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CHLOROPHYLL *a* FLUORESCENCE AND PHOTOCHEMICAL ACTIVITIES OF CHLOROPLAST FRAGMENTS

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SUMMARY

1. Comparative studies were made on the fluorescence characteristics of chlorophyll *a* at 20° and —193°, and quantum efficiencies for P 700 oxidation and $NADP^+$ reduction were measured in chloroplasts and chloroplast fragments obtained after incubation with 0.5 % digitonin.

2. Differences in the fluorescence yield of chlorophyll *a* in flowing and stationary suspensions of untreated chloroplasts and of the large fragments are indicative of light-induced photoreduction of the quencher Q of chlorophyll *a*, associated with pigment System 2 (chlorophyll a_2). The relatively low constant fluorescence yield of chlorophyll *a* in the small fragments indicates the absence of fluorescent chlorophyll a_2 from these fragments and suggests that the low fluorescence is due to chlorophyll *a*, associated with pigment System 1 (chlorophyll a_1). The ratio of the fluorescence yields of chlorophyll a_1 and chlorophyll a_2 is 0.45:1. In the large particles the concentration ratio of pigment System 1 and System 2 is 1:3.

3. The efficiencies of quanta absorbed at 673, 683 and 705 nm for $NADP^+$ reduction and P 700 oxidation in untreated chloroplasts and chloroplast fragments indicate that digitonin treatment results in a separation of System 2 from System 1 in the small fragments. Sonication does not cause such a separation. Under the conditions used P 700 oxidation and $NADP^+$ reduction in the small fragments separated after digitonin treatment, occurred with maximal efficiency of 0.7 to 1.0 and 0.7, respectively.

4. The constancy of the fluorescence yield of chlorophyll a_1 in the small fragments, under conditions at which P 700 is oxidized and $NADP^+$ is reduced, is interpreted as evidence either for the hypothesis that the fluorescence of chlorophyll a_1 is controlled by the redox state of the primary photoreductant XH, or alternatively for the hypothesis that energy transfer from fluorescent chlorophyll a_1 to P 700 goes *via* an intrinsically weak fluorescent, still unknown, chlorophyll-like pigment.

5. The low-temperature emission band around 730 nm is argued not to be due to excitation by System 1 only; the relatively large half width of the band, as com-

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PPNR, photosynthetic pyridine nucleotide reductase, a crude preparation of enzyme necessary for $NADP^+$ reduction (ref. 9); TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine hydrochloride; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

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pared to the emission bands at 683 and 696 nm, suggests that it is possibly due to overlapping emission bands of different pigments.

INTRODUCTION

A separation of the two photosynthetic pigment systems in spinach chloroplasts has been suggested[1,2]. Centrifugation of digitonin-incubated chloroplasts at different speeds yielded fragments with different activities for photochemical reactions specifically associated with one of the photosynthetic pigment Systems 1 or 2; the rate of photoreduction of $NADP^+$, driven by System 1 (ref. 3) was shown to be considerably higher in the small particles than in the larger ones, whereas the rate of photoreduction of 2,6-dichlorophenolindophenol (DCIP) driven by System 2, was higher in the large particles[1,2].

Recently it has been reported that in the small particles the concentration of P 700, which presumably is the primary photooxidant of System 1, relative to that of chlorophyll *a* is about twice as high as in the large particles[4]. This was interpreted as indicating that the light fraction contains relatively more of pigment System 1.

It has been shown by others that the intensities and the emission spectra of chlorophyll *a* fluorescence are different for different fractions of digitonin-treated chloroplasts[5–8].

This paper deals with results of fluorescence and absorbance studies on chloroplast fragments. Quantum efficiencies for the photooxidation of P 700 and photoreduction of $NADP^+$, emission spectra at 20° and —193°, relative quantum yields and spectra of light-induced changes in quantum yield at 2° of chlorophyll *a* fluorescence, have been estimated in different fractions of chloroplast fragments. The results strongly support the hypothesis that by digitonin treatment of chloroplasts at least a partial separation of System 1 from System 2 can be attained.

