Fluorescence of the Subchloroplast Particles Obtained by the Action of Triton X-100*

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ABSTRACT: The fluorescence spectra of a heavy and light particle obtained by fractionating spinach chloroplasts with Triton X-100 were examined at room and liquid-nitrogen temperatures. The fluorescence spectrum of the heavy particles at room temperature had a main peak at 682.5 nm. The fluorescence yield was strongly enhanced at −196°, and the spectrum developed two major peaks at 696 and 685 nm and a weaker broad band centered at 730 nm. The fluorescence was largely quenched by such electron acceptors as ferricyanide ion, 2,6-dichlorophenolindophenol, and ferricytochrome *c*. The fluorescence of the light particles was very weak at room temperature; the maximum was at 680 nm. The fluorescence was enhanced at −196°; one peak was at 678 nm and a relatively stronger new peak appeared at 726 nm. The fluorescence was quenched more by such electron acceptors as 2,6-dichlorophenolindophenol and ferricytochrome *c* than by such mixtures as (ascorbate plus phenazine methosulfate) or (ascorbate plus dichlorophenolindophenol plus nicotinamide–adenine dinucleotide phosphate plus ferredoxin plus nicotinamide–adenine dinucleotide phosphate reductase plus plastocyanin) which are known to bring about reactions involving P700 turnover. The fluorescence properties and the known photochemical properties of these particles indicate the 725- and 694-nm fluorescent peaks probably originated from the chlorophylls associated with pigment systems 1 and 2, respectively, of the chloroplasts.

The fluorescence properties of chloroplasts have long been used as a means for investigating energy transfer by the chlorophyll molecules in photosynthesis. Until recently, it has generally been assumed that a single form of chlorophyll *a* is responsible for the fluorescence in green plant chloroplasts. Along with the development of the concept of two pigment systems in plant photosynthesis, differences attributable to the two pigment systems have become increasingly noticeable also in the fluorescence spectra. Such differences in the fluorescence spectra were detected earlier in the blue-green (Bergeron, 1963; Kok, 1963) and red (Krey and Govindjee, 1964) algae, where the separation of the two pigment systems can easily be achieved experimentally. Evidence for the presence of different fluorescent species belonging to the two pigment systems of green plant chloroplasts was also reported recently (Govindjee and Yang, 1966).

Boardman and Anderson (1964) first demonstrated that the detergent digitonin fractionates spinach chloroplasts into subparticles which have properties resembling the two pigment systems. Recently Boardman *et al.* (1966) and Kok and Rurainski (1966) have also found differences in the fluorescence emission spectra of the particles. Since the subchloroplast particles obtained by Triton treatment also have properties characteristic of the two pigment systems (Vernon *et al.*, 1966), an investigation of the fluorescence spectra of these particles not only serves to further compare the properties of the subparticles obtained by different detergents, but may also furnish additional information on the nature and origin of the different fluorescence emissions.

Experimental Section

Fluorescence emission and excitation spectra were measured with a Cary (Model 56-231) three-port illuminator attached to the Cary Model 14R spectrophotometer, as shown schematically in Figure 1. The Cary monochromator was used to resolve the fluorescence spectrum. The light from a Bausch and Lomb 500-mm grating monochromator was used for excitation. For fluorescence measurements, the chopper in the illuminator was synchronized with that in the main spectrophotometer, so that fluorescence was detected during the same cycle the sample was illuminated. An EMI 9558 photomultiplier was installed in the usual light-source position at the back of the spectrophotometer. The output of the photomultiplier was fed through the electronics of the Cary spectrophotometer and recorded as a function of wavelength. The wavelength drive of the excitation monochromator was geared to the chart drive of the main spectrophotometer so that excitation spectra could also be recorded as a function of the excitation wavelength.

