STIMULATION OF DELAYED FLUORESCENCE OF CHLOROPHYLL A

IN SPINACH CHLOROPLASTS BY ELECTRICAL POTENTIALS AND pH GRADIENTS ACROSS THE THYLAKOID MEMBRANE INDUCED BY SALT OR ACID-BASE TRANSITION

G.P.B. KRAAN

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS, DR. W.R.O. GOSLINGS, HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE, TEN OVERSTAAN VAN EEN COMMISSIE UIT DE SENAAT TE VERDEDIGEN OP WOENSDAG 29 SEPTEMBER 1971 TE KLOKKE 15.15 UUR

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CONTENTS

CHAPTER I

INTRODUCTION

1.1 The photosynthetic apparatus

The photosynthetic apparatus in higher plants is situated in the thylakoid membranes. The thylakoids are flattened sacs[1] within the chloroplast.

1.1.1 Architecture. A schematic picture of the chloroplast in mesophyll cells is given in Fig. 1.1.

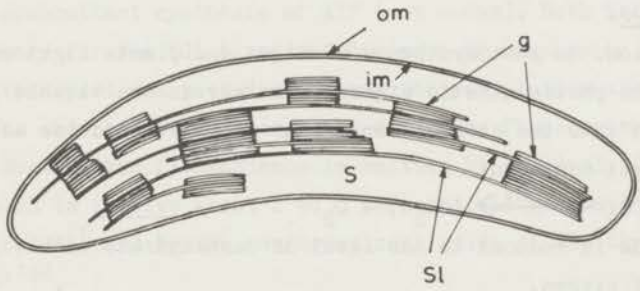

Fig. 1.1. Schematic representation of a chloroplast of higher plants. om - outer-membrane; im - inner-membrane; g - granum, stack of thylakoids; sl - stroma lamellae or large singular thylakoids; s - stroma.

The chloroplast is surrounded by two membranes which enclose the photosynthetic membranes and the stroma (s). The outer-membrane (om) is permeable to molecules of low molecular weight like sucrose in contrast to the inner-membrane (im) which is shown to have some specific ion translocation systems[2] just like in mitochondria. The photosynthetic lamellae are formed by the grana (g), or stacks of thylakoids, and by the stroma lamellae (sl), which consist of larger thylakoids and which interconnect the grana. All these lamellae or thylakoids are closed membranes with osmotic activity to ions and non-electrolytes[3,4].

Within these membranes the pigment systems (photosystems), the

electron transport chain and the photophosphorylation system are located. There is evidence that the thylakoids of the grana contain both photosystem 1 and photosystem 2, whereas the stroma lamellae have only photosystem 1 (ref. 5). The stroma or inter-membrane space contains enzyme systems for synthesis of carbohydrates, proteins and nucleic acids.

The length of an intact mature chloroplast varies from 3 to 10 μ and its thickness is 1 - 2 μ. The thylakoids of the grana have lengths from 200 to 600 nm (refs. 5, 6), whereas the thickness of the thylakoid membranes was found to be 130 Å (spinach[7]). The interlamellar distance (thickness of the internal thylakoid space) was reported to be about 200 - 220 Å (spinach[7,8]). The thickness of the thylakoid and its membranes decreases upon illumination by about 20 - 30 % (refs. 7, 8), at least in vivo.

1.1.2 Function. In photosynthesis of algae and plants light energy absorbed by the photosynthetic pigments, mainly in the visible region, is converted into the chemical energy of organic molecules according to the overall equation:

$$H_2O + CO_2 \xrightarrow{\text{light}} (CH_2O) + O_2 \qquad (1)$$

Carbondioxide is reduced to the level of carbohydrate with concomitant evolution of oxygen.

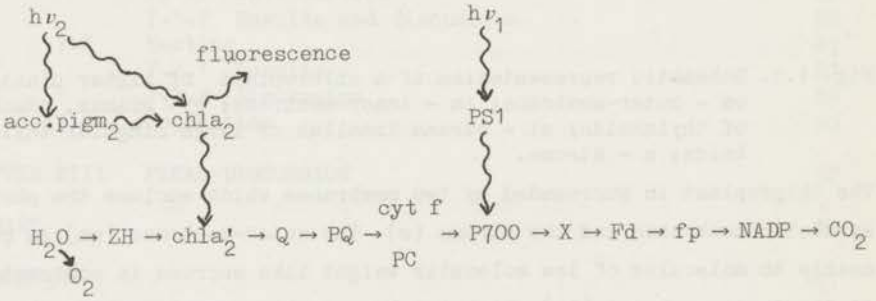

Fig. 1.2. Simplified scheme of the electron transport chain in photosynthesis of algae and plants. ZH - primary electron donor to PS2; $chla'_2$ - chlorophyll a of the reaction center of PS2; Q - primary electron acceptor of PS2; PQ - plastoquinone; cyt f - cytochrome f; PC - plastocyanin; P700 - chlorophyll a of the reaction center of PS1; X - primary electron acceptor of PS1; Fd - ferredoxin; fp - flavoprotein; NADP - nicotinamide-adenine dinucleotide phosphate.

The light energy ($h\nu_2$, $h\nu_1$) is absorbed by the accessory pigments and chlorophyll a in both pigment systems[21-23] (see Fig. 1.2). The excitation energy then is transferred by dipole-dipole interaction to the photoactive chlorophyll molecules, chlorophyll a_2' and P700, the reaction centers of pigment system 2 (PS2) and 1 (PS1), respectively. These molecules then become oxidized with concomitant reduction of the primary acceptors Q and X and are reduced in the subsequent dark reaction by the primary donor ZH and by cyt f or PC, respectively. The arrows indicate the direction of the electron transport. The electron carriers between the two reaction centers become reduced upon photochemical activity of PS2, and oxidized upon that of PS1. By means of the two photoacts[21-23] electrons are transported from H_2O to $NADP^+$ (refs. 9, 10) with concomitant synthesis of ATP (not shown). Both NADPH and ATP are necessary for the CO_2 fixation. For sake of simplicity cyclic electron transport and pathways to photophosphorylation are omitted. Part of the absorbed light energy is emitted as fluorescence of chlorophyll a. Most of the fluorescence is emitted by chlorophyll a_2. Its yield, which is *in vivo* about 2 to 8 per cent, is governed by the redox state of Q[26] and by the conformational state of the thylakoid membranes[141,142].

1.2 Delayed fluorescence

1.2.1 Early experiments. Luminescence* or delayed fluorescence emitted by photosynthetic preparations was discovered in 1950 by Strehler and Arnold[13] in an attempt to demonstrate formation of ATP with the firefly-luminescence assay system. Control experiments, however, showed that delayed light emission also occurred in the absence of the luciferin-luciferase system, that this light was emitted in the red instead of the green region of the spectrum, and that in contrast to the firefly-bioluminescence, which is induced by the high-energy compound ATP,

*Luminescence and delayed light emission from photosynthetic organisms have exactly the same meaning as delayed fluorescence, i.e. a reexcitation of the first singlet state of chlorophyll a_2 (algae and higher plants) from a metastable state induced by the light.

this luminescence was induced by light.

The light emission persisted for several minutes, i.e. long after the prompt fluorescence, which has a lifetime of the order of 1 nsec, had died out. Its emission spectrum as measured with Chlorella turned out to be similar to that of chlorophyll a fluorescence[13,14], which indicated that the luminescence was emitted by the first excited singlet state of chlorophyll a. The intensity of this delayed fluorescence from Chlorella was measured as a function of temperature between 0 and 50° C and was found to be optimal at about 37° C. The luminescence was measured about 0.2 to 1 sec after illumination. This indicated that (an) enzyme-catalyzed reaction(s) (was) were involved[13]. The activation energy was calculated to be about 19.5 Kcal ($\triangleq$ 0.85 eV). Following Duysens' discovery of light-induced changes in absorbance at 480 and 515 nm[15] Strehler and Lynch[16] demonstrated that the change at 515 and also the change at 648 nm paralleled in many respects delayed light emission with regard to the time course (induction) and dependency on illumination intensity. Strehler[17] also showed that the shape of the induction curve of luminescence in Chlorella was similar to that of fluorescence. The action spectra of delayed light of blue-green, red and green algae and of spinach chloroplasts[18,19] were also found to be similar to those of fluorescence[20] and showed a high activity of the phycobilins in the red and blue-green algae due to energy transfer[20] from these accessory pigments to chlorophyll a.

When evidence for the existence of two pigment systems in photosynthesis had been found[21-23] (see Fig. 1.2 for the overall scheme of photosynthetic electron transport) Goedheer[24] observed that light absorbed by the short-wavelength system, called pigment system 2 (ref. 21), caused luminescence whereas light absorbed by the long-wavelength system, called pigment system 1 (ref. 21), quenched luminescence. Bertsch[25] obtained similar results. At very low intensities of exciting light, however, the additivity[24,25] of the luminescence signals induced by both sorts of light given at the same time was demonstrated and no quenching effect could be observed. The action spectra of luminescence[24] of Chlorella and the blue-green alga Synechococcus were roughly similar to the corresponding spectra mentioned before[18,19].

1.2.2 Relation between luminescence and the redox state of the primary acceptor of pigment system 2. The yield of chlorophyll a fluorescence in pigment system 2 is postulated to be governed by the oxidation-reduction state of the quencher Q, the primary electron acceptor of this photosystem[26] (see Fig. 1.2). When all Q is oxidized, due to photochemical activity of photosystem 1 or to a long period of darkness, the yield of fluorescence is minimal. Upon absorption of light by photosystem 2, Q becomes reduced with a concomitant rise of the yield of maximally up to 4 times the base or minimal yield (see also refs. 27 - 29). Duysens[30] suggested that the luminescence measured by Goedheer[24] depended on two factors: (1) a chemical reaction, which excites chlorophyll a_2 and (2) the yield of prompt fluorescence. An increase in luminescence could be partly due to a higher yield of fluorescence of chlorophyll a_2. The quenching of the luminescence intensity[24] by action of pigment system 1 may then be due to oxidation of Q[26]. A similar suggestion was made by Franck and Rosenberg[31].

From measurements of delayed light from minutes up to very short darktimes (5.10^{-5} sec) Arnold and Davidson[32] concluded that luminescence, excited by saturating light, would constitute an appreciable part of the prompt fluorescence yield when the curve as represented in a log-log-plot was extrapolated to the lifetime of fluorescence. However, later on Arnold[33] found that the luminescence curve levelled off at short darktimes if the exciting light was non-saturating. The result of the new extrapolation was that at most 1 % of the prompt fluorescence might be rapidly decaying delayed light. Müller and Lumry[34] analyzed the prompt and the delayed fluorescence at very short darktimes ($t \ll 5.10^{-5}$ sec). Using an excitation beam modulated at very high frequencies they found that less than 10 % of the total emission, as compared to that obtained e.g. with fluorescein, which did not give luminescence, might be luminescence from bulk chlorophyll a_2 molecules, which were not reexcited via the reaction center of PS2. This luminescence, namely, was not changed by DCMU, hydroxylamine[35] or heat treatment[24], and therefore could only be part of the above mentioned minimal yield of fluorescence.

Evidence that delayed light does not occur in the absence of prim-

ary photochemistry was obtained by Clayton and Bertsch[36] and by Bertsch and coworkers[37]. A photosynthetically inactive mutant of the bacterium Rhodopseudomonas spheroides without P870 did not luminesce in contrast to the wild type[36], whereas two non-photosynthetic mutants of the green alga Scenedesmus obliquus, mutant 11 (no photoreaction 2) and mutant 8 (no photoreaction 1) emitted about 250 times less and 3 times more delayed light, respectively, than the wild type at 1 msec of darktime[37].

Azzi[38] measured very carefully the emission spectra of delayed (1 msec) and prompt fluorescence for several algae and found that the luminescence spectra of the green algae Chlorella and Scenedesmus were identical to the fluorescence spectrum showing the emission of chlorophyll a with a maximum at 685 nm. These results were in agreement with earlier results of Arnold and coworkers[13,14,18]. Whereas the fluorescence spectra of the blue-green alga Anacystis and the red alga Porphyridium also showed emission of the accessory pigments , the phycobilins, the relative contributions of which were dependent on the wavelength of the exciting light, the luminescence spectra showed the emission of chlorophyll a and at most only a minor contribution of phycocyanin.

The similar kinetics of the variable fluorescence and of the 3 msec-delayed fluorescence of the green bacterium Chloropseudomonas ethylicum and the algae Chlorella and Anacystis together with the different emission spectra between the initial and the variable fluorescence, the latter being proportional to the luminescence spectra[39], were explained by Butler[40]. By a reasoning similar to that of Duysens (see above) he proposed that the yield of luminescence, caused by re-excitation of chlorophyll a of system 2, should be, like the yield of the prompt fluorescence, dependent on the oxidation-reduction state of Q[26]. In the case of the bacterial emission spectra similar arguments were used[40] as those mentioned above (see Azzi[38]).

Lavorel[41,42] and Clayton[43] measured quantitatively the relationship between luminescence and fluorescence. Evidence was obtained by Lavorel that the intensity of delayed fluorescence of algae is proportional to the yield of prompt fluorescence and also, under certain conditions, proportional to the yield of variable fluorescence, which

suggests a linear relationship to the concentration of the reduced primary electron acceptor Q^-. Clayton[43] found the yield of luminescence in isolated chloroplasts to be proportional to what he called "live-fluorescence", which was assumed to be only slightly larger than the variable fluorescence. He also showed that ferricyanide added to chloroplasts illuminated with strong light initially decreased the 250 msec-delayed light. This decrease was proportional to the decrease of the "live" fluorescence. DCMU[26] added at low light intensities increased the delayed fluorescence[43] proportionally to the increased "live" fluorescence. Both phenomena are thus related to the amount of Q^-, which is depressed by reoxidation upon addition of ferricyanide and increased by DCMU. This inhibitor of electron transport prevents the reoxidation of Q^- by electron carriers between the two pigment systems[26]. Continued illumination in the presence of DCMU caused the luminescence to decline to a small fraction of its initial value, presumably due to inhibition of photochemistry which should exhaust the concentration of oxidized ZH.

However, at high light intensities and at very short darktimes (msec-range) addition of DCMU caused an immediate drop of delayed light emission. Here DCMU could not further increase the "live fluorescence yield" (amount of Q^-), so the remaining activity of DCMU is blocking stimulation resulting from electron transport and lowering the concentration of oxidized ZH (see above). Under the same conditions, but without DCMU, ferricyanide caused an initial inhibition of delayed fluorescence (5 msec) followed, however, by a stimulation higher than necessary to overcome only the initial inhibition. This stimulation is maybe explained by a completely different phenomenon which was recently discovered in different laboratories and which is described in Chapter V. This phenomenon is associated with a higher electrochemical potential difference across the thylakoid membrane caused by a rapid electron transport.

Taken together these results and the earlier mentioned, like the luminescence action spectra[18,19,24], which demonstrated high activity of light absorbed by pigment system 2 (see also refs. 24, 25), the need of functional reaction centers[36,37], and the various emission

spectra[38,39], these facts indicate that luminescence or delayed fluorescence of algae and higher plants might be caused by the back reaction of the primary photoproducts[42,47] of pigment system 2 causing reexcitation of chlorophyll a of this photosystem.

1.2.3 Relation between luminescence and the redox state of the donor(s) of pigment system 2. In the previous section it was described that in the presence of DCMU luminescence, excited by weak light and measured at 250 msec of darktime, finally was inhibited[43]. It was proposed that this inhibition was due to a diminished concentration of the oxidized primary donor to PS2 caused by a blocked photochemical reaction.

Recently Kok and coworkers[44] described a model to explain the relation between oxygen evolution and its precursers. Based on results obtained by Joliot and colleagues[45] they proposed a four linear step mechanism, in which the reaction center of PS2 or an associated donor, S, accumulates 4 +charges before one molecule of oxygen is evolved. Each of these 4 steps needs a photochemical turnover in the reaction center:

$$S_o \xrightarrow{h\nu} S_1^+ \xrightarrow{h\nu} S_2^{2+} \xrightarrow{h\nu} S_3^{3+} \xrightarrow{h\nu} S_4^{4+} \longrightarrow S_o$$

$$2H_2O \quad O_2 + 4H^+$$

Further they obtained evidence that in the dark 75 % of the reaction centers was in the stable state S_1^+ and 25 % in state S_o.

Jones[46] concluded from flash experiments that dark-adapted cells of Chlorella emitted only delayed fluorescence when two or more flashes had been received. More detailed information about the mechanism which causes this effect was obtained by Barbieri and coworkers[47]. They found that when a series of short flashes of light was given after a sufficiently long dark period, the 20 msec delayed fluorescence showed an oscillatory pattern with a periodicity of 4. This was apparently related to a similar oscillatory pattern observed for oxygen evolution[44,45]. The difference was that the luminescence was maximal after the second flash and that of oxygen evolution after the third flash. At shorter times (50 μ sec) 3 flashes were needed[48] to get the highest intensity of delayed fluorescence, just like oxygen evolution, so that S_4^{4+} might be the precursor of a very rapidly decaying luminescence component.

In terms of the hypothesis of Kok et al.[44] it might be that the oxidized donor to PS2 necessary for the luminescent reaction is S_3^{3+} if the darktime $t_d \geqslant 1$ msec and S_4^{4+} if $t_d \ll 1$ msec.

1.2.4 "Chemiluminescence" or stimulated delayed fluorescence. As would be expected from the early concept of Mitchell's chemiosmotic hypothesis[49] on the coupling of electron transport to phosphorylation Neumann and Jagendorf[50] demonstrated that unbuffered chloroplasts showed an increase in pH in the medium upon illumination, which disappeared after the light period. This light-induced change in pH, leading to an acidification of the interior of the thylakoid membrane was shown to be correlated with a so called high-energy state necessary for photophosphorylation, which was thought to be driven by effluxing protons through the membrane-bound reversible ATP-ase system[49]. A pH gradient across the thylakoid membrane, however, could also be formed artificially, and Jagendorf and Uribe[51] succeeded in the demonstration of a high yield of ATP formed by the disappearance of such a gradient, which was brought about by an acidification of the chloroplast suspension (down to about pH 4) followed by a rapid mixing with base, giving a final pH of about 8.5. Light was not needed for ATP formation in these experiments. The artificially induced phosphorylation[51] was also inhibited by the known uncouplers of photophosphorylation[52-54].

Meanwhile Mayne[55] had obtained some evidence that the delayed fluorescence, measured after 4 msec of darktime, was also related to the high-energy state. This evidence was based on the finding that uncouplers of photophosphorylation inhibited this delayed fluorescence. If instead of uncouplers the complete phosphate acceptor was added the luminescence intensity was lower, but in the presence of the so called "energy transfer inhibitors", which inhibit between the high-energy state and the phosphorylation of ADP, this inhibition of light emission was reversed.

These results[51,55] suggested to Mayne and Clayton[56] that the high-energy state may cause luminescence and that such an acid-base transition could bring about delayed fluorescence. This was found to be true and the pH jump caused a flash of luminescence, the emission spectrum of which was roughly similar to that of prompt fluorescence of chloro-

phyll a. The induced light emission was inhibited by uncouplers of photosynthetic phosphorylation and also by DCMU, which blocks electron transport close to the reducing side of pigment system 2 (ref. 26), but not the acid-base transition induced phosphorylation[51]. Later on Mayne[57] found, however, that preillumination was required for the acid-base induced luminescence which was measured 20 sec after switching off the light. This is understandable, because the energy of the emitted red light is about 1.81 eV per photon (685 nm), which corresponds to 42 kcal.einstein^{-1}, whereas a pH gradient of 4.0 pH units provides only 5.4 kcal.Mole^{-1} per hydrogen ion at 20° C. The luminescence spike might be the result of the pH gradient[58], which provided the activation energy for the delayed fluorescence[57]. "The pH transition triggers the release of light from some metastable state within the chloroplast"[57]. Another possibility proposed was the change of redox potentials of one or more intermediates in the electron transport chain causing reversed electron transport to the luminescence reaction[57]. A further discussion of these points is given in Chapter VIII. The action spectrum of the pH jump induced luminescence was suggested to be that of pigment system 2, because at wavelengths larger than 680 nm a red drop was observable[57]. Varying the intensity of exciting light Mayne[57] found that the metastable state needed for the induced spike of luminescence was formed via an apparent first order process, whereas its decay was second order.

Miles and Jagendorf[59] and independently Barber and Kraan[61] demonstrated that addition of salts to preilluminated chloroplasts also induced considerable intensities of luminescence emission (t_d = 10 sec) as did a sudden decrease of the pH below 4 (ref. 59). Both phenomena were inhibited by uncouplers of the photophosphorylation and by DCMU[59] just like the acid-base induced luminescence[57] and had about the same emission spectrum. A more detailed study of the effect of uncouplers will be reported in Chapters IV, V and VI (of this thesis). The results there will show that the inhibition by uncouplers was dependent on experimental conditions applied to bring about luminescence. A striking difference between the addition of salts and the acid-base transition was that in contrast to the latter[51,60] the former treatment did not cause ATP formation or ATP-ase activity[59]. No further conclus-

ions, however, were obtained by Miles and Jagendorf about the mechanism of the salt-induced luminescence. Evidence was also presented that the acid-base induced phosphorylation and luminescence must have at least partly different mechanisms and could be inhibited separately[59], although these processes were initiated by the same pH gradient.

In this thesis evidence will be presented that the stimulation of delayed fluorescence by addition of salt to chloroplasts is caused by a diffusion potential across the thylakoid membranes. This potential stimulates the back reaction between the charged photoproducts of system 2, which are located at opposite sides of the membrane. As will be discussed, the stimulation by a pH gradient, formed by an acid-base transition is probably due to a shift in pH-dependent equilibria of components on both sides of the membrane.

As will be shown the integrity of these membranes determines strongly the extent of the stimulation of delayed fluorescence.

Part of the results described in the following chapters have also been published elsewhere[61-63].

CHAPTER II

MATERIALS AND METHODS

2.1 Isolation of chloroplasts

Chloroplasts were isolated from leaves of spinach (Spinacea oleracea), obtained from market stores or grown in a greenhouse (var. "Nores" or "Glares"*). For some experiments the leaves were washed and macerated in a blendor at 0 - 4° in a solution of pH 7.8 containing 0.4 M sucrose and either 0.05 M Tris-HCl, 0.05 M N-tris(hydroxymethyl)-methylglycine (Tricine)-KOH or 0.05 M N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-KOH. The homogenate was filtered through two layers of nylon or perlon gauze (mesh width 60 - 80 μ), and the filtrate was centrifuged at 1000 x *g* for 5 min. The chloroplast pellet was resuspended in the same buffered sucrose solution and stored at 0° in the dark. Chloroplasts isolated in this way will be referred to as "normal chloroplasts".

For other experiments chloroplasts were isolated from freshly picked leaves (greenhouse) by a method described by Kraayenhof[64]. Briefly, the leaves were cut into small pieces in cold 0.4 M sucrose with 0.05 M Tricine, brought to pH 7.8 with NaOH or KOH, and the chloroplasts were gently squeezed from the leaf fragments by means of a ribbed perspex mortar and a ribbed teflon roller, filtered through two layers of perlon net (56 μ) and centrifuged at high speed (up to 8000 x *g*) for a short time. After gentle resuspension in the same buffer as used for the isolation the chloroplasts were stored at a high concentration (1.10^{-3} - 5.10^{-3} M chlorophyll) on ice in the dark. This procedure yielded at least 50 % Class I chloroplasts (as defined by Spencer and Unt[65]). Chloroplasts prepared by this method will be referred below to as "Class I chloroplasts".

The chlorophyll concentrations were determined according to Whatley and Arnon[66].

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* Thanks are due to D. van der Ploeg, Barendrecht, who kindly donated the seeds.

2.2 Apparatus and procedure

In order to study the stimulation of delayed light emission caused by a change in the concentrations of ions in the medium a mixing device was constructed. Two possibilities were considered. The first was to add the mixing solution to the preilluminated chloroplast suspension in front of the photomultiplier, which would enable a continuous observation of the luminescence but would also result in a less rapid and efficient mixing. The second one, which we chose, was to mix the suspension with the mixing solution outside the measuring cuvette, subsequently transport the suspension into the cuvette and measure with some small delay the luminescence intensity. This delay was, however, much shorter than the delay, which would have been occurred by the less efficient mixing in the first method.

