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FRACTIONATION OF THE PHOTOCHEMICAL SYSTEMS OF PHOTOSYNTHESIS

II. CYTOCHROME AND CAROTENOID CONTENTS OF PARTICLES ISOLATED FROM SPINACH CHLOROPLASTS

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SUMMARY

1. Spinach chloroplasts were fragmented by incubation with digitonin, and the resulting particles examined for their cytochrome and carotenoid contents. Cytochrome difference spectra were recorded both at 20°C and 77°K, usually without prior extraction of the chlorophylls.

2. It is concluded that at least three cytochromes are localized in the chloroplast; cytochromes *f* and b_6 with α bands at 554 and 563 mμ respectively, and another cytochrome with an α band at about 559 mμ at 20°C and 557 mμ at 77°K. The cytochromes are present in the approximate molar ratio of 1:2:2, respectively. It is assumed tentatively that cytochrome 559 (chloroplasts) is a *b*-type cytochrome. The chloroplast contains 430 molecules of chlorophyll per molecule of cytochrome *f* and 118 molecules of chlorophyll per molecule of cytochrome *b* (cytochrome b_6 *plus* cytochrome 559).

3. The results presented for the particles prepared by digitonin treatment of chloroplasts show that cytochromes *f* and b_6 are associated with Photosystem 1 and cytochrome 559 with Photosystem 2. Cytochromes *f* and b_6 are partly solubilized from the Photosystem 1 particle by the digitonin, and the method of preparation of the particles was modified to minimize this loss. Cytochrome 559 appears to be tightly bound to the Photosystem 2 particle.

4. The four major carotenoids, β-carotene, lutein, violaxanthin and neoxanthin were found to be present in both types of particles, but in varying proportions. The particles representative of Photosystem 1 were enriched in β-carotene.

INTRODUCTION

The studies of HILL and associates[1-3] showed that two cytochromes were localized in the higher plant chloroplast; cytochrome *f* (cyt *f*) which is a *c*-type cytochrome and cytochrome b_6 (cyt b_6). From spectroscopic evidence, LUNDEGÅRDH[4]

Abbreviations: cyt *f*, cytochrome *f*; cyt b_6, cytochrome b_6; cyt b_3, cytochrome b_3; cyt 559, cytochrome 559 (chloroplasts); cyt *b*, total cytochrome *b* (cyt b_6 *plus* cyt 559); chl *a*, chlorophyll *a*; chl *b*, chlorophyll *b*.

postulated that chloroplasts contained a further cytochrome of the *b* type, cytochrome b_3 (cyt b_3). The three cytochromes cyt *f*, cyt b_3 and cyt b_6, were characterized in the reduced state by α bands at 554, 559 and 563 mμ, respectively. Prior to LUNDEGÅRDH's observations, a soluble cytochrome with an α band at 559 mμ had been isolated from broad bean leaves[1] and named cyt b_3. Claims that cyt b_3 is present in microsomes[5], and mitochondria[6] have also been made, but these were based simply on the similarities of the absorption spectra to that of the soluble cytochrome of broad bean leaves. More recently, a soluble cytochrome with identical properties to cyt b_3 was isolated from etiolated mung bean seedlings[7]. On the basis of their oxidation–reduction potentials, HILL proposed that cyt *f* and cyt b_6 played a role in photosynthetic electron transport[2,3,8].

In the present study, we have reinvestigated the cytochromes of the spinach chloroplast, and determined the cytochrome composition of the particles obtained by digitonin fragmentation of the chloroplasts. The digitonin treatment causes a separation of particles representative of Photosystem 1 from particles which remain attached to the grana lamellae and are enriched in Photosystem 2 (refs. 9–13). From the results presented, it is postulated that cyt *f* and cyt b_6 are localized in Photosystem 1, and that cytochrome 559 (chloroplasts) (cyt 559) is associated with Photosystem 2. The cytochrome with an α band at about 559 mμ was named in this way to avoid confusion with the soluble cytochrome[1].

The distribution of the carotenoids, β-carotene, lutein, violaxanthin and neoxanthin between the two types of particles was also examined. It is concluded that all four carotenoids are present in both photochemical systems, but in varying proportions.

MATERIALS AND METHODS

Isolation of chlorophyll-containing particles

Spinach chloroplasts were prepared as described previously[11] and fragmented by incubation with 0.5 % digitonin for 30 min at 0°C. The chlorophyll-containing particles were separated by differential centrifugation under the following conditions: 1000 × *g* for 10 min, 10000 × *g* for 30 min, 50000 × *g* for 30 min and 144000 × *g* for 1 h. In some experiments, where indicated, the 144000 × *g* supernatant was centrifuged for an additional period of 3 h at 144000 × *g* to yield a further pellet. The pellet from each centrifugation was resuspended in 0.05 M phosphate buffer (pH 7.2).

In a few experiments, the mixture after incubation with digitonin was diluted with 9 vol. of 0.05 M phosphate buffer (pH 7.2) before differential centrifugation.

Determination of cytochromes

Difference spectra of the reduced and oxidized cytochromes were recorded, at both 20°C and 77°K with a Cary Model 14R spectrophotometer, fitted with a Cary Model 1462 scatter transmission attachment, containing an RCA type 6217 photomultiplier. The 0–0.1 slide wire was used. The cuvette assembly of BONNER[14] was modified to suit the optical system of the Cary (ref. 15). In our assembly, the cuvettes had an optical path-length of 2 mm and a volume of 0.28 ml. Absorption spectra at the temperature of liquid N_2 (77°K) were determined in 50 % glycerol, using the single freezing procedure[16].

Most of the cytochrome determinations on either chloroplasts or the various particles were carried out without prior extraction of the chlorophyll. The chlorophyll content of the suspensions varied from 0.4 mg/ml to 0.7 mg/ml. The absorbance baseline of the Cary over the wavelength range 500 mμ to 600 mμ was adjusted with equal volumes of a suspension in the sample and reference cuvettes, prior to the addition of oxidising or reducing agents. These were as follows: 0.1 M sodium ascorbate neutralized to pH 6.0; 0.1 M potassium ferricyanide, and solid sodium dithionite (May and Baker, laboratory-grade reagent). 20 μl sodium ascorbate or potassium ferricyanide were added to the cuvette and where necessary 20 μl of water were added to the reference cuvette. The method of calculation of cytochrome concentrations is given under RESULTS.

