ABSORPTION AND FLUORESCENCE OF CHLOROPHYLL *a* IN PARTICLE FRACTIONS FROM DIFFERENT PLANTS

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ABSTRACT Density-gradient centrifugation of disintegrated cells from a variety of plants gave two kinds of chlorophyll particles from all except the blue-green algae. As in previous procedures using detergents, the lighter Fraction 1 particles usually had greater absorption at longer wavelengths; they always had a lower ratio of short to long wavelength fluorescence at low temperature, and a lower fluorescence yield per chlorophyll than the denser Fraction 2 particles. Although only one kind of particle fraction was found in each blue-green alga, the fine structure of the chlorophyll *a* absorption band differed significantly among the three species measured.

INTRODUCTION

The absorption and fluorescence spectra of chlorophyll lipoprotein fractions prepared from a wide variety of plants have been measured, and a few examples are reported in this paper.

The detection and characterization of the various forms of chlorophyll *a* by means of absorption spectroscopy have often been discussed (1–10). It is still not known if the various forms are identical as to peak position and width in different species; nor is it clear how the forms recognized in absorption spectra are related to the components of fluorescence spectra. The absorption anomalies which can be present in spectra of pigmented particles measured in scattering samples have been reviewed by Butler (11).

Possible artifacts in fluorescence measurements are even more numerous than in absorption spectrophotometry (12, 13). For the following reasons it may be unprofitable to draw conclusions from comparisons of the shapes and relative peak heights of fluorescence spectra measured in different laboratories. (*a*) The most common source of trouble is reabsorption of fluorescent light within the sample. Neither the absorption nor fluorescence spectra of whole algae or of normally green chloroplasts can represent the sums of the individual spectra of the component pigments

because of reabsorption within single chloroplasts. Such distortion varies with the chloroplast size and pigment content (14). With disintegrated chloroplast particles that are themselves small enough to avoid distortion by internal reabsorption, it is possible to measure reliable fluorescence spectra if the concentration and sample thickness are small enough. If the sample in the measuring chamber is visibly green, we assume the fluorescence spectra will be erroneous. Only spectra that are proved to be free of reabsorption distortion by having identical shapes at concentrations or thicknesses varied by a factor of two, can be relied upon. (*b*) Fluorescence spectra are variously presented on the basis of either quanta or energy per unit of frequency, or of wavelength, i.e., $Q/\Delta\nu$, $Q/\Delta\lambda$, $E/\Delta\nu$, or $E/\Delta\lambda$. (*c*) The 680–685 nm band is so steep that the monochromator slit width is of critical importance in determining the shape of the curve. (*d*) Some published spectra are uncorrected for the wavelength variation of photomultiplier sensitivity and the monochromator transmission.

One purpose of this work was to provide comparative data by measuring spectra for particles of similar sizes prepared from a variety of plants, using a spectrophotometer designed to minimize errors due to light scattering.

The second purpose was to correlate absorption and emission bands, recording both spectra from aliquots of the same sample of finely disintegrated particles. Although a correlation between long and short wavelength absorption and emission in fractions from the same species was observed, the data offered no satisfactory explanation for the different positions of the long wavelength emission maxima in different plants. In addition to the fluorescence peak of chlorophyll *a* in vivo near 680 nm, most plants at low temperature have a long wavelength emission band (15) and a few show a small band near 700 nm (16). Although some of the forms of chlorophyll *a* seen in absorption spectra may be weakly or not at all fluorescent, presumably one of them corresponds to each emission band.

A third purpose of this study was to test density-gradient fractionation of disintegrated cells without using a detergent on a wide variety of algae and higher plants. Michel and Michel-Wolwertz (17) found that after breaking chloroplasts, suspended in a KCl-Tricine buffer, in a French press and after centrifugation in a sucrose density gradient, three particle-fractions could be recovered from green bands in the gradient. In terms of chlorophyll *a* to *b* ratio, fluorescence spectrum and yield, and photochemical activity, the lighter fraction and the two heavier fractions corresponded respectively to the lighter 144,000 *g* particles (system 1) and to the 10,000 *g* particles (system 2) isolated with the digitonin fractionation procedure of Boardman and Anderson (18).