The apparatus used consisted of the following parts (see Fig. 2.1a).

2.2.1 Preillumination chamber. This consisted of a one mm perspex cuvette, attached to a shutter on one side and to an identical one on the other side. These cuvettes contained the chloroplast suspension and the first mixing solution, respectively. For the acid-base experiment the first solution was the acid. For both the control and the salt experiments the cuvette was filled with buffer (pH 7.8).

2.2.2 Mixing device. After preillumination the contents of both vessels were mixed and transferred by means of pressed air and within 1 sec into a stainless steel syringe (see Fig. 2.1b). An identical one was mounted in a parallel position close to the first syringe and contained the second mixing solution, which in the sequence of the type of experiments, mentioned in Section 2.2.1 was base, buffer or salt solution. The exits were connected via stainless steel tubes with a Y-shaped tap made from a cylindrical piece of teflon surrounded by a stainless steel housing. When this tap was opened the contents of the syringes were expelled automatically into a common delivery pipe, by means of an air-driven piston attached to both plungers, and transported into the measuring cuvette at such a speed that turbulent mixing occurred in the mixing tube. The total mixing time used with most experiments described in this thesis was about 250 msec. In this time 2.0 ml chloroplast sus-

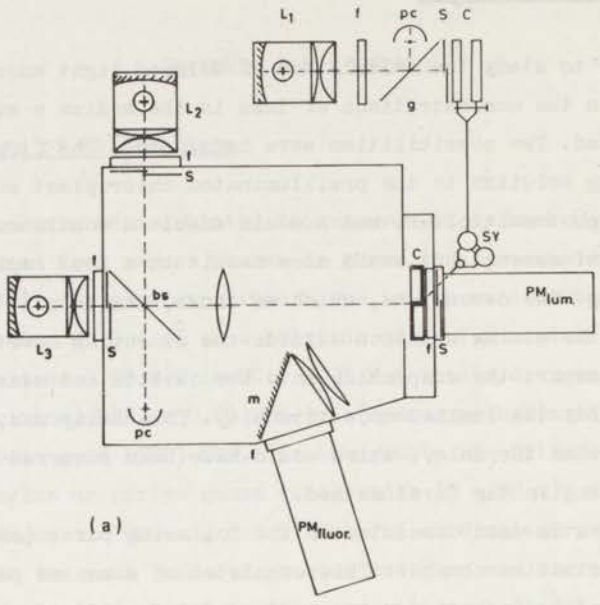

Fig. 2.1a. Survey of the apparatus (partly schematic) for measuring delayed and prompt fluorescence. PM - photomultipliers; m - mirrors; c - cuvettes; f - filters; s - shutters; g - a glass plate; bs - a beam splitter. The lamps L_1 and L_2 served to provide the preillumination and the fluorescence excitation light, respectively. L_3 provided preillumination in the experiments of Table 6.4 and the results described in Chapter V. The light intensities were measured by means of calibrated photocells (pc). Further details are given in the text.

pension and 2.0 ml of mixing solution were pressed from the syringes into to the measuring cuvette. Because the filling time of the part of the cuvette that was viewed by the photomultipliers was approximately 200 msec, the luminescence of each individual particle could not be registrated during about 50 msec after mixing. This was the time of transport of the mixed suspension from the mixing point (in the tap) just to the bottom of the observable part of the measuring cuvette (Fig. 2.1b). The average speed during this transport through the common delivery pipe (inner diameter 0.2 cm) in which turbulent flow occurred, necessary for satisfactory mixing, was about 5.10^2 cm.sec^{-1}.

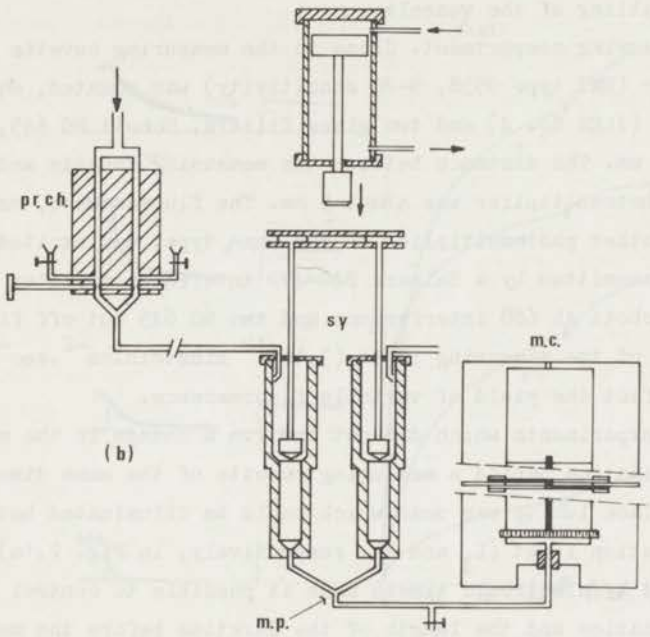

Fig. 2.1b. Details of the mixing procedure in a schematic drawing. pr ch - preillumination chamber; sy - metal syringes; mp - mixing point: the Y-shaped tap; mc - measuring cuvette. In the middle of the measuring cuvette a horizontal cross section through it is given. Further details are given in the text.

Just before the suspension came into view of the photomultipliers this speed was reduced to about 20 $cm.sec^{-1}$.

Some of the experiments were performed within a shorter time as reported earlier[61]. Then the two speeds were respectively about $1.8.10^{3}$ $cm.sec^{-1}$ and 70 $cm.sec^{-1}$.

2.2.3 Measuring cuvette. Because of the desirability to record both the prompt and the delayed fluorescence simultaneously a special cuvette was made (see Fig. 2.1b). It consisted of a gap of 4 x 4 cm^{2} in a sheet of stainless steel (2 mm), enclosed by two sheets of black perspex and divided by a thin wall of the same material into two identical and optically isolated halves with transparent perspex windows facing two photomultipliers. The bottom of the measuring vessel was a

2 mm layer of sintered glass situated 1 mm above the entrance to obtain uniform filling of the vessels.

2.2.4 Measuring compartment. Close to the measuring cuvette a photomultiplier (EMI type 9558, S-20 sensitivity) was mounted, equipped with a shutter (ILEX No. 4) and two glass filters, Schott RG 645, 3 mm and RG 630, 2 mm. The distance between the measuring cuvette and the front of this photomultiplier was about 5 cm. The fluorescence, measured with the other photomultiplier of the same type, was excited by blue light, transmitted by a Balzers B40-472 interference filter and filtered by a Schott AL 688 interference and two RG 645 cut off filters. The intensity of the measuring light (3.10^{-12} Einstein.cm^{-2}.sec^{-1}) was too low to affect the yield of variable fluorescence.

For experiments which did not involve a change in the medium after the illumination period a measuring cuvette of the same dimensions but without black lucite was used which could be illuminated both by actinic and excitation light (L_3 and L_2, respectively, in Fig. 2.1a). Shutters controlled by electronic timers made it possible to control the periods of illumination and the length of the darktime before the measurements of luminescence or fluorescence yields in the dark. The signals then were measured in separate experiments with the same photomultiplier close to the cuvette. The increase of the fluorescence yield caused by the actinic light beam was measured with the other photomultiplier.

The anode currents from the photomultipliers were fed into current-voltage converters and registrated by a 2-channel Clevite Brush strip-chart recorder or a Siemens oscillomink. Part of the delayed fluorescence signal was also fed into an electronic integrator connected to a recorder which plotted the integrated delayed fluorescence.

2.3 Some typical experiments

Some typical results obtained with the luminescence apparatus are shown in Fig. 2.2. The recordings in the left-hand column show the characteristic traces of delayed fluorescence. Curve a shows the delayed fluorescence in a control experiment, where the chloroplasts were diluted twice with the same buffer; upon the second mixing, 3 sec after

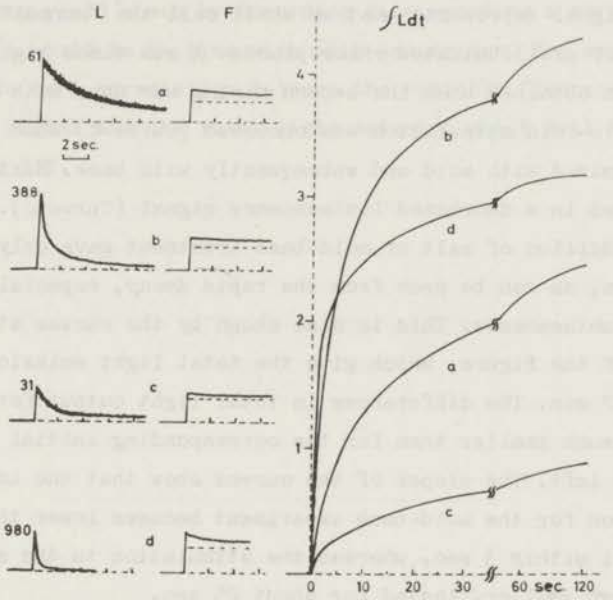

Fig. 2.2. Recordings of luminescence intensity (L), fluorescence yield (F) and integrated delayed light emission ($\int$Ldt) of normal chloroplasts (see Section 2.1). The chloroplasts, suspended in 0.4 M sucrose and 0.05 M Tricine, brought to pH 7.8 with KOH, were preilluminated for 5 sec with saturating red light. The first mixing was with the same sucrose-Tricine buffer (Recordings a, b and c) or with a solution containing 0.4 M sucrose and 0.02 M succinic acid (d, pH after mixing 4.2). The second mixing, after a darktime of 3 sec, was with buffer (a), NaCl, final concentration 0.3 M (b), succinic acid-sucrose solution (c), or with a solution of 0.3 M sucrose and 0.1 M Tris (d, pH after mixing 8.7). The final chlorophyll concentration was $7.5.10^{-5}$ M. Recording started immediately after the second mixing. The recordings of the left-hand row were made with different sensitivities of the apparatus; the numbers give the relative heights of delayed fluorescence immediately after the second mixing. The recordings for F and $\int$Ldt are given on the same relative scales. The fluorescence recordings were corrected for a contribution caused by delayed fluorescence. This correction was 29 % for the initial value of Recording d and correspondingly smaller for the other recordings. The dashed line indicates the fluorescence level after 2 min. The stimulation induced by mixing with NaCl varied with different batches of chloroplasts; it was usually 2 - 3-fold.

preillumination, the light emission was recorded (see Section 2.2). This signal represents what we shall call the "normal" delayed fluorescence of preilluminated chloroplasts. A six times higher signal (Curve b), was obtained when the second mixing was done with NaCl solution, and a 16-fold stimulation was observed (Curve d) when the suspension was first mixed with acid and subsequently with base. Mixing with acid alone resulted in a decreased luminescence signal (Curve c).

Addition of salt or acid-base treatment gave only an initial stimulation, as can be seen from the rapid decay, especially of the acid-base luminescence. This is also shown by the curves at the right-hand side of the figure, which give the total light emission, integrated over up to 2 min. The differences in total light output for Curves a, b and c are much smaller than for the corresponding initial luminescence levels at the left. The slopes of the curves show that the intensity of light emission for the acid-base experiment becomes lower than that of the control within 3 sec, whereas the stimulation in the salt experiment, although smaller, lasted for about 25 sec.

Recordings of the relative yield of chlorophyll fluorescence (middle column) showed much smaller differences than the luminescence recordings. Curve a shows the kinetics of fluorescence measured simultaneously with the luminescence for the control experiment. The fluorescence yield slowly decreased with time and after 2 min reached the level indicated by the dashed line. The latter level was approximately the same as that obtained without preillumination, and will be called "base" level. The difference between the total yield and the "base" yield will be called the "variable" fluorescence; at the beginning of the recording this amounted to about 55 % of the "base" fluorescence. The variable fluorescence is probably caused by Q^-, the primary electron acceptor of system 2 (ref. 26) which is reduced in the light and reoxidized in the dark. The base fluorescence and the initial yield of variable fluorescence were only slightly affected by salt or acid-base treatment. This indicates that the stimulated emission cannot be explained only by changes in the concentration of Q^- or in the fluorescence yield (see refs. 41-43), but is due to an increase in "intrinsic" delayed fluorescence, probably caused by a more rapid recombination of

Q^- and an oxidized product of the light reaction. The more rapid decay of the fluorescence yield shown by Recording d, suggesting a more rapid reoxidation of Q^-, would be in line with this assumption. For experiment c there appears to be a correlation between the variable fluorescence, the luminescence signal and the total light emission which will be discussed in Chapter III.

CHAPTER III

STIMULATION OF DELAYED FLUORESCENCE INDUCED BY AN ACID-BASE TRANSITION

3.1 Introduction

After the initial discovery by Mayne and Clayton[56] that an acid-base transition could bring about delayed fluorescence many aspects of this phenomenon were investigated. The most relevant fact was that this "oldest" type of stimulated luminescence was reported to exist only after preillumination[57]. This and further introductory details are discussed in Section 1.2.4.

In this chapter it will be shown that the intensity of the stimulated delayed fluorescence as induced by an acid-base transition, after saturating preillumination, is not only dependent on the size of the pH change in the medium but also on the time of acidification, the pH during the acid phase and on the type of buffer used. These latter three factors determine the pH inside the thylakoid membrane.

The permeability of the thylakoid membranes to protons is amongst other things dependent on the type of buffer used[67]. Tris-buffer and its zwitterionic analogue Tricine were used for the determination of the pH dependency of luminescence and of the acid-base jump induced stimulation of luminescence.

Chapter VI will give further information about the effect of the different buffers upon the luminescence phenomena and Chapter IV (Section 4.3) and VII will deal with other aspects concerning the stimulated luminescence brought about by an acid-base transition.

3.2 Results

3.2.1 pH dependence of delayed fluorescence. A decrease of pH after preillumination caused a decrease of delayed fluorescence. Fig. 3.1a shows the results of a series of experiments in which the chloroplasts, prepared in Tricine-KOH, pH 7.8, were subjected to sudden pH jumps by mixing with various concentrations of 2-(N-morpholino)ethanesulfonic acid

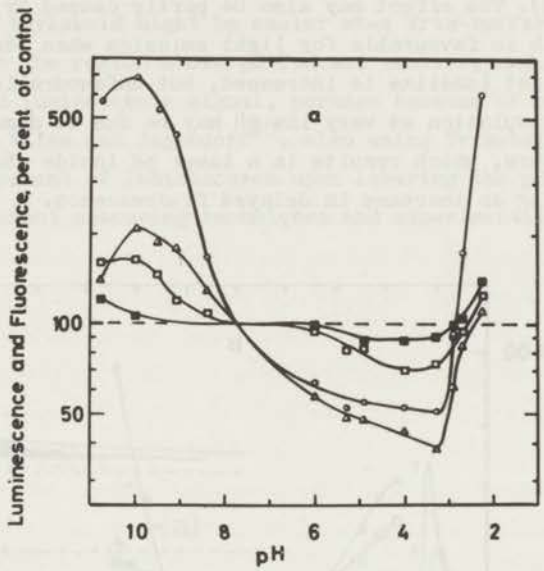

Fig. 3.1a. Effects of pH changes upon initial luminescence (O—O), integrated luminescence (Δ—Δ), base fluorescence (■—■) and variable fluorescence (□—□). The base fluorescence gives the fluorescence yields without preillumination. Initial luminescence and fluorescence were measured immediately after mixing with the solutions indicated below, as described for Fig. 2.2. Class I chloroplasts in 0.01 M Tricine-KOH buffer (pH 7.8). The pH was lowered with MES-HCl, or increased with Tricine-KOH. Darktime was 2 sec. Final chlorophyll concentration $2.5.10^{-5}$ M. The variable fluorescence yield at pH 7.8 was about 45 % of the base fluorescence yield.

(MES)-HCl or Tricine-KOH buffers. An increase of pH, up to pH 11, caused an increase in delayed fluorescence, and a decrease, down to pH 3, caused an inhibition. Decreasing to pH below 3 caused a strong temporary stimulation of delayed fluorescence. The same results were obtained when MES-HCl was partly replaced by succinic acid, in such a way that its total concentration was kept constant at 1.5 mM from pH 5 down to pH 2. The variable fluorescence was affected qualitatively in a similar way, but less strongly than the delayed fluorescence, whereas the level of base fluorescence was almost independent of pH.

It thus appears that part of the effect of pH on delayed fluores-

cence is due to a change in the yield of the variable fluorescence[43] (see Discussion). The effect may also be partly caused by the pH gradient generated, which is favourable for light emission when the pH outside of the chloroplast lamellae is increased, but unfavourable when it is lowered. The stimulation at very low pH may be due to damage of the lamellar structure, which results in a lower pH inside the thylakoid membrane, causing an increase in delayed fluorescence.

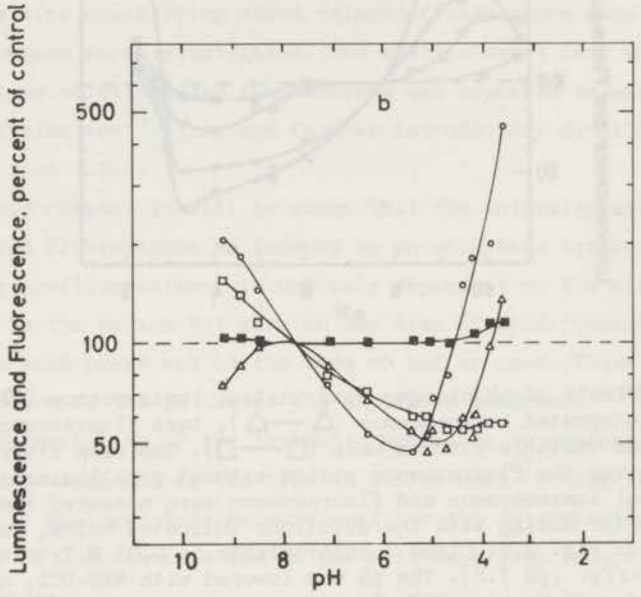

Fig. 3.1b. Effects of pH changes upon initial luminescence (O —O), integrated luminescence (Δ —Δ), base fluorescence (■ —■) and variable fluorescence (□ —□). The base fluorescence gives the fluorescence yields without preillumination. Initial luminescence and fluorescence were measured immediately after mixing with the solutions indicated below, as described for Fig. 2.2. Normal chloroplasts in 0.05 M Tris-HCl (pH 7.8). The pH was lowered with succinic acid, or increased with Tris. Darktime was 3 sec. Chlorophyll concentration 9.10^{-5} M. The osmolarity of the suspensions was kept constant at 0.4 M with sucrose throughout the experiments. The variable fluorescence yield at pH 7.8 was about 50 % of the base fluorescence yield.

Fig. 3.1b shows analogous experiments with Tris-buffers. The results were similar to those with Tricine, except that stimulation occurred

even below about pH 5.0 to 4.7, perhaps because acidification of the inside of the thylakoid might be easier when Tris-buffer[67] is used. The stimulation in the region above pH 7.8 was smaller, and was absent in the integrated luminescence signal, perhaps because of an uncoupler action by Tris. Miles and Jagendorf[59], also using Tris-buffer, observed only an enhancement of luminescence upon lowering the pH, possibly because of different measuring techniques and experimental conditions.

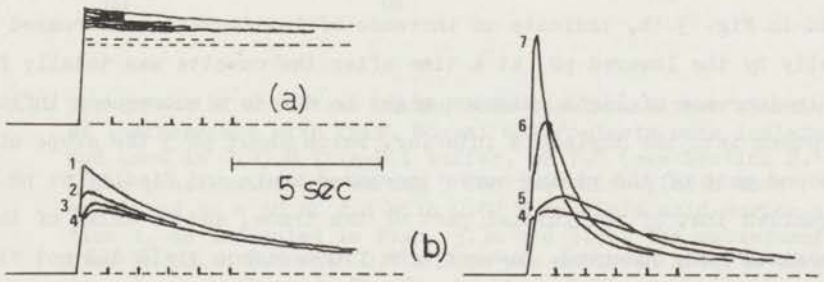

Fig. 3.2. Kinetics of luminescence and fluorescence after addition of acid to preilluminated chloroplasts in Tris-buffer. Normal chloroplasts in 0.05 M Tris-HCl buffer (pH 7.8). The pH was lowered with succinic acid. The maxima shown in (b) and in (a) indicate the luminescence values and the fluorescence yields the data of which are plotted in Fig. 3.1b. The curves in Fig. 3.2a indicate the fluorescence yield obtained (from the top downwards) at pH 7.8, 7.1, 6.3, 5.4, 4.6 and 4.2. The two dotted lines indicate the fluorescence yield without preillumination at pH 4.2 and 7.8. In Fig. 3.2b the luminescence signals are given. The numbers 1 - 7 indicate the curves at pH 7.8, 7.1, 6.3, 5.4, 5.0, 4.6 and 4.2. The other conditions as indicated in Fig. 3.1b.

From the kinetics of the fluorescence signals (see Fig. 3.2), measured after addition of acid to chloroplasts in Tris-buffer and obtained with a higher speed of the recorder, it appeared that the actual yield was decreased within the mixing time of about 50 msec. The corresponding luminescence signals showed biphasic kinetics during the filling time of the cuvette (50 - 250 msec). The initial slope (pH 6.3 - 4.6) was slightly lower than the control signal at pH 7.8, which demonstrates a small inhibition due to the lowered fluorescence yield. Subsequently the curve rose very slowly, starting just before the cuvette was filled completely and showed its maximum at $t_d \geqslant 0.25$ sec. In contrast to the control experiments, where the observed "maximum" coincided with a complete filling of the cuvette, these "delayed" maxima, plotted in Fig. 3.1b, indicate an increase of luminescence, decreased initially by the lowered pH, at a time after the cuvette was totally filled. This increase of light emission might be due to a subsequent influx of protons into the thylakoid interior. Below about pH 5 the slope of the second part of the rising curve increased again and finally at pH 4.2 equalled that of the initial part of the trace, and no delay of the "maximum" was observed. However, the fluorescence yield did not rise in this region of pH values.

A similar pattern was obtained with Tricine, although in the initial biphasic part of the luminescence curve at low pH values no change occurred unless below pH 3.5.

The slower kinetics, speeded up by a further decrease of pH or by the presence of an uncoupler (see Section 6.2), might be explained in a plausible way (see Discussion, Section 3.3). The initial slope, however, at a pH between 7.8 and 4.6 (Tris) can be understood satisfactorily in relation to the lowered fluorescence yield.

3.2.2 Effect of acidification time upon the stimulated delayed fluorescence induced by an acid-base transition. Fig. 3.3a shows the effect of the acid time on the stimulated luminescence from Tris-buffer chloroplasts induced by an acid-base transition. In comparison to the results of Fig. 3.3b where the time of acidification was kept constant and as

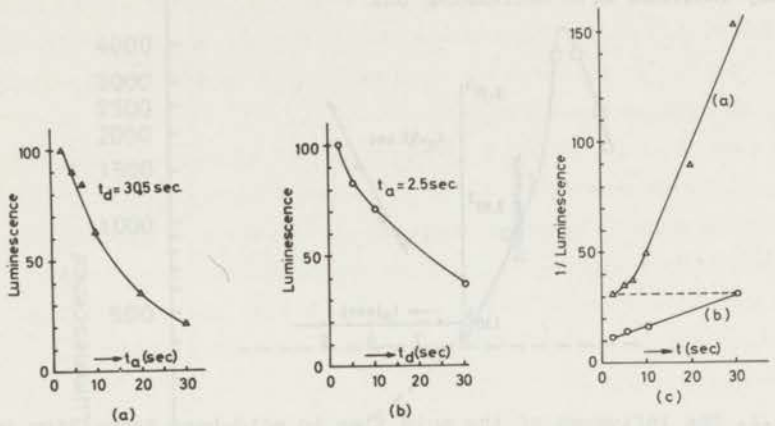

Fig. 3.3. The effect of acid time on the acid-base transition stimulated luminescence with Tris. Normal chloroplasts were isolated and used in 0.05 M Tris-HCl buffer, pH 7.8 (see Section 2.1). After preillumination during 5 sec the chloroplasts were acidified to a pH of 4.4 with 0.02 M succinic acid during a time t_a as indicated in Figs. 3.3a and 3.3b. The subsequent rise in pH to 8.6 with 0.1 M Tris was achieved after a darktime t_d as indicated. The final chlorophyll concentration was 6.10^{-5} M. Luminescence intensity is plotted in arbitrary units. In Fig. 3.3c the reciprocal luminescence intensities of Figs. 3.3a and 3.3b are plotted against the acid time (a) or darktime (b). Other details are given in Section 2.2.

short as possible, it can be concluded that the capacity for stimulated light emission decreased more strongly at pH 4.4 (3.3a) than at pH 7.8 (3.3b). This can be found too from the slopes shown in Fig. 3.3c, where the reciprocal intensities of the stimulated delayed fluorescence were plotted versus the time of acidification (a) or darktime (b).

