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ON THE IDENTITY OF THE 640-nm COMPONENT OBSERVED IN CHLOROPHYLL *b* ABSORPTION SPECTRA *IN VIVO*

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SUMMARY

Mathematical analysis of the results of mild acetone extraction of chloroplast-fragment preparations from *Ulva lactuca* demonstrates that the 640-nm shoulder on the short-wave side of the red chlorophyll *b* absorption band is not due to this chlorophyll.

The analysis furthermore provides rather strong evidence that the 640-nm shoulder is correlated with an absorption band peaking around 682nm. It is suggested that the 640-nm component is due to the reaction center pigment of Photosystem II.

INTRODUCTION

A shoulder at the short-wave side of the red absorption band of chlorophyll *b in vivo*, around 640 nm, has been observed by French[1] in *Ulva* for the first time. This shoulder, which need not be due to chlorophyll *b*, was also found in corn chloroplasts[2], bean leaf preparations[3,4], an *Oenothera* mutant[5], and spinach[6]. However, in *Chlorella* the occurrence of such a component could not be demonstrated[4,7]. In the latter species it may be absent, it may occur in such small amounts that its presence is masked by the overlap of the chlorophyll *b* absorption band, or its absorption maximum may be located at a wavelength other than 640 nm.

The presence of the 640-nm component is likely to be restricted to Photosystem II[5,6,8]. As the same may be true of chlorophyll *b*[9], the mentioned component might represent a minor chlorophyll *b* form. Though French *et al.*[5] and Michel and Michel-Wolwertz[6] remarked that a definite proof of such an identity is still lacking, French[10] concluded from curve analyses that the 640-nm absorption shoulder is likely to be due to a chlorophyll *b* type. According to French *et al.*[11], this shoulder may be due to free chlorophyll *b*, possibly dissolved in lipids. On the other hand, experiments on extraction with acetone, made Thomas[7] suggest that the 640-nm component may differ from chlorophyll *b*.

These contradictory suggestions may demonstrate that the identity of the

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component in question is not yet satisfactorily understood. The present study aims at obtaining more insight into this problem.

MATERIALS AND METHODS

As the 640-nm shoulder shows up most clearly in absorption spectra of *Ulva lactuca* at 77 °K, this species was chosen as an object. Batches of this seaweed were obtained from the Netherlands Institute for Sea Research at Den Helder and stored at −40 °C. Even after storage for more than one year, the spectrum did not change at this temperature. About 100 g of thalli were thoroughly rinsed in tapwater and subsequently washed in 0.02 M phosphate buffer, pH 7.3. Next, the thalli were macerated in an ice-cooled Sorvall omnimixer, operating at full speed, in portions of about 30 g for 4 min. The resulting suspension was filtered over a cottonwool-covered glass filter, and centrifuged at 48 000×*g* for 15 min. About equal portions of the sediment were taken up in demineralized water, or in 30, 40 and 50% reagent-grade acetone in demineralized water, called henceforth 30% acetone, and so on, and stored in the refrigerator at 4 °C for 3 h. The suspensions were stirred at intervals of about 0.5 h. After the extraction period, the samples were centrifuged at 48 000×*g* for 20 min. The sediments were resuspended in demineralized water, whereas the acetone concentration of the supernatants was raised to 80%.

Suspensions of chloroplast fragments from the chlorophyll *b*-free alga *Vischeria stellata* were prepared according to Thomas and Bretschneider[12].

Absorption spectra as well as absorption difference spectra were recorded in a Cary model 14R spectrophotometer. Approximate spectra of chlorophyll *b in vivo* at 77 °K were obtained by subtracting *Vischeria* spectra from those of *Ulva* in a way described earlier[12].

In order to prevent cracks in the glassy preparation at 77 °K, high-grade glycerol was added up to 65% to the suspensions, and 1-mm perspex cuvettes were used.