A few measurements were made on acetone-extracted powders prepared in the following way. The chloroplasts or fractions were resuspended in distilled water and lyophilized. The lyophilized powder was blended in 80 % acetone (1 ml solvent per mg powder) at −15°C for 30 sec, the suspension was centrifuged and the pellet re-extracted with 80 % acetone (0.1 ml/mg powder). After a second centrifugation the pellet was lyophilized immediately. Under these extraction conditions one would not expect to lose cytochromes to the extracting medium. For cytochrome determinations, the acetone-extracted powders were resuspended in a solution of 60 % glycerol and 40 % buffered sucrose (0.3 M sucrose–0.067 M phosphate buffer, pH 7.4), according to the methods of JAMES AND LEECH[17].

Cyt *f* was prepared from the leaves of parsley by the method of DAVENPORT AND HILL[2].

Determination of carotenoids and chlorophylls

The pigments were extracted into 80 % acetone and spectra were recorded with the Cary spectrophotometer. The amounts of chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*) were calculated by the method of ARNON[18]. The pigments were then transferred quantitatively to ether by adding an equal volume of ether and washing the ethereal layer with 1 % NaCl solution. The chlorophyll contents of ethereal extracts were checked using the equation of SMITH AND BENITEZ[19].

Total carotenoid in the ether extract was determined after saponification with methanolic KOH (1 vol. of 60 % aqueous KOH, added to 10 ml methanol); 2 ml of KOH reagent were used per 10 ml of ethereal extract. After saponification, the extracts were shaken several times with 4 % NaCl and dried over anhydrous Na_2SO_4. Spectra were recorded, and the carotenoid concentrations (μg/ml) obtained by dividing the absorbance values at 442 mμ by the factor, 0.24.

Both thin-layer chromatography and two-dimensional paper chromatography[20] were used to separate the various carotenoids. Ascending paper chromatography was carried out on Whatman 3 MM paper (25-cm squares) in the dark at 12–15°C. The developing solvents were : 2 % *n*-propanol (v/v) in light petroleum (b.p. 60–80°C) for the first dimension, and 30 % chloroform (v/v) in light petroleum (b.p. 60–80°C) for the second dimension. For quantitative measurements, the individual spots were cut out and eluted with diethyl ether. The identity, and the purity of the various carotenoids were checked from their absorption spectra. The following peak positions ($\lambda_{max.}$) were found: β-carotene (447 mμ), lutein (445 mμ), violaxanthin (440 mμ) and neoxanthin (436 mμ).

Concentrations of the individual carotenoids were determined, using an average specific absorption coefficient of 240 (l/g·cm).

Thin-layer chromatography was carried out on Kiesel gel G (Merck) on glass plates (20 cm × 50 cm) at the thickness of 0.25 mm; the plates were dried for 30 min at 75°C and cooled before use. After development with benzene–acetone (75:25, v/v) in the dark at 15°C, the bands were scraped off into small tubes and the pigment eluted rapidly with diethyl ether.

RESULTS

Cytochromes of chloroplasts

Reduced *minus* oxidized difference spectra of chloroplasts at 20°C and 77°K are shown in Fig. 1 and Fig. 2, respectively. The untreated *minus* ferricyanide-

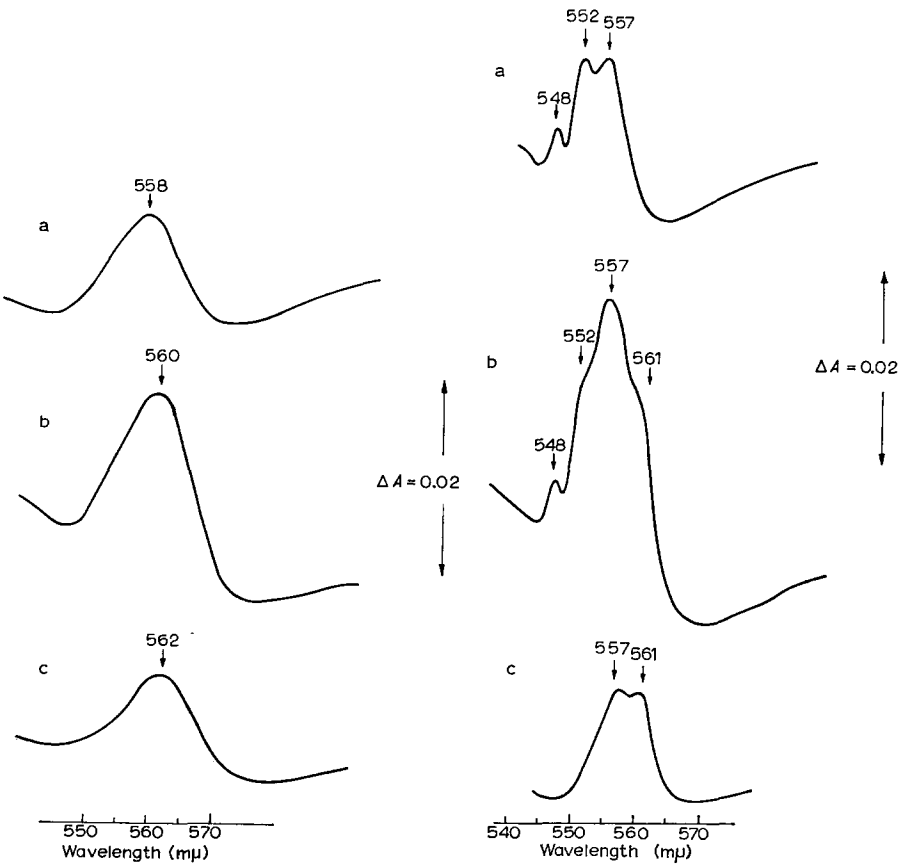

Fig. 1. Reduced *minus* oxidized difference spectra of chloroplasts at 20°C. (a) Ascorbate-reduced *minus* ferricyanide-oxidized (A–F). (b) Dithionite-reduced *minus* ferricyanide-oxidized (D–F). (c) Dithionite-reduced *minus* ascorbate-reduced (D–A). Chl *a plus* chl *b* concentration, 0.55 mg/ml. Optical path-length, 2 mm.