Fig. 3.3a further indicates that apparently no optimal time of acidification was observable. This was also found by Miles and Jagendorf[59]. The optimal acid time for chloroplasts in Tris-buffer at pH 4.4 (see also Fig. 3.1b) is apparently shorter than 2 sec, which was the limit of time in the mixing procedure. A higher pH during the acid period (see Fig. 3.1b) the optimal time of acidification may be longer

than 2 sec, because the permeability of the thylakoid membranes to protons may increase with decreasing pH.

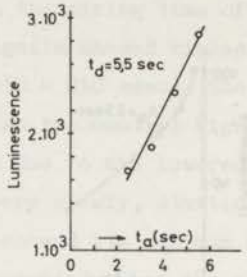

Fig. 3.4. The influence of the acid time in acid-base transition induced stimulation of luminescence with Tricine. Class I chloroplasts were isolated in 0.01 M Tricine-KOH, pH 7.8. After saturating preillumination for 5 sec the chloroplasts were acidified to pH 4.4 with MES-HCl during a time t_a as indicated. After 5.5 sec of darkness the pH was raised to pH 8.4 with Tricine-KOH. Final chlorophyll concentration was $2.5.10^{-5}$ M. The stimulated luminescence is given in arbitrary units. Other conditions are given in Section 2.2.

From Fig. 3.4 it is clear that under circumstances which were similar to those described for Fig. 3.3a the influence of the zwitterionic buffer Tricine is different from that of Tris. Instead of a decrease of stimulated luminescence with increasing time of acidification a steady increase was found. It is likely that due to a lower permeability of the thylakoid membranes to protons with Tricine the acidification of the interior space of the thylakoid is retarded in comparison to the effect with Tris.

3.2.3 pH optimum of the basic stage and influence of the acid pH on the stimulated delayed fluorescence during the acid-base transition. In Tricine the pH optimum of the basic stage in the acid-base experiment was found to be about 8.5. This was also reported for Tris preparations[59]. Fig. 3.5 shows that the stimulated light emission approximately equalled the normal luminescence intensity at pH 7.8 if the pH jump was from pH 4.4 to about pH 7. If the jump was up to pH 7.8 the stimulation factor was about 2. This indicates that the stimulation is not due to the

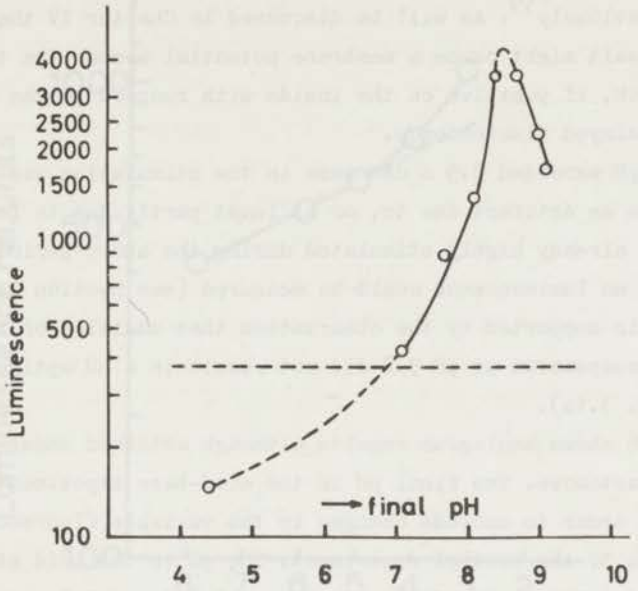

Fig. 3.5. The basic pH optimum of stimulated delayed fluorescence induced by an acid-base jump. Class I chloroplasts were isolated in 0.01 M Tricine-KOH, pH 7.8. Final chlorophyll concentration was $2.5.10^{-5}$ M. Immediately after preillumination the chloroplasts were acidified to pH 4.4 with MES-HCl. After 2.5 sec of darkness the pH was increased to the pH as indicated in the figure. The stimulated luminescence is given in arbitrary units in a semilogarithmic plot. The dashed horizontal line represents the luminescence value in the control experiment at pH 7.8. Other conditions are given in Section 2.2.

restoration of the variable fluorescence lowered during the acid phase (see also Fig. 3.6), but only to the pH gradient across the thylakoid membrane. This seems to be reasonable, because the pH inside was the same for all these experiments.

However, between pH 7.0 and 8.5 the increase of the stimulated delayed fluorescence was higher than one would expect from an exponential relationship between the luminescence and final pH (see Discussion in Chapter V). The extra increase was not due mainly to the variable fluorescence, which increased slightly with higher pH values (see Fig. 3.1a), but may be due also to increased concentrations of K^+-$Tricine^-$ (16 mM

at pH 8.4 and 5 mM at pH 7.0). This is in line with similar observations described previously[59]. As will be discussed in Chapter IV the sudden addition of salt might cause a membrane potential across the thylakoid membrane which, if positive on the inside with respect to the outside, stimulates delayed fluorescence.

If the pH exceeded 8.5 a decrease in the stimulation was observed. This might be an artifact due to, or at least partly due to loss of luminescence already highly stimulated during the short period (50 msec) during which no luminescence could be measured (see Section 2.2). This explanation is supported by the observation that addition of base to a chloroplast suspension at pH 7.8 did not result in a pH optimum at pH 8.5 (see Fig. 3.1a).

Fig. 3.6 shows analogous results although obtained under quite different circumstances. The final pH in the acid-base experiments was kept at pH 7.8 in order to exclude changes in the variable fluorescence yield in comparison to the control experiment. The pH in the acid stage was varied as indicated in the figure. Between pH 5.0 and 7.8 an exponential relation was obtained. The extra increase obtained if the previous acid pH was 4 or lower was possibly caused both by a slight increase of K^+-Tricine$^-$ concentrations and more likely by easier access of protons to the thylakoid interior during the acid phase.

3.3 Discussion

The results described in this chapter can be explained by the assumptions outlined below. The oxidized primary electron donor and reduced acceptor of photosystem 2 are responsible for the reexcitation of the first singlet state of chlorophyll a (ref. 42). This is a reversal of the photochemical act in pigment system 2:

$$h\nu + ZHchlaQ \rightleftarrows ZHchla^{*}Q \rightleftarrows ZH^{+}chlaQ^{-} \qquad (2)$$

In this equation chla stands for chlorophyll a in the reaction center of pigment system 2, ZH for the primary electron donor and Q for the primary electron acceptor. In general the excitation of the reaction center will take place by energy transfer from chlorophyll a_2 of the bulk of system 2 (see Fig. 1.2 and refs. 20 and 21). The emission of

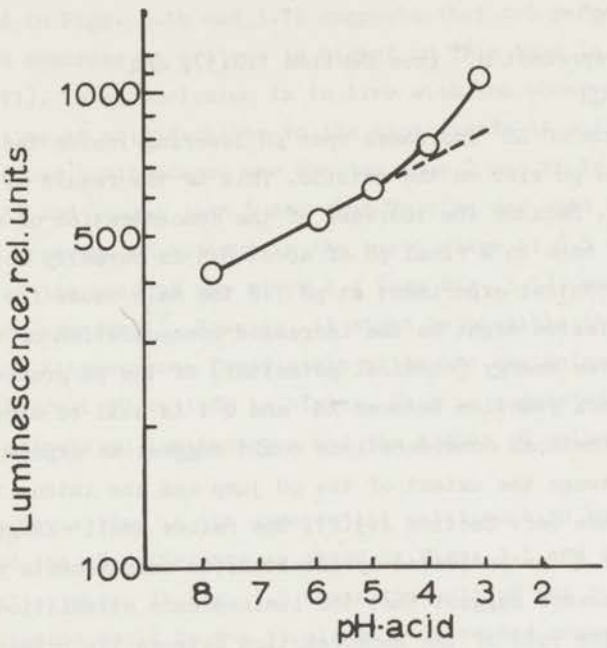

Fig. 3.6. The effect of the acid pH on the luminescence stimulated by an acid-base transition. Class I chloroplasts were isolated in 0.01 M Tricine-KOH, pH 7.8. The final chlorophyll concentration was $2.5.10^{-5}$ M. Immediately after illumination the chloroplasts were acidified with MES-HCl to the pH indicated in the figure. At a darktime of 2 sec the pH was increased to 7.8 with Tricine-KOH. The stimulated fluorescence is given in arbitrary units in a semilogarithmic plot. Other conditions are given in Section 2.2.

luminescence is the reverse of this reaction. Thus the reexcited singlet state of chlorophyll a of the reaction center will transfer its energy to the chlorophyll a of the bulk and this in general will emit the delayed fluorescence.

According to Mitchell's chemiosmotic theory of photoinduced electron transport and phosphorylation[68] and to conclusions of Witt and coworkers[69] the primary electron acceptor and donor are thought to be located on the outside and inside of the thylakoid membrane. Our results strongly support this location (see also Chapters IV and VI).

The results in this chapter suggest that the photoproducts react

in a pH dependent equilibrium, formally written as

$$ZH^+ \rightleftarrows Z + H^+ \qquad (3)$$

where ZH^+ may represent S_3^{3+} (see Section 1.2.3), and

$$Q^- + H^+ \rightleftarrows QH \qquad (4)$$

The concentration of ZH^+ increases upon pH lowering inside the thylakoid and of Q^- upon a pH rise on the outside. This is the result of an acid-base transition. Because the increase of the concentration of Q^- upon addition of the base to a final pH of about 8.5 is normally low with respect to the control experiment at pH 7.8 the main cause for the stimulated light emission might be the increased concentration of ZH^+ or, similarly, the free energy (chemical potential) of the pH gradient, which stimulate the back reaction between ZH^+ and Q^-. As will be discussed in Chapter VIII, kinetical considerations could suggest an exponential relation between the extent of the pH jump and the intensity of delayed fluorescence (cf. Section 4.3.2). The rather small changes in the concentration of Q^- as indicated by simultaneous measurements of the variable fluorescence suggest that the luminescence stimulation reflects an increase of the rate of the back reaction between the primary photoproducts, which is not primarily due to an increase in the concentration of the reduced electron acceptor Q^-, but probably to an increase in that of the oxidized electron donor Z^+. The small increase in Q^- may be caused by reversed electron flow.

From the results shown in Figs. 3.1a, 3.1b and 3.2 it is clear that acidification of preilluminated chloroplasts leads to lowering of the luminescence unless the access of the protons into the thylakoid is facilitated by further decrease of the pH outside, by uncouplers (see Chapter VI) or by detergents (Chapter VII). The lowering of luminescence is partly correlated with a concomitant decrease of the variable fluorescence (Section 1.2.2).

A sudden increase of pH from 7.8 to higher values after the preillumination stimulated the delayed fluorescence and also the variable fluorescence. This stimulated delayed light emission may be caused by an increase of the variable fluorescence, by a pH gradient across the thylakoid membrane and, to a minor extent, by the presence of an increased salt concentration (see Fig. 3.1a and Chapter IV).

The difference between the two buffers used in the experiments as described in Figs. 3.1a and 3.1b suggests that the permeability of the thylakoid membrane to protons is higher in Tris than in Tricine (see Chapter VI). This conclusion is in line with the observation that the optimal time of acidification in the experiments of acid-base transition induced luminescence was shorter than 2 sec if Tris was used (Fig. 3.3) and longer than 5 sec when Tricine was used (Fig. 3.4).

The apparent pH optimum in the basic stage at 8.5 which was observed, if the acid pH was at pH 4.4 (see Fig. 3.5), was also found by Miles and Jagendorf[59]. However, it might be possible that due to loss of delayed fluorescence immediately after the beginning of the second mixing the real pH optimum is higher. This is suggested by the relation between stimulated luminescence and the higher pH values shown in Fig. 3.1a.

The deviations in the exponential relationships between luminescence and the pH difference as shown in Figs. 3.5 and 3.6 might have different origins. In Fig. 3.5 where the acid pH was constant the extra light emission could be due to slightly increased concentrations of salt. It was not correlated with the variable fluorescence yield which did not change to such an extent. In Fig. 3.6, however, where the "basic" pH was kept constant at 7.8 the extra luminescence if the acid pH was below 4, was possibly caused by a more easy acidification of the thylakoid interior. This is only true if the permeability of the thylakoid membrane to protons increases with decreasing pH and might be related with the nett electrical charges of the structural proteins within the membranes. More details and discussion are given in Chapters VI and VIII.

CHAPTER IV

STIMULATED DELAYED FLUORESCENCE AS INDUCED BY ADDITION OF SALT

4.1 Introduction

Besides a sudden change in pH, another way to enhance delayed fluorescence after its excitation by light is to bring about a rapid increase of salt concentration outside the chloroplasts[61,59,62]. Results which will be described below[61,62] strongly suggest that the salt-induced enhancement is due to a development of a diffusion potential across the thylakoid membrane which is positive on the inside with respect to the outside. This membrane potential is the result of a higher passive permeability of the thylakoid membrane to the cations than to the anions of the added salts. From luminescence data as a function of concentrations of salts like potassium benzoate and potassium chloride it will be concluded that an exponential function of the membrane potential determines the lowering of the energy barrier (activation energy) between the light-induced energy level of the reaction of photosystem 2 and the first singlet state of chlorophyll a.

Some experiments reported in this chapter were done in cooperation with Dr. J. Barber (presently at the Imperial College of London) and are described in more detail elsewhere[61].

4.2 Relationship between membrane potential and ion distribution

A concentration gradient of ions across biological membranes has been measured for many types of tissues, microorganisms and cell organelles, like Chlorella[70], isolated chloroplasts[71,72], mitochondria[73,74,75] and axons[76] by use of radioactive isotopes. These concentration gradients give rise to a so-called membrane potential, which has been detected by means of microelectrodes in Chlorella[70] and Nitella[77].

The relation between the membrane potential and the concentration of all the ions present at both sides of the thylakoid membrane can be found from thermodynamic considerations. When pressure P and temperature

T are constant the difference in (Gibbs) free energy is defined as the electrochemical potential difference $\Delta\bar{\mu}$. For one particular monovalent cation, C^+, this difference is given by the sum of its chemical and its electrical potential difference:

$$\Delta\bar{\mu}_{C^+} = \Delta\mu_{chem} + \Delta\mu_{electr} \tag{5}$$

from which follows:

$$\Delta\bar{\mu}_{C^+} = RT\ln\frac{(C^+)_o}{(C^+)_i} + FE_{o-i} \tag{6}$$

where $(C^+)_o$ and $(C^+)_i$ denote outside and inside concentrations (or more precisely activities), respectively, of the C^+ - ion, E_{o-i} is the difference in electrical potential across the membrane, and the other symbols have their usual meanings. In general this electrochemical potential gradient in chloroplasts and mitochondria depends on the rate of electron transport. However, when there is thermodynamic equilibrium $\Delta\bar{\mu}_{C^+} = 0$, equation (6) converts into the well-known Nernst equation:

$$E_{i-o} = \frac{RT}{F}\ln\frac{(C^+)_o}{(C^+)_i} \tag{7}$$

When there is no equilibrium the Goldman equation[78] (equation 8) can be applied. This equation is based on the constant-field hypothesis[79], i.e. the electrical gradient within the membrane is assumed to be linear. It is restricted in its application on passive and independent ion fluxes and on the absence of a nett electrical current.

Assuming only monovalent ions to be present, the Goldman equation can be written as:

$$E_{i-o} = \frac{RT}{F}\ln\frac{p_{C_1}(C_1^+)_o + p_{C_2}(C_2^+)_o + \cdots\cdots p_{A_1}(A_1^-)_i + \cdots}{p_{C_1}(C_1^+)_i + p_{C_2}(C_2^+)_i + \cdots\cdots p_{A_1}(A_1^-)_o + \cdots} \tag{8}$$

where p_C and p_A are the permeability coefficients for the cations (C^+) and anions (A^-). The permeability coefficient was defined[78] approximately to be equal to

$$p_j = u_j RTk_j/a \tag{9}$$

where u_j is the mobility of j^{th} ion in the membrane, k_j is the partition coefficient between membrane and solution and a the thickness of the

membrane.

The membrane potential developed over the thylakoid membrane immediately after addition of a monovalent salt C^+A^- may be written as:

$$E_{i-o} = \frac{RT}{F} \ln \frac{a + p_C(C^+)_o}{b + p_A(A^-)_o} \qquad (10)$$

where a and b are determined according to equation (8) by the products of the permeability coefficients and the concentrations of ions inside and outside the membrane before salt addition, and $(C^+)_o$ and $(A^-)_o$ are the concentrations (activities) of the added ions outside the thylakoid. The change in membrane potential is positive if $p_C > p_A$.

4.3 Experimental results

4.3.1 Ionic permeabilities. Preliminary experiments showed that addition of salts to preilluminated chloroplasts enhanced the delayed fluorescence[61]. This was not due to a change in external osmolarity as appeared from maintenance of the sucrose concentration at 0.4 M. The kinetics were compared to those of the acid-base induced enhancement and it was found that the initial stimulation by salt addition was much less than by the acid-base jump, whereas its decay was much slower (see Figs. 2.2 and 4.1). Various salts were used in order to obtain an understanding of the mechanism of stimulation. These experiments showed that the stimulation by different alkali-chlorides was virtually the same except for NaCl which caused a larger enhancement (not shown). Similar experiments made by Miles and Jagendorf[59] showed that lithium chloride was less effective than sodium or potassium chloride, whereas choline chloride caused almost no stimulation of luminescence. A comparison of the results obtained by addition of various potassium salts showed that the magnitude of the enhancement of delayed light was dependent on the anion used[61] (see Table 4.1). The multiply-charged anions like sulphate and citrate effected a higher stimulation than monovalent anions like the halogenides and nitrate except for the organic monovalent species like acetate and benzoate. Similar results were obtained by Miles and Jagendorf[59]. Especially salts of benzoic acid were very effective in stimulating de-

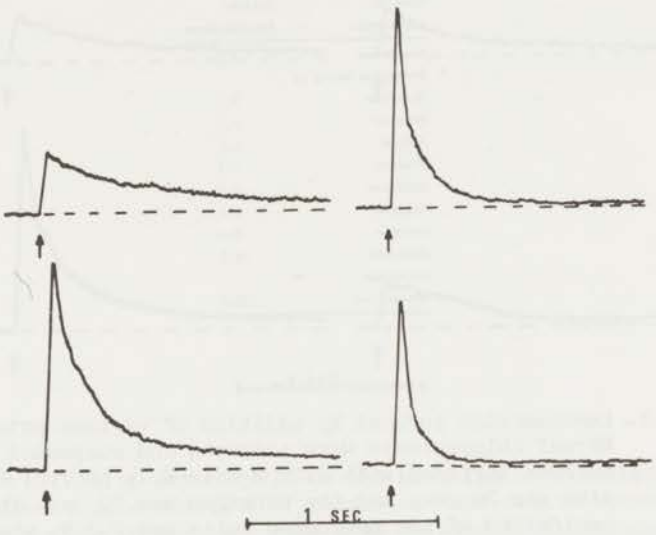

Fig. 4.1. The effect of gramicidin on the kinetics of the salt- and pH-induced enhancements of delayed light emission. Normal chloroplasts were prepared and used as indicated in Table 4.1, except for the pH which was 7.4. The curves on the left side represent the time courses of luminescence upon mixing with potassium benzoate (final concentration of 0.3 M), whereas those on the right side show the time courses of acid-base experiments, in which the pH was shifted from 7.4 to 4.1 durring the acid period and raised again to 8.8. For the experiments as shown in the lower curves chloroplasts were incubated for 20 min with 0.1 mg/l gramicidin before excitation by light. The other conditions were the same as given in Table 4.1.

delayed fluorescence[61]. Because the acid-base induced increase of luminescence[56] was inhibited by uncouplers of photophosphorylation, it was somewhat surprising to find that a compound like gramicidin[80,81] caused further enhancement of delayed light[61] upon salt addition to chloroplasts in Tris-buffer. This is shown in Fig. 4.1. The curves on the left side refer to stimulations by 0.3 M potassium benzoate with (lower) and without (upper) gramicidin, respectively. The same applies to the curves on the right side which represent the stimulations of light emission by an acid-base transition. This phenomenon is dealt with into more detail in

Type of experiment	Initial luminescence
Control	1.0
Potassium salts of	
Chloride	2.3
Bromide	2.3
Jodide	2.2
Nitrate	2.7
Sulphate	5.0
Citrate	6.2
Acetate	8.0
Benzoate	44.0
Acid-base transition	143.0

Table 4.1. Luminescence induced by addition of various potassium salts. Normal chloroplasts were prepared and suspended in 0.4 M sucrose buffered with 0.02 M Tris-HCl, pH 7.2. Preillumination time was 20 sec, and the darktime was 10 sec. The final concentration of the potassium salts was 0.3 M, whereas in the control experiment a final concentration of 0.6 M sucrose was used. For comparison an acid-base induced increase of delayed light is shown. Here the time of acidification at pH 4.1 was 8 sec and 10 sec after preillumination the pH shifted up to pH 8.7. The luminescence data are given in relative units. The optical density of the chloroplast suspension during preillumination was 0.66 per mm at 670 nm. The other conditions are described in Section 2.2.

the previous chapter and in Chapter VI.

Valinomycin strongly increased the effect of potassium-, but not of sodium salts (see Fig. 4.2). This, however, is in line with previous observations that valinomycin increases the permeability to potassium ions and not or slightly to sodium ions[82,83].

4.3.2 Experiments with mixtures of salt. The observations reported in the previous section may be explained by the assumption of a change in membrane potential across the thylakoid membrane due to a difference in permeability between the cation and anion used. If the membrane is more permeable to the cations the membrane potential is positive inside the membrane with respect to the outside. The high luminescence intensity observed with potassium benzoate may then well be caused by a very low permeability to the benzoate ion. A further increased permeability to the cations used by the cation translocators gramicidin and valinomycin,

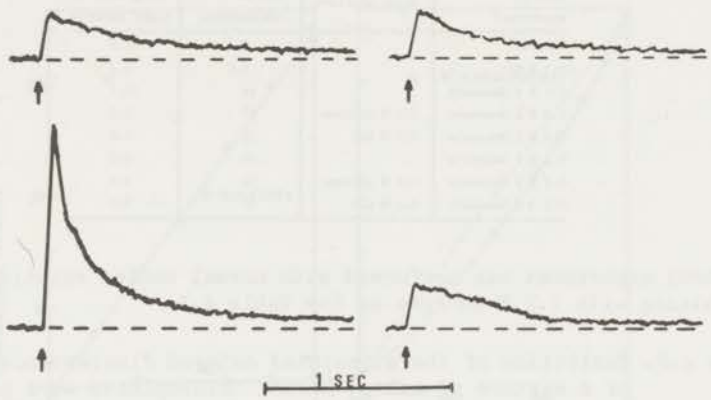

Fig. 4.2. The effect of valinomycin on the kinetics of stimulated light emission upon addition of potassium- and sodium benzoate. The curves on the left side show delayed fluorescence stimulated by 0.3 M potassium benzoate. The other two show corresponding time courses with 0.3 M sodium benzoate. The lower curves indicate the luminescence signals obtained from chloroplasts to which $3.3.10^{-6}$ M valinomycin was added 20 min before preillumination. The optical density of the suspension was 0.40 per mm at 670 nm. The other conditions were the same as given in Table 4.1.

will enhance this membrane potential as can be seen in equation (10).

