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IDENTIFICATION OF THE 120 μs PHASE IN THE DECAY OF DELAYED FLUORESCENCE IN SPINACH CHLOROPLASTS AND SUBCHLOROPLAST PARTICLES AS THE INTRINSIC BACK REACTION. THE DEPENDENCE OF THE LEVEL OF THIS PHASE ON THE THYLAKOIDS INTERNAL pH

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SUMMARY

After a 500 μs laser flash a 120 μs phase in the decay of delayed fluorescence is visible under a variety of circumstances in spinach chloroplasts and subchloroplast particles enriched in Photosystem II prepared by means of digitonin. The level of this phase is high in the case of inhibition of oxygen evolution at the donor side of Photosystem II. Comparison with the results of Babcock and Sauer (1975) Biochim. Biophys. Acta 376, 329–344, indicates that their EPR signal II_f which they suppose to be due to Z^+, the oxidized first secondary donor of Photosystem II, is well correlated with a large amplitude of our 120 μs phase. We explain our 120 μs phase by the intrinsic back reaction of the excited reaction center in the presence of Z^+, as predicted by Van Gorkom and Donze (1973) Photochem. Photobiol. 17, 333–342. The redox state of Z^+ is dependent on the internal pH of the thylakoids. The results on the effect of pH in the μs region are compared with those obtained in the ms region.

INTRODUCTION

The fast phases in the decay of delayed fluorescence which occur within the main turnover time of Photosystem II reflect stabilization steps of the reaction center after excitation. As such, a study of these fast phases has to give more detailed information on these different steps. Several investigators have now explored this field [1–6]. The reaction center of Photosystem II is assumed to consist of a three-component complex ZPQ, where Q is the primary photoreductant, P is the reaction center pigment P 680 the primary donor and Z the first secondary donor. After excitation of the reaction center pigment P^x a charge separation occurs leading to ZP^+Q^-. The back-reaction between P^+ and Q^- is normally prevented by a fast reduction of P^+ by the first secondary donor Z leading to Z^+PQ^-. Delayed fluorescence (luminescence)

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonylcyanide 3-chlorophenylhydrazone; Tris, tris(hydroxymethyl)aminomethane; Tricine, *N*-tris(hydroxymethyl) methylglycine; MES, 2-(*N*-morpholino) ethanesulphonic acid.

is thought to originate from the direct back reaction between P^+ and Q^-; as explained by Van Gorkom and Donze [7], this intrinsic recombination reaction should be stimulated if excitation of the reaction center occurs in a state where Z is already oxidized Z^+P^xQ. Therefore this state of the reaction center must be considered as inefficient; it could account for the "misses" in oxygen evolution [8]. During normal photosynthesis the state Z^+PQ^- of the reaction center relaxes at the donor side by charge movement of Z^+ to the S-states [8] and at the acceptor side by reoxidation of Q^- by the plastoquinone pool. Delayed fluorescence in the longer time range, > 1 ms, is understood to result from reversed electron flow to the reaction center in the already stabilized and relaxed state. This reversed electron flow may be driven either by the chemical (pH) or the electrical components of the membrane potential [9]. It is difficult to assign a phase in the decay of delayed fluorescence with certainty either to the intrinsic recombination, or to a stabilization step, or to a relaxation step (using these terms in the same sense as Lavorel [3]). The lifetime of a certain step may be changed by charge influence of an already existing dipole moment – due for instance to the pH gradient in the steady state or a local membrane potential – which may change the required activation energy for the back reaction. So independent parameters reflecting states of the reaction center would help to create more certainty. Fluorescence, originally proposed by Duysens and Sweers [10] to reflect the redox state of Q, is a candidate. However the decay of the fluorescence in the fast-time range is not likely to reflect only the redox state of Q, in view of recent results of Butler [11], Zankel [12], Etienne [13], A. Joliot [14] and Den Haan et al. [15] which propose the existence of a quenching state of the reaction center if P^+ is present. So, in the fast time range the $L - \Phi$ relationship originally proposed by Lavorel [16] has no clear validity (see also the discussion by Lavorel [17]). A plausible, independent measurement of Z^+ * seems to be the EPR signal II_f of Babcock and Sauer [18]. In this report we will compare our measurements in the fast time range of the decay of delayed fluorescence with those reported by Babcock and Sauer on EPR signal II_f, and we will show that the occurrence of the 120 μs phase in the decay is well correlated with that of EPR signal II_f under comparable conditions, which forces us to believe that the 120 μs phase is due to the stimulated intrinsic back reaction in the presence of Z^+. Furthermore we will extend our measurements to the ms time-range and also study the pH effect on the decay so as to compare our results for example with those obtained by Wraight and Crofts [19] and by Rottenberg et al. [20].

MATERIALS AND METHODS

Chloroplasts, Tris-washed chloroplasts and digitonin subchloroplast particles enriched in Photosystem II were prepared as already described [21] except that the isolation medium of the chloroplasts contained 0.1 % bovine serum albumin.

