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A STUDY OF CHLOROPHYLL *b IN VIVO*

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SUMMARY

1. The absorption spectrum of chlorophyll *b in vivo* at 77°K is presented as the difference spectrum between preparations of spinach and chlorophyll *b*-free *Vischeria stellata* chloroplasts.

2. A shoulder on this spectrum around 662 nm is due to a component different from chlorophyll *b*. This component may well be identical with the chlorophyll *a* form, chlorophyll *a* (665).

3. The 77°K chlorophyll *b* absorption spectra in the nonfractionated photosynthetic pigment apparatus and in fractions mainly representing Photosystems 1 or 2 are not significantly different.

4. The aerobic irreversible photobleaching of chlorophyll *b* was studied in the intact pigment complex as well as in fractions mainly consisting of Photosystem 1 or 2. A two-step photobleaching was observed in all cases. The time-course of this bleaching was not significantly different for chlorophyll *b* in both fractions.

5. These results do not indicate that more than a single chlorophyll *b* complex occurs *in vivo*.

INTRODUCTION

In previous studies[1,2] the irreversible aerobic photobleaching of chlorophyll *b* *in vivo* has been examined. In the first paper[1] it was suggested that, provided assumptions concerning the shape of the absorption spectra of chlorophyll *a* (670) and chlorophyll *a* (680) are correct, the time-course of chlorophyll *b* bleaching proceeds more or less exponentially. SHLYK AND NIKOLAYEVA[3] showed that *in vivo* chlorophyll *b* occurs in two physicochemically different states. These authors stated that freshly formed chlorophyll *b* complexes change various properties, such as resistance towards oxidative photobleaching, upon ageing.

The use of a technique different from that originally applied[1], namely establishing the chlorophyll *b* absorption spectrum *in vivo* as the difference spectrum of spinach chloroplast suspensions *vs.* a mixture of chlorophyll *b*-free algal preparations, showed that the chlorophyll *b* photobleaching indeed occurs at two rates in the chloroplast[2]. JUNGE AND WITT[4] observed biphasic absorption changes of chlorophyll *b* upon illumination. These changes are reversible and of a considerably faster rate than that of the irreversible oxidative photobleaching. The two phenomena, therefore, are not likely

to be related to each other. WITT *et al.*[5], however, considered the possibility that chlorophyll *b* functions at two sites in photosynthesis.

The two-step oxidative photobleaching of chlorophyll *b* might point to the occurrence of this pigment in two states *in vivo*. Except for sea lettuce[6], the room-temperature absorption spectra[2,7] do not suggest that the red chlorophyll *b* band is composed of more than one optically distinct component. As low-temperature spectra show a higher resolution, such spectra are established in the present study.

In photosynthesis at least two photochemical systems operate in series to maintain the electron flow (for a review *cf.* ref. 8). There is, however, no general agreement on the distribution of chlorophyll *b* over both photosystems. LOSADA *et al.*[9] suggested that chlorophyll *b* acts in the O_2-evolving System 2 exclusively. Fractionation[10–12] by detergents yielded two kinds of particles representing mainly System 1 and System 2, respectively. In both fractions chlorophyll *b* occurred, be it that the chlorophyll *a*: chlorophyll *b* ratio for System 1 was about 2–3 times higher than that for System 2. This ratio, however, cannot be taken as an indication of the purity of the separated photosystems[13,14]. Others believe that chlorophyll *b* occurs in both photosystems[8]. However, when using the detergent sodium dodecyl benzene sulfate in combination with electrophoresis in polyacrylamide gels, THORNBER *et al.*[15] succeeded in preparing chlorophyll–protein complexes derived from Photosystems 1 and 2 with chlorophyll *a*: chlorophyll *b* ratios about 10 times lower for the latter system than for the former one. Therefore (*cf.* also ref. 16), these experiments do not rule out the possibility that the occurrence of chlorophyll *b* is restricted to Photosystem 2.

A few preliminary experiments[2] did not yield convincing evidence of differences, if any, in oxidative photobleaching characteristics of chlorophyll *b* from System 1 or System 2 enriched preparations. Such experiments are repeated and expanded. Together with data from the low-temperature absorption spectra, these results are considered with respect to the complexity and distribution of chlorophyll *b in vivo*.