Fig. 2. Reduced *minus* oxidized difference spectra of chloroplasts at 77°K. (a) (A–F); (b) (D–F); (c) (D–A). Chlorophyll concentration and optical path-length as for Fig. 1.

TABLE I

WAVELENGTH MAXIMA IN DIFFERENCE SPECTRA OF CHLOROPLASTS AND FRACTIONS

Chloroplasts and fractions were prepared by the standard procedure. The 144000 × *g* supernatant was centrifuged for a further 3 h to give the 144_2 fraction and the 144_2 supernatant.

Fraction	*Wavelength maxima in difference spectrum (mμ)*					
	20°C			*77°K*		
	(A–F)	*(D–F)*	*(D–A)*	*(A–F)*	*(D–F)*	*(D–A)*
Chloroplasts	558	560	562	548, 552, 557	548, 552, 557, 561	557, 561
10000 × *g*	558	558–559	562	548, 552, 557	548, 552, 557, 561	557, 561
144000 × *g* (144_1)*	554	555, 562–563	562–563	548, 552, (557)**	548, 552, 557, 561	557, 561
144_2	554	555, 562–563	562–563	548, 552	548, 552, 557, 561	557, 561
144_2 supernatant	554	554, 562–563	562–563	—	—	—
cyt *f*	554	554	Nil	548, 552	548, 552	Nil
cyt b_6	—	563 (ref. 21)	—	—	—	—
cyt b_3	—	558–559 (ref. 4)	—	—	—	—

* The 144000 × *g* fraction prepared by the dilution technique gave the same maxima.
** The band at 557 mμ was barely detectable.

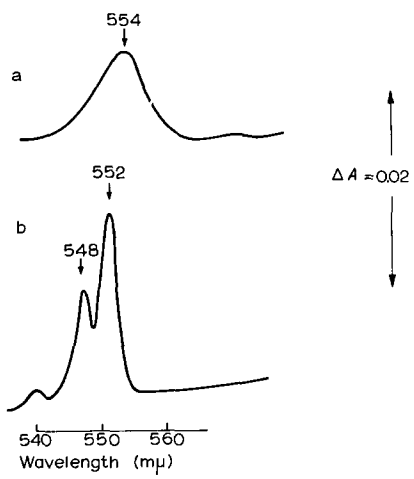

Fig. 3. Ascorbate-reduced *minus* ferricyanide-oxidized difference spectrum of cyt *f*. (a) 20°C; (b) 77°K.

oxidized (U–F) spectra were identical with the ascorbate-reduced *minus* ferricyanide-oxidized (A–F) spectra, which indicates that the cytochrome components seen in Figs. 1a and 2a were in the reduced state in the chloroplast. Reduced *minus* oxidized difference spectra of cyt *f* are shown in Fig. 3; they are in good agreement with those reported by HILL AND BONNER[21]. A comparison of the difference spectra of cyt *f* with the (A–F) spectra of chloroplasts (Figs. 1a and 2a) shows that in addition to cyt *f* a second cytochrome is reduced or partly reduced in chloroplasts. The absorption maximum of the (A–F) spectrum of chloroplasts at 20°C is located at 558 mμ, compared with 554 mμ for cyt *f* (Table I). At 77°K, the cyt *f* shows a splitting of the α band into two bands with maxima at 548 mμ and 552 mμ, while the chloroplasts in their (A–F) spectrum show in addition to the bands due to cyt *f*, a band at 557 mμ.

The dithionite-reduced *minus* ferricyanide-oxidized (D–F) spectra show the difference spectra of all cytochrome components in the chloroplast, and the dithionite-reduced *minus* ascorbate-reduced (D–A) spectra show the difference spectra of those cytochromes which are not shown in the (A–F) spectra *i.e.* cytochromes which are oxidized in the chloroplast. The absorption maximum of the (D–A) spectrum of chloroplasts at 20° C is at 562 mμ, which agrees fairly well with the reduced α band of cyt b_6 (ref. 3). Cyt b_6 is reduced by dithionite but not by ascorbate[17]. On lowering the temperature to 77° K (Fig. 2c), there is a partial splitting of the band to give two maxima of about equal extinction at 557 mμ and 561 mμ. It is assumed tentatively that both of these peaks are due to cyt b_6. The (D–F) spectrum at 77° K (Fig. 2b) shows an enhanced absorption at 557 mμ, but only a shoulder at 561 mμ. The shoulder at 552 mμ and the band at 548 mμ are presumably due to cyt *f*. It is assumed that the shoulder at 561 mμ and part of the band at 557 mμ are due to cyt b_6, while part of the band at 557 mμ is attributed to a third cytochrome. It is concluded that there are present in chloroplasts at least three cytochrome components; cyt *f* and cyt b_6 and a cytochrome with an α band at 557 mμ at 77° K. For reasons which are outlined in the DISCUSSION this latter cytochrome is believed to have an absorption maximum at about 559 mμ at 20° C. Unlike cyt b_6, it is present in the reduced state in the untreated chloroplasts (Fig. 2a). It is tentatively assumed that cyt 559 is a *b*-type cytochrome. The height of the (D–F) spectrum at 20° C (Fig. 1) is about twice that of the (A–F) spectrum, from which it is concluded that about half of the total cytochrome *b* (cyt *b*) is in the reduced state in the chloroplast.

In order to be certain that the presence of cyt 559 in the chloroplast fraction was not due to contamination by mitochondria or a soluble cytochrome, the chloroplasts were purified further. In a few preparations, the chloroplasts were purified by repeated resuspension in sucrose–phosphate buffer and sedimentation at 1000 × *g* for 10 min. The chloroplasts were pelleted six times, and such preparations will be designated, five times washed chloroplasts. A comparison of the difference spectra of five times washed chloroplasts with those of the standard preparation showed no significant differences, and it is concluded, therefore, that all the observed cytochromes are components of the chloroplast. CO had no effect on any of the spectra, indicating that none of the observed bands could be ascribed to a denatured cytochrome, a peroxidase, or a pigment similar to cytochrome *o*.