METHODS AND MATERIALS

Spinach was obtained from the local market. Chloroplasts were prepared from washed leaves by grinding them in a mixer (Starmix, type MXC) for 1 min at maximum speed in 0.05 M phosphate buffer (pH 7.2) containing 0.4 M sucrose and 0.01 M KCl. After filtration of the homogenate, cell debris was removed by centrifugation at 200 × *g* for 1 min. The supernatant was centrifugated for 10 min at 1000 × *g* and the sediment (denoted throughout this paper as the chloroplasts) was suspended either in phosphate buffer pH 7.2, or in 0.05 M phosphate buffer (pH 7.2) containing 0.01 M KCl and 0.5 % digitonin. Sub-chloroplast particles were usually fractionated from the latter preparation after 1 h incubation at 2°, by subsequent centrifugations at 1000, 10000, 50000 and 144000 × *g*. The procedure was essentially the same as that described by ANDERSON AND BOARDMAN[2]. Most of the experiments were carried out with the fractions sedimented between 0 and 10000 or between 1000 and 10000 × *g* (denoted as 10000 N fraction) and between 50000 and 144000 × *g* (144000 N fraction). By sonication of the 10000 × *g* sediment, suspended in 0.005 M phosphate buffer (pH 7.2) containing 0.01 M KCl and 0.5 M sucrose, for 8 min in a Branson sonifier (type S 125, output 8 A) a preparation was obtained (denoted as

10000 S fraction) from which by centrifugation a fraction was isolated sedimenting between 50000 and 144000 $\times$ g (denoted as 144000 S fraction).

The respective sediments were suspended in a standard 0.02 M phosphate buffer solution (pH 8.0), containing 0.035 M NaCl and 0.005 M $MgCl_2$, at such a concentration that for a 1-mm layer the absorbance at 680 nm was about 0.3.

For the experiments in which $NADP^+$ reduction and P 700 oxidation were measured, $NADP^+$, photosynthetic pyridine nucleotide reductase (PPNR), ascorbate, DCIP or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine hydrochloride (TMPD) were added to the buffer solution. The standard reaction solution contained (per 3 ml, in μmoles): phosphate buffer, 40; NaCl, 70; $NADP^+$, 0.6; ascorbate, 6; DCIP, 0.02 (or TMPD, 0.02) and about 0.82 ml of a PPNR preparation, prepared following method A of SAN PIETRO AND LANG[9]. In experiments with untreated chloroplasts 3(3,4)-dichlorophenyl-1,1-dimethylurea (DCMU) was added at a final concentration of 10^{-6} M.

Most of the fluorescence experiments were done with preparations suspended in the standard buffer solution (*i.e.* without the additions mentioned above). All procedures were carried out at approx. 2°.

$NADP^+$ photoreduction and P 700 photooxidation were measured by tracing the absorbance changes at 340 and 435 or 705 nm respectively, occurring upon actinic illumination. These experiments were carried out in a sensitive differential absorption spectrophotometer[10,11]. The wavelength of monochromatic actinic illumination was 673, 683 or 705 nm. The half width of the wavelength bands was approx. 20 nm. Reaction vessels of 1-mm internal thickness were used for these experiments, which were carried out at room temperature. The optical geometry was the same as that described in ref. 11.

Relative and absolute efficiencies of actinic light quanta of 673, 683 and 705 nm for bringing about $NADP^+$ reduction and P 700 oxidation were estimated from the initial slope of the light-induced absorbance change, the intensity of the actinic light, and the absorbance of the sample at the wavelength of the actinic light. The absorbance of the suspension at 673, 683 and 705 nm was measured with a Zeiss PMQ II spectrophotometer. Interference filters were placed in front of both the sample and reference cuvette in order to approximate the absorption of the suspension for light of the same wavelength band as that of the actinic light beam in the absorption difference measurements. In addition opal glass was placed behind the reference and sample cuvettes in order to diminish the effect of scattering[12]. A correction for the scattering was made by subtracting the apparent absorbance of the sample at 750 nm from the measured absorbance at each wavelength. For the 144000 N fractions this correction was negligible. The following absorptivities were used: for NADPH at 340 nm: 6.22 $cm^{-1} \cdot mM^{-1}$ (ref. 13); for P 700 oxidized *minus* reduced at 435 nm and 705 nm: 40 and 70 $cm^{-1} \cdot mM^{-1}$, respectively[14,15].