MATERIALS AND METHODS

Preparation

Suspensions of chloroplast fragments from spinach, either purchased at the local market or obtained from the botanical gardens, were prepared as described earlier[17], using 0.02 M phosphate buffer (pH 7.3). In fractionation experiments this medium was replaced by 0.05 M Tris buffer (pH 8.0). Fractionation into preparations enriched in either Photosystem 1 or 2 complexes was performed by sodium deoxycholate treatment as described by BRIL *et al.*[13] with the exception that the sodium deoxycholate-containing suspension was centrifuged at 10000 $\times$ *g* for 30 min, at 90000 $\times$ *g* for 30 min and, finally, at 183000 $\times$ *g* for 1 h in a Spinco centrifuge.

Suspensions of chloroplast fragments from the chlorophyll *b*-free algae *Vischeria stellata* and *Tribonema aequale* were prepared by harvesting these algae by centrifugation and filtration, respectively, washing with the mentioned buffers, sonication at 0.8 Mcycles/sec, output 250 W, and cooled with ice for 6 min with Vischeria and 15 min with Tribonema. Cell debris was removed by centrifugation at 2800 $\times$ *g* for 10 min.

Spectrophotometry

Absorption spectra of chlorophyll *b* at about 15° were recorded as difference spectra of spinach suspensions *vs.* chlorophyll *b*-free algal preparations in a Beckman

DK2 instrument. The absorbance of the preparations at the red chlorophyll *a* maximum was adjusted to about 0.7. In each experiment the concentration of the algal preparation was carefully adjusted in such a way that the absorption difference at the wavelengths of negligible chlorophyll *b* absorption around 668 nm equalled that at 750 nm. In this way scattering differences were accounted for reproducibly. This method has been earlier described in more detail[2]. In this paper it has also been mentioned that the chlorophyll *b* spectrum obtained in this way is only approximate. However, as progressive photobleaching widened the "zero region" in between the chlorophyll *b* band and that due to imperfect compensation of chlorophyll *a* absorption in such a way that chlorophyll *b* and chlorophyll *a* absorptions became clearly separated, the approximation seems to be satisfactory.

Absorption spectra of preparations to be compared at 77°K were successively recorded in a Cary Model 14R spectrophotometer, using slide wire 1480670 in the ranges 0–0.5 and 0.5–1.0. Next, the spectra were made to coincide around 668 nm. Then, the "algal" spectrum was substracted from the "spinach" one, and the difference spectrum was plotted. The preparations were cooled down to 77°K in the mentioned buffer media containing 60%, in some cases 65%, high grade glycerol. No cracks occurred when using 1-mm perspex cuvettes.

Bleaching

For simultaneous bleaching of the two kinds of suspensions, two Infolux 150H slide projectors were used. The light intensity at the front wall of the 1-cm glass cuvettes was $2 \cdot 10^6$–$3 \cdot 10^6$ ergs·cm^{-2}·sec^{-1}. The bleaching occurred in a refrigerated box with a perspex window at about 5°.

RESULTS

Low-temperature absorption spectra

An example of a low-temperature chlorophyll *b* spectrum in phosphate buffer is shown in Fig. 1. It represents a difference spectrum of spinach *vs.* Vischeria. The maxi-

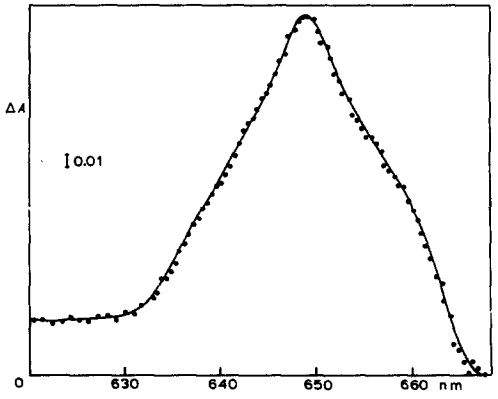

Fig. 1. Absorption spectrum of chlorophyll *b* in untreated spinach chloroplast fragments at 77°K. The plotted spectrum represents the difference spectrum of spinach *vs. V. stellata* preparations in phosphate buffer (pH 7.3). Note that, due to compensation of chlorophyll *a* absorption, no chlorophyll *a* vibrational band shows up around 625 nm. The same is shown in Fig. 3.

mum occurs at 648 nm, and a shoulder shows up around 662 nm. This shoulder is observed in all twelve chlorophyll *b* spectra of spinach chloroplast fragments. The same holds for three additional chlorophyll *b* spectra of *Aspidistra elatior* preparations. For both species the shoulder in less pronounced, though clearly present, if, instead of Vischeria, Tribonema is used as a reference. Therefore, at least part of the shoulder should be due to a component other than chlorophyll *b*.