RESULTS

Absorption spectra

In a previous report[7] it was mentioned that the 640-nm component virtually withstands a 3-h extraction period in 30% acetone, whereas the major part of it is removed upon extraction with 40% acetone. An example is shown in Fig. 1. Curve a represents the approximate absorption spectrum of chlorophyll *b* in *U. lactuca* at 77 °K, corrected for chlorophyll a_{662} absorption as described previously[8].

In this connection it is remarked that the mentioned chlorophyll *a* type was originally called C_a665, due to its apparent location on the major chlorophyll *a* band at room temperature. Curve analyses by French[10], as well as studies of low-temperature spectra[8] showed that the actual location of the absorption maximum of this chlorophyll *a* form should be around 662 nm.

Fig. 1 (Curve b) shows the short-wave side of the chlorophyll *b* absorption band in suspensions extracted with 30% acetone for 3 h, whereas Fig. 1 (Curve c) demonstrates the effect of extraction with 40% acetone for the same period. Since

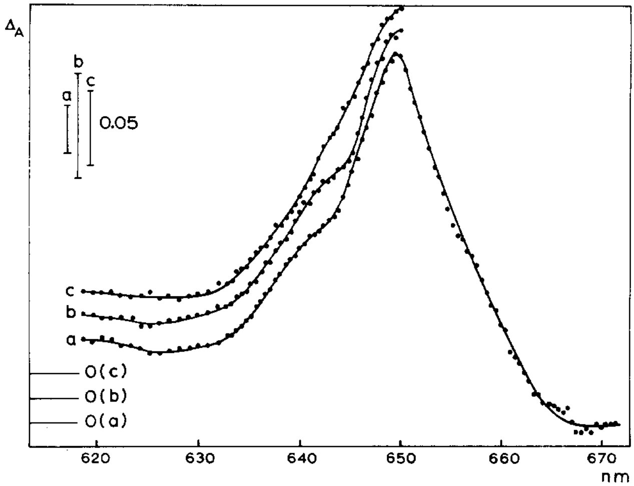

Fig. 1. Approximate red absorption band of chlorophyll *b* in *U. lactuca* at 77 °K. a, untreated; b, upon extraction with 30% acetone; c, upon extraction with 40% acetone. Reference: *V. stellata*. For details see text.

extraction of the various chloropyll *a* forms occurs at different rates, the shape of the long-wave side of the chlorophyll *b* band becomes less certain at increasing concentrations of acetone. Therefore, this side of Spectra *b* and *c* is not drawn. In any case it is clear from Fig. 1 that the major part of the 640-nm component is removed in 40% acetone, whereas it is still present in about the same proportion as chlorophyll *b* upon treatment with 30% acetone, as compared with the situation in the non-treated suspension. To facilitate comparison, the chlorophyll *b* maxima are made equally high, and the abscissae are shifted as indicated.

In order to check whether the 640-nm component shows up in the extracts, absorption difference spectra of the supernatants of preparations treated with either 30% or 40% acetone were established at room temperature. As acetone-containing preparations become snowy at 77 °K, it was not possible to obtain these spectra at low temperature. Before measuring, the acetone concentration in both extracts was raised to 80%. Fig. 2 shows that a pigment with maximum absorption around 633 nm is preferably extracted in 40% acetone.

The peak location around 633 nm differs from those of the absorption maxima of both main and vibrational bands of chlorophylls *a*, *b*, *c*, *d*, and protochlorophyll in acetone or ether[13,14]. Therefore, the spectrum in Fig. 2 possibly is due to the extracted 640-nm component. If so, extraction results in a blue shift of about 7 nm. The half-width value is about 20 nm at room temperature. Future research is needed to get more insight into the relationship between the 640-nm component and the extracted 633-nm pigment.