Fluorescence experiments were carried out with a fluorescence apparatus[16,17] equipped either with a flow attachment[15,17] or, for low-temperature experiments, with a Dewar flask with a transparant side window for the transmission of the excitation and fluorescence light beams. The 1-mm reaction vessel was in this case fixed on a piece of brass, the bottom part of which was plunged into liquid N_2. The experimental set-up of the flow apparatus was such that the fluorescence was excited in a suspension which was either flowing or stationary in a cuvette of 1-mm optical

path[15,17]. In the first case, due to the flow of the cells, the fluorescence emitted per fixed volume of cells can be considered to be excited by a light flash of about 1 msec (refs. 15, 17). The intensity of the exciting light was such that during flow (*i.e.* excitation by a flash) the fluorescence yield was low, whereas in the stationary suspension (*i.e.* excitation by continuous light) it increased due to a photochemical conversion of the energy trap(s) (*i.e.* the photoreduction of the primary oxidant Q of photosystem 2). Substraction of the emission spectra of the stationary and the flowing suspensions gives the fluorescence difference spectrum. The fluorescence spectra are shown as recorded, except for a multiplication factor, and are not corrected for the wavelength dependent transmission of the analyzing monochromator (Bausch and Lomb), or for the sensitivity of the photomultiplier (E.M.I. 9558 QC). Spectra were recorded at a half band width of 5 nm.

RESULTS

1. *Fluorescence characteristics*

Fig. 1 shows the room-temperature emission and fluorescence difference spectra of a heavy and a light fraction from digitonin-incubated chloroplasts. The spectra are reproduced from the original recordings by multiplying these by appropriate factors to match them at the maximum near 683 nm. At 20° the fluorescence emission above 695 nm relative to that at 683 nm is higher in the smaller fragments (144000 N fraction) than in the larger ones (10000 N fraction). The fluorescence difference spectra of the untreated chloroplasts and a 50000 N fraction were found to have approxi-

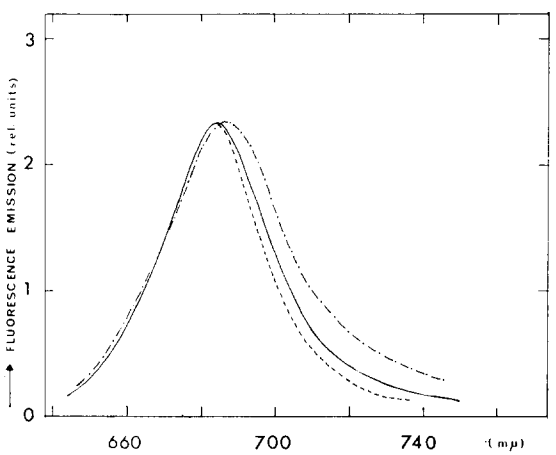

Fig. 1. Relative fluorescence emission spectra f of a 10000 N (———) and a 144000 N (–·—·–) chloroplast fraction of approx. equal absorbance at 20°, measured with the flow system under the same conditions (excitation light of 436 nm, intensity of the order of 10^{-9} einstein/cm^2·sec, duration of excitation flash t_e approx. 2 msec, due to the flow of the cells (see Fig. 2.3 of ref. 16)). The solid curve was recorded at a 0.45 lower sensitivity than the other one, in order to match the two spectra in the maximum near 683 nm. The fluorescence difference spectrum of the 10000 N fraction is given by the dotted curve (– – – –). This spectrum represents the difference in fluorescence in a flowing and in a stationary suspension, $s - f$, for actinic light of 436 nm of the same intensity (10^{-9} einstein/cm^2·sec). The original spectrum was multiplied by a factor of about 0.6 in order to match it at 683 nm with the fluorescence emission spectrum. The 144000 N fraction did not show measurable differences in fluorescence yield between flowing and stationary suspensions.

mately the same shape as that of the 10000 N fraction. As shown in Fig. 1 the fluorescence difference spectrum of the 10000 N fraction is relatively low in the wavelength region above 695 nm as compared to the emission spectrum. For the 144000 N fraction the fluorescence yield in continuous and short time excitation of the same intensity was the same. The first two columns of Table I show the relative fluorescence intensities at 683 nm for chloroplasts and chloroplast fragments in short flashes of excitation light (*i.e.* the "dark" yield of the fluorescence Φ_d, which is proportional to f (see Fig. 2)), and in continuous excitation light (*i.e.* the steady-state yield Φ_s,

TABLE I

RELATIVE "DARK" FLUORESCENCE YIELDS Φ_d AND CHANGES IN FLUORESCENCE YIELD AT 683 nm PER mg CHLOROPHYLL *a* IN CHLOROPLASTS AND CHLOROPLAST FRAGMENTS

The values in the table are an average over 5 experiments each with fresh preparations of equal absorbance. The experiments were carried out at 2° with the fluorescence flow apparatus. The "dark" fluorescence yield was measured in a flowing suspension under such conditions (*i.e.* duration of the excitation flash t_e about 5 msec, intensity of the flash of the order of 10^{-9} einstein/cm^2·sec), that the fluorescence yield Φ_d was minimal. Upon stopping the flow (*i.e.* $t_e \rightarrow \infty$) the fluorescence increased to a new steady state Φ_s.