Since the difference spectrum of red *vs.* far-red pre-irradiated spinach does not show this band, it seems less likely that phytochrome is the pertinent pigment.

Difference spectra of Tribonema *vs.* Vischeria at 77°K show a distinct band with its maximum around 667 nm, and a half-width value of about 9 nm (*cf.* Fig. 2). Therefore, a more probable possibility is that this pigment represents the chlorophyll *a* form, chlorophyll *a* (665)[18–21]. If so, it means that the chlorophyll *a* (665) content is higher in Tribenoma than in Vischeria.

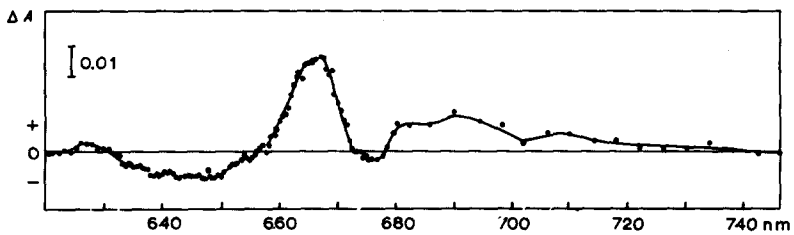

Fig. 2. Difference spectrum of *T. aequale vs. V. stellata* at 77°K in phosphate buffer (pH 7.3).

To find out whether the chlorophyll *b* absorption spectrum *in vivo* differs for both photosystems, particles enriched in either System 2 or System 1 were prepared as described under METHODS by sodium deoxycholate treatment in Tris buffer and subsequent centrifugation. Fig. 3 shows an example of chlorophyll *b* absorption spectra in untreated chloroplasts and both kinds of particle fractions. To facilitate comparison the spectra are adjusted to give the same peak height. For the untreated fragments, the 10000 × *g*, and the 183000 × *g* preparations, the main maximum is located at 649, 649, and 648 nm, respectively. In the same sequence, the half-width values are: 17, 17, and 18 nm. According to the slight scattering of the plotted dots, both the peak location and the half-width value cannot be considered significantly different for the three spectra.

The shoulder around 662 nm is clearly more pronounced in the 183000 × *g* fraction than in the 10000 × *g* one. In the nontreated sample it is intermediate. Four series of such spectra were established. The results, however, varied appreciably. In one series the relative size of the shoulder from the heavy fraction was even somewhat higher than that from the light one.

By subtracting the plotted values of Fig. 3B from those of Fig. 3C, the difference in shape of the chlorophyll *b* spectra between preparations enriched in Photosystem 1 and in Photosystem 2 is obtained. It is shown in Fig. 4. Due to a higher scattering in the preparation of Fig. 3C than that of Fig. 3B, the difference spectrum is distorted. By way of a rough approximation this scattering is indicated by the straight line drawn through the dots at wavelengths shorter than 653 nm. In this way it is shown that the spectrum of "System-1 chlorophyll *b*" does not significantly differ from that of "System-2 chlorophyll *b*" in this region. The band with its maximum at 666 nm is due

to the earlier-mentioned probably non-chlorophyll *b* component. This wavelength does not appreciably deviate from that of the major band maximum from the Tribonema *vs.* Vischeria difference spectrum, Fig. 2. The half-width values for both bands are 8 and 9 nm, respectively. Though no proof of it, these results do not conflict with the possibility that both bands refer to the same pigment.