In order to obtain further support for this assumptions we did experiments the results of which are given in Table 4.2 and Fig. 4.3b. If the membrane is more permeable to the chloride ions than to the benzoate ions, addition of KCl to potassium benzoate would inhibit the membrane potential according to equation (10). If the idea of the changed membrane potential were incorrect one should probably not expect to find inhibition but an extra enhancement of luminescence. Application of equation (10) shows that if the permeability to chloride is higher than to benzoate the denominator will increase more than the numerator:

$E(\text{K-benzoate}) > E(\text{K-benzoate + KCl})$, if $(p_K - p_{Benz}) > (p_K - p_{Cl})$

It must be mentioned that by replacing sucrose for KCl as an extra addition slight inhibition was obtained too, but this happened also for the control experiments, if sucrose solution was used instead of buffer

Type of experiment	Extra addition	Initial luminescence	Integrated light emission
Control*		2.6	6.0
0.3 M KCl		5.5	9.4
0.2 M K Benzoate		44	10.1
0.2 M K Benzoate	0.4 M sucrose	37	9.7
0.2 M K Benzoate	0.2 M KCl	32	8.8
0.3 M K Benzoate		59	9.6
0.3 M K Benzoate	0.4 M sucrose	50	9.6
0.3 M K Benzoate	0.2 M KCl	41	8.2

*Control experiment was performed with normal buffer solution instead of mixing with 1.2 M sucrose as for Table 4.1.

Table 4.2. Inhibition of the stimulated delayed fluorescence by addition of a mixture of salts. Normal chloroplasts were prepared and suspended in 0.4 M sucrose buffered with 50 mM Tris-HCl, pH 7.8. Final chlorophyll concentration was $1.1.10^{-4}$ M, whereas the final concentrations of the added salts or sucrose were as indicated. After illumination the suspension was mixed with a solution containing either benzoate or KCl alone or a mixture of benzoate and KCl or sucrose to the final concentrations indicated. The time of preillumination was 5 sec, and the darktime was 10 sec. The integrated light emission was registrated from 10 sec up to 2 min. The other conditions were as described in Table 4.1.

(see Table 4.6).

According to equation (10) the membrane potential increases upon addition of salt if $p_C > p_A$; the quotient after the logarithm increases linearly with the concentration of the cation when $b \gg p_A(A^-)_o$, as occurs with low concentrations of added salt or when p_A is relatively small. With potassium benzoate, which was assumed to have a low p_A, a linear relation was observed between the intensity of the stimulated luminescence and the concentration, up to a high concentration (see Fig. 4.3a). This suggested that the delayed fluorescence is proportional to the exponential of the changed membrane potential:

$$E = \frac{RT}{F}\ln\frac{a + p_K(K^+)_o}{b + p_{Benz}(Benz^-)_o} \approx \frac{RT}{F}\ln\frac{a + p_K(K^+)_o}{b} \qquad (11)$$

Thus, because the increase in luminescence by potassium benzoate is assumed to be proportional to the potassium concentration:

$$L \propto e^{E.F/RT} \qquad (12)$$

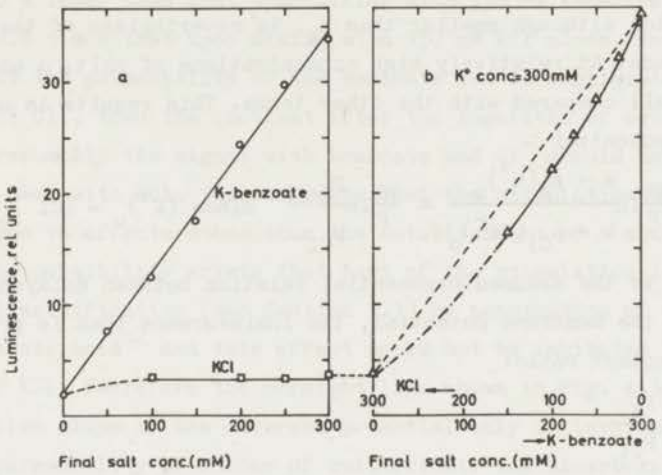

Fig. 4.3. Luminescence increased upon addition of various concentrations of potassium chloride and/or of potassium benzoate. Normal chloroplasts were prepared and suspended in 0.4 M sucrose buffered with 0.05 M Tris-HCl, pH 7.8. Final concentration of chlorophyll was $8.3.10^{-5}$ M (a) and $6.7.10^{-5}$ M (b). The salts were added after a darktime of 10 sec. The control experiment (in Fig. 4.3a) was performed by mixing finally with 1.2 M unbuffered sucrose solution instead of buffer, which gave a 20 % lower value than the "normal" control experiment (not shown). The indicated concentrations of salt were obtained by addition of an appropriate mixture of 1.2 M sucrose and 0.6 M KCl or 0.6 M K-benzoate (Fig. 4.3a), or of a mixture of 0.6 M K-benzoate and 0.6 M KCl with a constant K^+ concentration of 0.6 M (Fig. 4.3b). The final osmolarity was 0.8 M. The other conditions as indicated in Table 4.2.

and

$$L \propto \frac{a + p_K(K^+)_o}{b} \propto (K^+)_o \qquad (13)$$

With KCl, in contradistinction to observations of Miles and Jagendorf[59], we observed a saturation of the delayed emission below 0.1 M (Fig. 4.3a). The normally used buffered sucrose mixing solution for the control experiment was replaced by 1.2 M sucrose. This caused a lowering of the initial value by 20 %. Such an inhibition was also obtained if Tricine was used instead of Tris-buffer. The explanation might be that due to sudden halving of the outside concentrations of $R\text{-}NH_3^+$ (Tris) or Na^+

(Tricine) an efflux of these ions caused a membrane potential of opposite sign. The saturation with KCl could be explained by the assumption of a p_{Cl}, which although smaller than p_K, is nevertheless of the same order of magnitude. At relatively high concentrations of salt, a and b then become small compared with the other terms. This results in a constant membrane potential

$$E = \frac{RT}{F}\ln\frac{a + p_K(K^+)_o}{b + p_{Cl}(Cl^-)_o} \approx \frac{RT}{F}\ln\frac{p_K}{p_{Cl}} \qquad \text{since } (K^+)_o = (Cl^-)_o$$

According to the assumed exponential relation between delayed fluorescence and the membrane potential, the luminescence then is proportional to the constant ratio:

$$L \propto \frac{p_K}{p_{Cl}} \tag{14}$$

Fig. 4.3b shows data obtained upon addition of mixtures of potassium chloride and potassium benzoate in such a way that the final potassium concentration was the same for all experiments, so that the numerator in eq. (10) was constant. In agreement with the results of Table 4.2 it can be seen that the luminescence stimulated by K-benzoate is decreased by the KCl. Mathematically:

$$E = \frac{RT}{F}\ln\frac{a + p_K(K^+)_o}{b + p_{Benz}(Benz^-)_o + p_{Cl}(Cl^-)_o} \tag{15}$$

which, assuming $p_{Cl} \gg p_{Benz}$, reduces to

$$E = \frac{RT}{F}\ln\frac{c}{b + p_{Cl}(Cl^-)_o}\text{, where c is a constant.}$$

Finally

$$L \propto \frac{c}{b + p_{Cl}(Cl^-)_o} \tag{16}$$

which represents a hyperbolic function.

The results of Fig. 4.3b are in qualitative agreement with the above considerations, and show an approximately hyperbolic relation between the luminescence and the chloride concentration. However, the decrease of stimulation was smaller than would be expected (see eq.(15)).

E.g. mixing with 150 mM K-benzoate and 150 mM KCl gave a signal which was only 20 % lower than that upon mixing with 150 mM K-benzoate alone, and still 4.8 times that upon mixing with 150 mM KCl alone. Even if one assumes that the permeability of the benzoate ion is negligible compared with that of Cl^-, then the quotient after the logarithm of equation (15), and thus presumably the signal with benzoate and Cl^- should have been only twice that with KCl. This suggests that the stimulation by benzoate is partly due to effects other than the establishment of a membrane potential. The possibility exists that part of the stimulation is due to an internal acidification (see Section 3.3) by penetration of the uncharged benzoic acid[62] and this effect would not be inhibited by the presence of KCl. Therefore the straight line shown in Fig. 4.3a might have a smaller slope if the membrane potential only is involved.

4.3.3 The permeability of anions of zwitterionic and dicarboxylic acids. Jagendorf[84] expressed doubts about the assumed relatively low(er) permeability of the acetate (Table 4.1) and especially of the benzoate anion. The acetate ion was reported to be "rather" permeable[85-87]. Further it must be realized that the postulated low permeability of benzoate is in contrast to what has been concluded for related anions in the case of synthetic lipid membranes[88]. He also suggested[84] that according to the membrane potential theory the delayed fluorescence should be stimulated more upon addition of salts of aliphatic zwitterionic or dicarboxylic acids, the anions of which reportedly have a low permeability[89], than upon addition of salts of monovalent acids like hydrochloric acid. This prediction was correct as can be concluded from the data shown in Table 4.3. Sodium malate stimulated more than sodium chloride and potassium glutamate more than potassium chloride. Similar results were obtained by Barber[90]. Also potassium rhodanide (thiocyanate) increased the delayed fluorescence more than KCl, although from ion transport measurements in mitochondria it is known that the permeability of the inner mitochondrial membrane to the rhodanide ion is higher than that to potassium[91].

4.3.4 The effect of KCl and valinomycin on the negative membrane potential. In Section 3.3 it was proposed that the free energy of the chemical potential gradient of protons across the thylakoid membrane could stim-

Type of experiment	Initial luminescence (relative units)
Control	470
Potassium salts of	
Cl^-	690
CNS^-	880
Glutamate$^-$	1145
Sodium salts of	
Cl^-	890
Malate$^=$	1210
Benzoate$^-$	2610

Table 4.3. Effect of different potassium and sodium salts on stimulated delayed fluorescence. Class I chloroplasts were prepared and suspended in 0.4 M sucrose buffered with 0.01 M Tricine-NaOH, pH 8.2, and containing 3 mM $MgCl_2$. The indicated salts were solved in this buffered solution, from which sucrose was omitted, up to a concentration of sodium or potassium ions equal to 0.6 M, giving a final concentration of 0.3 M. The other conditions were as for Table 6.3.

ulate luminescence brought about by an acid-base transition. Because protons would tend to "leak" out the thylakoids down their concentration gradient after an acid-base jump, one may predict that a negative membrane potential, i.e. positive outside with respect to the inside of the thylakoid, would be generated.

According to this hypothesis the negative membrane potential could depress the stimulation of delayed fluorescence brought about by the pH gradient. Consequently this inhibition could be diminished by the presence of low concentrations of K^+ especially in the presence of valinomycin. The results are shown in Table 4.4. The relatively low stimulation by the acid-base transition, due to the rather small pH gradient, was considerably enhanced by K^+ ions and valinomycin added to the chloroplasts 10 min before preillumination. The explanation for this then would be that the negative membrane potential was almost prevented by an influx of the highly permeable potassium-valinomycin complex.

Table 4.4 also indicates that the NaCl-induced stimulated luminescence was inhibited in the presence of KCl and valinomycin, presumably

Type of experiment	No additions	KCl	Valinomycin	KCl + valinomycin
Control	73	82	70	74
NaCl	150	195	107	71
KCl	88	98	235	161
Sodium benzoate	-	-	300	170
Acid-base	260	310	280	465

Table 4.4. Effect of valinomycin and KCl upon stimulated delayed fluorescence. Normal chloroplasts were isolated and suspended in 0.05 M TES-NaOH, pH 7.8 ((Na^+) = 33 mM). Final chlorophyll concentration was 7.10^{-5} M. Valinomycin and KCl were added at a concentration of 2.10^{-6} and 10 mM, respectively before the preillumination. Final salt concentration was 0.3 M. The pH in the experiment of the acid-base transition shifted from 7.8 to 5.6 and finally to 8.5, brought about by 0.02 M succinic acid and 0.1 M Tris, respectively. Darktime was 3 sec. Other conditions as indicated for Fig. 2.2.

because the diffusion potential effected by NaCl was diminished by the efflux of the potassium-valinomycin complex. A similar result was obtained with sodium benzoate. The lowering by valinomycin alone (2^{nd} row) can be explained by the presence of a low concentration of endogenous K^+. The KCl-induced stimulation of delayed fluorescence was stimulated by valinomycin as mentioned in Section 4.3.1. If KCl was already present the stimulation by mixing with KCl was lower, probably because of a lower diffusion potential due to a smaller gradient of K^+ ions.

4.3.5 Preincubation with salts. According to equation (8) the size of the potential across the thylakoid membrane, induced by the added salt, is also dependent upon the concentrations of ions already present. Therefore it was desirable to study the delayed and stimulated delayed fluorescence after preincubation with various salts. Fig. 4.4 shows that the NaCl and KCl-induced enhancement of delayed light unexpectedly increased with increased salt concentration in the medium. This also applied to the normal delayed light emission and the initial fluorescence yield. However, the increase of the latter emission might explain the higher intensities of luminescence only to a minor extent.

It was previously reported that with isolated chloroplasts light-

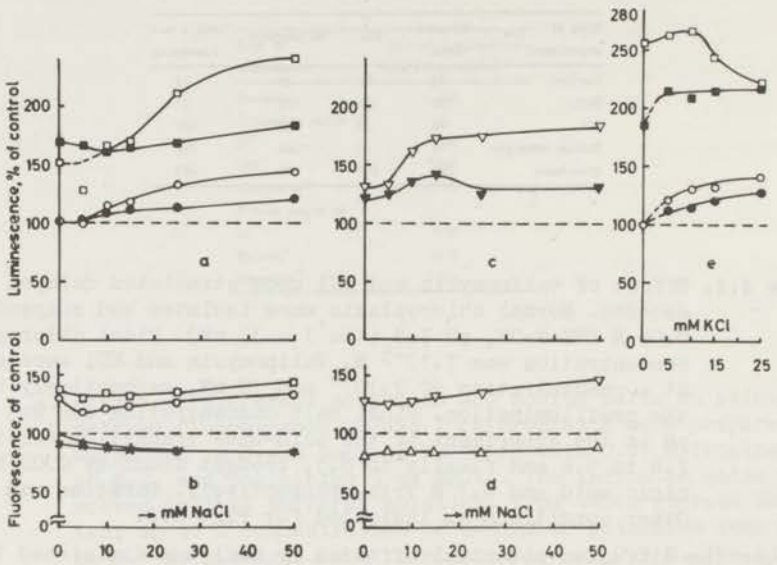

Fig. 4.4. Effect of the preincubation of salt upon delayed and stimulated delayed fluorescence, induced by NaCl and KCl. Chloroplasts were isolated and used as indicated in Table 4.2. Final chlorophyll concentration was 4.10^{-5} M (e) and 5.10^{-5} M (a - d). The time of preillumination was 5 sec, and the darktime was 3 sec. The chloroplasts were incubated in Tris-buffer to which NaCl or KCl was added to a concentration as indicated in the figures. The preincubation time was at least 10 min. The arbitrary intensities of luminescence, salt-induced stimulated luminescence as well as the initial yield of fluorescence and that after 2 min were plotted, relative to the values of the control experiments, as a function of concentration of salt used for the preincubation. The symbols used refer to the luminescence and total fluorescence ($F_o + \Delta F$) of O——O, control experiment; □——□, the experiment in which NaCl (0.3 M) was added in the dark; ▽——▽, the experiment in which KCl (0.3 M) was added in the dark; and to the F_o of +——+, control experiment; x——x, NaCl experiment; Δ——Δ, KCl experiment. The closed symbols refer to the corresponding values of integrated luminescence. The other conditions as indicated in Table 4.2.

induced electron transport, photophosphorylation, formation of the high-energy state (X_E), the change of absorption at 515 nm and the 3 msec

delayed light emission increased with higher concentrations of sucrose and salts of mono- or especially divalent cations[92-94]. Salts of the latter ions were found to increase also the variable fluorescence yield (see Discussion), both at room temperature and at the temperature of liquid nitrogen[95].

During preparation of this manuscript recent results[90] of Barber and Varley came to our attention, who observed a lowering of KCl-stimulated luminescence after preincubation with KCl, in qualitative agreement with what would be expected. The reason for this discrepancy is not clear at present, but may be due to the different experimental procedure and preparation of the chloroplasts.

From the Tables 4.5 and 4.6 it can be seen that an apparent stimulation of luminescence was also observed, when the chloroplasts were mixed before illumination with concentrations of NaCl, K-acetate or K-phosphate as high as the final concentration in salt jump experiments. If these high concentrations were kept constant during the two mixing periods, the luminescence intensity was higher than when the salt was added during the second mixing time (as in a "normal" experiment). However, the initial and the variable fluorescence yield also were found to be higher[106]. The Tables 4.5 and 4.6 also show the "intrinsic" luminescence, calculated by dividing the observed luminescence by the variable fluorescence, ΔF (see Section 1.2.2). It can be seen that these values for the incubation experiments are about the same or even lower than for the controls, in contrast to the results of salt jump experiments (see e.g. also Tables 6.2 and 6.3). This indicates that a "true" stimulation of luminescence does not occur. A further correction is possible by additionally dividing by the factor $F_o + \Delta F$, the actual fluorescence yield. This correction would be justified if (see Section 1.2.2) the luminescence yield is proportional not only to the concentration of Q^- (i.e. roughly proportional to ΔF) but also to the actual yield (see Fig. 6 of ref. 42). More details about this relationship are given in the discussion.

With NaCl gramicidin gave a stimulation of the intrinsic luminescence and the corrected intrinsic luminescence in a salt jump, but an inhibition in a preincubation experiment. The effect of gramicidin will

Type of experiment	Gramicidin D 10^{-6} M	Luminescence L	Integrated luminescence	Fluorescence yield	
				F_o	ΔF
Control	-	90 (100)	167 (100)	35.5	13.0 (100)
Control	+	112 (124)	202 (121)	34.5	17.5 (135)
NaCl	-	179 (199)	288 (172)	38.0	14.1 (108)
NaCl	+	663 (736)	331 (198)	38.5	15.6 (120)
Buffer/NaCl	-	182 (202)	280 (168)	42.0	26.1 (200)
Buffer/NaCl	+	154 (171)	326 (195)	42.0	34.2 (263)

continued	Gramicidin D 10^{-6} M	Intrinsic luminescence $L/\Delta F$	Luminescence corrected for fluorescence $10^2.L/(F_o+\Delta F)\Delta F$	Relative stimulation factor of luminescence	
				-Gram.D	+Gram.D
Control	-	6.92 (100)	14.3 (100)	1.00	
Control	+	6.40 (93)	12.3 (86)		1.00
NaCl	-	12.7 (184)	24.4 (171)	1.71[1]	
NaCl	+	43.3 (625)	80.0 (560)		6.50[2]
Buffer/NaCl	-	6.98 (101)	10.2 (71.4)	0.71	
Buffer/NaCl	+	4.64 (67)	6.09 (42.6)		0.50

1) If the "pure" stimulation factor by addition of NaCl is due to the membrane potential in Tris-buffer, $\exp(EF/RT) = 1.71$, according to equation (12), then the membrane potential at 20° is $E_{i-o} = 0.058 \log 1.71 = 13.5$ mV ($\hat{=}$ 0.31 Kcal.Mole^{-1}).

2) Analogously to 1) with gramicidin D $E_{i-o} = 0.058.\log 6.50 = 58.0.813 = 47$ mV ($\hat{=}$ 1.08 Kcal.Mole^{-1}).

Table 4.5. Influence upon luminescence by gramicidin D and NaCl present during the preillumination. Chloroplasts were prepared and used as indicated in Table 4.2. Final chlorophyll concentration was 4.10^{-5} M, and that of gramicidin D, if used, was 10^{-6} M. The experiments denoted "NaCl" refer to "normal" measurements where the chloroplasts were mixed with 0.6 M NaCl after preillumination. In the buffer/NaCl experiment the chloroplasts were incubated in a medium composed of buffer and 0.6 M NaCl in a ratio 1 : 1 (v/v) before the preillumination. After the light period the concentrations of the solutions were not changed during the two mixings so that the final concentration of NaCl was 0.3 M like in the normal NaCl experiment. The 3rd and the 4th columns give the initial and integrated luminescence, respectively. The 5th column gives the base and the variable fluorescence, the 8th column the intrinsic luminescence (see text), the 9th the intrinsic luminescence divided by the total yield of fluorescence. The

Table 4.5 (continued) last column gives the relative stimulation factors of luminescence. These were calculated from the ratios of luminescence by salt treatment and the corresponding values of the control experiment, as shown in the 9th column. The numbers in parentheses refer to the values normalized to the value of the control experiment in each column. Other conditions as indicated in Fig. 4.4.

be discussed more extensively in Chapter VI.

The presence of high concentrations of K-benzoate during the illumination period (see Table 4.6) caused a strong inhibition of the luminescence capacity, whereas the addition of this salt after the preillumination gave a high stimulation as was mentioned before. Another striking effect of benzoate was a strong, but slow, increase of the "base" fluorescence (measured 2 min after preillumination). This effect was independent of light and was also noticed when the mixing occurred after the preillumination.

If one assumes that the membrane potential and pH gradient induced by light has almost disappeared after 3 sec (as indicated by experiments discussed in Chapter V) one might suppose that the inhibited corrected luminescence in the presence of NaCl and other salts reflects a lower concentration of the oxidized primary electron donor to photosystem 2, ZH^+. The presence of NaCl might cause a higher rate of decay of this compound due to enhanced rate of electron transport. The rate of decay of ZH^+ might be accelerated further by the uncoupler gramicidin D.

Addition of NaCl to chloroplasts in Tris-buffer, preilluminated in the presence of gramicidin D (see Table 4.5), caused a strongly enhanced luminescence intensity as mentioned for KCl and K-benzoate in Chapter VI (see Table 6.3). This also applied to the luminescence corrected for the dependence on both the total and the variable fluorescence yield (see Discussion). If it is true that this corrected luminescence depends on the concentration of the oxidized primary electron donor of system 2, ZH^+ (S_3^{3+}, see Section 3.3), and exponentially on the free energy of the energy-rich state brought about by addition of NaCl, the shown stimulation factors could reflect the contribution of the NaCl-induced membrane potential. Calculation of these potentials according to equation (12) resulted in values of 13.5 mV and 47 mV without and with gramicidin, respectively (see Table 4.5).

Type of experiment	Luminescence L	Integrated luminescence	Fluorescence yield		Intrinsic luminescence	Luminescence corrected for fluorescence
			F_o	ΔF	L/ΔF	L/(F_o + ΔF)ΔF
Control	74 (100)	570 (100)	7.4	2.4	30.8 (100)	3.40 (100)
Sucrose	59 (80)	527 (93)	5.9	3.5	16.8 (55)	1.80 (53)
K-phosphate	128 (173)	905 (159)	6.8	2.8	45.7 (147)	4.76 (140)
Buffer/K-phosphate	121 (163)	895 (157)	6.4	3.5	34.6 (112)	3.49 (102)
K-acetate	246 (333)		6.7	2.0	123 (400)	14.3 (420)
Buffer/K-acetate	95 (128)		7.3	3.7	25.6 (83)	2.33 (69)
K-benzoate	525 (710)		9.5[1]	0	350 (1140)	- (-)
Buffer/K-benzoate	3 (4)		11.5[2]	-2.5	- (-)	- (-)

1) F_o increased continuously and independently of illumination. The initial fluorescence without preillumination was 8.0 and therefore ΔF was assumed to be 9.5 - 8.0 = 1.5.

2) The initial fluorescence after preillumination was 9.0.

Table 4.6. The influence of salts of weak acids added before or after preillumination. Normal chloroplasts were isolated and used as indicated in Table 4.2. Final chlorophyll concentration was 5.10^{-5} M. The pH of all salt solutions was brought to pH 7.8 with HCl and the K-phosphate solution was composed of 0.3 M K_2HPO_4 and 0.6 M KH_2PO_4 so that the pH was 7.8. Like in Table 4.5 buffer/salt refers to chloroplasts suspended in a medium composed of buffer and K-salt in a ratio 1 : 1 (v/v) before preillumination. Final K^+ concentration was 0.3 M. The other conditions as indicated in Table 4.5.