The three-port illuminator was operated in a double-beam mode. The reference signal was generated from an auxiliary photomultiplier (RCA 1P28) with a quantum counter consisting of Rhodamine B solution (Melhuish,

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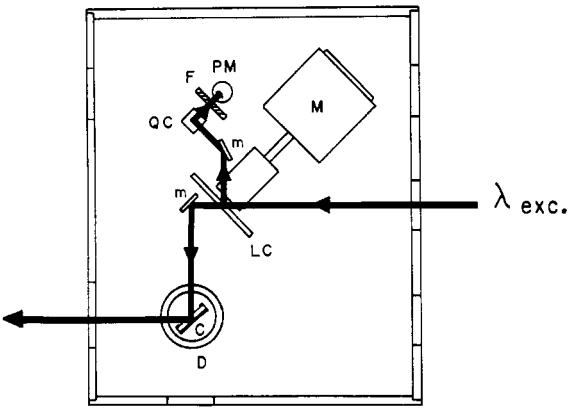

FIGURE 1: Schematic drawing of the three-port illuminator used in conjunction with the Cary Model 14R spectrophotometer for measuring fluorescence emission and excitation spectra. During operation the light chopper (LC) in the illuminator is synchronous with the light chopper in the spectrophotometer. Monochromatic light (λ_{exc}) from a Bausch and Lomb 500-mm grating monochromator was used for exciting the fluorescence. See text for detailed description of the short-path-length cuvet (C). For low-temperature measurements, the cuvet was cooled inside the dewar (D). M represents the chopper motor, m the front-surface mirrors, QC the quantum counter, PM an RCA 1P28 photomultiplier, and F the cutoff filter for isolating the Rhodamine B fluorescence.

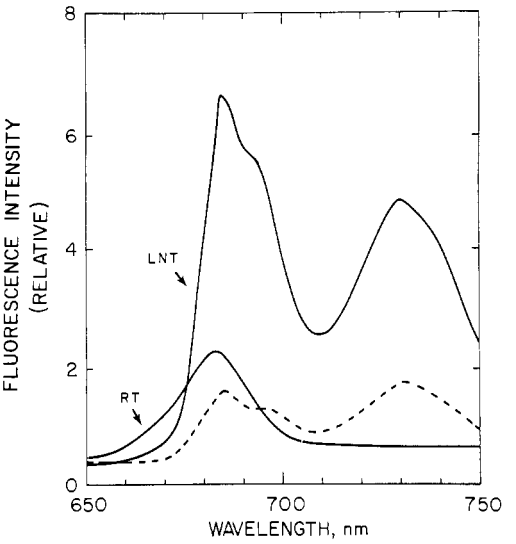

FIGURE 2: Fluorescence spectra of whole chloroplasts (spinach) at room (RT) and liquid-nitrogen (LNT) temperatures. The dashed spectrum was taken at liquid-nitrogen temperature in the presence of 0.1 mM DPIP.

1962). Since the quantum efficiency of Rhodamine B fluorescence has been shown to be independent of the excitation wavelength, this arrangement allows the true excitation spectra to be recorded (Weber and Teale, 1958). All fluorescence spectra shown in this paper are tracings of the original recordings, and were not corrected for the efficiency of the Cary monochromator or the response of the photomultiplier.

Room and liquid-nitrogen temperature fluorescence spectra were both measured in a metal cold-finger cuvet with a path length of 1 mm. The cuvet path length was formed by a microscope glass slide mounted to the metal wall with a rubber O ring. The polished metal back wall of the cuvet serves to increase the light-gathering efficiency. The cuvet was oriented 45° with respect to the excitation beam and the entrance slit of the Cary monochromator, as shown in Figure 1. For low-temperature measurements, the cuvet was cooled with liquid nitrogen inside the glass dewar.

The procedure for fractionating the chloroplasts into subparticles with Triton was described previously (Vernon *et al.*, 1966). Chemicals and cofactors used in this work are the same as those described in the preceding paper (Vernon *et al.*, 1967). For fluorescence measurements, the concentration of the suspension in the 1-mm cuvet was adjusted to give an optical density of 0.1 at the red absorption maximum. Unless otherwise mentioned, all fluorescence spectra were excited with a 5-nm wide excitation beam at a wavelength centered at the blue absorption maximum (437 nm). A constant slit width of 1.0 mm was used on the Cary monochromator.

Results

Fluorescence of Whole Chloroplasts at Room and Liquid-Nitrogen Temperatures. For comparison purposes, the fluorescence spectra at room and liquid-nitrogen temperatures for whole chloroplasts of spinach are shown in Figure 2. At room temperature, the fluorescence spectrum had a major band with the maximum at 682.5 nm. At liquid-nitrogen temperature, the fluorescence yield increased severalfold, the major-band maximum shifted to 685 nm, and a strong broad band developed at 730 nm. Also, a secondary peak developed at 694 nm.