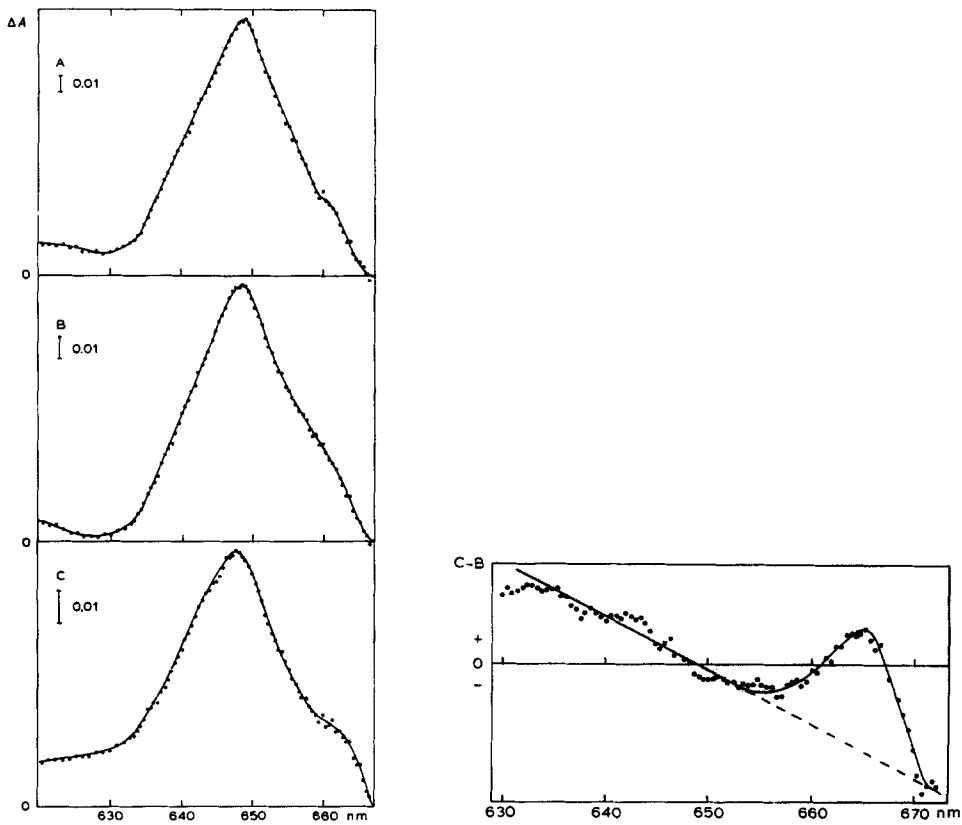

Fig. 3. Absorption spectra at 77°K of chlorophyll *b* in (A) untreated spinach chloroplast fragments and in fractions from sodium deoxycholate-treated samples obtained by centrifugation at (B) 10000 × *g* and (C) 183000 × *g*. The spectra are plotted as indicated in the legend to Fig. 1. Instead of phosphate, Tris buffer (pH 8.0) is used.

Fig. 4. Difference spectrum obtained by subtracting the plotted values of Fig. 3B from those of Fig. 3C. Except for the presence of the 665-nm component (probably non-chlorophyll *b*), these spectra do not show significant differences.

Bleaching

The time-course of the bleaching of chlorophyll *b* in phosphate buffer is shown in Fig. 5. Since these experiments only serve to check the earlier-observed[2] transition from "slow" into "fast" bleaching, the illumination period was not extended beyond 20 min when using phosphate buffer. In the present experiments this transition occurs upon about 12 min of irradiation, whereas formerly[2] it was noticed at about 17 min. The rate of the faster bleaching is about the same in both cases. However, the rate of the present slow bleaching, about 1.5 %/5 min, is half that in the former experiments.

Except for the reference preparation, the experimental conditions are the same. Consequently, this difference might be due to inadequate chlorophyll *a* absorption compensation. Therefore, the bleaching of the total chlorophyll *a* band was checked for both spinach and Vischeria preparations (Fig. 6). Contrary to that of spinach chlorophyll *a*, the bleaching rate of Vischeria chlorophyll *a* is high during the first minute of illumination. Then, it levels off and becomes slightly less than that of spinach. If the chlorophyll *a* compensation were insufficient, in particular the 5-min values (Fig. 5) should be off, and this might even suggest a transition point. However, the 0-, 5-, and 10-min values (Fig. 5) are on a straight line, and the transition occurs at about 12 min. Therefore, the lower rate of the initial bleaching in the present experiments as compared with that observed earlier[2] seems to be due to variations, possibly seasonal, in the properties of the used spinach.

