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Some properties of spinach chloroplast fractions obtained by digitonin solubilization

BOARDMAN AND ANDERSON[1] reported that when spinach chloroplasts are separated into fractions by digitonin solubilization followed by differential centrifugation, the resulting fractions are capable of performing efficiently either the NADP reduction (with reduced DCIP as the reductant) or the reduction of added DCIP (with water as reductant). It seems that the two pigment systems performing the two postulated light reactions in photosynthesis[2,3] had been separated. However, the separation of two pigment systems was only partial; in particular, the fraction which performs "Reaction II" appears to contain also "System I"—it does show NADP reduction with water as reductant.

We have examined the absorption and fluorescence properties of chloroplast fractions prepared by digitonin solubilization and fractional centrifugation, and the quenching of their fluorescence by oxidized cytochrome *c*. We measured the particle sizes in the several fractions, and the degree of polarization of chlorophyll emitted by them in polarized light, as evidence of orderly arrangement of the chlorophyll molecules.

Chloroplasts from spinach leaves were isolated and washed as described elsewhere[4], resuspended in a medium containing 0.5 M phosphate buffer (pH 7.2), 0.01 M KCl and 0.5 % digitonin[1], and allowed to incubate for 30–60 min. After incubation the chloroplasts were centrifuged at 1200 × *g* for 10 min precipitating Fraction 1, at 10000 × *g* for 30 min, producing Fraction 2, and at 50000 × *g* for 30 min, giving Fraction 3, in a Beckman-Spinco centrifuge. All pellets obtained were resuspended in the same buffer. The final supernatant liquid (Fraction 4) was also saved for analysis. The particle size was determined from electron photomicrographs made with a Siemens electron microscope. (These measurements were made by C. SHIMONY.) Measurements of the degree of polarization of chlorophyll *a* fluorescence, excited by polarized light, were made with WEBER's polarization meter[5]. Absorption measurements were made with a Bausch and Lomb spectrophotometer, equipped with an integrating sphere; fluorescence measurements, with a spectrofluorimeter built in our laboratory[6,7].

The size of the particles was estimated from chromium-shadowed electron micrographs. Particles in Fraction 4 consisted of small spherical particles (diameter 20 ± 5 nm) comparable with that of the macromolecules observed in the chloroplast lamellae. Fraction 3 consisted of discs, and clumps of discs about 100–200 nm in diameter, with very few of the small particles that formed the bulk of Fraction 4; these may be the "grana discs". Fraction 2 consisted of much larger discs (400–500 nm in diameter), in which the smaller discs of Fraction 3 were imbedded. In addition there were some thread-like filaments of 10-nm thickness, with lengths up to 100 nm. Some of the small round bodies (macromolecules?) that appeared in such great abundance in Fraction 4, were also scattered about in this fraction. Fraction 1 appeared to be made of particles only a little larger (about 700 nm in diameter) than the larger particles of Fraction 2. An occasional 100-nm disc or a 20-nm long "macromolecule" also appeared in Fraction 1. For the most part, the large discs lacked the smaller 100-nm discs observed within the particles in Fraction 2. (After the completion

TABLE I

Fraction number	*Polarization of fluorescence* (%)*	*Ratio of fluorescence intensities at 735 nm and 696 nm at −196°*
0	2.66	1.70
1	2.92	0.99
2	3.01	1.53
3	5.13	2.16
4	5.40	2.42

* The second column shows the degree of polarization of chlorophyll fluorescence when excited by polarized light of 420 nm.

of our work, we came to know of the excellent and more detailed work of SIRONVAL[8] and WESSELS[9] on the electron microscopic and biochemical characterization of chloroplast fragments prepared with and without digitonin, respectively.)

To judge the orderliness of the arrangement of chlorophyll *a* molecules in each fraction, measurements were made of the polarization of chlorophyll *a* fluorescence excited in them by polarized light. The smaller fractions show a higher degree of polarization (Table I, second column). This may mean that chlorophyll molecules in the small 20-nm particles prevalent in Fraction 4, are arranged in a more orderly way than in the larger particles, so that less depolarization is caused by energy migration. OLSON, BUTLER AND JENNINGS[10], and LAVOREL[11] have shown that degree of polarization of fluorescence is greater upon excitation of pigment System I than of System II. Perhaps Fraction 4 is enriched in System I.

Fig. 1A shows that chlorophyll *b* content is relatively increased in Fraction 1 (compared with "Fraction 0"), and it is relatively deficient in Fractions 3 and 4. At 480 nm, the absorption in the original material is due to about 55 % chlorophyll *b*, and about 45 % to the carotenoids. The distribution of absorption at 480 nm is, however, similar to that obtained at 650 nm, where chlorophyll *b* is the main absorber, suggesting that chlorophyll *b* and some carotenoids are associated in the same pigment system.