Chlorophyll–cytochrome ratio

Cyt *b* (cyt b_6 *plus* cyt 559) was determined from (D–F) difference spectra at 20° C using a difference molar extinction coefficient ($\varepsilon_{M,560\ m\mu} - \varepsilon_{M,575\ m\mu}$) of $2.0 \cdot 10^4$ for reduced *minus* oxidized cyt *b* (ref. 4). The absorbance difference between the maximum at 560 mμ and the minimum at 575 mμ was corrected for the small contribution of cyt *f* by subtracting 1/30 of the absorbance difference. This correction factor was derived because cyt *f* has a difference molar extinction coefficient ($\varepsilon_{M,560\ m\mu} - \varepsilon_{M,575\ m\mu}$) for its reduced *minus* oxidized spectrum of approx. $0.25 \cdot 10^4$, and it is estimated below that the ratio of cyt *b*/cyt *f* in the chloroplast is between 3.5 and 4.0. Chl *a* and chl *b* were determined[18], and the molar ratios of chl *a*/chl *b* and (chl *a* *plus* chl *b*)/cyt *b* calculated (Table II). The molar ratio of total chlorophyll to cyt *b* varied from 83 to 142, with an average of 118 from six preparations.

The molar ratio of total chlorophyll to cyt *f* could not be determined from the

TABLE II

MOLAR RATIOS OF CHLOROPHYLL/CYTOCHROME FOR CHLOROPLASTS AND THE 10000 × *g* AND 144000 × *g* FRACTIONS

Chloroplasts and fractions were prepared by the standard procedure as described in METHODS. (The chl *a* + chl *b*)/cyt *b* ratios were determined from dithionite-reduced *minus* ferricyanide-oxidized difference spectra of the chlorophyll-containing particles. The cyt *b*/cyt *f* ratios of the chloroplasts and the 10000 × *g* fraction were determined with acetone powders and that of the 144000 × *g* fraction on the chlorophyll-containing particles. The (chl *a* + chl *b*)/cyt *f* ratios were calculated from the ratios in columns 3 and 4. The number of separate determinations is given in parentheses.

Preparation	$\frac{Chl\ a}{Chl\ b}$	$\frac{Chl\ a\ plus\ chl\ b}{Cyt\ b}$	$\frac{Cyt\ b}{Cyt\ f}$	$\frac{Chl\ a\ plus\ chl\ b}{Cyt\ f}$
Chloroplasts	2.87	118 (6)	3.6 (5)	430
10000 × *g* fraction	2.34	120 (6)	6.1 (9)	730
144000 × *g* fraction	5.10	390 (5)	2.3 (5)	900

difference spectra of green chloroplasts, but it was obtained from measurements made on acetone-extracted chloroplasts. Difference spectra at 20°C of acetone-extracted chloroplasts are shown in Fig. 4. The (A–F) difference spectrum shows a maximum at 554 mμ corresponding to cyt *f*, instead of 558 mμ as with the green chloroplasts. This suggests that the cyt *b* seen in Fig. 1a had been altered by the acetone extraction so that it was no longer in the reduced state. Hence it was now possible to calculate the cyt *f* concentration from the (A–F) spectrum, using a difference molar extinction coefficient ($\varepsilon_{M,554\ m\mu} - \varepsilon_{M,575\ m\mu}$) of $2.5 \cdot 10^4$ (ref. 4). The cyt *b* was determined from the

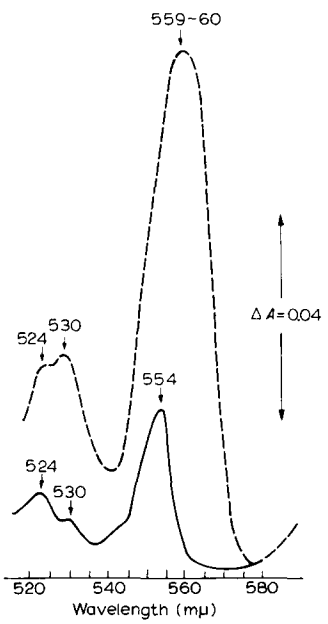

Fig. 4. Reduced *minus* oxidized difference spectra of acetone-extracted chloroplasts at 20°C. ———, (A–F); - - - - - - -, (D–F). Concentration of material, 51 mg/ml. Optical path-length, 2 mm.

(D–F) spectrum as described above, and thus the cyt *b*/cyt *f* ratio could be determined (Table II). An average value of 3.6 was obtained for the cyt *b*/cyt *f* ratio from five preparations.

Cytochromes of particles obtained by digitonin fragmentation

10000 × g fraction. The particles in this fraction have been shown to be enriched with respect to Photosystem 2 (refs. 9–13). Reduced *minus* oxidized difference spectra of the 10000 × *g* fraction generally resembled the corresponding spectra obtained with chloroplasts, but with the following differences. The maximum in the (D–F) spectrum at 20°C was located at 558–559 mμ, compared with 560 mμ for chloroplasts (Table I), and at 77°K the shoulders at 561 mμ and 552 mμ were less pronounced than with chloroplasts (Fig. 5). These differences show that the 10000 × *g* fraction contained a higher proportion of cyt 559, relative to cyt *f* and cyt b_6. The spectra of the 10000 × *g* fraction were not influenced by saturating amounts of CO. The (chl *a plus* chl *b*)/cyt *b* ratio was determined from the (D–F) difference spectra of the chlorophyll-containing fraction; an average value of 120 was obtained (Table II). This figure does not differ significantly from the average (chl *a plus* chl *b*)/cyt *b* ratio of chloroplasts. As with the chloroplasts, it was necessary to use acetone powders for the