In the presence of 10^{-4} M DPIP,[1] the fluorescence yield at both room and liquid-nitrogen temperatures decreased. The low-temperature fluorescence spectrum in the presence of DPIP is shown in Figure 2 by the dashed curve. Notice that the 685- and 694-nm bands were suppressed to a greater extent than the 730-nm band.

Fluorescence of the Heavy Particle Obtained by Triton Fractionation. The heavy particle (*e.g.*, P-1) fractionated from spinach chloroplasts with Triton is characterized by a low chlorophyll a:chlorophyll b ratio ($\cong$2) and a high lutein content relative to β-carotene (Ke *et al.*, 1966). With a sensitive difference spectrophotometer (Ke *et al.*, 1964) the P-1 fraction was found to be slightly

[1] Abbreviations used: DPIP, 2,6-dichlorophenolindophenol; NADP, nicotinamide-adenine dinucleotide phosphate; PMS, phenazine methosulfate.

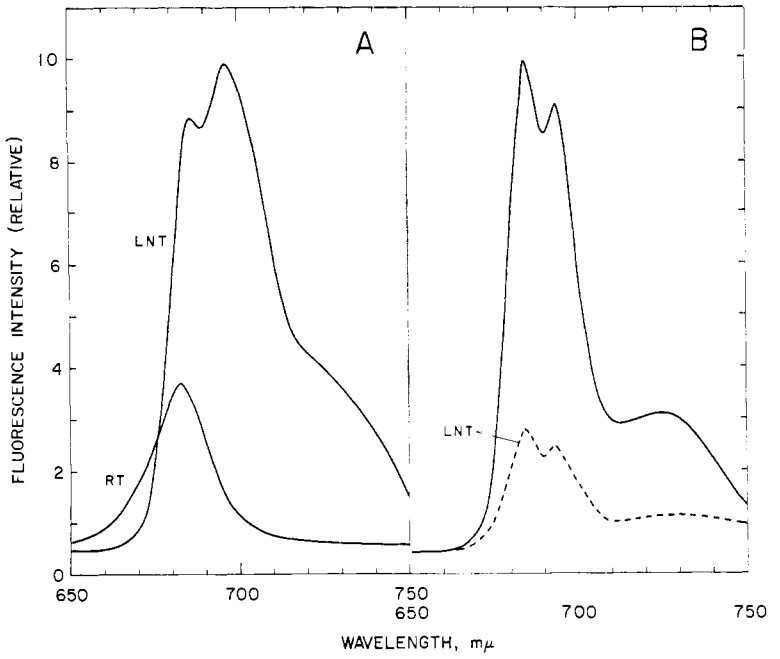

FIGURE 3: Fluorescence spectra of the heavy particle (P-1) fractionated from spinach chloroplasts with Triton. The room and liquid-nitrogen-temperature spectra of one preparation are shown at left. At right are the low-temperature spectra of another P-1 preparation and the same in the presence of 0.1 mM DPIP (dashed curve).

active in DPIP photoreduction with kinetics characteristic of pigment system 2 (B. Ke, unpublished experiments).

The fluorescence spectra of the P-1 particle at room and liquid-nitrogen temperatures are shown in Figure 3A. The room-temperature spectrum resembles that of whole chloroplasts, having the major-band maximum at 682.5 nm. At liquid-nitrogen temperature, the major band appeared at 696 nm, while a secondary peak remained at 685 nm. A weak shoulder appeared at 730 nm. With some preparations, the low-temperature spectrum assumed the profile shown in Figure 3B. The major band and the secondary band were now at 685 and 694 nm, respectively, plus a shoulder at 730 nm. In the case where there was more 694-nm emission, a greater combined band width also resulted, as evidenced in Figure 3A. In the presence of a number of electron acceptors such as ferricyanide, oxidized DPIP, or ferricytochrome *c*, both the room and liquid-nitrogen temperature fluorescence were partly quenched. This is shown by the dashed curve in Figure 3B for the case of 10^{-4} M DPIP at liquid-nitrogen temperature.

