCP (τ remains unaltered or rises, while φ drops, as compared to the initial level) may be attributed to multiple action of this agent: on one side, as a protonophoric uncoupler which (i) eliminates the energy-linked light-induced decline in τ and φ and (ii) causes a rise in τ and a drop in φ of the nanosecond recombination luminescence; on the other side, as an inhibitor of water cleavage, which disturbs electron transfer on the donor side of PSII and brings about fluorescence quenching unrelated to proton gradient collapse [16, 18]. Without taking into account the recombination luminescence, the disproportion in variations of τ and φ may be explained by suggesting that FCCP induces fluorescence quenching only in some part of the heterogeneous pigment system of a leaf.

Perhaps, clear data concerning the influence of the light-induced membrane potential on leaf fluorescence could be obtained with more specific agents, ionophorous antibiotics, but we failed to observe any effect of gramicidin D, as well as of nigericin in combination with valinomycin on leaf fluorescence. Possibly, penetration into leaf chloroplasts is hindered for these antibiotics.

The experiments with another uncoupling agent, TTFB, which had not been used earlier in fluorescence studies, were more successful. As seen from Fig. 6, exposure of an infiltrated leaf to the exciting light after keeping it during 20 min plunged into the buffer solution containing 100 μM TTFB leads to the increase in τ from ~2.1 to ~4.3 ns. The initial values of τ and φ practically coincide with their maximum values measured before addition of TTFB. The rise in τ is not accompanied by a

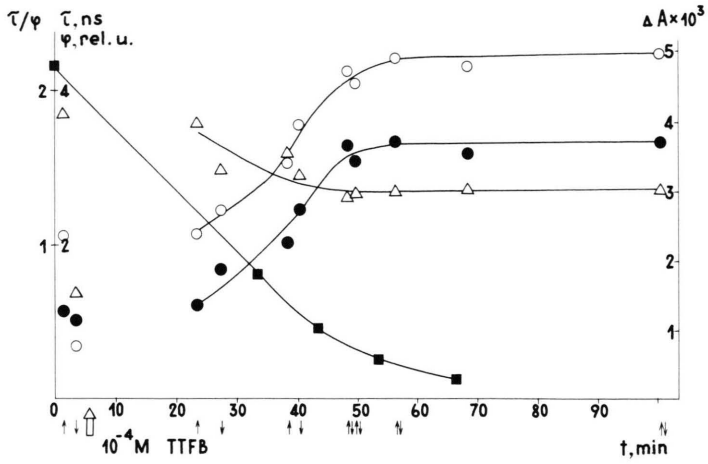

Fig. 6. The effect of TTFB on the fluorescence lifetime (τ, ○), on the fluorescence relative yield (φ, Δ), on the τ/φ ratio (●) and on the amplitude of the fast phase of the light-induced absorbance changes at 518 nm (ΔA, ■) of a bean leaf infiltrated with 0.05 M Tricine-NaOH buffer solution (pH 8). (↑) and (↓) denote the moments, at which a leaf and a reference cuvette with latex are exposed to the exciting light, respectively. The big arrow designates the moment of adding of TTFB to the incubation medium. (TTFB is added from 0.02 M solution in ethanol.)

corresponding increase in φ: while τ rises, the fluorescence intensity either slightly diminishes, or does not change at all, so that finally the φ value becomes by ~30% lower than the initial one and the τ/φ ratio increases by a factor of 2.5–3.0. The process is not reversed in the dark. As shown by measurements of the absorbance changes, a considerable part of the increase in τ proceeds in almost uncoupled membranes: by the beginning of the process, ΔA_{518} has already dropped by approx. 60%, probably as a result of the uncoupling action of TTFB (Fig. 6).

Similar results were obtained after infiltration of a leaf with the buffer solution containing 100 μM TTFB during ~90 min in the dark. After the onset of illumination the rather fast rise (with a halftime of ~30 s) in τ from 1.85 to 3.15 ns was observed, which was followed by the slower increase up to 4.3 ns (Fig. 7 A, B). The amplitude of the fast phase of ΔA_{518}, being partially suppressed after infiltration of the leaf with TTFB, slightly changed in the course of the increase in τ (Fig. 7 A). It is remarkable that the increase in τ, induced by intense illumination, starts from the low value, 1.85 ns, i.e. the prolonged keeping and even infiltration of a leaf with TTFB in the dark do not cause the increase in τ, but only accelerate the process of its light-induced rise (*cf.* Figs. 6 and 7 A). Thus, the discovered phenomenon of the increase in τ is the light-dependent process, hardly linked to changes in membrane potential. It has been noticed, by the way, that it is the blue, but not red, light that initiates the process.