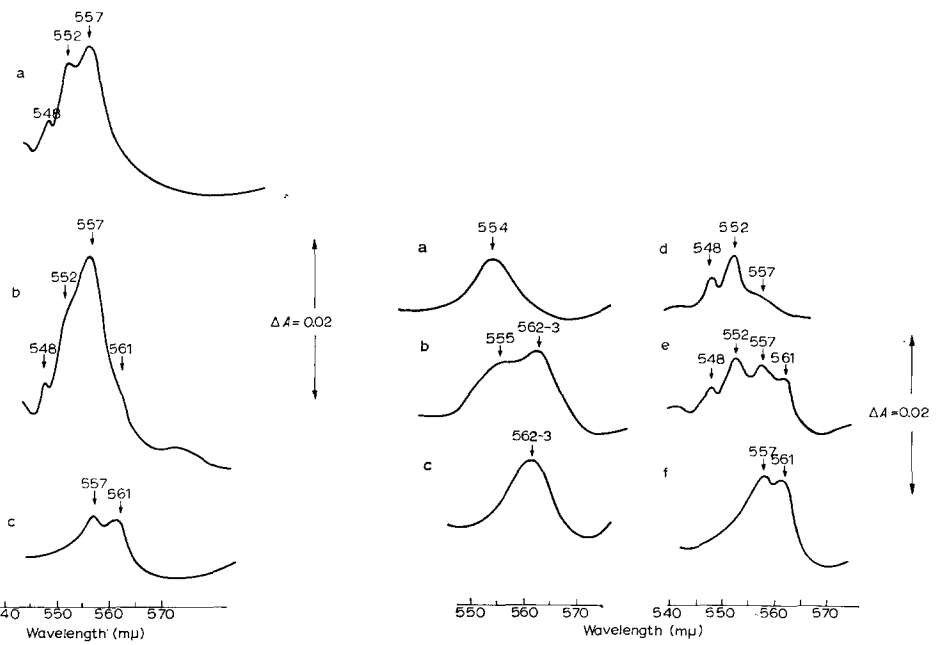

Fig. 5. Reduced *minus* oxidized difference spectra at 77°K of the 10000 × *g* fraction prepared by the standard procedure. (a) (A–F); (b) (D–F); (c) (D–A). Spectra recorded at 20°C resembled those of the chloroplasts, except for slight differences in the positions of the maxima (see Table I). Chlorophyll concentration, 0.48 mg/ml. Optical path-length, 2 mm.

Fig. 6. Reduced *minus* oxidized difference spectra of the 144000 × *g* fraction prepared by the standard procedure. Spectra with similar characteristics were observed when the fraction was prepared by the dilution technique. (a) (A–F), 20°C; (b) (D–F), 20°C; (c) (D–A), 20°C; (d) (A–F), 77°K; (e) (D–F), 77°K; (f) (D–A), 77°K. Chlorophyll concentration, 0.69 mg/ml. Optical path-length, 2 mm.

determination of the cyt *b*/cyt *f* ratio of the 10000 × *g* fraction. An average value of 6.1 was obtained from nine preparations (Table II).

144000 × g fraction (144_1). Particles in this fraction have been shown to be representative of Photosystem 1 (refs. 9–13). Difference spectra obtained with the 144_1 fraction are shown in Fig. 6. The (A–F) spectrum at 20°C shows a maximum at 554 mμ, and the low-temperature spectrum is that of cyt *f* with only a trace of a component at 557 mμ. In contrast with chloroplasts and the 10000 × *g* fraction, the untreated *minus* ferricyanide-oxidized (U–F) spectrum of the 144_1 fraction at 20°C did not resemble the (A–F) spectrum but was similar to the baseline (untreated *minus* untreated). It is concluded that in the 144_1 fraction all the cytochromes were initially present in the oxidized state.

The (D–F) spectrum at 20°C shows a maximum at 562 mμ and a pronounced shoulder at 555 mμ, while the (D–A) spectrum shows only the peak at 562 mμ. These room temperature spectra suggest the presence only of cyt *f* and cyt b_6 (refs. 1–3). At 77°K, the (D–F) spectrum shows four bands; at 548 mμ and 552 mμ (cyt *f*), and at 557 mμ and 561 mμ. Compared with chloroplasts the band at 557 mμ is greatly diminished. CO had no effect on these spectra.

The (chl *a plus* chl *b*)/cyt *b* ratios obtained with the 144_1 fraction are shown in Table II. An average value of 390 was obtained, but the variation (250–550) between preparations was much greater than for the chloroplasts or the 10000 × *g* fraction. An average value of 2.3 was obtained for the ratio of cyt *b*/cyt *f* in the 144_1 fraction; the concentration of cyt *f* was obtained from the (A–F) spectrum and the concentration of cyt *b* from the (D–A) spectrum. The average (chl *a plus* chl *b*)/cyt *f* ratio was 900. An analysis of acetone powders of preparations of the 144_1 fraction gave an average cyt *b*/cyt *f* ratio of 2.6, in good agreement with the chlorophyll-containing preparations.

Chlorophyll and cytochrome contents of the various fractions. Both the 10000 × *g* and 144_1 fractions had higher (chl *a plus* chl *b*)/cyt *f* ratios than the chloroplast, a result which suggests that some of the cyt *f* of the chloroplast was rendered soluble by the digitonin treatment. The high (chl *a plus* chl *b*)/cyt *b* ratio obtained for the 144_1 fraction suggests that some of the cyt *b* was also solubilized. Such a loss of cyt *b* was demonstrated in the experiment reported in Table III in which the chlorophyll and cyt *b* contents of all the fractions were measured. In this particular experiment, the 144000 × *g* supernatant was centrifuged for 3 h at 144000 × *g* to yield a further pellet, designated 144_2 fraction, and a supernatant, the 144_2 supernatant.

Table III shows the (chl *a plus* chl *b*)/cyt *b* molar ratios for all the fractions, and the (chl *a plus* chl *b*)/cyt *f* ratios for the 144_1 and 144_2 fractions and the 144_2 supernatant. It is immediately apparent that cyt *f* and cyt *b* (cyt b_6 in this case) are preferentially released into the 144_2 fraction and the 144_2 supernatant, as compared with the chlorophyll. Difference spectra for the 144_2 fraction resembled those of the 144_1 fraction. The (A–F) spectrum at 20°C showed a maximum at 554 mμ, the (D–F) spectrum a maximum at 562 mμ with a shoulder at 554 mμ, and the (D–A) spectrum had a maximum at 562–563 mμ. These spectra suggested the presence of cyt *f* and cyt b_6 in the 144_2 fraction. The (D–A) spectrum at 77°K showed a splitting of the 562-mμ band to give bands at 557 mμ and 561 mμ, similar to the behaviour of the 144000 × *g* fraction. Because of the low concentration of pigments the difference spectra at 20°C of the 144_2 supernatant were determined in cuvettes with 1 cm path-

length. They were similar to those reported for the 144_2 fraction, but the concentrations of cytochromes were too low for measurements at 77° K in the 2-mm cuvettes. Average ratios obtained by summing the chlorophyll and the cytochrome contents of the 50000 × *g*, 144_1 and the 144_2 fractions and the 144_2 supernatant were as follows: (chl *a plus* chl *b*)/cyt *b* = 156, (chl *a plus* chl *b*)/cyt *f* = 330, cyt *b*/cyt *f* = 2.1.