Just before the experiments the chloroplasts were diluted in 50 mM Tricine or 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer, depending on the pH required, 10 mM KCl and 2 mM $MgCl_2$. Spinach was either obtained on the local market or

* There may be some confusion about the symbol Z which is also used to denote the water-splitting system including S-states; here its use is restricted to the first secondary electron donor of Photosystem II.

grown in a greenhouse. Chlorophyll concentrations were routinely estimated simply by measuring the absorbance at 680 nm (opal glass method), calibrated by the extraction method of Arnon [22]. Delayed fluorescence was measured as reported by Lavorel [3, 6] using a continuous helium-neon 4 mW laser as light source (C. W. Radiation, S 405 R). Successions of actinic flashes were produced by a rotating chopper disc, producing flashes of 500 μs duration, at a frequency of 20 Hz. The chloroplasts, at 35 μg chlorophyll/ml, were contained in a 15 °C-thermostated glass semicapillary tube. The decays after several thousand flashes were averaged by means of a multichannel analyzer to obtain a sufficient signal-to-noise ratio; measurements were started only after the first induction phenomena had disappeared. Due to a postflash fluorescence artifact, the first 60 μs of the luminescence decay after the flash were not considered except if the luminescence intensity is high, as in the case of Tris-washed chloroplasts where the contribution of the artefact is negligible, the luminescence intensity immediately after the flash in a case where the artefact is negligible is taken as unity. The integrated luminescence between flashes could be followed by means of a strip chart recorder as a simple control to see for instance the induction phenomena or the exhaustion of added electron donors.

Fluorescence during the actinic flash could be monitored by means of the same photomultiplier but with a wide band amplifier instead of the pulse amplifier. The flash duration of 500 μs was chosen because at this duration complete saturation of fluorescence occurred. Certainly this flash duration allows several photochemical turnovers of the reaction center, a fact which it is important to take into account to explain our results.

The simple reagents we used were of the quality pro analysi; nigericin was the kind gift of Dr. Pettinga, Eli Lilly Int. Corp., U.S.A.; valinomycin was obtained from Calbiochem, U.S.A., bovine serum albumin fraction V, gramicidin D and carbonylcyanide 3-chlorophenylhydrazone (CCCP) from Sigma Chem. Co., U.S.A., 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) from Dupont de Nemours, U.S.A., tetraphenylboran from Merck, G.F.R. and semicarbazide from Fluka A.G., Switzerland.

RESULTS

In Fig. 1 the steady-state luminescence decay of a control sample is compared with samples uncoupled by means of valinomycin and nigericin in the absence and presence respectively of hydroxylamine and hydroxylamine+DCMU. When times longer than 1 ms are considered the most striking change is due to the effect of uncoupling; clearly the elevated level of luminescence without uncoupler is due to the existence of a pH gradient [19]. After about 1.5 ms the decays in all cases proceed approximately parallel. The decays in the presence of DCMU or hydroxylamine are not or are hardly influenced by the omission of the uncoupler (not shown). In these cases electron transport is inhibited, which prevents the formation of a pH gradient. So it seems wise not to compare the decay in the presence of hydroxylamine with the control but with the decay in the presence of uncoupler. In the presence of hydroxylamine the decay in the time range shorter than 1 ms is very regular (compare Lavorel [3]) with a $t_{\frac{1}{2}}$ of 105 μs (Fig. 1). Also in the case of uncoupling a regular decay predominates in this time range, $t_{\frac{1}{2}} = 110$ μs. In the presence of DCMU a phase with

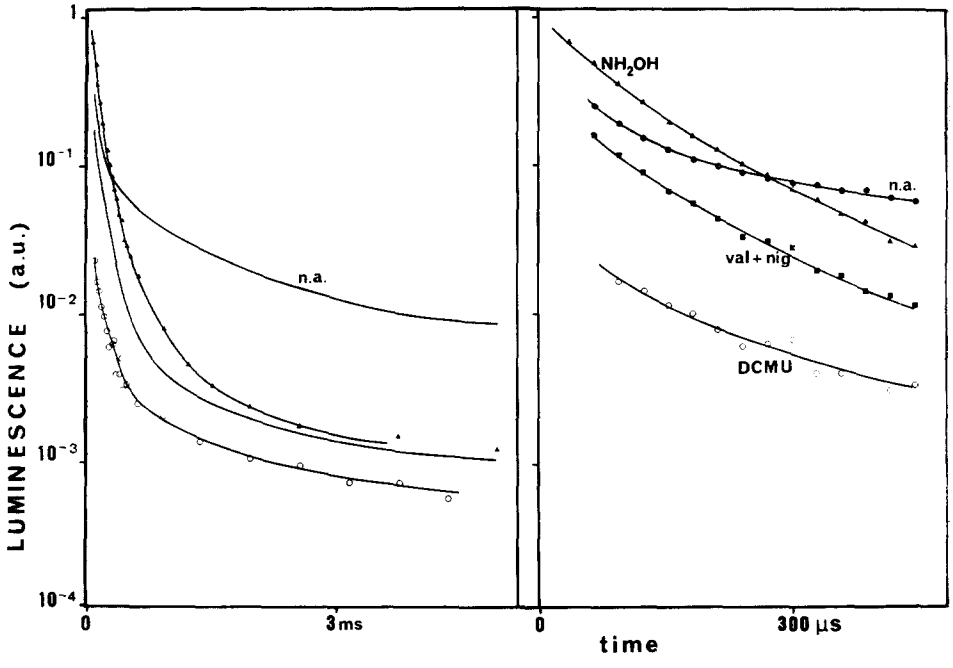

Fig. 1. Logarithm of luminescence intensity as a function of time after the flash of chloroplasts in Tricine buffer pH 7.8 with 1 mM H_2NOH; 10 μM DCMU+1 mM H_2NOH; 0.5 μM valinomycin +0.5 μM nigericin added where indicated. The same curves are presented on two different time scales. Each curve represents an accumulation of 10 000 decays. (a.u.: arbitrary units.) For reasons of clarity in some cases the measuring points are omitted and only the handdrawn curves are shown.