The 640-nm component may show absorption bands at additional wavelengths as well, but as acetone-containing preparations become snowy at 77 °K, this possibility was studied at low temperature by using sediments of extracted and non-extracted samples resuspended in demineralized water. An example of an absorption difference spectrum of a non-treated preparation *versus* a preparation

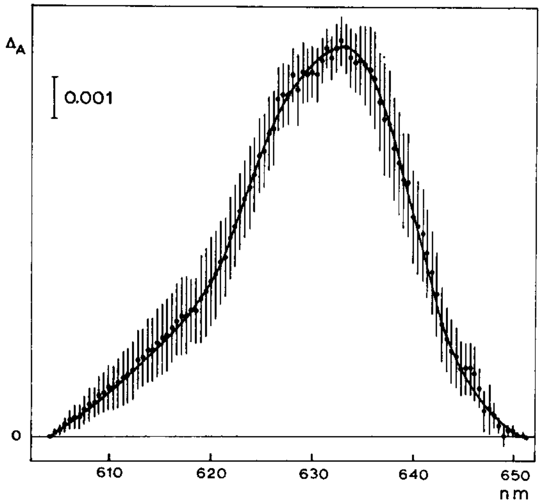

Fig. 2. Absorption difference spectrum of extracts from *U. lactuca* chloroplast fragments at room temperature by treatment with acetone in concentrations of 40% and 30% at 4 °C for 3 h. Prior to establishing the difference spectrum the acetone concentration in both samples was raised to 80%. Data are means, together with their standard deviations, of 10 experiments.

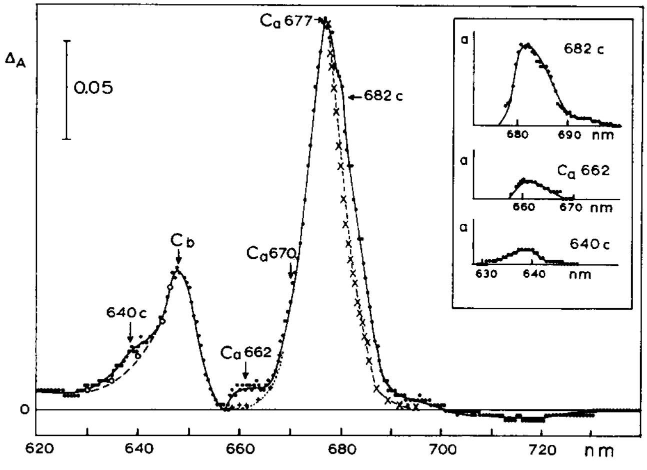

Fig. 3. Absorption difference spectrum at 77 °K of a non-treated chloroplast fragment suspension from *U. lactuca versus* a suspension extracted with 40% acetone at 4 °C for 3 h. Both samples are suspended in demineralized water. The original spectra are matched at 657 nm. ×---×, mirror-symmetrically plotted data of the short-wave side of the main chlorophyll *a* band; +---+, correction for C_a662 absorption (Thomas[8], Fig. 1); o---o, shape of the short-wave side of the chlorophyll *b* band free from the 640-nm component (Thomas[12], Fig. 4). Insert: differences (d) between measured and computed curves. For symbols see Table I.

extracted in 40% acetone is given in Fig. 3. The approximate heights of the various bands were obtained as follows. The heights of the chlorophyll *b* band and the main chlorophyll *a* one were read from the difference spectrum directly. For estimating the band height of the 640-nm component, chlorophyll *b* absorption was plotted, using the shape of the spectrum of this pigment free from the 640-nm shoulder, according to Fig. 4 from Thomas[15], and subtracted. The chlorophyll a_{662} band is corrected for contribution of the main chlorophyll *a* band by using the shape of the 662-nm band, according to Fig.1 from Thomas[8]. An approximation of the band height of a component with maximum absorption around 682 nm was established by subtracting a mirror-symmetrical plot of the short-wave side of the main chlorophyll *a* band from the measured long-wave side of this band. As it is a purely objective way of approximation, this procedure was chosen. Since in the difference spectrum the contribution of the chlorophyll a_{670} form is only rather poor, its absorption band was left out of consideration. The insert of Fig. 3 shows the spectra obtained by the mentioned substraction procedures. In this way 20 difference spectra were established and evaluated. The results are summarized in Table I.