It is concluded that cyt *f* and cyt b_6 are lost from the Photosystem I particle after its release from the chloroplast lamellae. Cyt *f* has a molecular weight of 245000 (ref. 22) and therefore some of this cytochrome would sediment to the bottom of the tube during a 3-h centrifugation at 144000 × *g*. The molecular weight of cyt b_6 is not known.

Cytochrome content of the 144000 × g fraction prepared by the dilution technique. In the normal procedure for preparing the fractions, the 144000 × *g* fraction is in contact with 0.5 % digitonin for a longer period than are the larger particle fractions, and this may account for its low cytochrome content. In order to minimize the loss of cytochrome from the 144000 × *g* fraction, the reaction mixture was diluted 10-fold at the end of the incubation period, before differential centrifugation. Digitonin at a concentration of 0.05 % is not effective in splitting the photochemical systems, and furthermore, chloroplasts allowed to stand in 0.05 % digitonin show HILL activities which are not significantly different from control chloroplasts.

Difference spectra of the 144000 × *g* fraction prepared by the dilution technique resembled the spectra of the 144000 × *g* fraction obtained by the normal procedure

TABLE III

MOLAR RATIOS OF CHLOROPHYLL/CYTOCHROME FOR THE FRACTIONS

Chloroplasts and fractions were prepared by the standard procedure. The 144000 × *g* supernatant was centrifuged for a further 3 h to give the 144_2 fraction and the 144_2 supernatant.

Fraction	$\frac{Chl\ a\ plus\ chl\ b}{Cyt\ b}$	$\frac{Chl\ a\ plus\ chl\ b}{Cyt\ f}$	$\frac{Cyt\ b}{Cyt\ f}$
Chloroplasts in digitonin	140	(430)	(3.6)
1000 × *g*	132		
10000 × *g*	130	(730)	(6.1)
50000 × *g*	350		
144000 × *g* (144_1)	510	1220	2.4
144_2	80	190	2.4
144_2 supernatant	27	44	1.6

TABLE IV

MOLAR RATIOS OF CHLOROPHYLL/CYTOCHROME FOR THE 144000 × *g* FRACTION PREPARED BY THE DILUTION TECHNIQUE

After incubation with 0.5 % digitonin for 30 min at 0°C, the mixture was diluted 10-fold before differential centrifugation. Cytochromes were determined on the chlorophyll-containing particles.

Preparation	$\frac{Chl\ a}{Chl\ b}$	$\frac{Chl\ a\ plus\ chl\ b}{Cyt\ b}$	$\frac{Chl\ a\ plus\ chl\ b}{Cyt\ f}$	$\frac{Chl\ a\ plus\ chl\ b}{Cyt\ b\ plus\ cyt\ f}$	$\frac{Cyt\ b}{Cyt\ f}$
1	5.5	182	380	125	2.1
2	5.7	191	345	122	1.8

but analysis (Table IV) showed that the chlorophyll/cytochrome ratios were much lower than those reported in Table II. Because of the very low concentration of pigments, it was not possible to determine chlorophyll/cytochrome ratios on the 144000 × *g* supernatant. The difference spectra of the 10000 × *g* fraction were similar to those obtained with the same fraction prepared by the normal procedure.

Chlorophyll and carotenoid content of fractions

The chlorophyll and carotenoid contents of chloroplasts and fractions are shown in Table V. Chlorophyll accounts for about 9 % of the dry weight of the spinach chloroplast. The chlorophyll content of the 10000 × *g* fraction is considerably higher than that of the chloroplasts, but this result is not unexpected. It is known that on fragmentation of chloroplasts, the soluble proteins of the stroma are released into the medium. The chlorophyll content of the 144000 × *g* fraction is much lower than that of the 10000 × *g* fraction. This is partly due to the sedimentation of some of the soluble proteins at 144000 × *g*. It seems likely, however, that the chlorophyll-containing particles in the 144000 × *g* fraction have less chlorophyll than the particles in the 10000 × *g* fraction. The low chlorophyll content of the 144000 × *g* supernatant is due to the colourless proteins of the chloroplast stroma. The chl *a*/chl *b* ratios were similar to those reported previously[9–13].

The total carotenoid content of the fractions follows the same trend as the chlorophyll content. The (chl *a plus* chl *b*)/carotenoid ratios of the 10000 × *g* fraction and the 144000 × *g* fraction are virtually the same, while the proportion of carotenoids in the supernatant is significantly higher.

Two-dimensional paper chromatography revealed that each of the fractions contained a mixture of the four carotenoids. The carotenoids could not be eluted quantitatively from paper, the overall recovery being about 80 %. With thin-layer chromatography, the recovery was 90–97 %. The carotenoid composition of chloroplasts and of the fractions are shown in Table VI. The composition obtained for the chloroplasts is in agreement with published figures[23,24]. Table VI indicates that the non-oxygenated carotenoid, β-carotene, accounts for a slightly higher proportion of the carotenoids of the 144000 × *g* fraction, and a lower proportion of those of the 10000 × *g* fraction, as compared with chloroplasts. The principal oxygenated carotenoid, lutein (which also contains zeaxanthin) is lower in the 144000 × *g* fraction and in the supernatant. These differences may be characterized by the xanthophyll/

TABLE V

CAROTENOID AND CHLOROPHYLL CONTENTS OF FRACTIONS

Chloroplasts and fractions were prepared by the standard procedure and were lyophilized prior to analysis.