$t_{\frac{1}{2}} = 130$ μs is present which runs fairly parallel to the decay in the presence of hydroxylamine. Its amplitude however is much smaller.

Other treatments, inhibiting at the donor side of Photosystem II, like hydroxylamine, have a comparable effect: both heat treatment (not shown) and Tris-washing (Fig. 4) of chloroplasts give rise to a rather regular decay with a high amplitude as in the hydroxylamine case. After addition of uncouplers or hydroxylamine to Tris-washed chloroplasts, the decay remains regular and the amplitude hardly changes. Also subchloroplast particles enriched in Photosystem II prepared by means of digitonin show a decay which is much like the decay of chloroplasts with hydroxylamine present.

Effect of changing the pH of the medium

In Fig. 2 the effect of changing the pH of the medium on the luminescence decay is shown. Looking at the ms time range (not shown) it is striking that the decays at the different pH values run parallel, even at the extreme pH values. In the shorter time range it is more complicated. Going down to lower pH values one obtains a more or less regular decay which at pH 5.1 is comparable with the Tris-washed case. The decays at neutral pH values are influenced by the pH gradient across the thylakoid membrane even at times as short as 200 μs and so the decay curves are polyphasic. The amplitudes are lowest at the higher pH values.

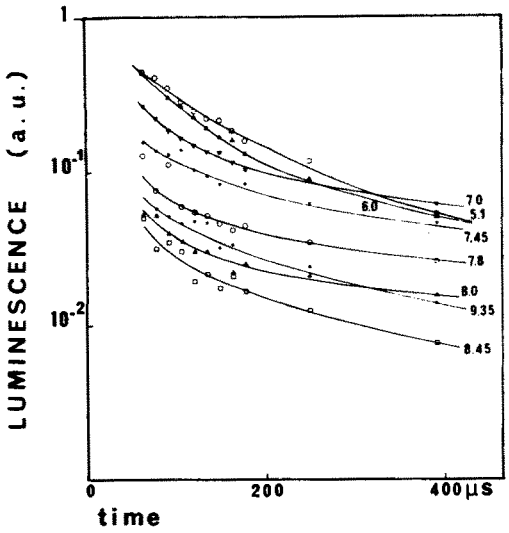

Fig. 2. Luminescence decays of chloroplasts at different pH values, pH as indicated. Each curve represents an accumulation of 2500 decays.

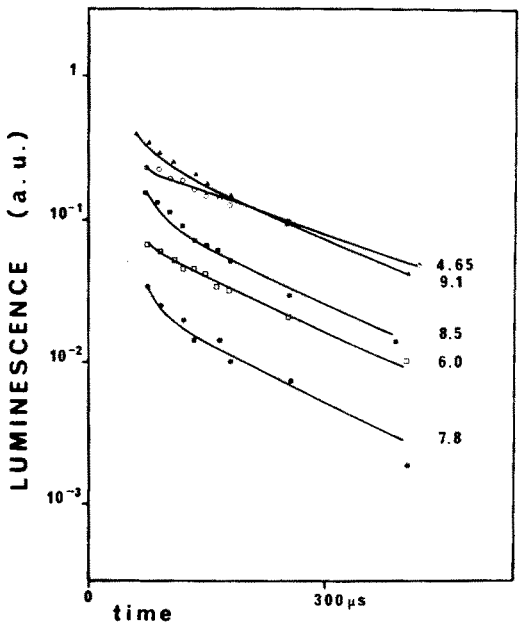

Fig. 3. Luminescence decays of chloroplasts at different pH values, as in Fig. 2, except for the addition of valinomycin + nigericin, 0.5 μM each.

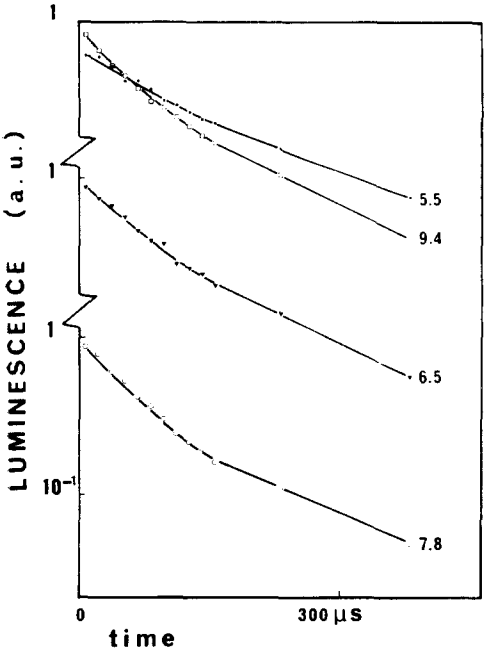

Fig. 4. Luminescence decays, as in Fig. 2, of Tris washed chloroplasts.