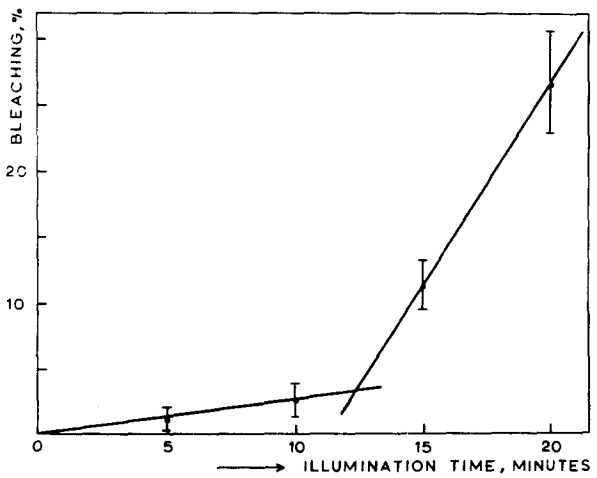

Fig. 5. Time-course of aerobic photobleaching of chlorophyll *b* in a washed suspension of spinach chloroplast fragments in phosphate buffer (pH 7.3). The chlorophyll *a* absorption is compensated for with Vischeria. Values are means of 10 experiments.

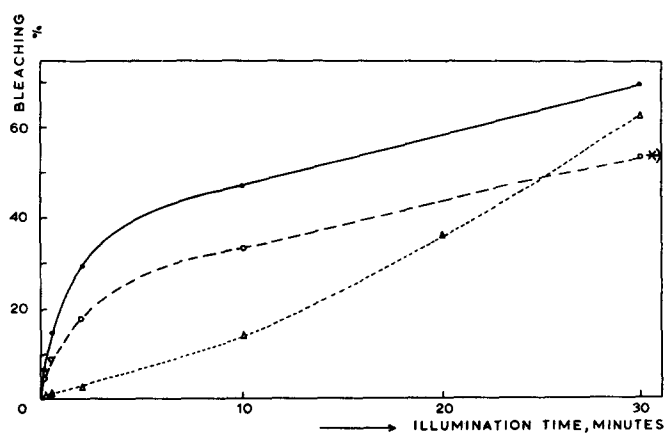

Fig. 6. Bleaching of the total chlorophyll *a* band of spinach (△-----△) and Vischeria (○- - -○), both in phosphate buffer (pH 7.3) and Vischeria in Tris buffer (pH 8.0) (●——●). *) denotes the mean of 2 experiments. Other values are means of 3 experiments.

The initial high-rate chlorophyll *a* bleaching of Vischeria is still more pronounced in Tris buffer (pH 8.0). The bleaching rate after the initial phase is nearly the same as that in phosphate buffer (pH 7.3). These effects will be studied further.

Upon 10 min of bleaching, the 662-mn shoulder on the chlorophyll *b* band (Figs. 1 and 3A) is relatively increased. This ratio remains more or less constant at prolonged bleaching (Table I). Obviously, the 662-nm shoulder starts to decrease later, or initially slower, than the chlorophyll *b* maximum. Even at prolonged bleaching both the location of this maximum and the half-width value of the chlorophyll *b* band, corrected for the 662-nm shoulder, did not change. In general it should be remarked that in the presence of glycerol the bleaching rate is clearly reduced.

TABLE I

RELATIVE INCREASE OF THE SHOULDER ON THE CHLOROPHYLL *b* BAND AROUND 662 nm UPON OXIDATIVE PHOTOBLEACHING

The size of the shoulder is measured as the distance of its maximum in vertical direction to a tangent in line with the slope of the long-wave side of the red chlorophyll *b* band. It is expressed in percent of this size in nonbleached preparations. The results refer to four independent series.