Two chlorophyll-containing particle fractions, differing in absorbance and fluorescence spectra, were isolated from several higher plants, a liverwort, several green algae, a red alga, and a diatom. No different chlorophyll fractions were apparent from the three species of blue-green algae, although the phycocyanin sepa-

rated almost completely from the chlorophyll particles. Similar fractionation experiments with spinach, barley, *Marchantia* and *Euglena* were presented in a previous report (19).

EXPERIMENTAL

Fractionation Procedure

Algae or chloroplasts were sedimented by centrifugation at 3000 *g* for 10 min, washed once with distilled water, and resuspended in 0.3 M KCl, 0.05 M Tricine[1] adjusted to pH 8. The suspension was forced through a needle valve by approximately 12,000 psi (8300 N/cm^2) pressure at least three successive times. Some algae required more passes in order to break a sufficient number of cells. The broken mixture was centrifuged as before to remove larger particles yielding the suspended homogenate. Ideally the homogenate should contain between 0.5 and 1 mg chlorophyll per ml, but with the algae which were difficult to break, homogenates were usually obtained with 100–200 μg per ml.

Linear, 12.5–50%, or step, 10–30–50%, top to bottom, gradients of sucrose dissolved in 0.15 M KCl, 0.05 M Tricine buffer at pH 8, were prepared in 34 ml centrifuge tubes. Two to four ml of homogenate were layered on top of the sucrose and spun at 64,000 *g* in the swinging-bucket rotor of a Spinco model L centrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) for 30–60 min depending on the plant species.

The green bands in the gradient were removed with a syringe and either dialyzed against dilute buffer overnight or analyzed immediately. All steps in the procedure were carried out near 4°C.

Spectral Measurements

The absorption spectrophotometer and special cuvettes have been described (20). Both were designed to measure a representative fraction of the scattered light. A metal finger of the cuvette was immersed in liquid nitrogen to cool the sample to −196°C. The samples were diluted with buffer to give an absorbance of less than 0.5 at 675 nm.

The spectrofluorimeter (21) automatically corrected the emission spectra for the spectral sensitivity of the appartus. Excitation at 435 nm, half-width 4.8 nm, was provided by a xenon lamp used with a Bausch & Lomb "high intensity" monochromator (Baush & Lomb Incorporated, Rochester, N.Y.) and a Corning 9782 (4–96) filter (Corning Glass Works, Corning, N.Y.) to remove traces of stray light in the 650–770 nm region. The light intensity on the sample was approximately 2×10^4 ergs/cm^2/sec^1. The half bandwidth of the analyzing monochromator was 4 nm and the plot produced is in terms of relative quanta per unit frequency interval, $Q/\Delta\nu$. The response of the apparatus was checked frequently against the emission of a Corning 3387 filter. The gain could be adjusted to correct for small changes in the lamp intensity, thus allowing a valid comparison of the fluorescence yield of samples recorded on different days. For comparisons of curve shapes the photomultiplier voltage was set to give curves of about the same size regardless of yield.

The low-temperature emission spectra were measured from a drop of sample (containing less than 5 μg chlorophyll/ml) placed in a 0.1 mm deep groove in an aluminum strip. The strip was held horizontally in a larger aluminum block cooled by liquid nitrogen.

The relative fluorescence yield measurements were determined at 23°C on two or more

[1] *N*-Tris (hydroxymethyl) methylglycine.

dilutions of a sample placed in a 0.2 mm deep groove in an aluminum strip. The height of the peak emission near 680 nm was divided by the relative response of the photomultiplier at the voltage used and by the chlorophyll concentration (determined according to Mackinney [22]) to give the relative fluorescence yield on a chlorophyll basis. Because these room temperature spectra all have the same shape, the peak emission value was proportional to the total emission.

Plant Material

The following plant species were used: spinach, barley (*Hordeum vulgare*), a barley mutant lacking chlorophyll *b* (23), *Atriplex semibaccata*, *Marchantia polymorpha*, *Chlamydomonas reinhardtii* No. 90 (−), *Scenedesmus obliquus* D_3, *Stichococcus bacillaris* No. 419, *Euglena gracilis* No. 752, *Botrydiopsis alpina* No. 295, *Porphyridium cruentum* No. 161, *Phaeodactylum tricornutum*, *Anabaena cylindrica* No. 381, *Anacystis nidulans* No. 625, and *Plectonema boryanum* No. 581.