Fraction	*Chl a plus chl b (mg/10 mg lyophilized powder)*	*Carotenoids (mg/10 mg lyophilized powder)*	*Chl a plus chl b / Carotenoids*
Chloroplasts	0.92	0.146	6.3
10000 × *g*	1.63	0.232	7.0
144000 × *g*	0.81	0.119	6.8
144000 × *g* supernatant	0.078	0.019	4.1

carotene ratio, which is higher in the 10000 × *g* fraction and lower in the 144000 × *g* fraction, as compared with chloroplasts. There was some variation between experiments in the relative proportions of the various xanthophylls, but the xanthophyll/carotene ratios remained fairly constant. Table VI shows that the xanthophylls, particularly violaxanthin, were preferentially released into the supernatant by the digitonin treatment.

TABLE VI

CAROTENOID COMPOSITION OF FRACTIONS

Chloroplasts and fractions were prepared by the standard procedure. The carotenoids were separated by thin-layer chromatography as described in METHODS.

Carotenoid	*Chloroplasts*	*Percentage composition*		
		10000 × g fraction	*144000 × g fraction*	*144000 × g supernatant*
β-Carotene	28	23	34	18
Lutein	45	46	33	33
Violaxanthin	17	16	23	37
Neoxanthin	10	16	10	12
Xanthophyll / Carotene	2.57	3.34	1.94	4.55

OGAWA, OBATA AND SHIBATA[25] determined the carotenoid composition of chlorophyll–protein complexes isolated from spinach chloroplasts by treatment with sodium dodecyl sulphate and gel electrophoresis. Because of the similarity in the ratios of chl *a*/chl *b* of their Components I and II, to those of the 10000 × *g* and 144000 × *g* fractions obtained by digitonin fragmentation[9], it was suggested that Component I was derived from System I and Component II from System 2. The carotenoid composition of Component II was similar to that of the 10000 × *g* fraction reported here, but Component I was devoid of neoxanthin and violaxanthin.

DISCUSSION

The molar ratio for (chl *a plus* chl *b*)/cyt *f* which we have calculated for spinach chloroplasts is in good agreement with ratios reported by DAVENPORT AND HILL[2] for parsley, and elder (Table VII). It is lower than the ratios obtained by LUNDEGÅRDH[4] with spinach chloroplasts, and JAMES AND LEECH[17] with *Vicia faba* chloroplasts, but higher than that given by SIRONVAL AND ENGELERT-DUJARDIN[26] for spinach. Most of the figures reported in Table VII were obtained with chloroplast preparations which had been extracted with acetone, and this may possibly account for the observed variations. If there is one molecule only of cyt *f* in a photosynthetic unit, then our figures show that the photosynthetic unit contains approx. 430 chlorophyll molecules. This is in good agreement with estimates of the size of the photosynthetic unit, made on the assumption that each unit contains one molecule of the long-wavelength chlorophyll, P700, which is considered to be the trapping centre for System I (ref. 27).

Our estimate for the molar ratio of cyt *b*/cyt *f* in chloroplasts is higher than the molar ratios reported by HILL AND BONNER[21], and SIRONVAL AND ENGELERT-DU-

TABLE VII

COMPARISON OF MOLAR RATIOS OF CHLOROPHYLL/CYTOCHROME FOR CHLOROPLASTS OR GREEN LEAVES

Material	$\frac{Chl\ a\ plus\ chl\ b}{Cyt\ f}$	$\frac{Chl\ a\ plus\ chl\ b}{Cyt\ b}$	$\frac{Cyt\ b_6}{Cyt\ f}$	$\frac{Cyt\ b_6}{Cyt\ b_3}$	$\frac{Cyt\ b}{Cyt\ f}$	
Parsley leaves	380					DAVENPORT AND HILL[2]
Elder leaves	430					DAVENPORT AND HILL[2]
Spinach chloroplasts			1.3			HILL AND BONNER[21]
Spinach or clover chloroplasts	550	111	2.3	0.85	5.0	LUNDEGÅRDH[4]
Spinach chloroplasts	200–300	150–230	1.0–1.4	2.3–2.9	1.4–2.0	SIRONVAL AND ENGELERT-DUJARDIN[26]
Vicia faba chloroplasts	580–1330	230–530	2.5			JAMES AND LEECH[17]
Spinach chloroplast; lamellar fraction	(230)	(230)	0.92			BIGGINS AND PARK[28]
Spinach chloroplasts	430	118			3.6	This paper

JARDIN[26], but lower than LUNDEGÅRDH's values (Table VII). JAMES AND LEECH[17] reported a ratio of 2.5 for *Vicia faba*. BIGGINS AND PARK[28] determined the molar ratio of cyt *b*/cyt *f* in a chloroplast lamellar protein fraction and obtained an average value of 0.92. LUNDEGÅRDH[4], and SIRONVAL AND ENGELERT-DUJARDIN[26] considered that chloroplasts contain two *b*-type cytochromes, cyt b_6 (α band 563 mμ) and cyt b_3 (α band 559 mμ), but the other investigators concluded that chloroplasts contain one *b*-type cytochrome *viz.* cyt b_6.

Our measurements with chloroplasts and the chlorophyll-containing particles derived by digitonin fragmentation support the view that chloroplasts contain, in addition to cyt *f* and cyt b_6, a third cytochrome with an absorption maximum (reduced *minus* oxidized) at about 559 mμ at 20° C. This cytochrome is named cyt 559, pending identification of its prosthetic group. In the present paper, we have tentatively assumed that it is a *b*-type cytochrome, and its concentration has been added to that of cyt b_6 to give a cyt *b* concentration.

The deduction that the α band of the third cytochrome is at approx. 559 mμ is based on the positions of the absorption maxima in the various room temperature difference spectra, and on our estimate of the relative proportions of the three cytochromes. From the relative heights of the bands in the (A–F) and (D–F) spectra of chloroplasts, it is concluded that the concentration of reduced cyt *b* is approximately doubled by dithionite treatment. On the assumption that cyt b_6 is oxidized in the chloroplast, and cyt *f* and cyt 559 mainly reduced, a figure of about 2 is obtained for the molar ratio of cyt 559/cyt *f*, *i.e.* half the value of the cyt *b*/cyt *f* ratio. Furthermore the molar ratio of cyt *b*/cyt *f* in the chloroplasts was about twice that of the 144_1 and 144_2 fractions which lacked cyt 559. It is estimated, therefore, that the three cytochromes in the chloroplast are present in the following approximate molar proportions: cyt *f*:cyt b_6:cyt 559 = 1:2:2. In making this estimate, it has been assumed that the molar extinction coefficient and band spread of the third cytochrome are similar to those of cyt b_6.