Illumination time (min)	*Intensity of shoulder* (%)
0	100
10	123
20	118, 117
40	110
70	109, 121
210	135

In fragmentation experiments with sodium deoxycholate, the phosphate buffer has to be replaced by Tris buffer (pH 8.0)[13]. In order to check the effect of the latter buffer as compared with that of the former one, a room-temperature difference spectrum of spinach as well as Vischeria in Tris *vs.* phosphate buffer is established (Fig. 7). It is evident that the changes are larger and more numerous in Vischeria than in spinach. This effect renders it impossible to match chlorophyll *a* absorption evenly in the region of chlorophyll *b* absorption, even if the former one is carefully balanced at wavelengths around 668 nm, where the chlorophyll *b* band starts to rise. The discrepancies decrease at prolonged irradiation. However, in the initial stage of bleaching it may erroneously suggest that an increase, not exceeding 5 %, of chlorophyll *b* absorption occurs.

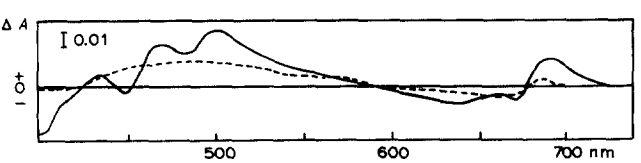

Fig. 7. Room-temperature difference spectra of Vischeria (———) and spinach (------) in Tris buffer (pH 8.0) *vs.* in phosphate buffer (pH 7.3).

Fig. 8 shows the time-course of bleaching for untreated preparations as well as for the 10000 × *g* and 183000 × *g* fractions from sodium deoxycholate treated suspensions. The mentioned effect in Tris buffer can be noticed upon 5 min of irradiation.

Though, therefore, the slope of the curves is not reliable during the initial bleaching stage, the following conclusions may be drawn. (1) The transition from "slow" to "fast" chlorophyll *b* bleaching occurs at about 13 min of illumination for both the untreated sample and the two fractions. In phosphate buffer it occurs at nearly the same time,

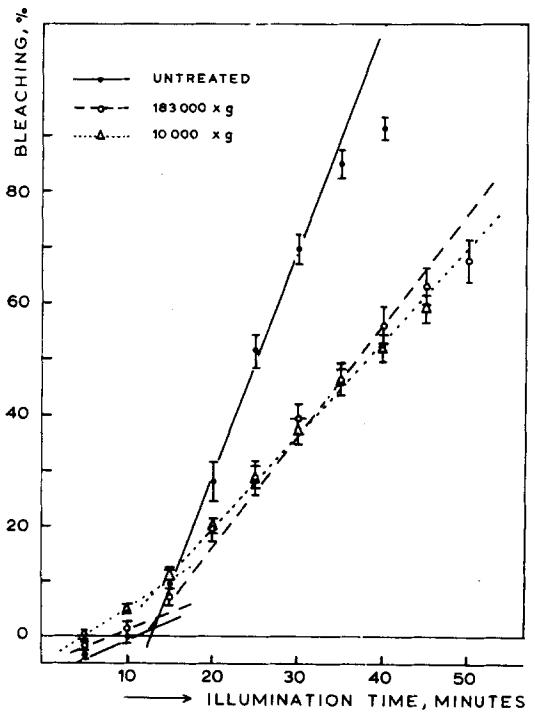

Fig. 8. Aerobic photobleaching of chlorophyll *b* in untreated spinach preparations and two fractions of sodium deoxycholate-treated samples. Each series consists of 10 experiments.

12.5 min (Fig. 5). (2) The bleaching rate of chlorophyll *b* in both fractions is lower than that of the untreated preparation. This effect may by due to sodium deoxycholate micelles embedding the particles and possibly retarding oxygen diffusion. (3) The bleaching rates of chlorophyll *b* in both fractions are not significantly different.

DISCUSSION

The low-temperature absorption spectrum of chlorophyll *b* shows a single peak around 648 nm. A shoulder around 662 nm is always present (Figs. 1 and 3A). It seems likely that this shoulder belongs to a pigment different from chlorophyll *b* for the following reasons:

(1) Its relative intensity depends on the choice of the chlorophyll *b*-free reference suspension.

(2) Location and half-width value of the band (Fig. 4) giving rise to the shoulder in question are the same as those of the main band from the difference spectrum between two chlorophyll *b*-free algae (Fig. 2).

(3) The initial bleaching rate of the shoulder is lower than that of the main, chlorophyll *b* band (Table I).