In Fig. 3 the same experiment as in Fig. 2 is shown except for the presence of uncouplers (valinomycin+nigericin, both 0.5 μM) [23]. In general the decays appear to be less polyphasic. The $t_{\frac{1}{2}}$ of the phase after disappearance of the fluorescence artefact is about 120 μs in the pH range from 5.5–8.5 but increases to 180 μs going down to pH 4.6 and to 240 μs at pH 4.3 (this last value is from an independent experiment).

In Fig. 4 the effect of pH on the decay of Tris-washed chloroplasts is shown. The decay does not show much sensitivity to pH except at the longer times (Fig. 5b) and at very low pH values ($\leqslant$ pH 5.5). At these low pH values even the initial level after the flash diminishes. After 100 μs a major 130 μs phase is dominant at pH values 6.0–9.4, at lower pH values this decay is slower. A large contribution of the fluorescence artefact is never apparent, probably it is masked by the large amplitude of the 130 μs phase. The luminescence intensity 100 μs after the flash may be taken as a measure of the amplitude of the 120 μs phase. This is done in Fig. 5a, summarizing the results of the three former figures. Also the luminescence intensity at 2 ms is plotted as a function of the pH to be able to compare these results. The high amplitude of the 120 μs phase seems to be correlated with a charge accumulation at the donor side of Photosystem II, as is obviously present in Tris-washed chloroplasts, where the water-splitting steps are inhibited. In normal chloroplasts the pH of the inside of the thylakoid, where the donor side of System II is supposed to be located, plays a crucial role in the ability of System II to accumulate a charge at the donor side.

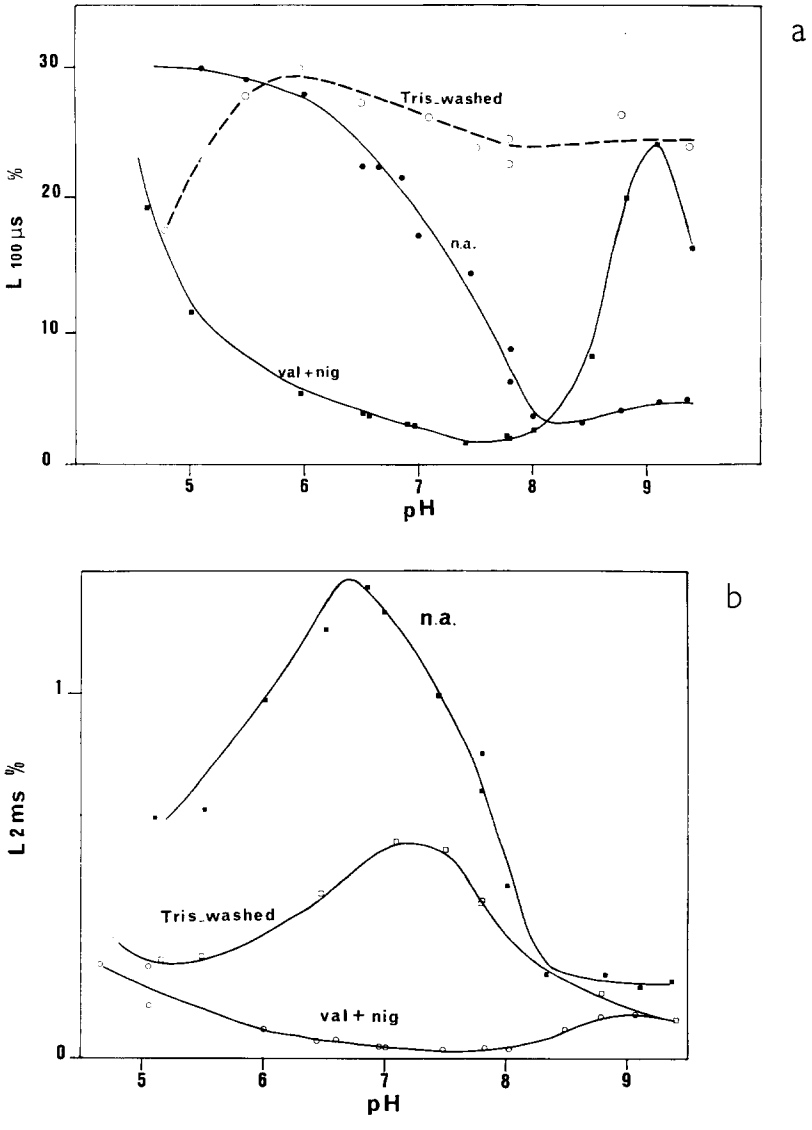

Fig. 5. Luminescence intensity in *a* at 100 μs, in *b* at 2 ms, of chloroplasts, Tris-washed chloroplasts and uncoupled chloroplasts (by means of 0.5 μM valinomycin + nigericin) as a function of pH. The intensity is expressed in percent of the level immediately after the flash of Tris-washed chloroplasts.