	Φ_d	Φ_s	$(\Phi_s - \Phi_d)/\Phi_d$
Chloroplasts	0.39 ± 0.02	1.00 ± 0.04	1.56 ± 0.07
10000 N	0.50 ± 0.02	1.40 ± 0.04	1.82 ± 0.09
144000 N	0.24 ± 0.01	0.24 ± 0.01	0.05

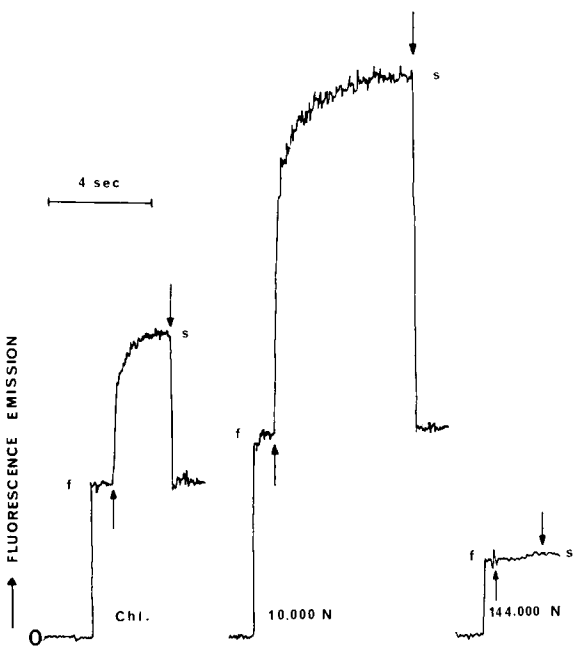

Fig. 2. Steady state fluorescence levels f and s, measured at 683 nm, of flowing and stationary suspensions, respectively, of chloroplasts and 10000 N and 144000 N chloroplast fragments. Flow conditions were approximately as described in the legend of Fig. 1. Excitation light was of a wavelength of 436 nm and of an intensity of approx. 10^{-9} einstein/cm^2·sec. Upward and downward pointing arrows mark the moments of stopping flow and starting flow, respectively.

which is proportional to s (see Fig. 2)). The data were obtained from experiments done with suspensions of chloroplasts and chloroplast fragments of nearly equal absorbance in the chlorophyll *a* absorption maximum near 679 nm. Fluorescence spectra were recorded at the same intensity of the exciting light, which was of a wavelength band around 436 nm ($\Delta\lambda$ about 5 nm). Because of slight variations in the absorbance of the different samples, appropriate corrections were applied in order to obtain the relative fluorescence emission in the maximum near 683 nm per absorbed quantum at 436 nm for the different fragments. In the third column the difference in fluorescence ($s-f$) in a stationary and a flowing suspension of chloroplasts or chloroplast fragments, measured at 683 nm, relative to f (see Fig. 2) (*e.g.* the relative change in fluorescence yield $\Delta\Phi/\Phi_d$ is given. The values given in the table are an average of several experiments, each with fresh preparations.

Fig. 3 gives fluorescence emission spectra of chloroplast fragments at liquid N_2 temperature. Excitation light was of a rather high intensity as a consequence of which the steady-state fluorescence was high in the heavier particles. The spectra were recorded at equal sensitivity settings of the apparatus for the different samples, which were of approximately equal absorbance at 680 nm. The spectra show significant differences in the relative emission of the bands around 683, 696 and in the region 720 to 730 nm. In the intact chloroplasts and the 10000 N fraction the low temperature fluorescence at 683 nm increased, after an initial level reached within the response time of the recorder, during a slower second phase to a steady-state level. This indicates the photochemical conversion of the reaction center at low temperatures[18]. The 144000 N fraction did not show such a second phase. It appears that the low-temperature emission of the 685- and 696-nm bands is lower in the smaller fragments, whereas the emission of the 720-nm band seems not to be so. The conclusion whether the