(4) The shoulder size differs for the heavy and light fractions (Fig. 3, B and C). The bleaching rate is equal for these preparations (Fig. 8). Apart from the shoulder, the shape and location of the red chlorophyll *b* band are the same in both fractions (Fig. 4). In view of (3) it is therefore rather unlikely that chlorophyll *b* from one of the fractions shows a complex red absorption band, namely a main peak and a shoulder, whereas that from the other one lacks the shoulder.

(5) The relative sizes of the shoulder in both fractions differs considerably in various preparations.

As to the nature of the pigment responsible for the shoulder in question, one might think of phytochrome in its form absorbing in the same regions[22-24]. According to C. J. P. SPRUIT (personal communication) this maximum occurs around 666 nm in the undamaged cell or tissue, whereas in aqueous extracts it is located at about 660 nm. However, a difference spectrum of red *vs.* far-red pre-illuminated spinach preparations did not show a trace of a phytochrome band.

The similarity of location as well as half-width value of the major bands at 77°K from the difference spectrum between two chlorophyll *b*-free algae and from that between chlorophyll *b* from both fractions (*cf.* Figs. 2 and 4, respectively), and after correction for the deviation from zero at the band bases, tentatively suggests that both bands refer to the same pigment. As the algae are chlorophyll *b*-free, this pigment, showing up as a shoulder on the low-temperature red chlorophyll *b* band, may be a chlorophyll *a* form, namely chlorophyll *a* (665)[1].

BRAINTAIS[21] observed chlorophyll *a* (665) in System I particles obtained with Triton X-100. However, if low-temperature spectra of both fractions separated with Triton X-100 or sodium deoxycholate are compared, a shoulder due to chlorophyll *a* (665) has been[21,25,13] and is observed in the present experiments again in the heavy 10000 × *g* fraction instead of in the light one. It might be that these seemingly controversial results are due to a smaller degree of overlap of absorption of chlorophyll *a* (680), chlorophyll *a* (670), and chlorophyll *b* in the heavy fraction compared with that in the light one, whereas chlorophyll *a* (665) occurs in both photosystems. Future study may elucidate this problem.

Apart from the shoulder, the 77°K red chlorophyll *b* absorption band in fractions enriched in Photosystem I material looks identical with that in System 2 enriched preparations. The time-course of oxidative photobleaching for chlorophyll *b* from both sources is the same. These results suggest that just a single form of chlorophyll *b* occurs *in vivo*. *In vitro*, chlorophyll *b* is capable of associating in various aggregates[26,27]. Apparently, such different types of aggregates do not exist *in vivo*.

The reason for the two-step character of the time-course of aerobic photobleaching still deserves further attention. It might be due to an increase of sensitivity towards this type of bleaching with the age of the chlorophyll *b* complexes as proposed by SHLYK AND NIKOLAYEVA[3]. It might also be due to the occurrence of both "exposed" and "protected" chlorophyll *b* in the system *in vivo*.

So far, no chlorophyll *b* complex free from chlorophyll *a* has been obtained. Chlorophyll *b*, therefore, is likely to be associated with the protein moieties of the chlorophyll *a* complexes. The chlorophyll *a*/chlorophyll *b* ratios from the literature[16] vary widely for Photosystems I and 2. This variety of values might be due to various

degrees of purity of the obtained fractions. In particular, the results of THORNBER *et al.*[15] suggest that chlorophyll *b* may be associated with a single photosystem, System 2, exclusively. Such would be in line with the present data showing that only a single shape of the red chlorophyll *b* absorption band as well as a single type of time-course for the aerobic photobleaching could be observed. In view of a possible spillover of energy between the photosystems, the occurrence of a chlorophyll *b* band in action spectra for both systems (*cf.* ref. 8) needs not contradict the above hypothesis.

Data on the function of chlorophyll *b* in photosynthesis, other than collecting quanta, are contradictory and too scarce to serve as additional support of the above conclusion. BOARDMAN AND HIGHKIN[28], working with chlorophyll *b*-free barley mutants, stated that chlorophyll *b* is not essential for the biochemistry of photosynthesis. According to ALLEN *et al.*[29], chlorophyll *b* may be involved in the production of the slow ESR signal upon illumination, whereas action spectra made by EGNÉUS[30] suggest that chlorophyll *b* functions in the production of the first spike in the O_2 evolution transient and, thus, in Photosystem 2.

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