4.3.6 The dependence of luminescence and intrinsic luminescence, changed by addition of NaCl, on the light intensity. The difference between the enhancements of delayed fluorescence by a high concentration of NaCl added before and after the preillumination using various intensities of light can be observed also in Fig. 4.5. It is clear that like the variable fluorescence yield in the dark (ΔF) both the normal delayed light and the two types of luminescence, enhanced by NaCl, were saturated at about 4000 erg $cm^{-2} sec^{-1}$ ($\approx$ 2 nEinstein sec^{-1}).

In contrast to the small difference in ΔF between the control experiment and that in which NaCl was added after the preillumination the difference between the intensities of the corresponding luminescence signals was rather high at low light intensities and low at relatively high intensities. This can be seen more clearly from the data of the intrinsic delayed fluorescence in Fig. 4.5c. Apparently the contribution

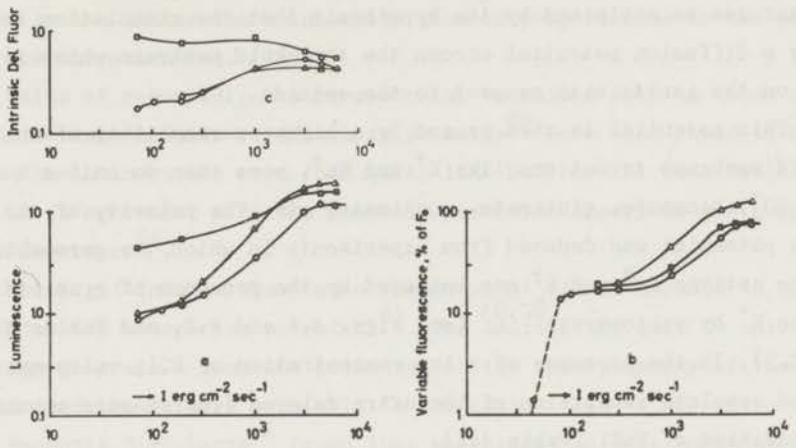

Fig. 4.5. Influence of the intensity of preillumination upon the variable fluorescence and the delayed fluorescence of chloroplasts in the presence of high concentration of NaCl. Chloroplasts were prepared and used as indicated in Table 4.2. Final chlorophyll concentration was 6.10^{-5} M. The intensity of preillumination was varied from 70 - 6000 erg $cm^{-2}sec^{-1}$ (4.10^{-11} - 3.10^{-9} Einstein sec^{-1}). The NaCl experiments were performed like those described in Table 4.5. O——O, control; □——□, NaCl; Δ——Δ, buffer/NaCl. Other conditions as indicated in Fig. 4.4.

by the NaCl-induced membrane potential to the stimulation is higher if the chloroplasts are weakly illuminated.

Saturation of the three types of intrinsic luminescence seemed to occur at lower light intensity than that of the observed luminescence values. It can be seen also that the increase of the different types of intrinsic delayed fluorescence is relatively low or negative in the range from about 100 up to 6000 erg $cm^{-2}sec^{-1}$. These observations might suggest that the formation of the oxidized primary electron donor saturates, at least in Tris-buffer chloroplasts, at a somewhat lower light intensity than that of Q^-, as measured by the variable fluorescence yield.

4.4 Discussion

Upon addition of salt to preilluminated chloroplasts the intensity

of delayed fluorescence is stimulated. The results mentioned in this chapter can be explained by the hypothesis that the stimulation is caused by a diffusion potential across the thylakoid membrane which is positive on the inside with respect to the outside.

This potential is then caused by a higher permeability of the thylakoid membrane to cations like K^+ and Na^+, more than to anions such as Cl^-, $SO_4^=$, benzoate, glutamate, succinate, etc. The polarity of the membrane potential was deduced from experiments in which the permeability to the cations Na^+ and K^+ was enhanced by the presence of gramicidin[80,81] and to K^+ by valinomycin[82,83] (see Figs. 4.1 and 4.2, and Tables 4.4 and 4.5). In the presence of a low concentration of KCl, valinomycin caused complete inhibition of the extra delayed fluorescence caused by the addition of NaCl (Table 4.4).

Application of the Goldman equation (8) (ref. 78) and the derived equation (10) support these results and conclusions. Moreover the membrane potential theory as pointed out above is also supported by the results shown in Table 4.2 and Fig. 4.3b. The experiments described there were performed with a mixture of salts consisting of 0.6 M potassium benzoate and 0.4 M potassium chloride (Table 4.2) or with various mixtures of these salts in such a way that the final concentration of potassium was kept constant at 0.3 M (Fig. 4.3b). Equation (15) can explain the lowering of the membrane potential due to potassium benzoate if potassium chloride is present too. This is based on the assumption that the permeability to the benzoate anion is much smaller than that to chloride, which is in line with the results shown in Fig. 4.3a. If the stimulation of luminescence were not related to the diffusion potential but only to the concentration of K^+, one should not expect to observe inhibition but an extra enhancement of delayed fluorescence if KCl was added to potassium benzoate (Table 4.2).

The straight line in Fig. 4.3a shows the linear relationship between luminescence by potassium benzoate and its concentration. According to equation (10) and the assumption that the quotient after the logarithm increases linearly with the potassium concentration when the variable term in the denominator is very small (see above and eq. (11)), it is likely that the delayed fluorescence is proportional to the ex-

ponential of the changed membrane potential (eq. (12)), because the increase in the luminescence intensity then is proportional to the potassium concentration (eq. (13)).

Salts of reportedly low permeability to anions such as those of aliphatic zwitterionic or dicarboxylic acids[89] were also found to stimulate the luminescence more than salts like NaCl or KCl (see Table 4.3). This is in agreement with the membrane potential theory as was suggested by Jagendorf[84].

The light-induced absorbance change at 515 nm is assumed to be an indicator for a membrane potential[81]. The addition of salt to chloroplasts also may generate a membrane potential of the same polarity if the thylakoid membrane is more permeable to the cation than to the anion used.

Recently Strichartz[96] found that salt addition to a suspension of chloroplasts in the dark caused changes in absorbancy at 515 nm. The difference spectrum obtained in this way by addition of potassium salts, especially in the presence of valinomycin, was identical to the photo-induced one. Moreover, under the latter conditions the absorbance changes were proportional to the logarithm of the concentration of the added salt. This result seems to confirm the exponential relationship between delayed fluorescence and the membrane potential (eq.(12)) as induced by salt addition.

It was suggested that salt present during the light phase might give a better control of electron flow to the energy conserving mechanism[94] in chloroplasts and induce a conformational change[92] in the thylakoid membrane together with a configurational change[7]. Indeed a light-induced decrease of the thickness of the thylakoid membrane (conformational change) and of the internal membrane distance within the thylakoid (configurational change) was observed recently by means of electron-microscopy and microdensitometric measurements[7,8,97]. This decrease was found in the presence of NaCl and phenylmercuriacetate or sodium acetate[7] if isolated chloroplasts were used. The same effect without added salt was observed in vivo with the algae Ulva and Porphyra[97]. The results obtained on the enhanced yield of fluorescence in broken chloroplasts by low amounts of divalent cations were explained by the hypothesis that the light-induced movement of ions caused a modification of the

membrane structure which upon illumination by light of pigment system 2 favoured the transfer of excitation energy to this photosystem[99]. The typical high intensities of delayed fluorescence caused by preincubation with salts could be fully explained by the equally increased variable fluorescence yield (Figs. 4.4 and 4.5 and Tables 4.5 and 4.6). Table 4.5 indicates that the intrinsic delayed fluorescence as obtained from chloroplasts incubated with a high concentration of NaCl before the illumination was equal to the normal intrinsic delayed fluorescence in contrast to the observed luminescence.

This table also shows that if that part of the luminescence intensity was considered which is not related to the total and to the variable yield of fluorescence (see Sections 1.22 and 3.3) the ratio of the thus corrected luminescence increased by NaCl to that without salt addition, which is called the stimulation factor, might be related to the contribution of the NaCl-induced membrane potential. Calculations according to equation (12) resulted in values of about 15 and 50 mV without and with gramicidin D, respectively. Lavorel's conclusion, however, that the luminescence intensity divided by the total fluorescence yield is linearly related to the variable fluorescence yield[41,42] is not confirmed by the results obtained by Clayton[43] (see Section 1.2.2). However, as far as NaCl addition after the preillumination is concerned, the stimulation factors mentioned in Table 4.5 do not change in a relevant way if the ratios were taken from the intrinsic luminescence values, because the differences between the base fluorescence in the control and that in the NaCl experiments and similarly the differences between the corresponding values of the variable fluorescence were rather small. Also the variable fluorescence was smaller than the base fluorescence. For these reasons the membrane potentials do not change too and in fact the indicated method to calculate these potentials is rather independent on the use of one of the two mentioned columns (see Table 4.5).

The influence of Tris-buffer and also of gramicidin D on the salt-induced stimulation of delayed fluorescence will be dealt with more fully in Chapter VI.

CHAPTER V

RELATION BETWEEN DELAYED FLUORESCENCE AND THE LIGHT-INDUCED HIGH-ENERGY STATE

5.1 Introduction

From the previous two chapters and the general introduction it is obvious that delayed fluorescence can be stimulated by an "energized state" of the thylakoid membrane brought about by an acid-base transition or salt addition. The delayed fluorescence is also relatively high if an energy-rich state induced by light exists[55].

After prolonged illumination the intensity of delayed light decays about 100-fold between 1.10^{-3} and 1.10^{-1} sec (refs. 32, 100). This fast decay is remarkable in view of the much slower disappearance of reduced Q as indicated by the decrease of the fluorescence yield in the dark (see Fig. 5.3b and Table 6.1), especially for chloroplasts without addition of an electron acceptor, and of the disappearance of the various oxidized forms of Z, as deduced by Joliot et al.[45,47] from measurements of oxygen evolution.

However, according to the theories of Mitchell[68] and Witt et al.[69] the preillumination generates an electrochemical potential difference, consisting of a positive membrane potential and a pH difference. Both these would, according to the results and discussions of Chapters III and IV, strongly stimulate delayed fluorescence. After switching off the light the stimulation factor then decreases upon decay of the membrane potential and proton gradient which are dependent on the degree of coupling between electron transport and photophosphorylation and on phosphorylation conditions. This is supported e.g. by the decay kinetics of the light-induced absorbance change at 515 nm which seems to be an indicator for the membrane potential[81,99].

In this chapter a few experiments will be described, which show that the luminescence measured after 2 or 3 sec and after shorter dark-times, respectively, have different saturation intensities and behave

differently to uncouplers like gramicidin D. A similar effect by the uncoupling agent Triton X-100 will be shown in Chapter VII.

5.2 Results

In order to get more information about the influence of the stimulation factor induced by light, the effect of the preillumination intensity was studied on delayed fluorescence measured at different darktimes (see Fig. 5.1). The insert shows that the yield of the variable fluorescence measured at the end of the preillumination in the absence of an artificial electron acceptor was saturated at a rather low intensity. This also applied to the delayed light emission measured after 2 or 3 sec (see the two lower curves). The top curves show that at shorter darktimes the saturation intensity was much higher and the delayed fluorescence measured after 40 msec did not saturate, although the highest light intensity used was about 25 times that of the intensity which yielded saturation of the variable fluorescence. The top curve (40 msec) also indicates that the increase in the luminescence at about 10^3 erg.cm^{-2}.sec^{-1} was larger than the increase of the 3 sec luminescence. This is presumably due to a higher variable fluorescence left at 40 msec than at 3 sec of darkness (see Fig. 5.3b, top curve).

Separate experiments (not shown) indicated that the change in absorbance at 515 nm which is an indication of the membrane potential[81] was not saturated at intensities between 10^3 and 10^4 erg.cm^{-2}.sec^{-1}. Thus the "rapid" but not the "slow" phase of delayed fluorescence might be stimulated by a membrane potential generated during the preilluminat-ion.

The uncoupling compound gramicidin D is known to inhibit strongly the absorbance change at 515 nm (ref. 69), and with continuous illumination no change at all was observed in the presence of 10^{-6} M gramicidin (unpublished observation). The effect of this antibiotic on delayed fluorescence is shown in Fig. 5.2. It can be seen that the apparent inhibition of the luminescence by this compound decreases with increasing time of darkness. This seems to indicate that without gramicidin D the contribution of the membrane potential to the luminescence intensity is

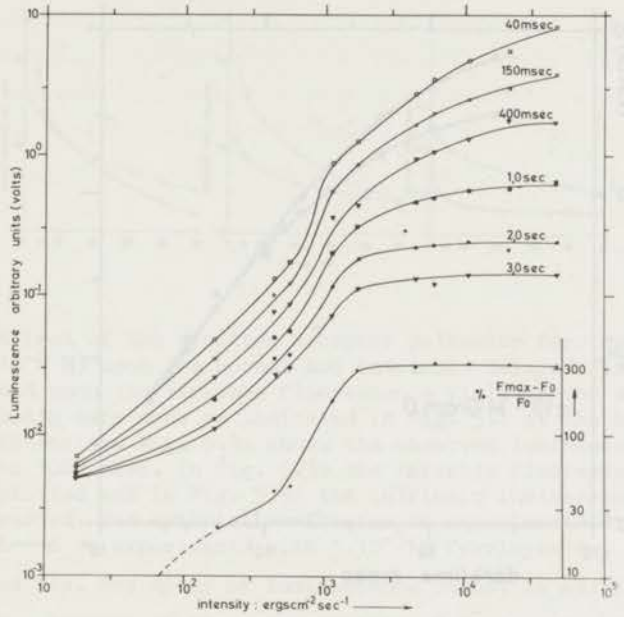

Fig. 5.1. Effect of preillumination intensity upon delayed fluorescence at different darktimes and upon the increase of the fluorescence in the steady state. Class I chloroplasts were suspended in a solution containing 0.03 M Tricine-NaOH, pH 7.8, 3 mM $MgCl_2$ and 0.4 M sucrose. Final concentration of chlorophyll was $2.5.10^{-5}$ M. The chloroplasts were preilluminated for 5 sec in the measuring cuvette with blue light of indicated intensity, transmitted by a Balzers Calflex C, and Corning 4-96 and Schott 18-2 colour filters and varied by neutral grey filters. Luminescence was measured from 40 msec up to 3 sec after preillumination. Fluorescence (insert at the bottom) was measured just before ending the preillumination.

diminishing with increasing darktime.

A third indication that a high-energy state may contribute to the luminescence intensity was obtained by the measurements of luminescence and the "dark" fluorescence yield induced by high light intensities in the presence of potassium ferricyanide (see Fig. 5.3). The electron acceptor, which has a higher oxidation-reduction potential (E'_o = +430 mV)[101] than the primary electron acceptor of PS2, Q (E'_o = -35 mV)[102],

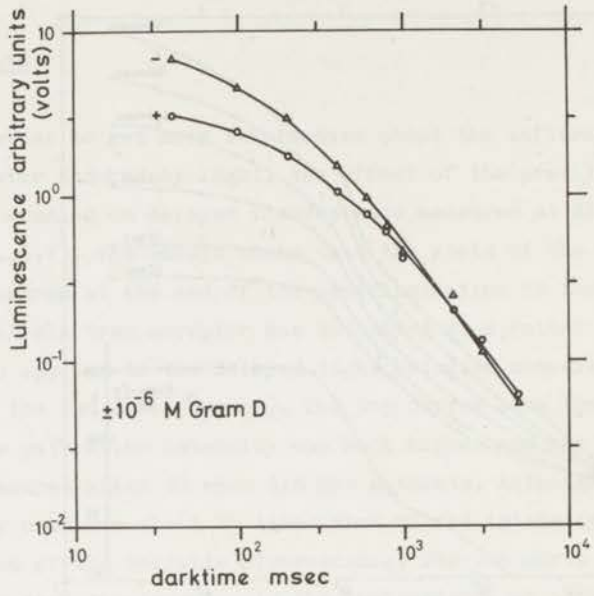

Fig. 5.2. Inhibition of delayed fluorescence by gramicidin D ($1.0.10^{-6}$ M) as a function of darktime. Measurements were done as indicated in Fig. 5.1 at the highest light intensity.

kept at least during the short period of illumination (5 sec) the concentration of Q^- below its maximal value. This is shown in Fig. 5.3b, where the variable fluorescence yield measured at the end of the illumination and during the subsequent sec of darkness is plotted. Also the rate of reoxidation of Q^- was higher in darkness with the electron acceptor present than in its absence. However, the luminescence excited in the presence of ferricyanide (Fig. 5.3a) was higher at darktimes below about 200 msec than in its absence, and the intrinsic luminescence ($L/\Delta F$) (section 1.2.2 and Chapter IV) was higher up to about 1 sec.

The higher luminescence in the presence of ferricyanide may be due to two causes. Firstly the possibly higher concentration of ZH^+ (S_3^{3+}) in the presence of the electron acceptor is due to the higher rate of electron transport. Secondly the high-energy state or electrochemical potential gradient coupled to the higher rate of electron transport may

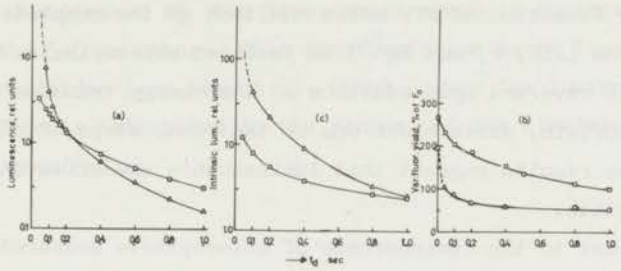

Fig. 5.3. Effect of the electron acceptor potassium ferricyanide ($5.0.\ 10^{-5}$ M) upon the normal and intrinsic delayed fluorescence and upon the variable fluorescence yield in the dark. Measurements were done as indicated in Fig. 5.1 at the highest light intensity. Fig. 5.3a shows the observed luminescence from 40 to 1000 msec. In Fig. 5.3b the variable fluorescence yield is plotted and in Fig. 5.3c the intrinsic luminescence is represented. The symbols □——□ refer to experiments without and Δ——Δ to experiments with 5.10^{-5} M ferricyanide.

be increased too. The decay of luminescence cannot be attributed to that of ZH^+ only, because if the concentration of Q^- is not maximal, its halftime of decay in chloroplasts is about 30 sec (ref. 103). The rate of disappearance of the 515 change[99], which has been attributed to the disappearance of the membrane potential is of the same order of magnitude as the difference in decay between the intrinsic luminescence signals. The results shown in Fig. 5.3c are consistent with the assumption that the contribution of the high-energy state to the luminescence has disappeared in about 1 sec of darkness.

5.3 Discussion

Consideration of some results previously described in the literature have led to indications about the influence of the so-called high-energy state on delayed fluorescence. Although at that time the absorbance change at 515 nm (refs. 15, 16) was not interpreted as a high-energy state[81] it was found that delayed fluorescence (Chlorella) paralleled in many respects this absorbance change[16], both measured about

0.1 sec after actinic illumination (see Section 1.2.1). The 4 msec delayed fluorescence was inhibited either by uncouplers of photophosphorylation or by formation of ATP after addition of the complete phosphate acceptor system (ADP, P_i and Mg^{2+}) to isolated chloroplasts. The latter inhibition was reversed upon addition of the energy transfer inhibitors[55] DIO-9 and phlorizin, which block one of the final steps of photophosphorylation. These results suggest that luminescence was affected by the high-energy state.

In contrast to the luminescence of chloroplasts measured after about 0.2 sec or longer time (see also Fig. 5.3a), which was lower in the presence of an electron acceptor such as potassium ferricyanide[43,104], the corresponding values measured in the msec range were found to be higher if electron acceptors were present, e.g. ferricyanide[43,55,105], methylviologen[105] or NADP + ferredoxin[105] (see Fig. 1.2). The lowering of luminescence at longer times[43,104] was caused by a lower fluorescence yield[43]. The higher luminescence intensity at shorter times can be explained by the accumulation of the high-energy state due to increased electron transport in the presence of the electron acceptors. This is in agreement with Clayton's conclusion that high luminescence is associated with an increased photochemical turnover[43].

Fig. 5.3b shows that the fluorescence and thus Q^- decreased only little in 40 msec of darkness in the absence of ferricyanide. The top curve of Fig. 5.1 which has been measured also after 40 msec in the absence of the electron acceptor shows a "rapid" increase in luminescence at an intensity of about 10^3 erg.cm^{-2}.sec^{-1}. This increase also occurred in the variable fluorescence measured in the steady state (see bottom insert). Thus this increase in luminescence is probably largely due to the accumulation of Q^-. For intensities higher than 10^3 erg.cm^{-2}.sec^{-1} a "slow" but large increase in the 40 msec luminescence was found while the fluorescence stayed constant. This increase also occurred in the absorbance change at 515 nm under these conditions. The latter increase in the luminescence is attributed to the appearance of an electric field across the thylakoid membrane. The luminescence measured after 3 sec shows a much less pronounced increase due to the accumulation of Q^-, because at about 10^3 erg.cm^{-2}.sec^{-1} the fluorescence has decreased after

3 sec as indicated in Fig. 5.3b. No increase in the luminescence measured after 3 sec occurs at higher intensities. Apparently the electric field has disappeared at this time, which again is in agreement with the decay of the 515 nm change. This interpretation is in line with the effect of gramicidin (see Fig. 5.2), which lowers the luminescence after 40 msec. This is attributed to the removal of the electric field by the uncoupler gramicidin.

CHAPTER VI

EFFECT OF SUSPENSION MEDIUM, MEMBRANE PERMEABILITY MODIFYING AGENTS AND DCMU ON THE VARIOUS TYPES OF LUMINESCENCE

6.1 Introduction

In Section 3.3 it was mentioned that the intensity of stimulated delayed fluorescence was dependent on the type of buffer used. Therefore it was important to study the effect of Tris and of the zwitterionic buffers, Tricine and TES upon the several types of luminescence. The effect of compounds, which enhance the permeability of thylakoid membranes to monovalent cations, like gramicidin D, FCCP and DNP, will also be reported in this section.

6.2 Results

6.2.1 Effects of Tris, Tricine and DCMU on the variable fluorescence yield. Table 6.1 shows that the fluorescence yields of chloroplasts suspended in Tricine and Tris-buffers (with or without added KCl) are, except for those in the second column, basically the same. The fluorescence level in weak light (the base level, F_o, when Q is presumably largely oxidized), and in stronger light with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)[26] (the maximal level, F_1, when Q is probably completely reduced) were approximately the same in both buffers. The main difference was in the fluorescence level in stronger light without DCMU, which may indicate a higher level of reduction of Q in Tris than in Tricine buffer. Upon cessation of illumination the variable fluorescence level decreased to approximately 50% in about 2 sec in most experiments.

6.2.2 The effects of the various buffers on fluorescence and on the several types of luminescence. Tables 6.2 and 6.3 demonstrate the effects of suspension medium on luminescence and fluorescence. The main difference between chloroplasts suspended in TES, Tricine and Tris-buffers was in the stimulation of the observed luminescence induced by NaCl or KCl. The stimulation induced by NaCl or KCl was larger TES or in Tricine buf-

Medium	No additions			1.10^{-6} DCMU		
	F_o	F_1	$F_{(2\ sec)}$	F_o	F_1	$F_{(2\ sec)}$
Tricine-KOH	9.4	18.9	14.1	10.0	28.7	17.3
Tris-HCl + KCl	9.7	29.7	17.9	11.7	30.5	22.6
Tris-HCl	9.7	24.4	17.5	12.1	30.2	22.9

Table 6.1. Relative fluorescence yields of chloroplasts suspended in different media. Class I chloroplasts were isolated in Tricine-KOH buffer (see Materials and Methods) and suspended in the various buffers indicated (pH 7.8). The concentration of K^+, if present, was 15 mM, that of chlorophyll was 5.10^{-5} M. Fluorescence was excited by weak modulated blue light (472 nm, 4.10^{-11} Einstein.cm^{-2}.sec^{-1}). The yield of fluorescence was modified by additional actinic light (487 nm, 1.10^{-9} Einstein.cm^{-2}.sec^{-1}) in a way similar to that described by Duysens and Sweers[26]. The steady-state fluorescence yield without actinic light is denoted by F_o; F_1 denotes the steady-state yield with actinic light, and $F_{(2\ sec)}$ the yield, 2 sec after cessation of actinic illumination. The fluorescence yields are expressed in arbitrary units, different from those of the other tables and figures.

fer than in Tris. However, as for the intrinsic luminescence or for the luminescence corrected for both the variable and the total fluorescence yield, the difference between the light emission stimulated by NaCl or KCl addition and the normal luminescence was smaller. The changed variable fluorescence yields in the salt experiments may be due to an unknown mechanism, which might be related to ion-induced structural change of the thylakoid membranes[106]. The significance of the corrections applied for the two last columns of both tables was discussed already in Chapter IV (see Table 4.5).