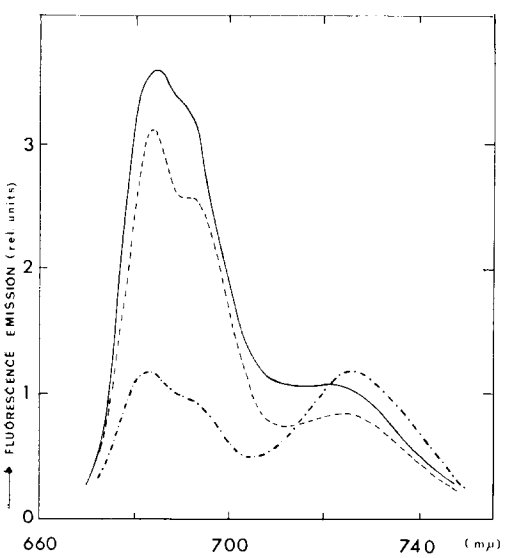

Fig. 3. Relative fluorescence emission spectra of chloroplast fractions of approximately equal absorbance at liquid N_2 temperature. Excitation light of 436 nm and about 10^{-9} einstein/cm^2·sec intensity. – – – –, digitonin fraction sedimented at 10000 × *g* (10000 N); –.—.–, digitonin fraction sedimented at 144000 × *g* (144000 N); ———, fraction sedimented at 144000 × *g* (144000 S) after sonication of the 10000 N fraction.

fluorescence yield of the different chlorophylls is related to the particle size or to the relative content of the two pigment systems in the particles, or alternatively to both, awaits a study of the photochemical activities of the different fractions.

2. Photochemical activities

Table II summarizes for a number of experiments the efficiencies of quanta absorbed at 673 and 683 nm relative to those absorbed at 705 nm in bringing about $NADP^+$ reduction and P 700 oxidation in chloroplasts and chloroplast fragments.

TABLE II

EFFICIENCIES, Φ_λ, (ELECTRON EQUIVALENTS PER ABSORBED QUANTUM), OF QUANTA ABSORBED AT 673, 683 AND 705 nm FOR P 700 OXIDATION AND $NADP^+$ REDUCTION IN CHLOROPLASTS AND CHLOROPLAST FRAGMENTS

The measurements were done with freshly prepared fragments from fresh chloroplasts (Expts. 1–5) or from chloroplasts which were kept for several weeks in a refrigerator at −30° (Expts. 6 and 7). Quantum efficiencies were calculated from the initial slope of the light-induced absorbance changes occurring at 340 nm ($NADP^+$) and 430 nm (P 700), the intensity of the actinic light, and the absorbance of the samples. The specific molecular extinction coefficients applied were 6.22 $cm^{-1} \cdot mM^{-1}$ for $NADP^+$ at 340 nm, and 40 $cm^{-1} \cdot mM^{-1}$ for P 700 at 430 nm. It was verified that for the actinic intensities used (10^{-11}–10^{-10} einstein/$cm^2 \cdot$sec) the reaction rates were linearly dependent on the intensity. The standard error of each experiment in the table is about 10–15%. The measurements were carried out at 20°. The concentrations of the sample constituents are given in METHODS AND MATERIALS. In Expts. 4 and 5 TMPD was used instead of DCIP, and in Expt. 5 a chloroplast extract from a 200000 × *g* supernatant was added in addition to PPNR. The average values of Φ_λ/Φ_{705} of all experiments are given at the bottom of the table. The value in parentheses in the fourth column is the average value of Φ_{683}/Φ_{705} when it is assumed that the value of Expt. 2 should have been about 1.0 instead of 1.6 (see text).

Expt. No.	*Fraction*	*P 700 oxidation*			*$NADP^+$ reduction*		
		Φ_{705}	Φ_λ/Φ_{705}		Φ_{705}	Φ_λ/Φ_{705}	
			683	*673*		*683*	*673*
1	10000 N	0.55	0.3	—	—	—	—
1	144000 N	0.28	1.2	—	—	—	—
2	10000 N	0.71	0.4	0.3	—	—	—
2	144000 N	0.58	1.6	—	—	—	—
3	10000 N	0.16	0.6	—	—	—	—
3	144000 N	0.28	1.1	1.0	—	—	—
4	Chloroplasts	—	—	—	0.95	0.5	0.4
4	144000 S	—	—	—	0.10	0.4	0.4
4	144000 N	—	—	—	0.20	0.7	—
5	Chloroplasts	—	—	—	0.95	0.5	—
5	10000 S	—	—	—	0.22	0.6	—
5	144000 N	—	—	—	0.14	0.8	—
6	Chloroplasts	—	—	—	0.59	0.5	0.5
6	10000 N	—	—	—	0.17	0.6	0.5
6	144000 N	0.67	0.8	0.6	0.69	0.8	0.7
7	Chloroplasts	—	—	—	0.67	0.4	0.4
7	144000 N	—	—	—	0.44	0.8	0.8
1–7	Chloroplasts	—	—	—	—	0.5	0.4
	10000 N 10000 S 144000 S	—	0.4	—	—	0.5	—
	144000 N	—	1.2 (1.0)	—	—	0.8	0.75