Effects of CCCP

The effects of the poison CCCP are summarized in Table I. At low concentrations (0.2 μM) of CCCP its effects are quite like those of uncouplers like valinomycin +nigericin: at pH values below 8 the amplitude of the 120 μs phase is smaller compared to that for coupled chloroplasts whereas at high pH this phase is greatly stimulated. At a high concentration of CCCP the decay during the first half ms is clearly monophasic $t_{\frac{1}{2}} = 160$ μs. The amplitude of this phase is small at all pH values:

TABLE I

THE EFFECT OF CCCP ON THE LUMINESCENCE EMISSION OF CHLOROPLASTS

pH	Luminescence intensity at 100 μs, in % of the initial level after the flash with Tris-washed chloroplasts.		
	no addition	0.2 μM CCCP	5 μM CCCP
6.0	32	20	6.5
7.5	13	8	4
9.1	11	31	1

very evidently at high pH values but also at pH 6.0 or 7.5. The amplitude is always smaller than in the uncoupled state alone (e.g. effected with gramicidin). CCCP at high concentration also profoundly reduces the level of the 120 μs phase (see Table 2) of Tris-washed chloroplasts where uncouplers like valinomycin+nigericin have no effect. These effects of CCCP are explained by its ability to deactivate the charge accumulation at the donor side of Photosystem II. CCCP as such is already known to deactivate steps of the water-splitting system [13, 24].

Effects of DCMU

The effect of DCMU on the luminescence decay at a saturating concentration is shown in Fig. 6. It strongly depresses the amplitude of the 120 μs phase which is also retarded to $t_{\frac{1}{2}} = 160$ μs. The luminescence intensity 100 μs after the flash in the

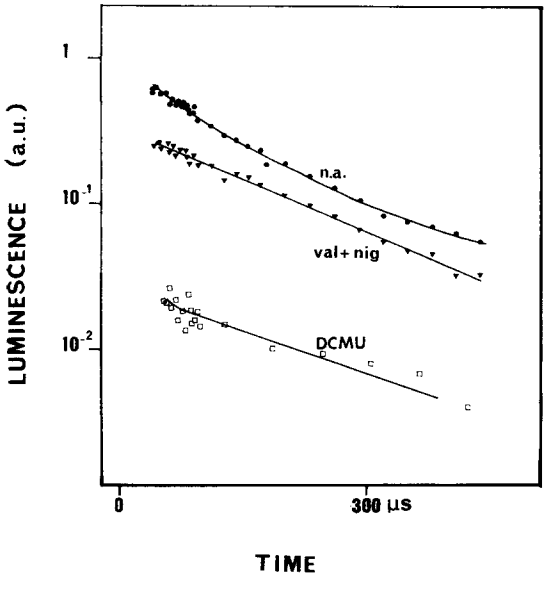

Fig. 6. Luminescence decays of chloroplasts in MES buffer pH 6; 20 nM valinomycin, 20 nM nigericin and 10 μM DCMU added where indicated. Each curve represents an accumulation of 12 000 decays.

presence of DCMU is, at pH values above 5, always about 1.5 % of the original level after the flash in Tris-washed chloroplasts; at pH values below 5 it increases markedly, to 13 % at pH 4.3, but at this pH the luminescence intensity of uninhibited chloroplasts is high (70 %, $t_{\frac{1}{2}} = 240\ \mu s$).

Effects of electron donors

The effect of an artificial electron donor for Photosystem II on the decay of

TABLE II

THE EFFECT OF SEMICARBAZIDE ON THE LUMINESCENCE DECAY OF TRIS-WASHED CHLOROPLASTS COMPARED WITH THE EFFECT OF CCCP

The Tris-washed chloroplasts were suspended in Tricine buffer of pH 7.8. The luminescence intensity is taken relative to the original level immediately after the flash in Tris-washed chloroplasts without additions.

Addition	Luminescence intensity at 100 μs in %	$t_{\frac{1}{2}}$ in μs
–	26	115
10 mM Semicarbazide	3.9	120
5 μM CCCP	3.0	110

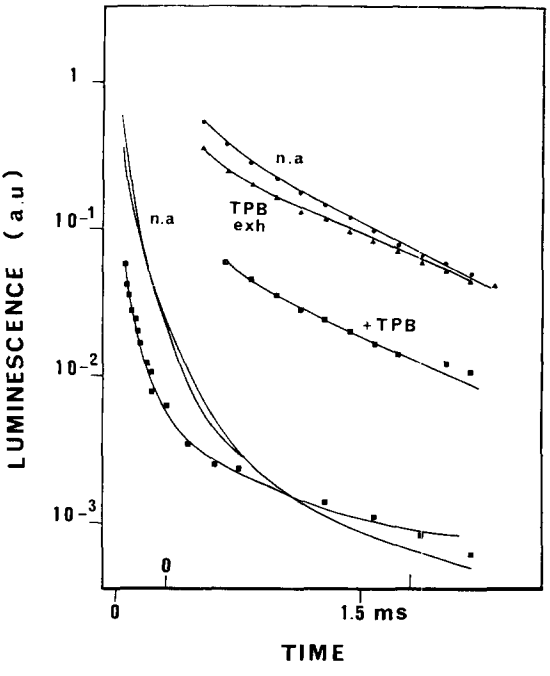

Fig. 7. Luminescence decays of System II particles in phosphate buffer pH 7.8. 5 μM tetraphenylboran (TPB) is added where indicated. The $t_{\frac{1}{2}}$ of the phases, with and without tetraphenylboran, on the fast time scale is 110 μs. After exhaustion of the donor (TPB) the luminescence returns approximately to its original level, but the $t_{\frac{1}{2}}$ is slightly retarded. Each curve represents an accumulation of 10 000 decays. The same curves are presented on two different time scales.