Another difference between chloroplasts suspended in the buffers mentioned above was found in the luminescence upon pH lowering. In agreement with the results shown in Figs. 3.1a and 3.1b acidification of the chloroplasts caused a decrease or an increase of luminescence if the suspension contained the zwitterionic buffers or Tris, respectively. The intrinsic luminescence at low pH turned out to be more increased than the observed luminescence (Tris experiments), less decreased (TES) or even slightly higher than the control (Tricine) (see Tables 6.2, 6.3

Type of experiment	Luminescence L	Integrated luminescence	Fluorescence yield		Intrinsic luminescence	Luminescence corrected for fluorescence
			F_o	ΔF	$L/\Delta F$	$L/(F_o + \Delta F)\Delta F$
TES-KOH						
Control	65 (59)	119 (110)	13.0 (13.0)	3.0 (4.0)	21.6 (14.7)	1.35 (0.87)
NaCl	155 (130)	172 (142)	13.0 (12.0)	5.0 (6.5)	31.0 (20.0)	1.72 (1.08)
Acid	24 (36)	37 (55)	13.0 (13.0)	1.5 (2.5)	16.0 (14.0)	1.10 (0.90)
Acid-base	500 (610)	143 (97)	13.0 (12.5)	5.0 (5.5)	100 (111)	5.5 (6.17)
Tris-HCl + KCl						
Control	70 (69)	120 (116)	13.0 (13.0)	7.5 (8.0)	9.34(8.62)	0.45 (0.41)
NaCl	88 (151)	173 (173)	15.0 (15.0)	7.0 (8.0)	12.6 (18.9)	0.57 (0.82)
Acid	96 (164)	71 (81)	14.0 (14.0)	4.0 (4.5)	24.0 (36.5)	1.33 (1.97)
Acid-base	980 (860)	141 (108)	14.5 (15.0)	7.5 (6.0)	130 (143)	5.9 (6.80)

Table 6.2. Influence of different buffers and gramicidin on the various types of luminescence, after rapid mixing. Normal chloroplasts were isolated in TES-KOH buffer and suspended either in 0.05 M TES-KOH (pH 7.8) or in 0.05 M Tris-HCl (pH 7.8) with added KCl (30 mM). Final chlorophyll concentration was $2.5.10^{-5}$ M. In the acid experiments the pH was lowered to 5.6 (TES) and 4.2 (Tris), respectively, by addition of 0.02 M succinic acid during the second mixing period. In the acid-base experiments the pH lowered during the first mixing period (see acid exp.) was subsequently raised to 8.5 and 8.6, respectively, with 0.1 M Tris. The darktime was 3 sec. The numbers in parentheses refer to experiments with 1.10^{-6} M gramicidin D, added 10 min before preillumination. NaCl concentration was 0.3 M. The meanings of the various columns are described already in Chapter IV, Table 4.5. The other conditions as given in Fig. 2.2.

and 6.5). This might be due to a partial increase of luminescence not correlated to the lowered variable fluorescence yield but caused by penetration of protons into the interior, which is favourable for the delayed fluorescence (see Section 3.2, Fig. 3.2 and Section 3.3, eq. (3)). An increase in luminescence caused by an increase in pH was found for both types of buffer, in line with results of Figs. 3.1a and 3.1b. The corresponding increase in variable fluorescence yield was more pronounced if Tris was used instead of Tricine. This also applied to the decrease in the experiments in which the pH was lowered (Table 6.3).

In normal luminescence signals ("control") and considering the smaller pH jump in the case of the TES experiment, the acid-base signals

Type of experiment	Luminescence L	Integrated luminescence	Fluorescence yield		Intrinsic luminescence	Luminescence corrected for fluorescence
			F_o	ΔF	$L/\Delta F$	$L/(F_o + \Delta F)\Delta F$
		Tricine-NaOH				
Control	71 (81)	490 (540)	10.3 (10.2)	4.7 (6.3)	15.1 (13.0)	1.01 (0.79)
KCl	175 (167)	700 (640)	10.3 (11.2)	6.0 (6.1)	29.1 (27.3)	1.78 (1.58)
K-benzoate	325 (650)	475 (665)	10.3* (11.2)*	6.0 (7.0)	55 (93)	3.3 (5.10)
Acid	50.5(125)	275 (335)	11.2 (10.2)	3.0 (5.3)	16.8 (23.6)	1.18 (1.52)
Base	138 (72)	470 (360)	12.1 (12.3)	4.9 (5.0)	28.1 (14.4)	1.65 (0.83)
		Tris-HCl				
Control	71 (90)	450 (605)	12.6 (12.2)	6.3 (7.4)	11.3 (12.1)	0.60 (0.62)
KCl	120 (640)	680 (1110)	12.6 (13.5)	6.0 (8.1)	20.0 (79)	1.08 (3.66)
K-benzoate	480 (1990)	605 (1105)	12.6* (13.5)*	7.6 (5.0)	63 (398)	3.12 (21.5)
Acid	105 (580)	375 (653)	15.0 (14.0)	1.4 (3.4)	75 (170)	4.5 (9.75)
Base	133 (97)	550 (512)	12.3 (13.0)	9.1 (10.1)	14.6 (9.6)	0.68 (0.42)

*Like in Table 4.6 F_o slowly increased independently of illumination. Therefore the F_o values of the KCl experiment are given in this column and also used in the last two columns.

Table 6.3. The absence of KCl in the Tris suspension and the effect of gramicidin on luminescence affected by a pH jump. Normal chloroplasts were prepared in 0.05 M Tris-HCl buffer, pH 7.8, and suspended either in 0.05 M Tricine-NaOH, pH 7.8, or in the isolation buffer. Final chlorophyll concentration was 5.10^{-5} M. In the acid experiment the pH was lowered with an equal volume of a sucrose solution containing 0.02 M succinic acid to pH 4.2 (Tricine) and 4.0 (Tris), respectively, whereas in the basic experiment the pH was raised from pH 7.8 to pH 9.1 (Tricine) and to pH 9.0 (Tris) by addition of an equal volume of sucrose solution plus 0.1 M Tris. The darktime was 3 sec. The numbers in parentheses refer to experiments with 1.10^{-6} gramicidin D, added 10 min before the measurement. The other conditions as for Table 6.2.

in Table 6.2 were of comparable magnitude.

6.2.3 Gramicidin D. This uncoupler in general had little effect on the normal luminescence as pointed out in Chapter V (see Fig. 5.2 at 3 sec of darktime), and also on the base fluorescence. However, gramicidin slightly increased the variable fluorescence yield. This observation cannot be explained in line with equation (4) (Section 3.3):

$$Q^- + H^+ \rightleftarrows QH \qquad (4)$$

If acidification would cause a lowering of the concentration of Q^-, which is supposedly a precursor of luminescence (Section 3.3, eq. (4)), an increase in pH then must give an increase in Q^-. In almost all experiments, the uncoupler enhanced the variable fluorescence yield. This

may suggest that the effect is not related to pH-induced changes in the concentration of Q^-.

From Table 6.3 it can be seen that gramicidin increased the luminescence values at low pH and decreased the delayed light emission at high pH. This was also true for the intrinsic luminescence values. Both changes are possibly related to the uncoupling effect of gramicidin[80,81]. The antibiotic increases the permeability to all monovalent cations. Therefore it may enhance the acidification of the thylakoid interior and accelerate the decay of the proton gradient in the case of the low and of the higher pH experiments, respectively.

In general the acid-base induced increase of delayed fluorescence was inhibited by gramicidin D (see Tris experiments in Tables 6.2 and 6.5, and the Tricine experiment in Table 6.5), apparently due to the accelerated decay of the proton gradient as can be seen also from the integrated luminescences. As for the Tris experiments the higher the enhancement of the acid-induced increase of luminescence in the presence of the uncoupler, the smaller was the stimulation of luminescence in the corresponding acid-base experiment (see Tables 6.5 and 6.2). Besides the accelerated decay of the pH gradient here the lowering of delayed fluorescence is also due to loss of luminescence capacity during the acid period.

With Tris the NaCl and KCl-induced increase of delayed light was stimulated by gramicidin (see Section 4.3.5, Table 4.5) and even more strongly if the medium did not contain KCl added previously. According to the explanation that the increase of stimulation by gramicidin is due to the enhanced permeability to Na^+ and to K^+ (see Chapter IV, eq. (10)), the stronger stimulation can be explained by the larger gradient of the cation, which corresponds to a larger membrane potential (see Chapter IV, eq. (8)). The latter reasoning can be applied also to the corresponding experiments without the uncoupler. In contrast to the results with Tris-buffer the NaCl or the KCl-induced increase of luminescence in TES or Tricine buffer was slightly inhibited by the uncoupler (see Tables 6.3 and 6.5). In the presence of gramicidin[80,81] the permeability of the thylakoid membrane to both H^+ and Na^+ or K^+ ions is much higher, although it might be possible that the increase of permeability

to protons by gramicidin is lower in Tris than in the zwitterionic buffers (see below). Therefore it could be possible that in contrast to Tris-buffer the increase of membrane potential induced by NaCl or KCl in TES or Tricine is roughly cancelled by an increased rate of effluxing protons in the presence of the uncoupler. The stimulated delayed fluorescence induced by potassium benzoate in Tris was highly increased by gramicidin. This is in agreement with the results shown in Fig. 4.1. However, gramicidin did not inhibit the corresponding stimulation in Tricine. This could mean that potassium benzoate partly stimulates luminescence by an internal acidification caused by penetration of uncharged benzoic acid[62] (see Section 4.3.2).

6.2.4 The uncoupling effect of Tris. The differences between the results with zwitterionic buffers and Tris are not quite clear, but may be related to the well known uncoupling action of Tris[67]. In Tris buffer without gramicidin but with KCl present (Table 6.3) the stimulation by salt addition is smaller than in Tricine or TES. This indicates that the diffusion potential upon addition of NaCl (or KCl) may be lowered due to effluxing protons driven by the electric field. To accept this suggestion one has to assume that the permeability to protons in Tris is higher than in TES, caused by cyclic permeation of Trisonium cation and free Tris base[110]. This was also found by Miles and Jagendorf[84].

The inhibiting effect of Tris upon the salt-induced increase of luminescence was not dependent on a long time of incubation. If Tris buffer replaced Tricine after the preillumination period, the KCl-increased light emission was lowered.

The effect of gramicidin on the normal luminescence was much more conspicuous after a shorter darktime (0.1 sec) as shown in Table 6.4. Here the luminescence signal was considerably higher in Tricine than in Tris-buffer. Gramicidin gave a more than two-fold inhibition of the signal in Tricine. However, it had a much smaller effect on the luminescence in Tris-buffer, dependent on the presence of added KCl. In comparison to the lowering of luminescence by gramicidin as shown in Fig. 5.2 after 0.1 sec which was slightly less than 50 %, the inhibition here in Tricine was larger, presumably due to the lower intensity of actinic light. Inhibition of photophosphorylation by gramicidin increases with

Medium	Gramicidin	Luminescence L		Integrated luminescence		Fluorescence yield			
						F_o		ΔF	
		-	+	-	+	-	+	-	+
Tricine-KOH		4120	1650	1270	810	8.0	7.7	6.7	7.0
Tris-HCl + KCl		1570	1120	960	760	9.0	8.5	14.5	14.5
Tris-HCl		2180	2490	930	930	9.0	8.0	11.3	11.0

Table 6.4. Effects of gramicidin on the prompt and delayed fluorescence measured after 0.1 sec. Chloroplasts were isolated in Tricine-KOH buffer, pH 7.8, and suspended in the various buffers indicated (pH 7.8). Final chlorophyll concentration was 5.10^{-5} M. The chloroplast suspension was preilluminated with blue light (see Fig. 5.1) at an intensity of $1.5.10^{3}$ erg.cm^{-2}.sec^{-1} (≈ 0.6 nEinstein.cm^{-2}.sec^{-1}) in the measuring cuvette. After a darktime of 0.1 sec luminescence was measured. The fluorescence yield was measured in a separate experiment with the luminescence photomultiplier (see Section 2.2.4). ΔF denotes the variable fluorescence yields measured after 0.1 sec. The concentration of gramicidin was $1.0.10^{-6}$ M. The integrated luminescence was recorded up to 100 sec. Further conditions as given in Fig. 2.2.

decreasing light intensity[107]. The lower yield of delayed light in Tris and the smaller inhibition by gramicidin is in line with the discussed uncoupling action of Tris.

6.2.5 The various effects of the uncouplers 2,4-DNP and FCCP on luminescence. The above explanation for the different effects of the buffer used on the normal and salt-induced increase of luminescence is supported by results of similar experiments in the presence of 2,4-DNP (see Table 6.5). This compound[68,108] is known to enhance the permeability to protons only. Addition of NaCl to chloroplasts in Tricine buffer in the presence of DNP ($7.5.10^{-5}$ M), which was added after the preillumination, caused a smaller stimulation of delayed fluorescence than without DNP. The inhibition by DNP is slightly higher in the salt experiment than in the control one, not only when NaCl but also when Na-benzoate (not shown) was added. An explanation is that DNP^- anions driven by the electrical field[68] due to NaCl addition enter the thylakoid interior, and reduce the membrane potential. This could furthermore result in a slight in-

Type of experiment	Luminescence L			Integrated luminescence			Fluorescence yield F_o			Fluorescence yield ΔF			Intrinsic luminescence $L/\Delta F$			Luminescence corrected for fluorescence $L/(F_o + \Delta F)\Delta F$		
		G	D		G	D		G	D		G	D		G	D		G	D
Tricine-KOH																		
Control	61	50[1]	52	120	114	88	10.5	12.5[1]	11.3	4.5	3.0[1]	4.0	13.6	16.7	13.0	0.91	1.08	0.85
NaCl	370	200[1]	246	214	160	130	12.5[2]	12.5	12.2	2.5[2]	5.5	3.6	148[2]	36.4	68	10[2]	2.29	4.32
Acid	32	58[1]	43	43	60	46	12.0	12.0	12.0	2.0	3.5	2.0	16.0	16.6	21.5	1.14	1.07	1.54
Acid-base	950	780	310	150	105	44	12.0	12.0	12.0	3.0	4.5	6.0	317	173	52	21	10.5	2.88
Tris-HCl + KCl																		
Control	82	81	85	137	151	105	12.0	12.0	12.5	7.5	8.0	8.0	10.9	10.1	10.6	0.56	0.50	0.52
NaCl	135	225	125	228	219	151	14.0	14.0	14.0	6.5	7.5	6.5	20.8	30.0	19.2	1.01	1.39	0.94
Acid	115	275	228	85	114	80	14.0	13.0	16.0[3]	2.5	4.5	0[3]	46	61	114[3]	2.79	3.49	7.1[3]
Acid-base	980	520	490	163	56	55	13.5	14.0	14.0	7.0	5.5	10	140	95	49	6.83	4.85	2.0

[1] First mixing after illumination was performed with Tris-buffer instead of Tricine.

[2] Very often the fluorescence after 2 min in the NaCl experiment was relatively not so much higher than the corresponding value in the control experiment. Therefore the NaCl-induced luminescence values as given in the last two columns might be about 30 % lower.

[3] At the low pH in Tris-buffer DNP enhanced the base fluorescence yield independently of preillumination. Therefore the calculations in the last two columns are performed with the F_o of the NaCl experiment.

Table 6.5. The effects of 2,4-DNP and gramicidin on the various types of luminescence from chloroplasts in different buffers. Normal chloroplasts were isolated in Tricine-KOH, pH 7.8, and suspended either in 0.05 M Tricine-KOH, pH 7.8, or in 0.05 M Tris-HCl (pH 7.8) + 0.016 M KCl. Final chlorophyll concentration was $2.5.10^{-5}$ M. In the acid experiments the pH was lowered with 0.02 M succinic acid to pH 4.2 (Tricine) and 4.0 (Tris), respectively. In the acid-base experiments the pH was subsequently raised to pH 8.7 with both buffers used. The data given in the "subcolumns" G and D refer to experiments with 1.10^{-6} M gramicidin D, added 10 min before preillumination, and to 2,4-DNP, added immediately after preillumination during the first mixing. Its final concentration was $7.5.10^{-5}$ M. The other conditions are given in Table 6.2.

crease of the internal pH, if DNP^- picks up a proton (possibly within the hydrophobic membrane) and leaves the thylakoid as an uncharged molecule. This latter process, however, must be limited, due to the neutral pH inside.

Upon acidification DNP increased slightly the low luminescence sig-

nal of chloroplasts in Tricine buffer. This can be explained by an accelerated lowering of the pH on the inside of the thylakoid membrane. This would also result in a higher pH gradient upon mixing with the base (Tris), which, however, would rapidly dissipate because of transport of the free acid out of the membrane down to the proton gradient. Therefore the acid-base induced increase of delayed light was expected to be lower with the 250 msec mixing time used, including a 50 msec "dead" period. A strong inhibition, especially of the integrated signal, was indeed observed.

With Tris-buffer DNP also gave a slightly inhibited stimulated luminescence upon salt addition. This is in contrast with gramicidin D. This observation supports the hypothesis that inhibition by DNP is due to DNP^- influx. Both DNP and gramicidin stimulated the luminescence signal in Tris upon acidification, which without these uncouplers was higher than the control signal (see Section 3.2.1). The acid-base transition induced increase of delayed fluorescence was inhibited also, although the inhibition here (with Tris present in the acid phase) might be partly due also to extra loss of luminescence capacity during the acid period. The integrated luminescence signals in both tables support the conclusions described above. DNP had no significant effect upon the base and variable fluorescence in these experiments.

The effect of various concentrations of DNP and FCCP on luminescence was studied for the two types of pH jumps in Tris-buffer. The results were related to the uptake or release of protons and/or of undissociated acid molecules[109]. FCCP[107,111] is a strong uncoupler of photophosphorylation, especially at low light intensities[111], whereas DNP[108], at least at neutral pH[112], is not.

At relatively low concentrations both weakly acidic compounds, which increase the membrane permeability to protons, had similar effects on the acid or acid-base stimulated luminescence, as demonstrated for DNP in Fig. 6.1 and for FCCP in Table 6.6. Like in Table 6.5 (Tris-buffer) normal delayed light (see Fig. 6.1) was not affected initially by addition of 2,4-DNP, whereas the integrated signal was inhibited 20 to 25 % at 1 to 2.10^{-4} M DNP. The kinetics of the integrated normal luminescence (not shown) showed that the inhibitory action started only after about

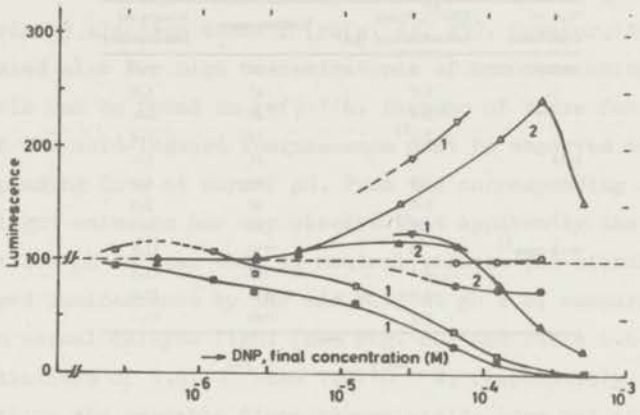

Fig. 6.1. The effects of various concentrations of 2,4-DNP on different types of luminescence. Normal chloroplasts were isolated and suspended in 0.05 M Tris-HCl sucrose, pH 7.8 (see Materials and Methods). Final chlorophyll concentration was $8.5.10^{-5}$ M, whereas that of 2,4-DNP is indicated on the abscissa. Addition of this compound occurred after the preillumination during the first (1) or second (2) mixing period, as indicated at each curve. Open symbols refer to initial luminescence and closed symbols to integrated luminescence values. Except for the acid-base experiments (70 msec) the second mixing time was 250 msec. ○,●, normal delayed light; △,▲ (2);▽,▼(1), luminescence upon acidification with 0.02 M succinic acid to a final pH of 4.2; □,■ (1), acid-base induced increase of luminescence to a final pH of 8.6 after mixing with 0.1 M Tris sucrose. Darktime after preillumination was 3 sec. The other conditions: Sections 2.2 and 2.3. The data with DNP were normalized to those without DNP, which were in relative units:

	Luminescence	Integrated luminescence
Control	47	10.2
Acid	67	6.3
Acid-base	950	10.3

15 to 20 sec.

The luminescence upon addition of acid was further increased with increasing concentrations of DNP, up to about $2.5.10^{-4}$ M. The integrated light emission was increased too, but to a lesser extent, and was inhibited at concentrations higher than 10^{-4} M. At no concentration of DNP the total light emission was higher than the corresponding values at neutral pH. The stimulation may be caused by an increased rate of

Type of experiment	FCCP[1], final concentration (µM)	Luminescence	Integrated luminescence
Control	0	52	9.8
	1.0	47	5.0
	5.0	42	2.2
	2.5[2]	14	1.4
Acid	0	61	7.1
	1.0	69	6.0
	5.0	68	3.9
	2.5[2]	23	3.1
Acid-base[3]	0	1110	13.7
	0.1	1190	13.5
	0.5	1230	12.6
	2.5	1195	10.1

1) Addition of FCCP after preillumination during second mixing.
2) " " " " " " first "
3) These experiments were performed with the 70 msec mixing procedure.

Table 6.6. Influence of FCCP on various types of luminescence. Chloroplasts were isolated and suspended in Tris-buffer. Final concentration of chlorophyll was $6.9.10^{-5}$ M. Further conditions as for Fig. 6.1.

proton uptake (via the free acid), whereas the inhibition of the integrated signal correlated roughly with a decreased yield of prompt fluorescence (not shown). The decrease in stimulation of the acid-changed luminescence at high concentration of DNP was not caused by delay in registration (50 msec), because at the maximal stimulation of this type of delayed fluorescence by DNP ($2.5.10^{-4}$ M) no difference was obtained if the more rapid (70 msec) or the slower (250 msec) mixing procedure was used.

The above results are partly similar to corresponding data obtained with the uncoupler FCCP (see Table 6.6). Here the integrated light emission in the acid-base experiments was inhibited less than the integrated normal delayed light. Normal delayed light was inhibited immediately by FCCP in contrast to DNP, whereas the integrated light emission was more strongly affected, indicating that FCCP not only reduces the rate of back reaction in the reaction center of PS 2, but also the amount of luminescence capacity. It may be that FCCP[113,114] besides its uncoupling

activity also inhibits electron transport between H_2O and PS2 by increasing the rates of de-activation of the different stages between H_2O and the primary electron donor Z (refs. 44, 45). However, this effect was published also for high concentrations of ammonium chloride[115]. More details can be found in ref. 116. Because of these facts the stimulation of the acid-induced luminescence must be observed in relation to corresponding data at normal pH. From the corresponding data of integrated light emission one may observe that apparently the inhibition by FCCP at low pH is less than at neutral values. The stimulation of acid-changed luminescence by DNP and FCCP at pH 4.2, compared to their effects on normal delayed light (see Fig. 6.1 and Table 6.6), was equal at concentrations of $1.0.10^{-5}$ and $1.0.10^{-6}$ M, respectively. At these concentrations the variable fluorescence yields were not affected by the uncouplers. On the assumption that the extra increase of delayed light emission is due to penetration of the protonated molecules of DNP and FCCP, and taking into account the p_{K_a} values[117] (approximately 4.1 and 6.1, respectively), this suggests that the permeability to FCCP might be 4 - 5 times higher than that to DNP.