TABLE I

BAND HEIGHTS OF COMPONENTS IN ABSORPTION DIFFERENCE SPECTRA OF CHLOROPLAST FRAGMENT PREPARATIONS FROM *ULVA LACTUCA* AT 77 °K

The difference spectra are obtained by subtracting spectra of fragments extracted with 40% acetone (40%) from either those of blanks, or spectra of fragments extracted with 30% acetone (30%). Extraction at 4 °C for 3 h. C_a677 and C_a662, chlorophyll *a* forms; C_b, chlorophyll *b*; 682*c* and 640*c*, components absorbing around 682 and 640 nm, respectively. Heights of absorption bands are expressed in per cents of those of the main bands, C_a677, or, in order to obtain the ratios C_a677/640*c*, in relative units (mm).

Band height						*Ratio*				*Type of difference spectrum*
C_a677 (mm)	*640c (mm)*	*640c (%)*	*682c (%)*	*C_a662 (%)*	*C_b (%)*	*C_a677/640c*	*682c/640c*	*C_a662/640c*	*C_b/640c*	
118.0	2.0	1.7	6.8	5.9	46.6	59.0	4.0	3.5	27.4	Blank *versus* 40%
82.0	2.0	2.4	14.6	9.8	48.2	41.0	6.1	4.1	20.1	
92.5	4.0	4.3	29.0	3.8	28.6	23.1	6.7	0.9	6.7	
111.0	3.5	3.1	16.2	7.2	47.7	31.7	5.2	2.3	15.4	
81.0	2.0	2.5	21.0	3.7	43.2	40.5	8.4	1.5	17.5	
80.0	3.0	3.8	21.5	5.1	36.1	26.7	5.7	1.3	9.5	
80.5	2.5	3.1	22.5	8.1	40.6	32.2	7.3	2.9	13.1	
71.0	2.0	2.8	18.3	5.6	35.2	35.5	6.5	2.0	12.6	
73.0	1.5	2.1	12.3	9.6	28.7	48.7	5.9	4.6	13.7	
60.5	2.5	4.1	24.6	10.7	34.6	24.2	6.0	2.6	8.4	
55.0	1.0	1.8	7.3	10.9	58.2	55.0	4.1	6.1	32.3	30% *versus* 40%
46.5	0.7	1.6	10.6	13.0	61.7	66.4	6.6	8.1	38.6	
50.0	2.0	4.0	14.0	6.0	40.0	25.0	3.5	1.5	10.0	
68.0	2.0	2.9	10.3	8.8	64.7	34.0	3.6	3.0	22.3	
51.0	2.0	3.9	25.5	11.7	62.7	25.5	6.5	3.0	16.1	
33.5	2.0	6.0	34.3	8.8	46.3	16.7	5.7	1.5	7.7	
52.5	1.0	1.9	7.4	7.6	61.0	52.5	3.9	4.0	32.1	
50.0	1.2	2.5	8.0	4.0	42.0	41.7	3.2	1.6	16.8	
51.0	1.5	2.9	13.7	10.8	35.3	34.0	4.7	3.7	12.2	
46.0	1.5	3.3	23.9	8.7	37.0	30.7	7.2	2.6	11.2	

Mathematical analysis

In order to investigate the relationship between the heights of the various peaks, rank correlation coefficients[16] (Spearman's *r*) were computed. The general idea is as follows. If there is a dependence between the heights of two peaks then it may be expected that in one experiment the values of the two peaks are low, and in another experiment both are high. The above-mentioned correlation coefficient can be considered as a measure of dependence. When $r=1$ the dependence is as high as possible in the sense that the two highest values occur in the same experiment, the two second highest peaks in another experiment, and so on. The two smallest values, then occur also in one experiment. There is another form of dependence, *i.e.* the case where the highest value of one peak occurs in the same experiment as the lowest value of the other peak, the second highest value of the one peak in the same experiment as the second lowest value of the other peak, and so on. For this case $r=-1$. When there is no relation between the two peaks, r is zero. The higher the absolute value of r, the stronger is the relationship between the two peaks. The results of such an analysis are given in Table II.