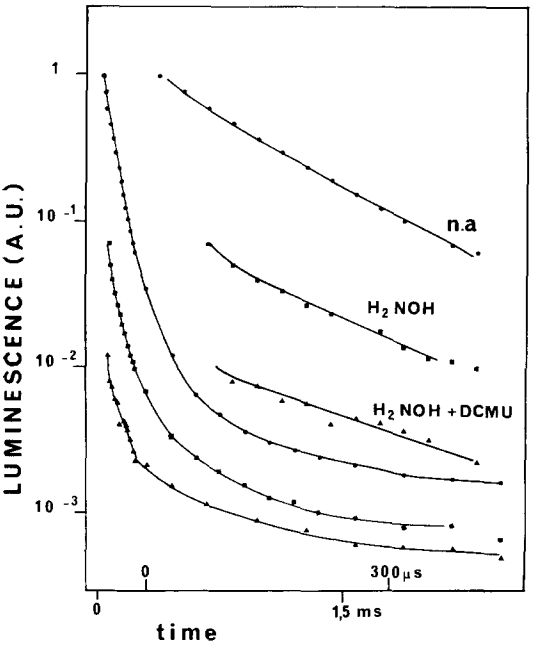

Fig. 8. Luminescence decays of System II particles (70 μg chlorophyll/ml) in phosphate buffer pH 7.8. 1 mM H_2NOH and 10 μM DCMU are added where indicated. The $t_{\frac{1}{2}}$ of the phases on the fast time scale is, respectively, 105, 130 and 160 μs for the control sample, the sample with hydroxylamine and the DCMU inhibited sample. Each curve represents an accumulation of 10 000 decays. The same curves are presented on two different time scales.

Tris-washed chloroplasts is shown in Table II. Here semicarbazide is used which needs a rather high concentration compared with more efficient donors like tetraphenylboran [25] or hydrazobenzene [21] but the latter substances would be exhausted during the experiment due to a high rate of electron transport. The effect is very like that of CCCP and may simply be explained by the ability of electron donors to reduce directly the accumulated charge at the donor side of Photosystem II.

In System II particles prepared by means of digitonin, which exhibit a much lower rate of electron transport without added electron acceptor the effect of tetraphenylboran on the decay could be observed (Fig. 7). As with Tris-washed chloroplasts the amplitude of the 120 μs phase is markedly reduced. It should be noted that in System II particles hydroxylamine also depresses the 120 μs phase (Fig. 8) which suggests that in these particles hydroxylamine acts as a much more active electron donor than with chloroplasts, where it is rather its action as inhibitor which prevails. That hydroxylamine is a bad electron donor was already noted by Lavorel [3] and Etienne [13]. Another feature of an efficient electron donor is that whereas in DCMU inhibited Tris-washed chloroplasts the 120 μs phase still reaches a certain level, after the first flashes this effect is absent if tetraphenylboran is added. This indicates that with Tris-washed chloroplasts even in the presence of DCMU some charge accumulation at the donor side is possible resulting in the luminescent back reaction which reopens the reaction centers so offering the next flash the possibility of provoking a

TABLE III

THE EFFECT OF FERRICYANIDE ON THE LUMINESCENCE INTENSITY AT 100 μs OF UNCOUPLED CHLOROPLASTS

pH	The luminescence intensity at 100 μs in % of the original level of Tris-washed chloroplasts immediately after the flash		
	no addition	0.1 μM gramicidin D	0.1 μM gramicidin D +0.1 mM ferricyanide
5.15	–	14	27
6.0	22	10	28
7.0	9	6	19
8.0	4	6	20
9.1	6	29	25

renewed charge separation and so on until the charge accumulation at the donor side is eliminated by a slow reduction by a secondary donor (compare Ref. 5). In the presence of tetraphenylboran this charge accumulation at the donor side is annihilated by a fast reduction even during the first flash but this may well be due to the fact that the flashes are too long: one might suppose that a first single turnover flash should have given some luminescence even in the presence of tetraphenylboran.

Effect of the electron acceptor ferricyanide

The effect of an electron acceptor is just the opposite of that of an electron donor: if ferricyanide is added the 120 μs phase is stimulated (Table III). Here the uncoupled case is chosen to be sure that pH effects could not interfere. The stimulation of the luminescence by ferricyanide stops when the acceptor is exhausted due to Hill activity; the time needed for this offers a simple test for activity under the employed conditions: about 140 μEquiv. mg chlor^{-1} · h^{-1} at pH 8.0 and about 65 μEquiv. mg · chlor^{-1} · h^{-1} at pH 6.0. To explain the stimulation of the 120 μs phase by electron acceptors in the uncoupled case is difficult. It may be due to a change of the redox state of intermediates at the donor side of Photosystem II, thus facilitating charge accumulation there.