6.2.6 The effect of DCMU on luminescence. It was assumed that the stimulation of luminescence in the acid-base experiment was caused by increased concentrations of Q^- and ZH^+ on the outside and the inside of the thylakoid membrane, respectively (see ref. 62 and eqs. (2) - (4) in Section 3.3). To test this hypothesis experiments were performed with DCMU. The effect of DCMU ($1.3.10^{-5}$ M) on delayed fluorescence is summarized in Table 6.7.

As shown in Table 6.1 the variable fluorescence in Tricine remaining after 2 sec of darktime was higher in the presence of DCMU. The normal delayed fluorescence was found to be higher approximately to the same extent (see also ref. 43).

The NaCl and the acid-base transition induced increase of delayed light emission was partially inhibited. This is in agreement with earlier reports[57,59]. Like with the control experiment, the variable fluorescence was enhanced by DCMU. The mechanism of the inhibition is uncertain. If one assumes that the concentration of ZH^+ after 2 sec is unaffected by DCMU (see control experiment) the inhibitory activity must

Type of experiment	- DCMU				+ DCMU			
	Luminescence	Integrated luminescence	F_o	ΔF	Luminescence	Integrated luminescence	F_o	ΔF
Control*					34	158	42	47
Control	49	236	58	56	64	321	62	73
NaCl	104	363	68	47	80	304	70	72
Acid (2)	22	110	65	32	34	271	62	80
Acid (1,2)	19	120	70	30	42	242	62	78
Acid-base	515	438	87	52	247	224	65	64

Table 6.7. The effect of DCMU on the various types of luminescence and on the variable fluorescence. Class I chloroplasts were isolated in Tricine-KOH, pH 7.8, and suspended in the same medium without or with DCMU in such a way that its final concentration was $1.3.10^{-5}$ M. Final chlorophyll concentration was $2.5.10^{-5}$ M. In the acid experiments the pH was lowered with MES-HCl to a final pH of 4.1 during the second mixing (Acid (2)) or during the first mixing after which the pH was kept constant during the second mixing (Acid (1,2)). In the acid-base experiment the pH was increased to 9.5 by addition of Tricine-KOH. Acid (1,2) is the control experiment for the acid-base jump experiment. Darktime was 2 sec. F_o denotes the base fluorescence yield without preillumination. Control* indicates the normal experiment without DCMU. The experiments in the left and in the right part of the table were performed with different batches of chloroplasts. The other conditions as for Fig. 2.2.

be on the reversed electron transfer on the acceptor side of PS2, i.e. from PQH_2 to Q.

An important difference between the luminescence and the variable fluorescence was obtained on lowering of the pH after preillumination in the presence of DCMU. Although a lowering of the luminescence intensity was observed, the variable fluorescence remained constant. This might be explained as outlined below. One may assume that DCMU does not affect the electron donor S in its various states as influenced by addition of salt or acid. Further the pH sensitive redox reaction between Q^- and PQ is involved in delayed fluorescence:

$$Q^- + H^+ + 1/2\ PQ \underset{\text{DCMU}}{\rightleftharpoons} Q + 1/2\ PQH_2 \qquad (17)$$

From this equation the inhibition of the lowering of the variable fluorescence yield upon acidification is understandable. On the other hand, the decrease in luminescence by lowering the pH is not inhibited by DCMU. This cannot be explained by the above equation, but can be accounted for by the earlier proposed equilibrium reaction (see Section 3.3 and ref. 62):

$$Q^- + H^+ \rightleftarrows QH \,, \qquad (4)$$

which is not blocked by DCMU and in which only Q^- is a precursor of luminescence.

The above considerations thus suggest that both mechanisms operate in normal and stimulated delayed fluorescence.

All these assumptions may account for a remaining stimulation of luminescence by an acid-base transition or by salt addition.

6.3 Discussion

In section 6.2.6 it is concluded from acidification experiments that both eq. (4) and eq. (17) can be used for describing the fluorescence and luminescence phenomena. DCMU blocks the reversed electron flow from PQH_2 to Q (eq. (17)) but not the shift from QH to Q^- (eq. (4)). Both processes give rise to stimulation of luminescence. The increase of the concentration of ZH^+ due to pH lowering on the inside is presumably not affected by DCMU. Although it is possible that in the presence of DCMU the concentration of ZH^+ during the acidification is somewhat lowered due to reaction with Q^-, the inhibition by DCMU of the stimulated luminescence in the acid-base experiment may be due to a faster decay of Q^-. This is caused by the possibly increased rate of back reaction between Q^- and ZH^+ (see Section 3.3, eq. (2)) and the inhibition of reduction of Q by PQH_2. A similar reasoning can be applied for the inhibition by DCMU of stimulated delayed light in the salt experiment.

As stated, the absence of inhibition by DCMU of the normal delayed fluorescence, measured 2 sec after a 5 sec lasting preillumination period (see Table 6.7 and also Figs. 3 and 7 in ref. 43) and of the intrinsic luminescence might be explained by the assumption that the concentration of ZH^+ remains rather unaffected and has a low rate of decay. On the

other hand in the presence of 5.10^{-5} M DCMU, used in the luminescence experiments, no oxygen evolution can be observed if the foregoing dark-time is long enough to exhaust the S_3^{3+} state[119]. Therefore one may conclude that the oxidized primary electron donor to PS2, ZH^+, reacts with Q^- in producing luminescence. ZH^+ is the same as Z^+ in Duysens and Sweers' scheme[26]. It is assumed here that Z^+ oxidizes secundary donor(s), giving rise to the states S_n^{n+} of Kok[44] (Section 1.2.3). The concentration of ZH^+ can be "buffered" by S_3^{3+} after switching off the light.

The above conclusion which is in line with results recently published[120], is confirmed by results from investigations concerning the relation between the 0.1 sec luminescence and the pH of incubation of chloroplasts in zwitterionic buffers. The actinic light was very low in order to exclude contribution of the high-energy state (see Chapter V) to the luminescence and the fluorescence yield in the light increased only a few per cent at neutral pH. The observed luminescence was very low at pH values between 5 and 7, but increased strikingly below pH 5. This was not caused by a corresponding increase of the variable fluorescence yield. Because electron transport is inhibited at these low pH values, the oxidation of the S_n^{n+} states by ZH^+ is blocked. Therefore the relatively high concentration of ZH^+ in this pH region (see Section 3.3, eq. (3)) caused a relatively high luminescence intensity[121]. These results will be published elsewhere.

The salt-induced membrane potential (see Chapter IV) may be lower in Tris than in Tricine or TES due to cyclic permeation of Trisonium cation and free Tris base, which cause an efflux of protons (see Tables 6.2, 6.3 and 6.5). In the presence of gramicidin D the increase of the membrane potential due to enhanced Na^+ or K^+ permeability may be almost cancelled in the zwitterionic buffers in contrast to the Tris experiments. This then is due to a relatively more increased permeability to protons in the former buffers than in Tris in the presence of gramicidin.

The weak acid 2,4-DNP, which inhibits in both types of buffers the stimulated light emission induced by salt (see Table 6.5), lowers presumably the membrane potential due to influx of DNP^- anions driven by the electrical field.

The stimulated luminescence on acidification in Tris in the presence

of DNP or FCCP is maybe caused by increased penetration of protons into the thylakoid interior. The inhibition of luminescence in the acid-base experiments by DNP may be interpreted by the assumptions that the pH gradient upon addition of the base, although higher, decays faster and that the capacity to luminesce is lowered during the acid period, because increased luminescence was observed.

CHAPTER VII

DAMAGING OF THE THYLAKOID MEMBRANE

7.1 Effects of the non-ionic detergent Triton X-100

7.1.1 Introduction. Another way to obtain information about the correlation between the integrity of the thylakoid membrane system and the stimulation of the delayed fluorescence was to study the influence of the non-ionic detergent Triton X-100. This is a water soluble polyether[122,123] (isooctylphenoxypolyethoxyethanol) with 9 or 10 oxyethylene groups in sequence per molecule resulting in an average molecular weight of 630.

At very low concentrations it uncouples photophosphorylation[123-125] with concomitant increased rate of electron transport[123,124]. If the amount of the detergent is increased the electron transport between water and NADP is inhibited[123,124]. Electron transport can be restored upon addition of suitable electron donors for system 1 or 2 (refs. 123, 126). Still higher concentrations effect a partial separation into particles with photochemical activities belonging to photosystem 1 (ref. 127) and to photosystem 2 (ref. 126), respectively.

7.1.2 Results and Discussion. In order to get a well defined experimental procedure the chloroplast suspension (see legend of Fig. 7.1) was mixed thoroughly with an equal volume of buffer containing the detergent and kept for 5 min at room temperature before preillumination started. This method prevented variations in Triton concentration larger than a factor of two for each individual chloroplast during the mixing. Triton concentrations were determined by absorption measurement at 275.5 nm, on basis of a molar extinction coefficient of $1.33.10^3$ (Rohm and Haas Cy, see ref. 123).

Fig. 7.1 shows the effect of increasing concentrations of Triton X-100 upon the various types of delayed fluorescence. The salt-induced stimulation of luminescence (NaCl) was affected already at concentrations slightly higher than 0.16 molecule Triton per chlorophyll molecule (0.001 % (v/v)). The enhanced luminescence due to an acid-base transition showed a sharp drop at 0.01 %. Above 0.02 % the acid-base signal was al-

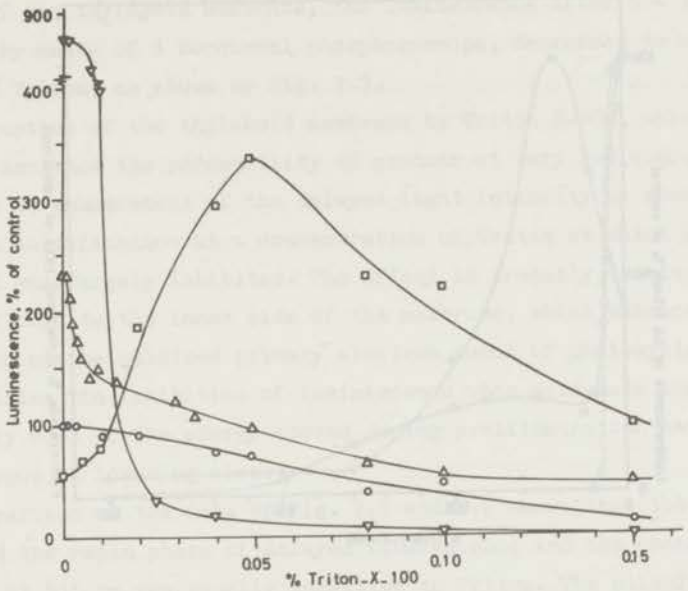

Fig. 7.1. Effect of different concentrations of Triton X-100 upon various types of luminescence. Class I chloroplasts (chlorophyll concentration 1.10^{-4} M) were suspended in a solution of 0.4 M sucrose and 0.01 M Tricine-KOH (pH 7.8), and preincubated for 5 min at room temperature with the concentration of Triton indicated. A concentration of 0.10 % corresponds to a molar ratio of Triton to chlorophyll of 15.9. O—O, normal delayed light; Δ—Δ, NaCl-induced luminescence; □—□, luminescence upon acidification with MES-HCl to a final pH of 4.6; ▽—▽, acid-base induced luminescence (final pH 8.8 obtained by mixing with Tricine-KOH). The darktime after preillumination was 2 sec. Further conditions as for Fig. 2.2.

ready lower than the slightly inhibited "normal" delayed light, whereas some salt-induced stimulation persisted at much higher concentrations.

Measurement of the activity of the Hill reaction with ferricyanide for the same chloroplast preparations (Fig. 7.2) at various concentrations of detergent gave results similar to those reported by Vernon and Shaw[123], and Izawa and Good[124]. The stimulation of electron transport, maximal at 0.02 %, has been attributed to uncoupling of photophosphoryl-

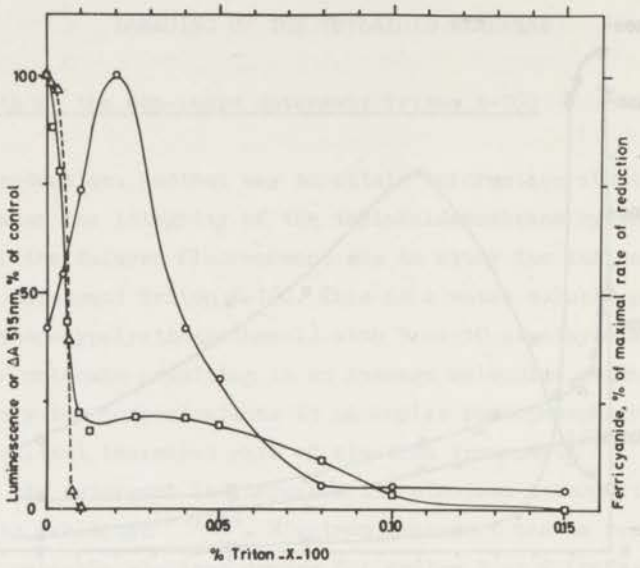

Fig. 7.2. Effect of Triton X-100 upon the rapid phase of delayed fluorescence (□——□), ferricyanide Hill reaction (o——o) and absorption increase at 515 nm (Δ——Δ). Delayed fluorescence was measured with a Becquerel phosphoroscope. The wavelength of exciting light was 481 nm, the intensity 3.10^{-9} Einstein.cm^{-2}.sec^{-1}, the average darktime 5 msec. Ferricyanide reduction was measured spectrophotometrically as the initial rate of reduction in saturating light of 656 nm (12.10^{-9} Einstein.cm^{-2}.sec^{-1}); without Triton the reduction rate was 40μ moles/mg of chlorophyll per h. The concentration of ferricyanide was 5.10^{-4} M. The steady-state absorption increase at 515 nm was measured with the same intensity and wavelength of actinic light. The other conditions were as for Fig. 7.1.

ation[123-125], the inhibition at higher concentrations to disruption of the electron transport system. The uncoupling of photophosphorylation, the inhibition of the luminescence stimulated by the acid-base shift and the partial inhibition of the NaCl effect are probably due to disruption of the structure of the thylakoid lamellae. Parallel measurements showed that the light-induced increase in absorption at 515 nm, thought to be an indicator of membrane functioning[81], was completely inhibited

at a Triton concentration of 0.01 %. In contrast to the delayed fluorescence after 2 sec, which was apparently largely independent of the integrity of the thylakoid membrane, the luminescence after 5 - 10 msec, measured by means of a Becquerel phosphoroscope, decreased to about 20 % at 0.01 % Triton, as shown by Fig. 7.2.

Disruption of the thylakoid membrane by Triton X-100, which was shown to increase the permeability to protons at very low concentration[125], gave rise to enhancement of the delayed light intensity by about a factor of 7 upon acidification at a concentration of Triton at which electron transport was largely inhibited. The effect is probably due to easier access of acid to the inner side of the membrane, which enhances the concentration of the oxidized primary electron donor of photosystem 2. It also explains the inhibition of luminescence upon acid-base transition. Apparently most of the energy stored during preillumination had been dissipated upon pH lowering alone.

Comparison of the data of Fig. 7.1 and 7.2 shows that the NaCl-induced and the rapid phase of delayed fluorescence and the changes of absorption at 515 nm are equally sensitive to Triton. The stimulation of luminescence by the acid-base transition appears to be somewhat less sensitive, suggesting that the membrane potential collapses earlier than the artificial pH gradient with increasing Triton concentrations. The pH gradient at the low concentrations of detergent might initially be even larger than without detergent due to more rapid acidification inside.

In agreement with observations of Sauer and Park[112] Triton at concentrations above 0.02 % was found to enhance considerably the base fluorescence yield (F_o). This complicates a comparison of the yield of delayed fluorescence at high concentrations of Triton. The Triton concentration at the maximum of acid-induced luminescence caused a base fluorescence yield which at the low pH was only 50 % higher than that of the control experiment at the same concentration of Triton. This base fluorescence yield was 2 times higher than that in the control experiment without Triton. The NaCl-induced fluorescence yield was about 25 % higher than the corresponding value for the control experiment. Apparently the higher intensities of luminescence in the acid experiments cannot or only for a small part be ascribed to higher yields of the base fluorescence

yield. This also applied to the increased variable fluorescence yield, which in all types of experiments was enhanced enormously. The effect may be due to high pressure or friction during the second mixing and the presence of the detergent, especially above 5.10^{-2} %. Experiments performed on a laboratory constructed apparatus without a mixing device, and adapted to fluorescence experiments[128] with a modulated excitation beam made it clear that, for a stationary suspension of chloroplasts in buffer, with increasing concentrations of Triton the increase in fluorescence yield by actinic light and thus the reduction of Q was inhibited (not shown).

Type of experiment	Triton X-100 5.10^{-3} %	Luminescence	Integrated luminescence	P_o^*	ΔF^*
Control	-	405	1910	60	51
	+	410	2030	74	57
NaCl	-	1690	4510	70	53
	+	940	3490	65	61
Acidification	-	181	1205	60	38
	+	192	1350	75	43

*corrected for a small increase of fluorescence yield observed without preillumination which was apparently due to the mixing procedure in the presence of Triton X-100.

Table 7.1. Effects of Triton X-100 added after the preillumination on prompt and delayed fluorescence. All conditions are the same as for Fig. 7.1 except that addition of the detergent occurred immediately after the light period (first mixing).

Table 7.1 shows the effect of 5.10^{-3} % Triton, added immediately after preillumination, on luminescence and fluorescence upon addition of salt or acid. Here the time of incubation was slightly less than 2 sec. The effects agree with both the luminescence intensities as shown in Fig. 7.1 and the integrated light signals measured for those experiments. The data show that Triton reacts rapidly with thylakoid membrane[125], and indicate that the phenomena shown in Fig. 7.1, at least at low concentration, are not due to an effect of the detergent occurring in the preillumination phase. The base and variable fluorescence were not sig-

nificantly affected by Triton at this concentration (5.10^{-3} %).

7.2 Heating

7.2.1 Results. It is well known that heating of isolated chloroplasts will affect the Hill activity[129-134], and possibly also the structural integrity of the thylakoid membrane[134]. The loss of capacity to evolve oxygen by heat treatment may be due to loss of Mn^{2+} (refs. 133, 135), whereas the decrease of membrane integrity seems to be indicated by the increase of Hill reaction rate[134] if the heating time is very short, suggesting uncoupling.

Experiments on normal and stimulated luminescence performed after heating of chloroplasts at 45° indicated that both these phenomena were observable. Upon heating at 45° the inactivation of normal delayed fluorescence started immediately as can be seen in Fig. 7.3, whereas the stimulation of the delayed fluorescence by a KCl gradient was inhibited too. The luminescence in the acid-base transition, however, showed a further enhancement, at least if compared to the heated control values, which seemed to indicate that a slightly higher permeability to protons could effect a more decreased pH inside the thylakoid membrane during the short acidification time (3 sec), resulting in a higher pH gradient. The increase in luminescence upon acidification (Tris-buffer) was also enhanced which supports the above explanation.

Upon longer heating the stimulation by the acid-base shift (the same applied to K-benzoate) was more strongly inhibited than the control values at pH 7.8.

From the integrated signals (not shown) a similar pattern was observed. The values of integrated delayed light upon acidification of heated chloroplasts were higher in relation to those of the control experiments, even after 5 min of heating.

The base fluorescence scarcely increased with heating time (see also ref. 132). The variable fluorescence was found to be slightly higher (not shown) but the effect was insufficient to explain the initial increase, if any, in luminescence. The effect, like with Triton, may be an artifact due to the mixing, since for a stationary suspension it has

been reported that the variable fluorescence is inhibited by heat treatment[132].

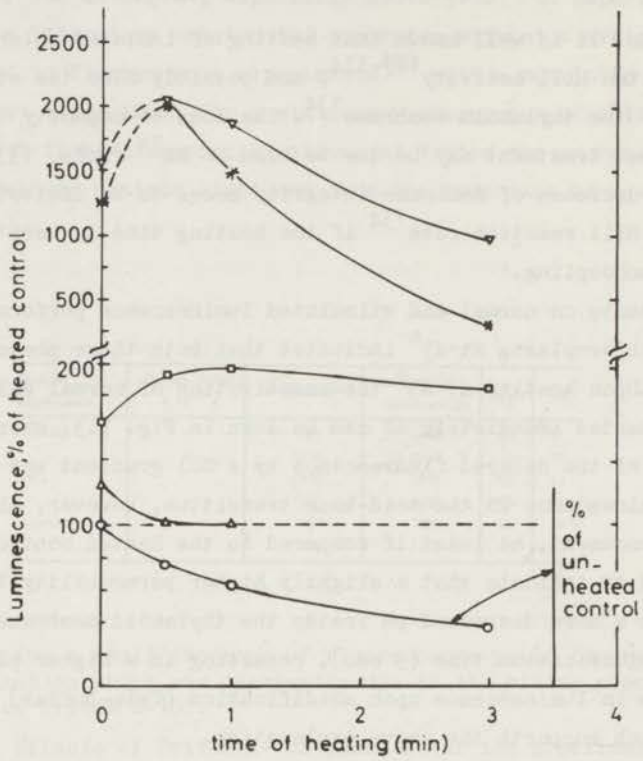

Fig. 7.3. Effect of heating on delayed and stimulated delayed fluorescence. Normal chloroplasts were isolated and suspended in 0.05 M Tris-HCl, pH 7.8. Final chlorophyll concentration was 4.10^{-5} M. Just before heating chloroplasts were diluted from the stock suspension in 10 ml of buffer to a concentration of $1.6.10^{-4}$ M of chlorophyll. The vessel containing this suspension (0°) was heated in about 15 sec to 45° under rotation in a water bath at 60° C. Heating was continued in another bath at 45° during the time indicated in the figure. After heating the vessel was quickly cooled down to 0°. The pH of the acid experiment was 4.6 by addition of 0.02 M succinic acid and the subsequent rise in pH to 8.5 in the acid-base experiments was performed with 0.1 M Tris. Except for the luminescence in the control experiment, O——O, which is plotted as the percentage of the unheated control, all other luminescence data are given

Fig. 7.3 (continued) as percentage of the luminescence of the heated control: KCl, Δ—Δ; K-benzoate, ✱—✱; acid, □—□, and acid-base, ▽—▽. All other conditions are as indicated in Fig. 2.2.

7.2.2 Discussion. The heat inactivation of normal and stimulated luminescence might be caused by inhibition of electron transport between water and photosystem 2 due to loss of Mn^{2+} which is an intermediate electron carrier[133,135]. The result of this depletion could be a lower yield of the variable fluorescence[133], caused by insufficient transfer of electrons to Q, and therefore lower intensities of luminescence.

The relative increase of the stimulated delayed fluorescence induced by an acid-base transition might be explained by an increase of the stimulating pH gradient. This could be due to an increase of the permeability of the thylakoid membranes to protons[136]. At longer times of heating the stimulated delayed fluorescence was found to be inhibited more strongly than the control values at pH 7.8. It is likely that this is due to a more rapid collapse of the pH gradient after adding the base, and to loss of luminescence capacity during the preceding acid period.

The fact that even 5 min of heating inhibits only partly the luminescence emission and especially the relatively increased integrated luminescence upon acidification, indicates that the primary photochemistry of PS2 is rather insensitive to mild heating. This conclusion is supported by the restoration of the variable fluorescence yield of heat inactivated chloroplasts, if DCMU or hydroxylamine is present[133].

The extent of heat inactivation of both membrane integrity and electron transport was dependent not only on the heating time and temperature, but also on the concentration of chlorophyll used. Further the presence of serum albumin[137] which retards the inhibition of both the rate of uncoupled electron transport and of photophosphorylation efficiency (aging) and is known to bind unsaturated fatty acids released from biological membranes[137,138], prevented largely the heat-induced decrease of normal luminescence at pH 7.8 as measured after 0.1 sec of darkness[139].