The time courses of the light-induced increase in τ varied in different experiments, probably due to changes in the amount of TTFB having penetrated into leaf chloroplasts. Addition of ethanol together with TTFB is of no importance, as we have obtained similar results while using an alkaline water solution of TTFB (not shown).

It is noteworthy that the increase in τ up to 4.3 ns is not accompanied by appreciable changes in a room temperature fluorescence spectrum of a leaf (not shown). Moreover, the values of τ, measured with the use of a glass filter transmitting almost the whole spectrum of leaf fluorescence ($\lambda > 620$ nm), and those obtained with a filter transmitting only the long-wavelength part of the spectrum ($\lambda >$ 730 nm) are practically the same, though the corresponding fluorescence intensities differ by a factor of ~3.5. When taking into account that in the absence of TTFB the kinetics of the light-induced decline in τ of leaf fluorescence at $\lambda > 730$ nm is analogous to that at $\lambda > 620$ nm (Fig. 8), the conclusion may be drawn that TTFB produces the same effect on τ in the short-wavelength and in the long-wavelength parts of the fluorescence spectrum.

In the course of this investigation we have not obtained clear data concerning the effect of the light-induced membrane potential on nanosecond luminescence of chlorophyll in chloroplasts and whole leaves. Most probably, the magnitude of $\Delta\psi$ maintained across a thylakoid membrane by photosynthetic electron flow is too small [53], so that the effect of $\Delta\psi$ on the nanosecond luminescence cannot be detected.

As to the effect of TTFB on leaf fluorescence, two phases are discernable in the process of the τ increase after infiltration of a leaf with this agent: (i)

the fast rise in τ from ~ 2 to ~ 3 ns observed in the time range of seconds and tens of seconds, and (ii) the slow increase in τ from ~ 3 to ~ 4.3 ns with a halftime of ~ 30 min. If TTFB is simply added to the incubation medium of an infiltrated leaf the monotonous slow rise from ~ 2 to 4.3 ns is observed. The fast rise is similar to the change in τ, which occurs after infiltration of a leaf with 100 μM DCMU (not shown) or after addition of 5 μM FCCP together with 100 μM DCMU to the incubation medium (Fig. 3 D). Possibly, these changes have the common nature. The first phase of the increase in τ is characterized by a decrease in φ, as compared to the initial level, by 20–30% in the case of TTFB and by $\sim 50\%$ – in the case of FCCP plus DCMU. During the second phase observed with a TTFB-infiltrated leaf, φ remains unaltered.

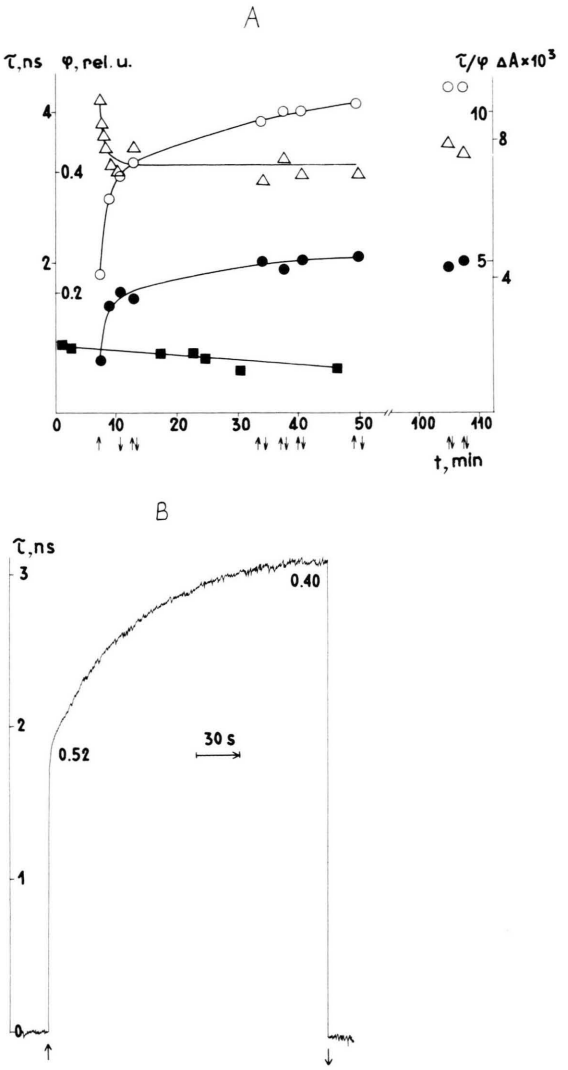

Fig. 7. (A) The time course of the fluorescence lifetime (τ, ○), the fluorescence relative yield (φ, Δ), the τ/φ ratio (●) and the amplitude of the fast phase of the light-induced absorbance changes at 518 nm (ΔA, ■) of a bean leaf infiltrated in the dark for ~ 90 min with 0.05 M Tris-HCl buffer containing 100 μM TTFB. (B) The fast phase of the kinetics of τ and φ (see numbers above the trace of τ) shown in (A).