Fluorescence of the Light Particle Obtained by Triton Fractionation. The light particle (P-D10) fractionated from spinach chloroplasts with Triton is characterized by a high chlorophyll a:chlorophyll b ratio ($\cong$7) and a high β-carotene content (Ke *et al.*, 1966). The P-D10 particle is extremely active in pigment system 1 reactions, namely, photoreduction of NADP or viologen dyes and photooxidation of plastocyanin and ferrocytochrome *c* (Vernon *et al.*, 1967). It gives light-induced electron spin resonance (esr) and absorbancy-change signals representing P700 photooxidation. With a sensitive difference spectrophotometer (Ke *et al.*, 1964) the P-D10 particle has also been found to reduce DPIP with rapid rates of onset and decay (B. Ke, unpublished experiments).

At room temperature, the P-D10 particle shows extremely weak fluorescence (Vernon *et al.*, 1966), with an emission peak located at 680 nm. As seen in Figure 4A, the fluorescence was enhanced at $-196°$; the first peak was now located at 678 nm and a larger new peak appeared at 725 nm. In different preparations of P-D10 particles, no fluorescence peaks at 685 and 695 nm were observed at liquid-nitrogen temperature. The presence of such electron acceptors as ferricyanide, ferricytochrome *c*, or DPIP also quenched both the room and liquid-nitrogen temperature fluorescence, as shown in Figure 4B for 10^{-4} M ferricytochrome *c* at both room and liquid-nitrogen temperatures. On the other hand, in the presence of a complete donor (ascorbate, DPIP, and plastocyanin) and acceptor (NADP, ferredoxin, and NADP reductase) system, in which NADP is known to be photoreduced at a high rate, both the room and low-temperature spectra were affected only very slightly. As reported in the preceding paper (Vernon *et al.*, 1967), the P-D10 particle in the presence of ascorbate and PMS allows a light-induced absorbancy-change signal at 430 nm to take place completely independent of temperature down to $-196°$, indicating a very rapid turnover of P700. Yet the fluorescence spectra at both room temperature and $-196°$ were little affected under such conditions.

Fluorescence of the 144,000g Supernatant Fraction. The supernatant material from the 144,000*g* centrifuga-

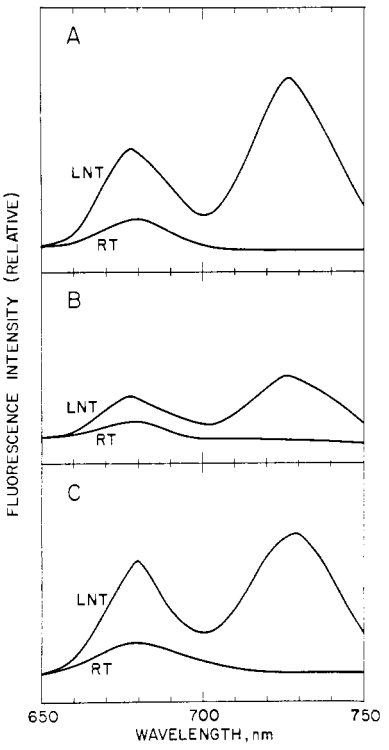

FIGURE 4: Fluorescence spectra. (A) The fluorescence spectra of light (P-D10) particles at room and liquid-nitrogen temperatures. (B) The corresponding spectra in the presence of 0.1 mM ferricytochrome *c*. (C) The corresponding spectra in the presence of a complete donor (ascorbate, DPIP, and plastocyanin) and acceptor system (NADP, ferredoxin, and ferredoxin–NADP reductase). Concentrations of the cofactors are the same as described in Figure 2 of the preceding paper.

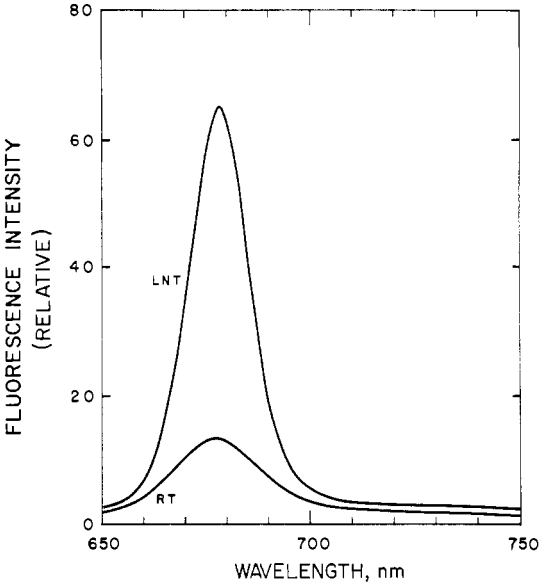

FIGURE 5: Fluorescence spectra of the 144,000*g* supernatant material at room and liquid-nitrogen temperatures.