DISCUSSION

The presence of a phase in the decay with a $t_{\frac{1}{2}}$ of 120–160 μs is easily recognized in nearly all cases. As is shown, its amplitude is correlated with a charge accumulation at the donor side of Photosystem II. This charge accumulation clearly takes place in Tris-washed chloroplasts which are inhibited at the donor side; it is accompanied by pigment bleaching [27] as it is in subchloroplast fragments enriched in Photosystem II [21] which also show an elevated level of the 120 μs phase in their luminescence decay. This bleaching is inhibited by electron donors or DCMU and enhanced by ferricyanide. Recent results of Babcock and Sauer [18] on EPR measurements of Tris-washed chloroplasts show a fast-decaying EPR signal (their signal II_f) which accompanies a charge accumulation at the donor side. They offer arguments that this signal II_f is well correlated with the presence of the oxidized form of the first secondary donor, Z^+, and it seems reasonable to compare their results with ours. Fast transients

of signal II_f are observed in normal chloroplasts (pH 7.6) [28] whereas the signal becomes evident after a treatment destroying oxygen evolution. These treatments also give rise to an elevated level of the 120 μs phase in the luminescence decay. In the presence of DCMU the signal II_f may be observed in Tris-washed chloroplasts during the first flashes until the inactive state ZPQ^- is reached whereas in the presence of an efficient electron donor, (they use phenylenediamine or hydroquinone) only during the first flash is the signal II_f apparent. If one compares this experiment with the one reported here with tetraphenylboran, the resemblance is striking: the 120 μs phase in the luminescence is still present after the first flashes with Tris-washed chloroplasts in the presence of DCMU, whereas after addition of tetraphenylboran as a donor, this luminescence is absent.

A difference between their measurements and ours however is that at normal pH we could not find relief from the DCMU inhibition by a high concentration of ferricyanide as they did. At pH 4.3 we found an approx. 50 % restoration of the amplitude of the medium phase ($t_{\frac{1}{2}} = 240$ μs at this low pH) by ferricyanide, indicating a strong pH dependence of a redox mediator close to Z (this point is currently under investigation). A high amplitude of the 120 μs phase correlated with Z in its oxidized form can easily be explained by assuming the 120 μs phase to be caused by the "intrinsic" back reaction between P^+ and Q^- which is highly favoured if Z is oxidized. Van Gorkom and Donze [7] explain Zankel's [1] 200 μs phase in this way. Zankel himself has more prudence in the explanation of his 200 μs phase; he also allows a correlation with only the decay of Q^- – the intrinsic back reaction must be assumed to be much slower in that case – because in the fluorescence decay he finds a 200 μs component (see Zankel [12]). In our case an explanation like this is improbable because the steady state level of fluorescence during the flashes in Tris-washed chloroplasts is very low (compare Ref. 29), close to f_0. One might rather ask the question is there a minimum level of Q^- required to give the intrinsic back reaction? Apparently the presence of Z^+ is much more important, the much faster stabilization steps at the donor side underlie this phenomenon, re-excitation of the reaction center in the state Z^+PQ in our experiments is possible due to the long flash duration.

Nevertheless, variations in the Q^- level do not permit more than qualitative assumptions here. The hypothesis that a high amplitude of the 120 μs phase in the decay indicates a relatively high concentration of Z^+ is very useful also for explaining our pH experimental results. Oxidation of water leads to proton liberation at the inside of the thylakoid [30]. Conversely, when the inside of the thylakoid already has a low pH, the water oxidation is retarded or inhibited and it must be assumed that positive charges are stocked at the donor side of System II leading to a relatively high concentration of Z^+, and this in its turn to a stimulation of the 120 μs phase in the luminescence decay (this argument is comparable with the one proposed by Kraan et al., [31]). The difference between uncoupled and normal chloroplasts (Fig. 5a) may well be explained by the internal acidification of the thylakoid in the latter case, which causes a higher level of the 120 μs phase in the pH region 5–7.5 compared to the uncoupled case. The high level of the 120 μs phase in uncoupled chloroplasts at pH > 8.5 is due to inhibition of the water-splitting site, by the direct exposure of this site to the high pH (compare Ref. 32). Harth et al. [33] showed that the inhibition of electron transport by uncouplers at high pH could be restored by simple washing or by the addition of an electron donor (benzidine-ascorbate). In our experiments, Table 1, CCCP at a