The algae came from the Indiana Culture Collection, Department of Botany, Indiana University, Bloomington, Ind. (24) except *Phaeodactylum* that was obtained from the Woods Hole Oceanographic Institute, Woods Hole, Mass. The algae were cultured autotrophically in 1–2 liter batches of the appropriate medium in Fernbach flasks shaken over fluorescent lamps and supplied with 3% CO_2 in air. The size of the inoculum and the light intensity were adjusted to give a dense suspension in 5–7 days. Spinach was purchased in a local market. Barley was grown in a green house. *Marchantia* and *Atriplex* were collected locally by Dr. O. Björkman.

RESULTS

Particles from all the plants except the blue-green algae separated into two distinct, green layers in the step gradient; one was located just above the 30% and the other just above the 50% sucrose. Similar results were observed in the linear gradients except that the top band was usually broader than the bottom band, and with spinach a third band appeared near the center of the tube. The centrifugation is not designed to come to equilibrium but to produce well separated bands in a reasonable amount of time. Empirically this time was found to be 30, 45 or 60 min with different species.

Although we routinely measured absorption spectra between 400 and 600 nm of all the plants and we always observed small differences in carotenoid absorption between Fractions 1 and 2, here we discuss only the red chlorophyll bands.

Green Algae and Leaf Chloroplasts

The absorption spectra of corresponding fractions (top band = Fraction 1, bottom band = Fraction 2) from the higher plants were similar to those of *Chlamydomonas* shown in Fig. 1. The peak near 650 nm is from chlorophyll *b*; the peaks at 677 nm and shoulders near 670 nm are from chlorophyll *a*. The shapes of the chlorophyll *a* bands are different in the two fractions, causing Fraction 1 to absorb relatively more between 680 and 700 nm. A small band near 698 nm was evident in spectra of the homogenate and Fraction 1 from *Scenedesmus* but was not evident in Fraction 2.

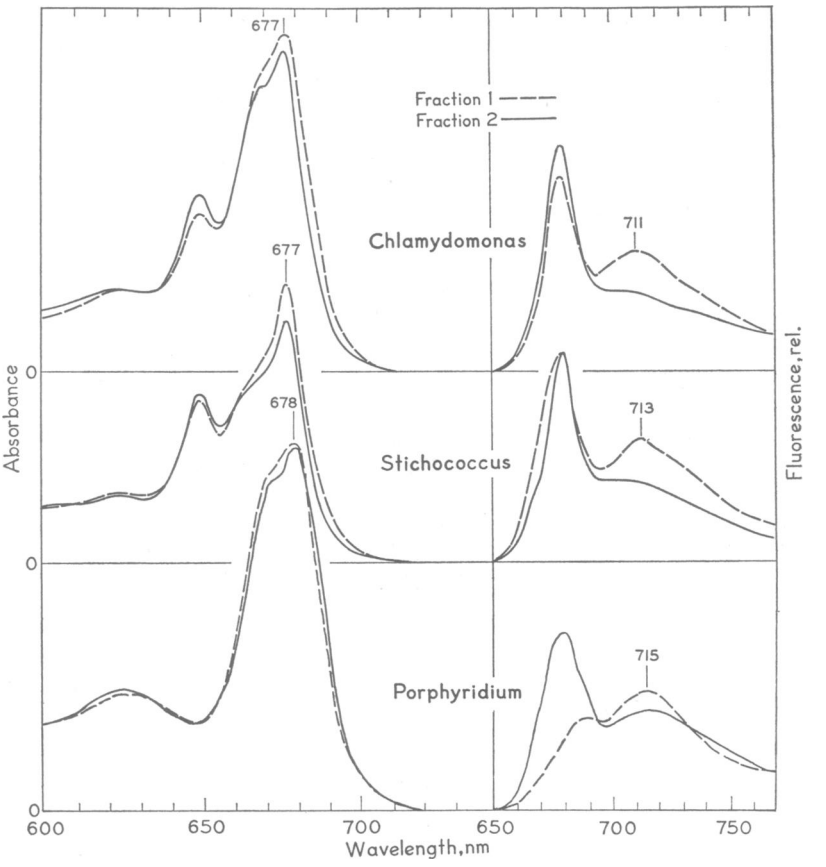

FIGURE 1 Absorption and fluorescence spectra of Fractions 1 (- - -) and 2 (——) prepared from *Chlamydomonas* (top), *Stichococcus* (middle) and *Porphyridium* (bottom) recorded at −196°C. Excitation was at 435 nm.