tion consists mainly of Triton-dispersed chlorophyll and is highly fluorescent. As shown in Figure 5, both the room and low-temperature fluorescence spectra have only one major peak at 678 nm. The long-wavelength fluorescence is completely missing in the detergent-dispersed chlorophyll.

Excitation Spectra. Low-temperature excitation spectra for the fluorescence of the heavy and light particles were measured in the blue absorption region of the chlorophylls and carotenoids. One typical spectrum is presented for each subparticle in Figure 6. For the heavy particle (P-1), the excitation spectrum was taken for the fluorescence maximum at 696 nm (Figure 6A), and for the light particle (P-D10), it was measured at 725 nm (Figure 6B). Excitation spectra measured at other fluorescence maxima have similar profiles for each subparticle. Note that with the quantum counter arrangement, the excitation spectrum is automatically corrected to equal incident quanta.

The excitation spectrum for P-1 particle fluorescence at 696 nm shows one band at 435 nm corresponding to chlorophyll a, a stronger band at 465 nm corresponding to chlorophyll b, and a prominent shoulder at 485 nm, presumably corresponding to one or more of the carotenoids. On the other hand, the excitation spectrum for the 725-nm fluorescence of the P-D10 particle has a stronger 435-nm band and a weaker 465-nm band and there is no indication of excitation in the carotenoid region.

Discussion

The liquid-nitrogen-temperature fluorescence spectrum of chlorophyll in chloroplasts displaying the 685-, 695-, and 720–730-nm bands similar to that shown in Figure 2 was first reported by Litvin *et al.* (1960) and later by Goedheer (1964), who also attributed the fluorescence bands to different forms of chlorophyll a. More recently, several studies on the steady-state fluorescence spectra of blue-green and red algae as well as green plant chloroplasts led to the suggestion that the 695-nm fluorescence band may be associated with the chlorophyll molecules transferring energy to the trap of pigment system 2. Among these studies (Bergeron, 1963; Kok, 1963; Krey and Govindjee, 1964, 1966) are variation of the wavelength of the excitation light allowing selective activation of the two pigment systems; study of the nonlinear dependence of fluorescence in *Porphoridium cruentum* at high intensities of light which activates pigment system 2; and the study of the effect of DCMU on the steady-state fluorescence spectrum of *Porphoridium*.

The 720-nm fluorescence was first observed by Brody (1958) in *Chlorella* and in a concentrated solution of chlorophyll, and was attributed to aggregated chlorophyll. In 1961, Butler reported a 730-nm fluorescence band in green leaves at liquid-nitrogen temperature. He

also measured the excitation spectrum and found a band at 705 nm, which he attributed to a chlorophyll form absorbing at 705 nm (C705) and receiving energy from the bulk chlorophyll. The C705 band was also observed directly in the low-temperature absorption spectrum. More recent experiments relating the 720–740-nm fluorescence to the chlorophyll molecules of pigment system 1 were those on the blue-green (Bergeron, 1963; Kok, 1963) and red algae (Krey and Govindjee, 1966) by selective excitation of the pigment system 1 chlorophyll. In spinach chloroplasts, excitation with 400- or 635-nm light which is absorbed more by chlorophyll a gave stronger 740-nm fluorescence than with 490- or 650-nm light which is absorbed mainly by chlorophyll b (Govindjee and Yang, 1966).