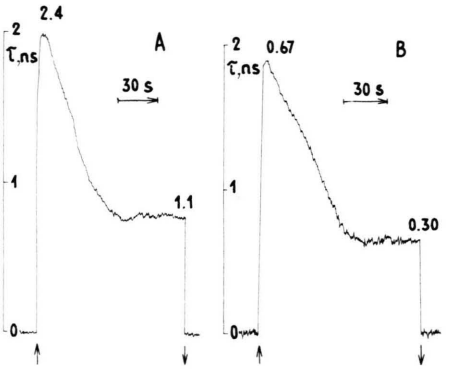

Fig. 8. The kinetics of the fluorescence lifetime (τ) and the fluorescence relative yield (see numbers above the traces of τ) of a bean leaf infiltrated with 0.05 M Tris-HCl buffer solution. The trace A is detected through a glass filter transmitting fluorescence in the range of $\lambda > 620$ nm and trace B – through a glass filter transmitting the $\lambda > 730$ nm range.

The effect of TTFB on leaf fluorescence may be tentatively explained from two viewpoints, both seeming plausible to us:

I. When believing that the recombination luminescence of PSII makes the major contribution to the chlorophyll fluorescence of green plants under high light conditions, one may suppose that a light-dependent conformational change takes place upon binding of TTFB with the 32-kDa protein, regulating electron transfer between plastoquinones Q and B. This leads to a decrease in the rate constant of radiative recombination of charges in the ion pair $P680^+Ph^-$ as a result of, *e.g.*, an increase in the distance between P680 and Ph. In this case, τ and φ may actually change in opposite directions, as it is observed during the first phase.

II. When assuming the nanosecond emission of chlorophyll in higher plants to be due to the functionally separated light-harvesting chlorophyll *a*/*b* complex [37, 55, 43], it may be suggested that (i)

the 32-kDa protein plays an important role in mediating excitation energy transfer from LHC to the core antenna of PSII, and the inhibitor-induced conformational change in the protein lowers the efficiency of this process, or (ii) the TTFB-induced conformational change involves not only the 32-kDa protein, but also the proteins of LHC, and thereby disturbs excitation energy transfer from LHC to other pigment-protein complexes, which results in the considerable increase in τ and φ. In order to make the hypothesis II self-consistent, an additional assumption should be made that the enhancement of fluorescence of LHC is accompanied by quenching of fluorescence of some other complexes. Otherwise, the increase in the τ/φ ratio observed in the presence of TTFB would be unclear.

The biphasic character of fluorescence changes, possibly, is related to the predominant effect of TTFB at first on the electron transfer (the hypothesis I) and then on the excitation energy transfer (the hypothesis II).

In fact, the use of TTFB unexpectedly gave us the first opportunity to observe with an intact photosynthetic object, a mature green leaf, the high values of the mean fluorescence lifetime (4.0–4.3 ns), approaching those measured for a dilute solution of chlorophyll *a* [56]. Similar values of τ (up to 5 ns) were measured earlier only with etiolated leaves [42]. According to the data of phase and pulse fluorometry, the maximum mean values of the chlorophyll fluorescence lifetime in green photosynthetic objects, such as leaves, algae and chloroplasts, at room temperature hardly exceed 2 ns (see [2, 35] and refs. therein, [48]), whereas τ measured for the monomeric form of the isolated LHC is of the order of 3–4 ns [55, 57, 58]. Possibly, we were lucky to observe an unusual state of LHC *in vivo*, when there is practically no excitation transfer from chlorophyll of this complex to any quenching centers. This is of interest for elucidation of the state of LHC in a thylakoid membrane and, in general, the state of chlorophyll *in vivo*. The fact is that in solution with pigment concentration as high as that in a leaf, chlorophyll fluorescence is highly quenched ($\tau < 1$ ns [59]). Thus, interaction with proteins prevents the concentration quenching of chlorophyll fluorescence and provides the special state of chlorophyll, characterized by low nonradiative losses of excitation energy.

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