high concentration caused a large decrease of the amplitude of the 120 μs phase at high pH, whereas CCCP at uncoupling concentrations showed the same effects as the ionophores. CCCP at high concentration was shown to have an effect quite similar to that of the electron donor semicarbazide (Table II). Apparently it acts as a catalyst, providing electrons to the donor side of Photosystem II, probably from the plastoquinone pool [13, 24]. The notion that the internal pH – and also ΔpH – are important as controlling factors in electron transport is now well established [20, 34]. It explains for instance the shifts in pH optimum of steady-state oxygen evolution upon uncoupling (see the discussion by Trebst [41]). Our experiments here provide additional evidence: the internal pH of the thylakoid has an important influence on the redox state of steps leading to water oxidation (Fig. 5a) which will often be rate-limiting steps for electron transport via Photosystem II. If one compares the level of the 120 μs phase at for instance pH 7.5 in normal chloroplasts with the same level in uncoupled chloroplasts one has to go down 2.5 pH units indicating an internal acidification of at least 2.5 pH units in the coupled chloroplasts. At pH 6.0 this decrease is about 1.5 pH units (compare refs 20, 35). At pH 8–9 in normal chloroplasts the amplitude of the 120 μs phase is minimal indicating a fast reduction of Z^+ by the water-splitting system. At these external pH values an optimal internal pH of about 5.5 seems to be established [20]. This optimum internal pH in uncoupled chloroplasts is established at an external pH of about 7 where the amplitude of the 120 μs phase is also minimal. According to Bamberger et al. [34] it is not merely the internal thylakoid pH that is rate controlling but rather a "membrane pH" which may be understood from our experiments to be the pH localized in the thylakoid membrane close to the sites where positive charges are accumulated. At a very low external pH (< 4.5) uncoupling has no further influence, probably at this low pH the thylakoid membrane has become leaky to protons.

Our findings are in agreement with those of Lumpkin and Hillel [4] on Chlorella, who found, after various treatments, a distinct 110 μs phase in their luminescence decays. Qualitatively we are also in agreement with the results of Lavorel [3, 6] on Chlorella; he found however a faster decay in the presence of hydroxylamine, maybe in part due to the difference in the temperature employed. Further research to compare and characterize the decays in Chlorella and spinach chloroplasts more precisely are in progress.

If our assumption that the 120 μs phase is due to the intrinsic recombination, a 120 μs component should also be visible in P680 and Q. Decay times close to 120 μs have in fact been reported [12, 36] for both P and Q. A difficulty is the 35 μs component in the P680 decay reported by Gläser et al. [37]. This decay time, which is explained by the reduction of P^+ by Z is too slow compared with the 120 μs for the intrinsic back reaction to permit efficient photosynthesis. From this point of view the proposal of Den Haan et al. [15] and Duysens et al. [38] of a reduction time of P^+ by Z shorter than 1 μs seems more reasonable.

Nevertheless there is much evidence for the existence of a 35 μs component [1, 12, 14], which may be due to a secondary charge movement at the donor side of System II.

The millisecond emission of luminescence

The effects influencing the 120 μs phase extend also to longer times, but here

the effects may be masked or complicated by reversed electron flow from pools where the charges generated by the photoactivity are stored during the relaxation of the reaction center. As is shown in Fig. 5b the luminescence emission at 2 ms of normal chloroplasts (compare Ref. 19) is higher at all measured pH values, apparently due to their ability to form a pH gradient. Also Tris-washed chloroplasts which do not form a pH gradient have a higher ms luminescence emission than uncoupled chloroplasts; in this case we must assume that this is primarily due to the charge accumulation at the donor side of System II reflected as the electrical component of the membrane potential.

Uncoupled chloroplasts apparently are not able to build up either the chemical (ΔpH) or the electrical component, the latter being prevented by electron donation by the water-splitting system. At more extreme pH inhibition of electron transport via Photosystem I may also play a role.

It seems interesting to compare our results with those obtained by Wraight et al. [39]. They observe a stimulation of the 100 ms luminescence at low pH, $\leqslant$ 5.0, and at high pH in uncoupled chloroplasts, which they attribute to an inhibition of electron flow as is done here, but in the presence of DCMU the 100 ms luminescence is inhibited above about pH 8, as opposed to pH 5–7. This latter observation is in contrast with our observations on the 120 μs phase in the presence of DCMU, which has a constant level over the pH range 5–9. They explain the differences in their results on uncoupled chloroplasts with those on coupled chloroplasts [19] by the pH difference at the inside of the thylakoid between coupled and uncoupled chloroplasts: the low internal pH in coupled chloroplasts is supposed to change the redox environment at the donor side. As is shown here this is also true in the 100–400 μs region, dominated by the 120 μs decay. Wraight et al. [39] also did measurements on the behaviour of the O_2 evolving system: using flash series illumination they showed that in uncoupled chloroplasts at extreme pH values there was a build up of the oxygen precursor state S_3. This result is quite compatible with our assumption of charge accumulation at the donor side of System II under such conditions.

Interesting results were obtained by Velthuys [40], who showed, by means of flash sequence studies, that 40 ms luminescence, when the pH was increased to 10 before the last flash of a series, had the same flash number dependence as Zankel's [1] 200 μs component. If the pH was decreased to a low value, $\approx$ 4.5, the same phenomena were observed but also the dark luminescence increased. This may mean that inhibition by low or high pH isolates the accumulated charge including S_3 from the water-splitting system but not from the primary donor. The increase of luminescence in the dark upon acidification may be due to a pH-dependent redox potential of an intermediate where this positive charge is stored.

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