Each experiment, indicated by a number in the first column, was done with freshly prepared fragments from fresh chloroplasts (numbers 1–5) or from chloroplasts which were kept for several weeks in the dark at −30° (numbers 6 and 7). The highest quantum efficiencies for P 700 oxidation and $NADP^+$ reduction were about 1.0 (683 nm) (not given in Table II) and 0.95 electron or hydrogen equivalents per quantum (705 nm), for chloroplasts, and 1.0 (683 nm) and 0.69 (705 nm) for the 144000 N fractions. In other cases lower values (down to 0.14) for $NADP^+$ reduction in the light particles were found. The variability of the results with different preparations is presumably due to non-optimal conditions of some of the sample assays, most probably caused by some loss of enzymes or of other intermediates of the electron transport chain in the final preparation. However, in the samples which apparently reduced $NADP^+$ with low efficiency P 700 was found to be oxidized with a nearly maximal efficiency. In Expts. 4 and 5 the efficiency of $NADP^+$ photoreduction and P 700 oxidation was measured in a 10000 N and a 144000 N fraction, and in a 144000 S fraction, sedimented at 144000 × *g*, obtained after 8–10 min sonication of the 10000 N digitonin fraction. The ratio 1.6 for the 144000 N fraction in Expt. 2 (Table II) is surprisingly high and would be expected not to be higher than 1.0. The discrepancy may be caused by the fact that in the small particles the absorbance at 705 nm is partly due to an inactive pigment with a relatively high absorbance above 700 nm, which in this sample was present in a relatively high concentration, causing an apparently low efficiency of 705-nm quanta. This inactive pigment may be solubilized chlorophyll *a* of which the absorption maximum is in the 670-nm region. This would also explain the lower relative efficiency of 673-nm quanta, which was occasionally observed.

The ratio of chlorophyll *a*/P 700 of the different fractions (*i.e.* 10000 N, 144000 N, 144000 S) and of the chloroplasts was estimated to be 600, 230, 520 and 480 respectively. The fluorescence emission spectra of the fractions are those given in Fig. 3. It was found, in accordance with similar conclusions of KATOH AND SAN PIETRO[19], that the rate of photoreduction of $NADP^+$ was higher for the 144000 S than for the 10000 N fractions. Fig. 4 gives recorder tracings indicating that the fluorescence yield of chlorophyll *a* in a 144000 N fraction does not change upon illumination, whereas the same illumination causes a photooxidation of P 700. The experiments were done in the same apparatus, immediately after each other. The sample also showed a change n absorbance at 340 nm, due to $NADP^+$ reduction (not shown in Fig. 4).

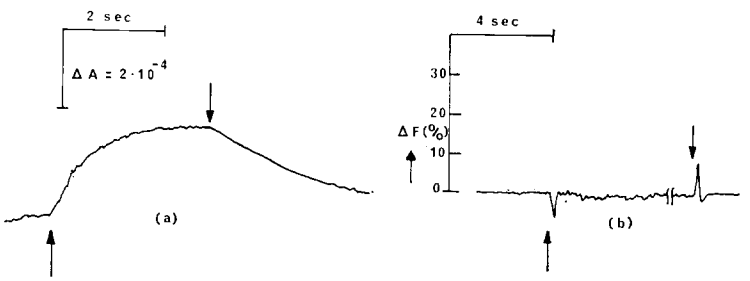

Fig. 4. Recorder tracings of the absorbance change at 705 nm (a) and change in the yield of fluorescence emission (> 680 nm) (b), occurring upon actinic blue (λ = 436 nm) illumination in a 144000 N chloroplast fraction. Upward deflections indicate a decrease in absorbance due to P 700 oxidation (a) and an increase in fluorescence yield (b), respectively. Intensity of actinic light was approx. $3 \cdot 10^{-10}$ einstein/cm^2·sec.