With *Chlamydomonas* the ratio of chlorophyll *a* to *b* (determined in acetone extracts) was 3.4 in Fraction 1 and 2.5 in Fraction 2. More experiments were performed with spinach than with any other plant, and often the *a*:*b* ratio of Fraction 1 was 2–3 times greater than that of Fraction 2.

Stichococcus has a higher proportion of chlorophyll *b* (*a*:*b* = 1.6) than other green algae and relatively less of the chlorophyll *a* form absorbing near 670 nm. However, as shown in Fig. 1, fractionation of this species produced neither a more nor a less complete separation of the chlorophylls.

From all the plants the amount of long wavelength fluorescence relative to that emitted near 680 nm was greater in Fraction 1 than in Fraction 2. The emission spectra in Fig. 1 illustrate this. However, the peak position of the long wavelength maximum that appeared at 711 nm in *Chlamydomonas* varied between 711 and 735

TABLE I

RELATIVE FLUORESCENCE YIELD OF CHLOROPHYLL *a* IN FRACTIONS 1 AND 2 AT 20°C AND LONG WAVELENGTH EMISSION MAXIMUM OF FRACTION 1 AT −190°C

Plant material	Fluorescence yield		Fluorescence peak
	Fraction 1	Fraction 2	Position
			nm
Spinach	2.2	7.1	734
Barley	0.74	3.1	—
Barley mutant (minus chlorophyll *b*)	0.68	3.1	721
Atriplex semibaccata	0.72	2.2	735
Marchantia polymorpha	0.62	3.7	721
Chlamydomonas reinhardii	2.9	5.9	711
Scenedesmus obliquus	5.0	9.2	720
Stichococcus bacillaris	2.0	6.3	713
Euglena gracilis	2.2	3.8	726
Botrydiopsis alpina	1.4	2.5	715
Phaeodactylum tricornutum	1.9	9.6	no peak
Anacystis nidulans	0.54		715
Anabaena cylindrica	0.91		726
Plectonema boryanum	0.24		728

nm in different species listed in Table 1. No obvious correlation was found between the absorption and emission bands of different plants.

The fluorescence yield per unit of chlorophyll *a* was always greater in Fraction 2 (Table 1). The emission spectra measured at room temperature were all similar in hape with a peak near 680 nm and a tail extending to long wavelengths but without other peaks. The total fluorescence of Fraction 2 from each species was greater than that of Fraction 1 at low temperature also, but this was more difficult to measure quantitatively because of the path length increase caused by ice crystals.

The spectra and fluorescence yield values of the homogenates were intermediary between those of the fractions, indicating no interaction of the particle-fractions before separation in the gradient or any significant effect of sucrose concentration. The fluorescence yields decreased slowly with time of storage at 4°C. In one experiment with *Scenedesmus* particles, the yields of the homogenate and of Fraction 1, decreased by 25% and of Fraction 2 by 50% in three days.

A Red Alga

A *Porphyridium* homogenate was centrifuged in the step gradient for 45 min; the phycoerythrin remained at the top of the tube and two brownish-green bands separated at lower levels. The absorption spectra of the two fractions shown in Fig. 1 were slightly different near the peaks, but by contrast with the green plants,

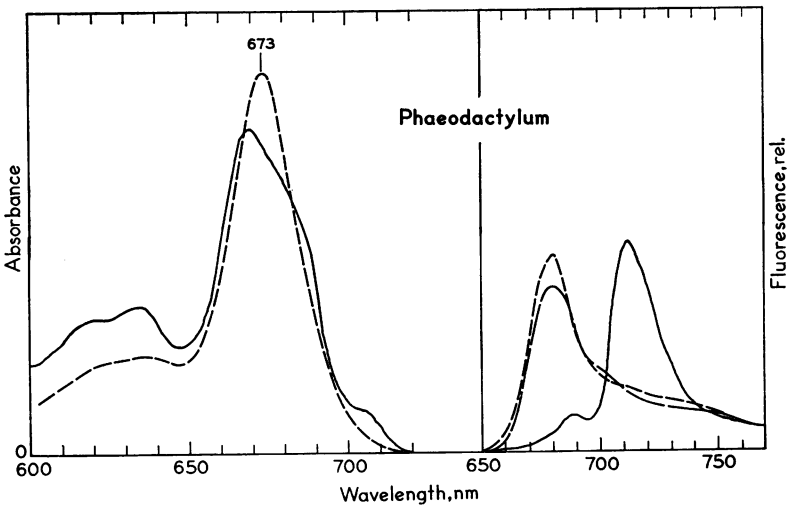

FIGURE 2 Absorption and fluorescence spectra of *Phaeodactylum* recorded at −196°C. Excitation was at 435 nm. The symbols are: cells (——), Fraction 1 (- - -), and Fraction 2 (— —).