Although low-temperature spectra cannot be used for the quantitative estimation of the cytochromes due to variable intensification of the bands on freezing, they are extremely useful for identification of the components. The bands at 552 mμ and 548 mμ are ascribed to cyt *f*, while the bands at 557 mμ and 561 mμ in the spectra of the 144_1 and 144_2 fractions may represent the splitting of the α band of reduced cyt b_6 at low temperature. The splitting of the α bands of several reduced cytochromes of the *b*-type has been reported by ESTABROOK[29]; in contrast to cyt *c* of heart muscle and cyt *f* of chloroplasts, where the band of lower extinction is on the short-wavelength side of the major band, it is on the long-wavelength side in cytochromes of the *b* type. The spectrum reported for reduced cyt b_2 has been confirmed by Dr. C. A. APPLEBY in this laboratory with a twice recrystallised preparation. The α band of cyt b_2, which is at 556 mμ at room temperature gives at 77° K two bands, at 552 mμ (major band) and at 556 mμ. A final decision as to whether the band at 557 mμ in the $144000 \times g$ fraction is in fact due to cyt b_6 or to a third cytochrome in the fraction must await the purification of cyt b_6. From the (D–F) spectrum of chloroplasts at 77° K, we conclude that the α band of cyt 559 shifts to 557 mμ on lowering the temperature.

From previous work[9–13], the conclusion was reached that the $144000 \times g$ fraction was representative of Photosystem 1, and the $10000 \times g$ fraction enriched in Photosystem 2. From the present study, it is proposed that cyt *f* and cyt b_6 are

associated with Photosystem 1 and cyt 559 with Photosystem 2. The (chl *a plus* chl *b*)/cyt *f* and cyt *b*/cyt *f* ratios reported for the 10000 × *g* fraction are consistent with this postulate, if it is assumed that the 10000 × *g* fraction contains 70 % Photosystem 2 and 30 % Photosystem 1. A similar conclusion as to the composition of the 10000 × *g* fraction was reached by ANDERSON, FORK AND AMESZ[12] from measurement of the amounts of P700 in chloroplasts and the fractions. The average (chl *a plus* chl *b*)/P700 ratio of the 10000 × *g* fraction was 690, as against a (chl *a plus* chl *b*)/cyt *f* ratio of 730 reported here. The (chl *a plus* chl *b*)/P700 ratio of the 144000 × *g* fraction was 205 and of the 144000 × *g* supernatant, 980. In contrast, the (chl *a plus* chl *b*)/cyt *f* ratios which we have obtained for the 144000 × *g* fraction are higher than the (chl *a plus* chl *b*)/P700 ratio, while the (chl *a plus* chl *b*)/cyt *f* ratios of the 144000 × *g* supernatant are considerably lower than the corresponding (chl *a plus* chl *b*)/P700 ratio. The lowest ratio which we have obtained for a 144000 × *g* fraction is 345, when the fraction was prepared by the dilution technique. Whereas P700 appears to be tightly bound to the Photosystem 1 complex, cyt *f* and cyt b_6 are to a large extent lost from the complex if it is allowed to stand in the digitonin solution. This solubilization of the cytochromes of Photosystem 1 can be minimized by a dilution of the reaction mixture immediately after incubation. It seems likely, however, that the (chl *a plus* chl *b*)/cyt *f* and (chl *a plus* chl *b*)/cyt b_6 ratios in the intact Photosystem 1 particle are even lower than those reported in Table IV. The good correspondence between the (chl *a plus* chl *b*)/cyt *f* and (chl *a plus* chl *b*)/P700 ratios for the 10000 × *g* fraction suggests that cyt *f* is not lost from the Photosystem 1 particle until after the latter is released from its close association with Photosystem 2. Cyt 559 appears to be firmly bound to the Photosystem 2 particle.

Measurements of light-induced absorbance changes[30] in chloroplasts and algae have established that cyt *f* is rapidly oxidized by light absorbed by Photosystem 1, and there is good evidence to support the conclusion that cyt *f* is closely associated with P700 in Photosystem 1, possibly in a charge-transfer complex[31]. Cyt *f* oxidation in the fractions obtained by digitonin fragmentation of chloroplasts was studied by ANDERSON, FORK AND AMESZ[12]. The amount of light-induced oxidation of cyt *f* was greater in the 144000 × *g* fraction than in the 10000 × *g* fraction, but it was lower than that expected if P700 and cyt *f* were present in equal amounts. This result again reflects the loss of cyt *f* from the Photosystem 1 particle. From a kinetic study of the photooxidation of plastocyanin in detergent-treated chloroplasts, KOK AND RURAINSKI[31] proposed that plastocyanin is also closely associated with P700 and that it may act in parallel with cyt *f*. Such a parallel formulation may explain why the molar concentration of cyt *f* in the chloroplast is only one-half that of cyt 559 of cyt b_6.

The involvement of cyt b_6 or cyt 559 as electron carriers has not been established in an unequivocal way. OLSON AND SMILLIE[32] observed slow absorbance changes due to the reduction of cyt b_6 in fragments of Euglena chloroplasts. More recently, RUMBERG[33] reported absorbance changes at 435 mμ and 412 mμ in spinach chloroplasts, which he attributed to cyt b_6, but these changes may possibly have been due to cyt 559. On the basis of inhibitor studies with chloroplasts, ARNON, TSUJIMOTO AND MCSWAIN[34] proposed that cyt b_6 is involved in ferredoxin-mediated photophosphorylation. This type of cyclic phosphorylation is thought to be catalysed by Photosystem 1.

From present experimental evidence, it is tempting to speculate that cyt 559 is

a component of the electron-transport pathway which connects the two pigement systems, whereas cyt b_6 is involved in a cyclic flow of electrons associated with Photosystem 1.

The present study indicates that the major carotenoids are present in both photochemical systems, but in different proportions; in particular, the Photosystem 1 particle contains a higher proportion of β-carotene. Recent fluorescence measurements show that the carotenoids are functional in both photosystems[13]. An examination of the fluorescence properties of the 10000 $\times$ g and 144000 $\times$ g fractions revealed the presence of excitation bands in the carotenoid region of the spectrum with both fractions.

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