DISCUSSION

Relative efficiencies of quanta of different wavelengths for $NADP^+$ reduction and P 700 oxidation in chloroplasts and chloroplast fragments were measured in order to establish whether the high rate of $NADP^+$ reduction[1,2] and methyl red reduction[19,20] observed in small fragments of digitonin-treated chloroplasts, is solely due to a separation of pigment System 1 from System 2, or not.

Recent results, reported by KATOH AND SAN PIETRO[19] and by VERNON *et al.*[20,21], do not exclude the possibility of an additional effect related to the decrease of the particle size.

In chloroplasts the two pigment systems appear to absorb about an equal number of quanta in the wavelength region 670–690 nm, whereas above 690 nm System 1 becomes the predominant absorber[22–24]. If the 144000 N fraction contains particles of pigment System 1 only, one would measure approximately equal efficiencies of 705-, 683- and 673-nm quanta, whereas in the chloroplasts (containing both systems) the efficiencies of 683- and 673-nm quanta would be approximately one half those of 705 nm. Our results show that in the 144000 N fraction the efficiency of 683-nm quanta for the reduction of $NADP^+$ and oxidation of P 700 is 80 to 100 % that of 705-nm quanta, whereas in the chloroplasts it is 50 %. This indicates that the 144000 N fraction contains particles, predominantly, of pigment System 1. In spite of attempts to effect uniform processing there were significant fluctuations in the results of the various experiments. These might be primarily due to an, as yet, still uncontrolled destructive effect of digitonin, presumably due to a partial destruction of the chlorophyll–protein complex. The low quantum efficiencies for $NADP^+$ reduction in some samples, as discussed, may be caused by a loss of enzyme activity during the preparation. The efficient photooxidation of P 700, which was usually observed under such non-optimal conditions of samples for $NADP^+$ reduction, indicates that the pigment system and the reaction center are functionally intact. The observation that in such preparations the rate of $NADP^+$ reduction with a TMPD–ascorbate donor couple was higher than with the DPIP–ascorbate couple may indicate that in the preparations plastocyanin[19,25,26] or plastoquinone C (ref. 27) was lost.

It was found that at pH = 8.5 subparticles of a 10000 N fraction obtained after sonication (10000 S) showed a much higher rate of $NADP^+$ reduction in light of 683 nm than the 10000 N fraction; at pH = 7.0 the effect was much less pronounced (L. SLOOTEN, unpublished). The rate of photoreduction of $NADP^+$ in 10000 S and 144000 S fragments at pH = 8.5 and 7.0 is still lower, however, than the reduction rate in the 144000 N fragments. In addition, the efficiency for $NADP^+$ reduction of 683-nm quanta relative to that of 705-nm quanta in the sonicated 144000 S fragments was found to be approximately equal to that in the 10000 N fraction, indicating that sonication did not cause a separation of the pigment systems present in the 10000 N fraction. The P 700/chlorophyll *a* ratio and the fluorescence low-temperature emission spectra of these different fractions (Fig. 3) give further experimental support for this statement. In the 10000 N, 10000 S and 144000 S fractions the relative efficiencies of 683- and 673-nm quanta for $NADP^+$ reduction and P 700 oxidation, which were found to be in the range of 0.3 to 0.6 (see Table II), are rather high. By calculation, the relative concentration of System 1 in these fractions is 23 to 29 %, which would mean that the relative efficiencies for these photochemical reactions should not have

been higher than 0.3. The most probable explanation for this discrepancy is that the 22-nm band width of the 705-nm filter, which was used for the preferential absorption by pigment System 1, was too broad, and thus transmitted too much light of shorter wavelengths, which is largely absorbed by System 2. This causes a lower apparent efficiency of 705-nm quanta, and, consequently, higher relative efficiencies of quanta of shorter wavelengths in fractions, which contain particles of both pigment systems. The effect is strongest in fractions with the highest concentration ratio of System 2 relative to System 1 (*i.e.* 10000 N, 10000 S and 144000 S).