The subchloroplast particles obtained by Triton fractionation offer an additional and more direct means of differentiating the fluorescent species belonging to the two pigment systems. Results reported here on the heavy and light subchloroplast particles appear to be consistent with the current considerations. Our data indicate that the 685- and 695-nm fluorescence bands belong to the chlorophyll forms of pigment system 2 and the 725-nm fluorescence band to that of pigment system 1. The 678-nm fluorescence band observed for the P-D10 particles is most likely owing to a trace of Triton-solubilized chlorophyll. As seen from Figure 5, the Triton-solubilized chlorophyll is highly fluorescent; thus a slight contamination of the P-D10 particle during its separation from the 144,000*g* supernatant material could easily account for the observed fluorescence at 678 nm. Also, additional chlorophyll could become solubilized as a result of continued incubation of the P-D10 particle in a Triton medium.

The excitation spectrum shown in Figure 6 apparently indicates that chlorophyll b as well as the carotenoids are relatively more efficient in activating the 695- and 685-nm fluorescence in the heavy particle, while chlorophyll a is relatively more efficient for the 725-nm fluorescence in the P-D10 particles. This is also consistent with the current concept that the function of chlorophyll b is associated with pigment system 2. However, considering the high chlorophyll a:chlorophyll b ratio in the light particles, chlorophyll b is actually highly efficient for producing fluorescence in both the heavy and light particles.

Hill reagents such as ferricyanide and DPIP act as electron acceptors for pigment system 2, thus reducing the probability of energy dissipation through fluorescence. The assignment of the 685- and 695-nm fluorescence bands to the chlorophyll molecules serving as energy traps for pigment system 2 is consistent with fluorescence quenching by these Hill reagents.

Analogously, and consistent with Butler's (1961) earlier suggestion, the 725-nm fluorescence band of the P-D10 particles may be attributed to the chlorophyll molecules (or C705) serving as energy collectors, rather than the reaction center chlorophyll, P700, of pigment system 1. Quenching of the far-red fluorescence by ferricytochrome *c* or DPIP would be expected as a result of electron flow from the highly reducing "X" (see Vernon

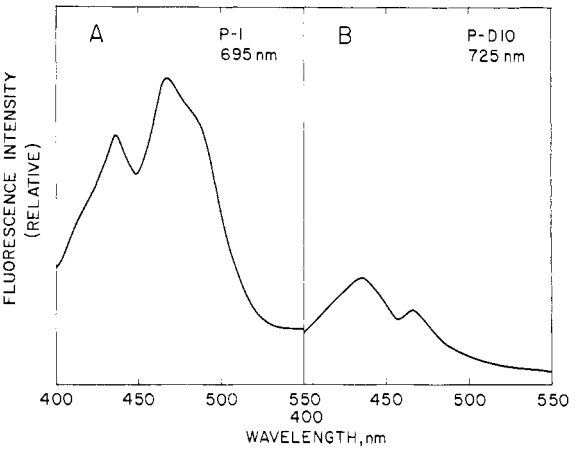

FIGURE 6: Excitation spectra of the heavy (A) (fluorescence measured at 695 mm) and light particle (B) (fluorescence measured at 725 nm) at −196°.

et al., 1967). The lack of fluorescence quenching when a complete NADP reduction system or an ascorbate–PMS mixture is present cannot yet be explained. The lack of fluorescence quenching was also reported by Boardman *et al.* (1966) for digitonin light particles under similar conditions.

We have also examined the fluorescence emission and excitation spectra of the digitonin-fractionated chloroplast particles at room and liquid-nitrogen temperatures and found results very similar to those of the Triton particles. Some heavy-particle preparations obtained by digitonin fractionation show a stronger 695-nm band while some others showed a stronger 685-nm band at −196°, but they always had only a weak shoulder at 725 nm. The digitonin light particles showed a stronger fluorescence at 725 than at 680 nm (note that the fluorescence maximum of the Triton P-D10 particles was at 678 nm).

The response of both particles to the various electron acceptors and their excitation spectra are also similar to those of the Triton particles. However, the digitonin heavy particles reported by Boardman *et al.* (1966) had a much stronger fluorescence band at 735 nm. The 144,000*g* supernatant material from their digitonin fractionation showed a very strong 735-nm fluorescence band, whereas the Triton supernatant material was almost completely free of it. In the excitation spectra reported by Boardman *et al.* for both the heavy and light particles, the chlorophyll a peak was always stronger than the chlorophyll b peak. Also, both excitation spectra showed a prominent shoulder in the 480–490-nm region. Some of these discrepancies obviously arise from slight differences in the procedures of particle fractionations.

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