TABLE II

CORRELATION BETWEEN THE HEIGHT OF THE 640-nm ABSORPTION BAND AND THOSE OF THE OTHER MAXIMA GIVEN IN TABLE I

Rank correlation between the 640-nm component and	r	P
C_b	-0.39	0.1
C_a662	-0.15	>0.1
C_a677	-0.16	>0.1
682*c*	0.84	<0.001

Of course, the correlation r possibly could be due to chance. Therefore, the null hypothesis, holding that in case of relation the population correlation ϱ is zero, was tested, *cf.* ref. 16. The results are given in the same table. The meaning of P, the level of significance, is, loosely speaking, the probability that in the experiments a value greater than, or equal to, $|r|$ is found, given that the population correlation $\varrho=0$. The implication is that the smaller P is, the less one is inclined to believe that the computed value of r is due to chance. In practical statistics one usually considers experimental results with P values smaller than 0.05 to be "significant", in the sense that one is prepared to believe that there is some relationship between the two peaks in the present analysis. From the data in Table II it can be concluded that there is very strong evidence that the 640-nm component and the 682-nm component are related. There is no reason to assume any relationship between the 640-nm component and the remaining components in the table.

DISCUSSION

Calculations of rank correlation coefficients, r, and levels of significance, P, given in Table II, demonstrate that no correlation exists between the band heights around 640 nm and those of C_b at 648 nm. It is therefore concluded that the pigment absorbing around 640 nm differs from chlorophyll *b*.

On the other hand, such calculations show a rather significant correlation between the band heights of the 640- and 682- nm components. Consequently, both latter bands can be considered to belong to one and the same pigment.

According to Döring *et al.*[17] the reaction center pigment of Photosystem II, in spinach chloroplasts, shows absorption maxima at 640 and 682 nm. However, the data of these authors and the present ones, refer to room temperature and 77 °K spectra, respectively. Therefore, and because of the fact that different species are involved, if both studies actually deal with the same pigment, the exact peak locations may somewhat differ. The means for these wavelengths from 20 low-temperature difference spectra are 638.0 ± 0.2 and 681.6 ± 0.6 nm. The values represent means togther with their standard deviations.

Because of the different temperatures to which the spectra in both studies refer, as well as the fact that the shape of the 682-nm band is only approximately established in the present investigation, the proportion of the heights of both bands can be compared only roughly. An estimation of the band-height ratio 682/640 nm in Fig. 3 from Döring and co-workers[17] yields about 6.5, whereas this ratio as a mean of the 20 present experiments amounts to about 5.5. Since in the present study, see Fig.3, the 682-nm band height is approximated by subtraction of the mirror-symmetrically plotted data of the short-wave side of the main, red, chlorophyll *a* band from the measured values around 682 nm, the latter ratio is somewhat too low. For, as the slope of the long-wave side of this chlorophyll *a* band is steeper than that of the short-wave one, the band height of the 682-nm component will be found to be somewhat decreased. Still it can be concluded that in both studies, the 682- and 640-nm bands are major and minor ones, respectively.

In combination with the fact that the occurrence of the 640-nm component is likely to be restricted to Photosystem II[5,6,8], these results suggest that the pigment in question probably represents the reaction center pigment of this photosystem.

Attemps to isolate the extracted pigment, see Fig. 2, have not been successful so far. Norris *et al.*[18] concluded from the asymmetry of ESR signals that the reaction center chlorophyll of Photosystem I in algae is held in a rigid matrix. McElroy *et al.*[19] arrived at the same conclusion for reaction center bacteriochlorophyll. If this property also holds for the reaction center pigment of Photosystem II, the absorption band depicted in Fig. 2 might refer to a pigment–protein complex which, at least for some time, persists in 80% acetone. If, actually, such were the case, a related, major band at longer wavelenghts would be expected to occur in the extracts as well. Since, however, in the latter region the amounts of extracted bulk chlorophyll *a* differed considerably in the various preparations, whereas the pigment in question occurs only in minor quantities, a reliable analysis is not possible. Future research may elucidate this problem.

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