From fluorescence studies with intact algae, it was concluded that the bulk of the chlorophyll *a* fluorescence is excited by System 2 (chlorophyll a_2), and that chlorophyll *a*, excited by System 1 (chlorophyll a_1) is relatively weakly fluorescent[28,15,17]. The relatively low dark fluorescence yield of chlorophyll *a* at 683 nm at 20° in the 144000 N fraction, indicates that in these particles much less fluorescent chlorophyll a_2 is present. The changes in the fluorescence yield, known to be caused for the main part by chlorophyll a_2 (refs. 28, 15), are only observed in the chloroplasts and in the heavier particles. This too indicates that the fluorescent chlorophyll a_2 is present predominantly in the 10000 N and is absent from the 144000 N fraction. Assuming that chlorophyll a_2 is absent from the 144000 N fraction, that the fluorescent chlorophyll *a* is chlorophyll a_1, and that the fluorescence yields of both chlorophyll a_1 and a_2 are independent of the particle size, *i.e.* are constant in all fractions, and finally that in untreated chloroplasts both pigment systems contain equal concentrations of chlorophyll a_1 and chlorophyll a_2 with equal absorptivities at 680 nm, the relative concentration of the pigment systems in the different fractions can be calculated from the fluorescence data given in Table I. It is estimated that in the 10000 N fraction the ratio of the concentrations of pigment System 1 and System 2 is about 1:3 (with an uncertainty of 10–15 %). The estimated concentration ratio of the pigment systems in the 10000 N fraction agrees fairly well with that calculated by ANDERSON, FORK AND AMESZ[4], on the basis of the chlorophyll *a*/P 700 ratio, and fits with the data on the chlorophyll *a* content of different chloroplast fractions, given by BRIANTAIS[5]. It follows further from these calculations that the "dark" fluorescence yield of chlorophyll a_1 at 683 nm at 2° is about 0.45 ± 0.04 of that of chlorophyll a_2.

The fluorescence spectra at low temperature (Fig. 3) show that the emission of the 696-nm band is relatively higher in the chloroplasts and in the heavy fractions, which suggests, in accordance with conclusions obtained by others[30,31] that this is an emission of a chlorophyll-like pigment, probably the chlorophyll *a* C_a 680 (ref. 29), predominantly excited by pigment System 2. In the spectrum at −193° of the 144000 N fraction a small hump around 696 nm was observed, indicating that in this fraction F 696 is excited, however, with less efficiency than in the particles, which contain both systems (*e.g.* the chloroplasts, 10000 N and 144000 S fractions). The low temperature emission band around 720 to 730 nm was suggested to be due to a chlorophyll-like pigment C 700 of pigment System 1 (refs. 31, 32). KOK[32] reported that the emission was largely absent in the heavy fractions. Our results suggest that at least part of the emission around 730 nm is not excited by System 1. In our samples the 730-nm emission of the 144000 N fraction was up to 1.5 times as large as that in the 10000 N fraction, whereas for the same samples it was found that the ratio P 700/chlorophyll *a*, which in KOK's[31] scheme should parallel the concentration ratio C 700/chlorophyll *a*, was at least two times as large. BOARDMAN, THORNE AND

ANDERSON[8] also concluded that in the heavy particles the 730-nm emission was too high as compared to the 730-nm emission in the light particles, to account for excitation by System I only. DUYSENS[33] argued from fluorescence studies in *Schizothrix calcicola* that in algae at least part of the emission of 730 nm comes from System 2. The relatively large half width of the 730-nm emission band, which is about twice as large as that of the 683- and 696-nm emission bands (see Fig. 3), suggests that this band is composed of at least two overlapping emission bands with different maxima.

The constancy of the fluorescence yield in the light fraction at 20° and at —193°, which was observed under conditions at which P 700 is oxidized (*cf.* Fig. 4) and $NADP^+$ is reduced by light, indicates that there is no direct transfer of energy from the chlorophyll-like pigments with the fluorescence bands at 683, 696 and 720–730 nm to the reaction center of pigment System I. For photosynthetic bacteria it was shown[34] on basis of a certain model with energy transfer between photosynthetic units[33,35] that the fluorescence yield of bacteriochlorophyll is quantitatively correlated with the photoconversion of the reaction center. In intact algae an increase in the fluorescence yield of chlorophyll a_1 was found, which was not correlated with redox changes of P 700 (refs. 15, 17). These fluorescence changes were suggested to be correlated with the redox changes of the (quenching) primary oxidant X of System I (ref. 17). Because of the constancy of the fluorescence yield of chlorophyll a_1 in the light particles of System I in chloroplasts, however, it should be assumed that during electron flow from the electron donor couple to $NADP^+$ no accumulation of reduced X (XH) occurs in the light, due to a high turnover rate. An alternative explanation, given by DUYSENS[36], is that in the chloroplast energy transfer from chlorophyll a_1 to P 700 occurs only *via* an intrinsically weakly or non-fluorescent chlorophyll a_1' present in a relatively small concentration. In such a model the fluorescence yield would be essentially constant during and after the bleaching of P 700.

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