The locations of the red absorption maxima are plotted in Fig. 1B. (The wave-

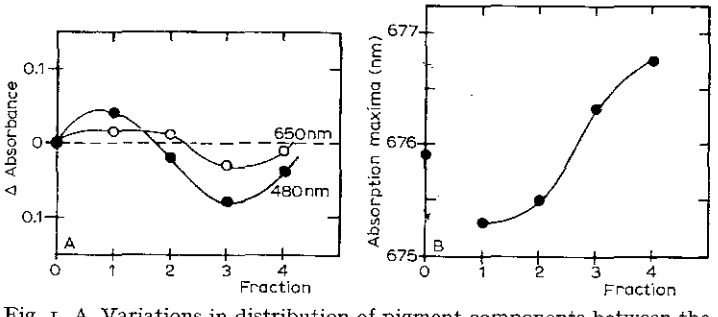

Fig. 1. A. Variations in distribution of pigment components between the 1–4 digitonin-solubilized fractions as compared with the original suspension (Fraction 0). The absorption curves of all fractions were "normalized" at the peak of chlorophyll *a* band. The differences were measured at 650 nm (the region of high chlorophyll *b* absorption) and at 480 nm (region of high chlorophyll *b* and carotenoid absorption). B. Location of the red chlorophyll *a* absorption band in different fractions of digitonin-solubilized chloroplasts.

length maxima were measured very carefully, and, although their accuracy is not better than $\pm$ 0.5 nm, their precision is $\pm$ 0.2 nm.) The red absorption maximum of intact chloroplasts lies at 676 nm; those of Fractions 1 and 2 at 675.5 nm, and those of Fractions 3 and 4 at 676.5 nm. This could mean that the lighter particles are enriched in the "long-wavelength" chlorophyll *a* complexes, while the heavier particles are enriched in the "short-wavelength" ones (as well as in chlorophyll *b*). However, these shifts are so slight that they could be due to differences in chlorophyll *b* content, and may be explained without invoking differences in the chlorophyll *a* bands. THOMAS[12] has also shown shifts in absorption maxima in different fractions of chloroplasts.

At liquid nitrogen temperature, complex fluorescence spectra appear (Fig. 2). (For previous observation of the complex structure, see refs. 7, 13–18.) The ratio of F735 to F696 is shown in Table I (third column). A decreased ratio (F735/F696) may be indicative of enrichment of System II and an increased ratio of System I. In the lighter-particle fractions (Fractions 3, 4), there is more of the 735-nm component compared to the 695-nm component, as shown by a high ratio (>2) of F735/F696, whereas in the heavier-particle fractions (Fractions 1, 2), the same ratio is lower (<2). The 695-nm component (F696) is clearly present in the heavier fractions (Fractions 1, 2), but absent from the lightest Fraction 4.

In experiments with Porphyridium[18], a dominant 712-nm (F720) band is observed upon excitation of System I (chlorophyll *a*) and a F696 band upon excitation

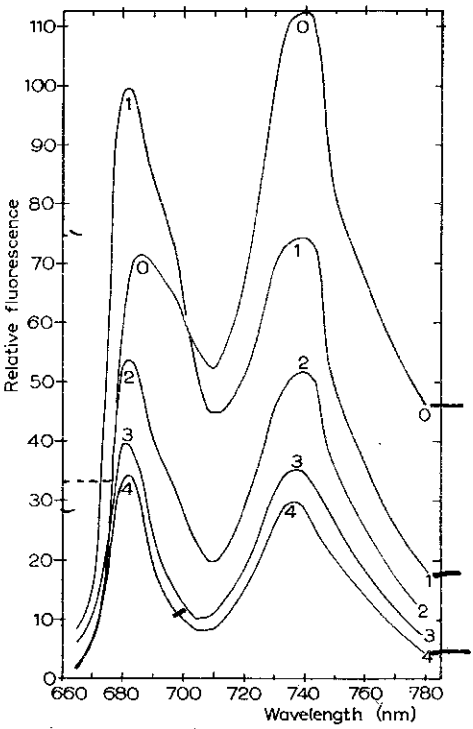

Fig. 2. Fluorescence spectra (at −196°) of digitonin-solubilized spinach chloroplast fractions.

of System II (phycoerythrin), suggesting that F696 belongs to System II and F720 to System I.

Fluorescence-quenching experiments of the several fractions give some information about their photochemical properties. The fluorescence of the heavier fractions (Fractions 1, 2) was quenched up to 2.6% by $6 \cdot 10^{-4}$ M oxidized cytochrome *c*, whereas those of the Fraction 4 were not quenched. DCMU (10^{-4} M) abolished the effects of cytochrome *c*. The effect of reduced cytochrome *c*, with and without NADP, was too slight (less than 0.5%) for meaningful interpretation.

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