Fraction 1 did not absorb significantly more at longer wavelengths. Nevertheless the emission of Fraction 1, as usual, was much greater than that of Fraction 2 at long wavelengths.

A Diatom

The absorption spectra of intact cells or chloroplasts of the plants discussed thus far were qualitatively similar to their respective homogenates, with allowance for broadening of the main band and the higher absorbance at shorter wavelengths caused by scattering, the high optical density of whole chloroplasts, and the sieve effect. But with *Phaeodactylum* breaking the cells produced marked changes in the absorption and fluorescence spectra as illustrated in Fig. 2. The shoulder on the absorption spectrum at 685 nm, the small band near 705 nm, and the large emission peak at 712 nm all disappeared when the cells were broken.

After 35 min centrifugation in the sucrose gradient, these diatom particles separated into two brown bands. Both fractions contained fucoxanthin, but there were some small differences in carotenoid absorption between 400 and 600 nm. The absorption spectrum of Fraction 1 shown in Fig. 2 was similar to spectra of both the homogenate and Fraction 2, although the latter absorbed slightly less near 685 nm.

The emission spectra of the two fractions also differed only slightly, but the fluorescence yield of Fraction 2 was five times greater than that of Fraction 1 (Table 1) in one experiment and three times greater in another experiment.

Blue-Green Algae

When homogenates prepared from *Anacystis*, *Anabaena*, and *Plectonema* were centrifuged in the gradient, nearly all of the phycocyanin remained at the top of the tube, and a diffuse layer of chlorophyll was spread from about 1 cm below the phycocyanin down to the 50% sucrose. Green samples removed from different levels in the tube had identical absorption and fluorescent properties.

The absorption and fluorescence of chlorophyll particles from the three blue-green

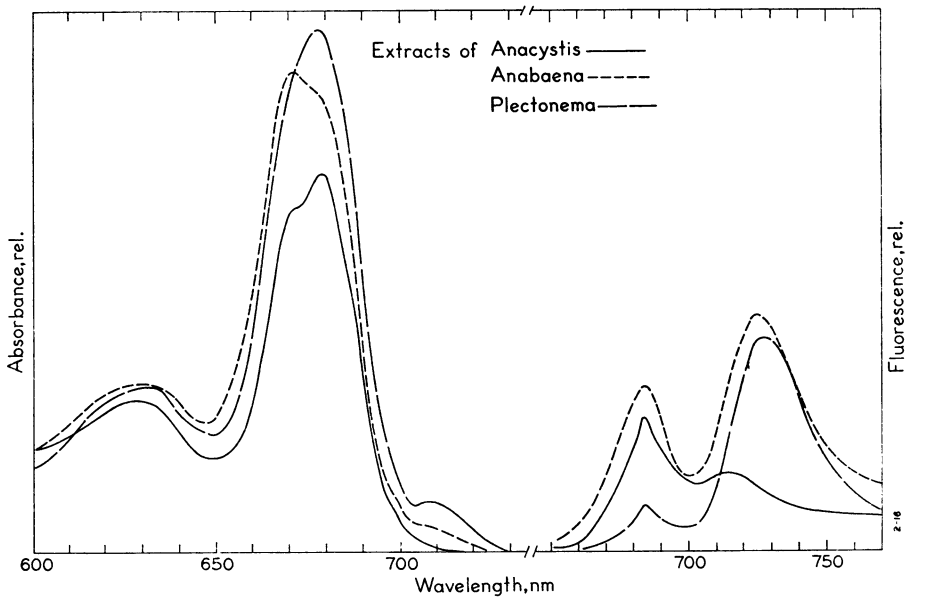

FIGURE 3 Absorption and fluorescence spectra of chlorophyll particles from *Anacystis* (——), *Anabaena* (– – –), and *Plectonema* (— —) recorded at −196°C. Excitation was at 435 nm.

algae are compared in Fig. 3. The absorption spectrum of the chlorophyll from *Anacystis* is similar to that from most other plants with a peak near 678 nm and a shoulder at shorter wavelengths; *Anabaena* chlorophyll has a peak near 672 nm and a shoulder at longer wavelengths; and *Plectonema* has a nearly symmetrical main chlorophyll *a* band with a distinct smaller band near 710 nm. The emission spectra of the three species also differ.