Type of experiment	Control chloroplasts				Sonicated chloroplasts			
	Luminescence	Integrated luminescence	F_o	ΔF	Luminescence	Integrated luminescence	F_o	ΔF
Control	54	215	10	6.5	39	210	20	12
Acid (2)	51	130	14	3.2	250	293	20	13
Acid (1,2)	26	140	13	5.8	36.5	138	19	10
Acid-base	224	269	12	10.9	160	118	21	8

Table 7.2. The effect of sonication on luminescence and fluorescence. Class I chloroplasts were isolated in Tricine-NaOH buffer, pH 7.9 in the presence of 10 mM KCl. Final chlorophyll concentration was $1.3.10^{-5}$ M. Part of the concentrated chloroplast suspension (see Section 2.1) was sonicated for 5 min at 0° (Branson sonifier; 20 kHz, full power). Addition of 0.03 M succinic acid in 0.4 M sucrose lowered the pH to 4.3. Acid (2) means: acidification during 2nd mixing, whereas acid (1,2) stands for acidification during 1st mixing and addition of a same acid mixture during the second mixing without any change in pH. In the acid-base experiment the pH was increased to 8.6 by addition of 0.1 M Tris + 0.3 M sucrose. Acid (1,2) was the control experiment for the acid-base experiment. The darktime was 2 sec, whereas the acid time outside the measuring cuvette was 1.5 - 2 sec.

7.3 Sonication

Chloroplasts frozen and stored at about -30° possess damaged thylakoid membranes as do chloroplasts which were sonicated (Table 7.2) at 0° for 5 min in a Branson sonifier (20 kHz). Both types of chloroplast preparations showed a marked increase in luminescence intensity upon mixing with acid and a decrease in acid-base induced stimulation as compared with normal chloroplasts.

Like with the other treatments, mentioned in this chapter, a higher permeability to protons caused by damaging of the thylakoid membranes may lead to an accelerated and extended acidification and to a decrease of the size of the pH gradient (acid-base experiment) together with an increased decay of this gradient. However, closer examination of Table 7.2 suggests in addition the possibility that with sonicated chloroplasts the damaged thylakoid membranes are partly inside-out like with mito-

chondria[140], because the total light emission upon acidification is higher than in the control experiment, whereas the corresponding value in the acid-base experiment is lower. This phenomenon is in contrast to what was observed after treatments with uncouplers or up to 0.03 % of Triton X-100, where the integrated signal upon acidification was lower than the control one. The other indications are obtained by measurement of the variable fluorescence yield.

The variable fluorescence yield of the control experiment in sonicated chloroplasts was higher than that of normal chloroplasts. Upon acidification no lowering of the variable fluorescence yield was observed in contrast to the corresponding value of the control sample, whereas the variable yield in the acid-base experiment was lower than that of the control experiment. These observations suggest an alternative explanation, similar to that discussed in Section 6.2.6, if it is assumed that sonication acts in the same way as DCMU. The low yield of variable fluorescence in the acid-base experiment may be due to a fast decay of Q^- then caused by blocked electron flow from PQH_2 to Q (see eq. (17)). The increased luminescence upon acidification is different from the corresponding value in the DCMU experiment (see Table 6.7) and is caused, as pointed out above, by penetration of protons into the "leaky" thylakoids.

Experiments with salt addition (e.g. KCl with valinomycin) may distinguish between these two explanations.

CHAPTER VIII

FINAL DISCUSSION

In Chapter I, Section 1.2.2 arguments are given according to which delayed fluorescence in algae and higher plants is caused by reexcitation of chlorophyll a due to a reversed reaction of the primary photoproducts of PS2, Q^- and ZH^+. Its intensity is dependent on temperature during both preillumination and measurement[13,100], and on the redox state of the primary electron acceptor of PS2, Q, which governs the variable fluorescence yield[26] and also the yield of delayed fluorescence[30,42,43]. It has also been demonstrated that luminescence depended on the redox state of electron donor(s) of PS2 (refs. 48, 120).

Five years ago an unspecified correlation was discovered between luminescence and the energy conservation mechanism which leads to photophosphorylation[55]. A first indication that also "chemical" treatments caused an increase of luminescence came from acid-base transition experiments[56,57]. The stimulus for these experiments came from the observation of ATP formation in the dark by such a treatment[51]. However, the stimulated luminescence needed preillumination[57] (see Section 1.2.4).

It was the purpose of the investigations reported in this thesis to find more information about the relationship between changes in conditions of the preilluminated chloroplasts and the stimulated delayed fluorescence.

After the discovery that salt added to preilluminated chloroplasts led also to stimulation of delayed light[59,61] it was proposed that the increase was caused by a diffusion potential across the thylakoid membrane[61]. This membrane potential, positive inside with respect to the outside, was attributed to a higher passive permeability of the thylakoid membrane to the cations than to the anions of the added salts. From a linear relationship obtained between luminescence and various concentrations of potassium benzoate it was concluded that luminescence is exponentially dependent on the membrane potential[62,143] which is generated on addition of salt. This conclusion is only correct if one assumes that the Goldman and derived equations (see Chapter IV, eqs. (8) - (16))

are valid for the thylakoid. This result and other indications, as described in Chapter IV, led to a direct support of that part of Mitchell's chemiosmotic hypothesis[68] according to which the primary electron donor ZH and acceptor Q of PS2 are located on the inside and on the outside of the thylakoid membrane, respectively. Upon illumination a charge separation in the reaction center of PS2 results in the formation of Q^- and ZH^+. The potential generated in this way can be observed, according to conclusions of Witt and colleagues, by the absorbance change at 515 nm[69]. This change, however, is partly caused by a similar charge separation in PS1. The half-rise time of the absorbance change upon excitation by laser flashes was found to be less than 20 nsec, which seems too fast to be associated with secondary, non-photochemical reactions. The decay of the 515 change is very much dependent on the degree of coupling between electron transport and photophosphorylation; the presence of uncouplers, aging of the chloroplasts, Tris-buffer etc., increased this rate. In tightly coupled chloroplasts its halftime of decay was reported to be about 230 msec[99]; upon complete uncoupling the decay time may become a few msec.

The generation of a membrane potential by salt addition of the same polarity, as caused by illumination, therefore will stimulate the reaction rate between Q^- and ZH^+, and thus luminescence. Additon of salt to chloroplasts in the dark was found to cause a change in absorbance at 515 nm (ref. 96). The difference spectrum obtained upon addition of KCl in the presence of valinomycin was stated to be identical to the light-induced difference spectrum . Moreover these latter changes were proportional to the logarithm of the concentration of added KCl (ref. 96).* These results confirm the idea of the stimulation of delayed fluorescence by a membrane potential generated by salt addition. Analogous absorption changes on bacterial chromatophores[149] were earlier reported and similarly interpreted.

The acid-base induced stimulation of luminescence occurs upon the establishment of a temporary pH gradient such that the pH inside the thylakoid is lower than outside. This process has been described in

*A preprint[148] received after completion of this manuscript casts some doubt on this point.

Chapter III, where it is pointed out that the low pH inside and the high pH outside favor a shift of the equilibrium of the reactions shown in eqs. (3) and (4) towards the formation of ZH^+ and Q^-, and consequently enhance the rate of reversal of the photochemical reaction in PS2 (see eq. (2)). The results and suggestions described here can be summarized in the following scheme (see Fig. 8.1).

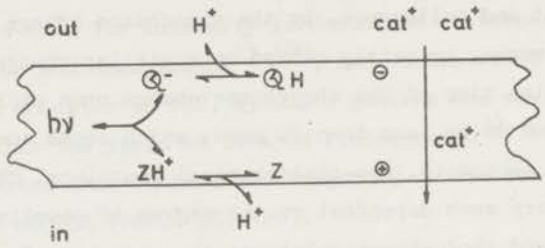

Fig. 8.1. Hypothetical scheme for delayed and stimulated delayed fluorescence as discussed in Chapters III and IV.

In this model one may expect that the luminescence intensity L is proportional to

$$L \propto [Q^-] \cdot [ZH^+] \cdot e^{(-E_a + EF)/RT} \qquad (18)$$

in which E_a is the activation energy and EF the potential energy. If relation (18) is correct, the above mentioned linear relation between luminescence and concentration of potassium benzoate confirms the validity of the Goldman equation for the thylakoid membrane.

Acidification of the preilluminated chloroplasts would tend to diminish delayed fluorescence due to a decrease of the concentration of Q^-. The effect is apparently not fully compensated by the production of ZH^+ which may be assumed to occur when the acid subsequently penetrates into the thylakoid (see Fig. 3.2). If the pH outside is below about 3.0 in Tricine and about 5.0 - 4.7 in Tris (see Figs. 3.1a and 3.1b) or if the membrane integrity is affected by the presence of uncouplers, Triton X-100 or by heating the increase in ZH^+ is high enough to overcome the inhibition of luminescence by a decreased fluorescence yield.

The variable fluorescence yield is lowered in the absence, but not in the presence of DCMU upon acidification of the preilluminated chloroplasts. The luminescence, however, is lowered, independently of DCMU

(see Table 6.7). The explanation for this apparent discrepancy was that besides the shift from Q^- to QH which decreases luminescence due to a decrease in the concentration of Q^-, but which does not cause a change in the variable fluorescence yield, the acid-induced shift in the redox reaction with plastoquinone (eq. (17)) is blocked by DCMU. Upon addition of base to chloroplasts treated in this way the concentration of Q^- would then be restored initially but would decrease more rapidly by reaction with ZH^+ than without DCMU, because the electron transport inhibitor blocks the reduction of Q by PQH_2. This mechanism may explain the partial inhibition of the pH jump stimulated luminescence. In Section 6.3 it is also argued that in contrast to previous assumptions ZH^+ must be the reactant of the luminescent reaction and not S_3^{3+}, although the concentration of the former is dependent on that of the latter. The stimulation of this type of luminescence is caused by increased concentrations of Q^- and ZH^+, brought about by shifts in the pH sensitive redox reactions with the secondary electron acceptor and donor, respectively. One may expect that the product of the concentrations of Q^- and ZH^+, $[Q^-] \cdot [ZH^+]$, then is exponentially dependent on the pH difference. Therefore the stimulated luminescence must depend exponentially on the pH gradient. Our experiments (see Chapter III) indicate that this is the case in a first approximation.

Besides the argument derived from the DCMU experiments, the masking of the possible stimulation of luminescence in the acid-base experiment by changes of the redox potentials of Q/Q^- and Z^+/Z may be explained by the assumption that going from pH 7.8 finally to about 8.5 (see legend of Fig. 2.2) the actual increase in Q^- as indicated by the variable fluorescence is low.

If the revised mechanism is real, this is a further difference between the stimulated luminescence and the ATP formation by an acid-base transition (cf. ref. 59). Although it was shown that in the presence of reduced cytochrome c, acidification caused a reduction of cytochrome b reversed by addition of base[145], the ATP formation in this way was not inhibited by the simultaneous presence of electron transport inhibitors DCMU and e.g. Antimycin A (ref. 146). DCMU, however, is demonstrated to inhibit partially the stimulation of luminescence in an acid-base ex-

periment.

The generation of an electrochemical potential gradient[68] by addition of salt (membrane potential) or by a pH gradient (chemical potential gradient of protons), which both stimulate delayed fluorescence, is preceded by a light-induced one, the size of which is dependent on the light intensity, time of illumination and the presence of an electron acceptor etc. Therefore it is suggested that during the lifetime of this electrochemical potential gradient or high-energy state in the dark the luminescence intensity is "intrinsically" increased. The results are given in Chapter V, where it is shown that the intensity for saturation of luminescence decreases with increasing darktime (see Fig. 5.1) up to 2 or 3 sec, which is the limit of lifetime of the 515 change in the dark[99]. Illumination in the presence of potassium ferricyanide caused a further increase in luminescence, the absorbance change at 515 nm, and in the pH gradient. This also supports the interpretation given above (Fig. 5.3).

The dependence of the light-induced stimulation of delayed fluorescence on the membrane potential and the pH gradient across the thylakoid membrane is also strongly indicated by the induction curve of the 1 msec luminescence[144], which showed a variation of the luminescence during illumination of high intensity in the presence of an electron acceptor. Two phases have been distinguished. A rapid rise due to both the increase of fluorescence yield and membrane potential and a slower phase due to an increase of pH gradient. The conclusions were deduced from the use of ionophorous antibiotics, because the rapid rise was largely reduced by valinomycin, whereas the slow increase was completely inhibited by the presence of sufficient nigericin[144].

The mechanisms discussed above also present an explanation for the observed rapid decay[32,100] in intensity of delayed light shortly after preillumination by about 100-fold or more between 1.10^{-3} and 1.10^{-1} sec. This fast decay cannot be deduced from the rate of reoxidation of Q^- as indicated by the decrease of variable fluorescence yield (see e.g. Fig. 5.3b) and from the disappearance of the various oxidized intermediates between H_2O and ZH as indicated by measurements of oxygen evolution[45,47].

The above explanation also accounts for the much higher sensitivity towards Triton X-100 treatment of the 5 msec than of the 2 sec phase of

delayed fluorescence (Chapter VII, Fig. 7.1). A similar sensitivity to Triton was found for the salt and the acid-base transition induced luminescence and also for the absorbance change at 515 nm (Fig. 7.2).

Along the above lines one can also explain why gramicidin and other uncouplers of photophosphorylation inhibit the fast phase of luminescence (see ref. 55 and Fig. 5.2, and Table 6.4) much more strongly than the slow phase. Gramicidin also enhances the decay of the 515 nm change[81].

An investigation was done on the kinetics of luminescence after 0.1 sec excited by strong light in the presence of several uncouplers[147]. It was found that $2.5.10^{-7}$ M FCCP decreased the initial (0.1 sec) luminescence to 20 % and the integrated light emission (up to 2 min) to 30 %. Also 1.10^{-7} M S-13 (5-Cl-,3t-butyl-2'-Cl,4'-NO_2 salicylanilide (Dr. Ph. C. Hamm, Monsanto Cy, St. Louis)) caused a deerease to about 30 % and 40 %, respectively, and 1.10^{-2} M methylamine to about 70 % and 20%, respectively. From these data one might conclude that FCCP and S-13, although less pronounced, inhibit not only the rate of the back reaction giving luminescence but also the total light output, in contrast to methylamine. On the other hand 1.10^{-4} M 2,4-DNP caused an inhibition which increased with increasing time of darkness as could be concluded from the lowering of the respective signals to 80 % and 60 %. The inhibition of the stimulated luminescence in an acid-base experiment by compounds like gramicidin D and DNP increases with both a lower pH and with incubation time of the preceding acid period and depends also on the type of buffer. This conclusion was based on the results reported in Chapters III and VI. This may account for the larger inhibition of luminescence in acid-base experiments reported by Mayne[57] and also by Miles and Jagendorf[59], who applied a much longer acidification period (10 sec). During this time much of the luminescence capacity may have been dissipated, owing to the luminescence stimulation by e.g. gramicidin at low pH (see Tables 6.2, 6.3 and 6.5).

The lowering of stimulated luminescence in Tricine by gramicidin or DNP after addition of NaCl or KCl was proposed to be caused by abolishment of the increase of membrane potential either by an increased efflux of protons (gramicidin D) or by an influx of DNP^- anions (Chapter VI).

The saturation of stimulated delayed fluorescence, which is

induced by an acid-base transition (not shown; cf. ref. 57), or by a salt jump (Fig. 4.5) occurs at the same light intensity as the normal long lived delayed fluorescence. This supports the conclusion that salt and acid-base treatments may only stimulate the normal luminescence reactions.

Finally, as shown e.g. in Fig. 2.2, the higher total light emission induced by these treatments indicates that during the measurement of the normal luminescence radiationless decay pathways occur.

SUMMARY

In this thesis the results are described of a study of stimulated delayed fluorescence of chlorophyll a in isolated chloroplasts.

Chapter I gives an introduction to the subject and a review and discussion of the pertinent literature. Various factors which determine the yield of delayed fluorescence are discussed, including the concentration of primary photoproducts in PS2 and the existence of a light-induced high-energy state or electrochemical potential gradient across the thylakoid membrane. The phenomenon of stimulation of the light emission due to treatments (acid-base transition or salt addition) after the preillumination is also briefly considered in relation to older experiments.

Chapter II gives a description of the apparatus and of the experimental procedure.

In Chapter III a possible mechanism of the acid-base induced stimulated luminescence is investigated together with the phenomenon of a change in luminescence intensity due to the preceding acid treatment. Amongst other things the results suggest that the luminescence intensity is exponentially dependent on a pH gradient across the thylakoid membrane.

Chapter IV deals with the stimulation of delayed fluorescence upon addition of salts of monovalent cations. It is suggested that the stimulation is caused by a diffusion potential which is positive inside, with respect to the outside of the thylakoid membrane, and which is due to a higher permeability of the membrane to the cations than to the anions used. This conclusion is supported amongst other things by experiments with valinomycin, which increases the permeability for potassium, but not for sodium ions.

Chapter V describes the relation between delayed fluorescence and the intensity of preillumination. Different saturation intensities were observed for a rapid phase of delayed fluorescence on the one hand, and for a slow phase (as for the acid-base and salt-stimulated delayed fluorescence) and the prompt fluorescence on the other hand. These results and the effects of uncouplers and electron acceptors are explained by the generation of an electrochemical potential gradient across the mem-

brane by the preillumination which decays in about 2 sec.

In Chapter VI, the effects of Tris-buffer and its zwitterionic analogues Tricine and TES are studied on the various types of luminescence and on fluorescence. It also describes the effects of membrane modifying agents such as gramicidin D, FCCP and DNP.

On basis of experiments with DCMU and results described in Chapter III a model is discussed to explain the stimulation of delayed fluorescence by an acid-base transition. It is concluded that two kinds of mechanisms occur which cause the stimulation. The first one is based on the occurrence of pH dependent redox reactions of the primary reactants of PS2 with plastoquinone and with intermediates between water and PS2, respectively. The second one is based on pH dependent non-redox equilibrium of both the oxidized primary electron donor and the reduced electron acceptor of PS2.

Chapter VII deals with the effects of damaging the thylakoid membranes by the detergent Triton X-100, by mild heating at 45° and by sonication at 20 kHz. The results seem to indicate that the inhibition by these treatments of the various stimulations of delayed fluorescence is related to the increased permeability of the thylakoid membrane to protons and to an inhibition of electron transport.

Chapter VIII gives a final discussion of the main aspects of our investigations.

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ABBREVIATIONS

DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DNP	2,4-Dinitrophenol
FCCP	Carbonyl cyanide p-trifluoromethoxyphenylhydrazone
MES	2-(N-morpholino)ethane-sulphonic acid
pK_a	negative logarithm of the acid-dissociation constant
PS1	Pigment system 1 (photosystem 1)
PS2	Pigment system 2 (photosystem 2)
TES	N-tris(hydroxymethyl)methyl-2-aminoethane-sulphonic acid
Tricine	N-tris(hydroxymethyl)methyl-glycine
Tris	Tris(hydroxymethyl)aminomethane

CHEMICALS

The grade of purity of all the chemicals used was as high as possible. MES was obtained from Calbiochem; Tris from Merck. Gramicidin D, TES and Tricine were purchased from Sigma. All other chemicals were obtained from BDH.

Dr. P.G. Heytler kindly donated FCCP to Professor Duysens and the valinomycin was a gift from Dr. B.C. Pressman and from Dr. C.W. Pettinga (Eli Lilly and Cy.).

CURRICULUM VITAE

Na het behalen van het diploma Gymnasium β in 1953 aan het St. Bonifacius Lyceum te Utrecht begon ik in 1957 met de studie in de scheikunde aan de Rijksuniversiteit te Utrecht.

In november 1963 legde ik het kandidaatsexamen f en g af. Hierop volgde in juni 1966 het doktoraalexamen biochemie met als bijvak biofysica.

Vanaf juni 1966 was ik als wetenschappelijk medewerker verbonden aan het Laboratorium voor Biofysica van de Rijksuniversiteit te Leiden, in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO). Onder leiding van Prof.Dr. L.N.M. Duysens en Dr. J. Amesz werd sindsdien het hier beschreven onderzoek verricht.

S T E L L I N G E N

I

Bestudering van vertraagde fluorescentie leidt mogelijkerwijs tot een beter inzicht in zowel de fotochemie van pigmentsysteem 2 als in de eigenschappen van biologische membranen; gestimuleerde luminescentie kan dienen als indikator van de integriteit van het thylakoidmembraan.

R.H.Ruby, Photochem.Photobiol., 8 (1968) 299;
dit proefschrift, hoofdstuk VI en VII.

II

De eenvoudige relaties tussen de intensiteiten van direkte en van vertraagde fluorescentie, zoals geformuleerd door Lavorel en Clayton, gelden slechts als er geen veranderingen optreden in het thylakoidmembraan.

J.Lavorel, in H.Metzner, Progress in Photosynthesis Research, H.Laupp Jr., Tübingen, 1969, Vol.2, p.883
R.K.Clayton, Biophys.J., 9 (1969) 60
C.A.Wraight and A.R.Crofts, Eur.J.Biochem., 19 (1971) 386
Dit proefschrift, hoofdstuk V, VI en VII.

III

Een acceptabele methode om de fotochemische activiteit van geïsoleerde systeem 2 deeltjes te testen is wellicht het meten van de door sterk aanzuren verhoogde luminescentie.

Dit proefschrift, hoofdstuk VII.

IV

Het is zeer wel mogelijk, dat de verlaging van luminescentie door verhitting of veroudering van chloroplasten ondermeer te verklaren is door de aktiviteit van vrije onverzadigde vetzuren.

R.E.McCarthy and A.T.Jagendorf, Plant Physiol., 40 (1965) 725
M.Friedlander and J.Neumann, Plant Physiol., 43 (1968) 1249
P.A.Siegenthaler, Plant and Cell Physiol., 10 (1969) 811
Dit proefschrift, hoofdstuk VII.

V

Teneinde een vergelijking van de mechanismen van de door een zuur-base overgang geinduceerde fosforylatie en gestimuleerde luminescentie zinvol te maken mag de overweging gelden, dat de snelheid van de ATP produktie een betere parameter zou zijn dan de totale hoeveelheid zo geproduceerd ATP.

C.D.Miles and A.T.Jagendorf, Arch.Biochem.Biophys., 129 (1969) 711.

VI

De door Skulachev genoemde "laatste barrière" om de chemiosmotische theorie voor de fosforylatie in zijn geheel te aanvaarden vormt juist het fundamentele onderscheid tussen deze en de chemische theorie.

V.P.Skulachev, FEBS Letters, 11 (1970) 301.

VII

Het fotosynthese-actiespectrum gemeten door Balegh en Biddulph geeft onvoldoende aanwijzingen om gedeeltelijk verlaagde aktiviteit van chlorofyl b te veronderstellen; de hoge aktiviteit voor CO_2 opname in het gebied van 600-640 nm is niet zonder meer verklaarbaar.

S.E.Balegh and O.Biddulph, Plant Physiol., 46 (1970) 1.

VIII

De wijze waarop Kunderd, Spencer en Budde experimentele gegevens

hebben geinterpreteerd om de bruikbaarheid van een nieuwe massa-spectrometrische methode voor sekwentie-analyse van peptiden aan te tonen is aan bedenkingen onderhevig.

A.Kunderd et al., Anal.Chem., 43, 1086 (1971).

IX

Er is vooralsnog geen dwingende reden om aan te nemen, dat koolstofverbindingen een essentiële rol spelen bij N_2-fixatie in planten.

W.D.P.Stewart, Nitrogen fixation in plants, The Athlene Press, London, 1966.

X

Een konsekwente toepassing van de aan velen uit hun klassieke opleiding bekende spreuk: "Non scolae sed vitae discimus" zou kunnen leiden tot een nog beperkter aantal uren Latijn of Grieks op Gymnasium β en Atheneum.

XI

Sinds de wolfskwint op een aanvaardbare manier verdwenen is door de "komma van Pythagoras" gelijkelijk te verdelen over alle twaalf kwinten is er geen magistralere kompositie verschenen betreffende de gelijkzwevende temperatuur van klavierinstrumenten dan Bach's Wohltemperirte Clavier (1722).

Hans Brandts Buys: Het Wohltemperirte Clavier van Johann Sebastian Bach, Van Loghum Slaterus, Arnhem, 1955.

XII

Een zinvolle meerjaren-planning op ieder niveau van het universitair bestuur is in feite onmogelijk zolang het wetsontwerp Posthumus niet is behandeld door het parlement.

XIII

Indien het op de lagere scholen als verplicht vak gegeven muzikale vorming in het verleden minder was ondergewaardeerd, zou Radio Veronica wellicht een lagere luisterdichtheid gehaald hebben.

G.P.B.Kraan.