DISCUSSION

It has been assumed that the lighter, system 1 particles absorb at longer wavelengths than the heavier, system 2 particles because the former contains proportionally more C*a* 680. The definitive spectra illustrated above suggest another hypothesis. Each fraction may contain a different C*a* 680 with the same peak positions but different

bandwidths. The greater long wavelength absorption by Fraction 1 would result from its C*a* 680 having a broader bandwidth.

French (25) has attempted to match similar experimental spectra with the sums of gaussian component curves. Because such a very narrow band is required to fit the 677 nm peak of Fraction 2, he suggests that the shape of this band may be influenced by wavelength-selective scattering (26, 27) caused by anomolous dispersion. Since the particle fractions differ in density, the chlorophyll concentration within the particles may also differ, causing a larger contribution by scattering in one fraction than in the other. On the other hand *Phaeodactylum* fractions had different densities but similar absorption spectra.

The differences in low temperature fluorescence between the two chlorophyll fractions were usually larger and thus easier to measure than the absorption differences. Especially fractions from those plants lacking chlorophyll *b* had very similar chlorophyll *a* absorption spectra but different fluorescence spectra. Fraction 1 from all the plants had relatively greater long wavelength emission than Fraction 2. When considering only the results from higher plants and green algae except *Scenedesmus*, one is tempted to ascribe the long wavelength emission to the form of C*a* 680 in Fraction 1.

However, *Scenedesmus*, *Euglena*, *Phaeodactylum*, *Plectonema*, and *Anabaena* have distinct small absorption bands between 695 and 710 nm which could also be sources of long wavelength emission. Perhaps a similar long wavelength form of chlorophyll is present in all plants, but in amounts too small to detect in absorption spectra. An additional confusing finding was that particles from *Scenedesmus* mutant No. 8 (28), that has no 700 nm absorption band, showed relatively even more emission at 720 nm than particles from the parent strain. We must conclude that the data do not yet suggest how chlorophyll particles can have such similar absorption spectra but different long-wavelength emission maxima. Differences in the efficiency of energy transfer from one form of chlorophyll to another may well be complicating the picture.

The lower fluorescence yield of system 1 particles compared to system 2 particles fractionated with digitonin has been noted (29). Since the addition of digitonin caused a 70% increase in yield of the chloroplast fluorescence before centrifugation, the fractionation procedure of Michel and Michel-Wolwertz is probably safer, particularly for yield studies of these complexes.

Breaking *Phaeodactylum* destroyed the unusual 710–714 nm emission maximum (30); nevertheless two particle fractions that differed in fluorescence yield were recovered from the gradient. Mann and Myers (31) attempted to fractionate *Phaeodactylum* with several detergents and found that the detergents caused a shift to shorter wavelengths in fucoxanthin absorption, which was seen also in solvent extraction. Although our procedure failed to remove fucoxanthin from chlorophyll, the pigment apparently did remain in its native state on both particles.

The failure to find different chlorophyll particles in the blue-green algae may or may not be significant. Shimony et al. (32) found two kinds of particles following treatment of *Anacystis* with 0.6% digitonin and Ogawa et al. (33) separated two different chlorophyll-protein components from *Anabaena* following solubilization with sodium dodecyl sulfate and electrophoresis. Our procedure may just not be applicable to this kind of alga. Alternatively, the removal of phycocyanin might destroy the Fraction 2 type of chlorophyll *a* or there may be very little of this type present originally in these cells. The chlorophyll particles that were recovered had the low fluorescence yields characteristic of Fraction 1.

CONCLUSIONS

One reason that the physical-chemical nature of the absorbing forms of chlorophyll *a* has remained obscure is because of the difficulty in separating them without destruction. The procedure described above effects a partial separation and is applicable to a wide variety of plants. These very small chlorophyll-lipoprotein particles which differ in their chlorophyll absorption should be excellent material for further study to determine the aggregation and lipid or protein binding of chlorophyll in its native functional state.

Since the same fractionation procedure produced two corresponding chlorophyll particles from so many different plants, the basic arrangment of the chlorophyll-lipid-protein molecules is probably similar in these plants.

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