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STUDIES ON THE RED ABSORPTION BAND OF CHLOROPHYLL *a* *IN VIVO*

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## SUMMARY

It was studied whether certain earlier observed weak shoulders on the red absorption band of chlorophyll *a in vivo* might represent anomalies due to overlap of absorption bands.

The results are suggestive of the fact that no such anomalies occur. It is therefore concluded that the present study supports the concept holding that, at least, 6 rather than 4 chlorophyll *a* forms may be present in the state *in vivo*.

The true position of the C<sub>a</sub>680 absorption maximum is suggested to occur at a wavelength at least 4 m $\mu$  longer than that actually measured in spectra of cells and chloroplast suspensions.

The data suggest that C<sub>a</sub>667 is likely to function in the Hill reaction with *p*-benzoquinone as an oxidant at least as efficiently as C<sub>a</sub>670.

The possible significance of the chlorophyll *a* forms other than C<sub>a</sub>670, C<sub>a</sub>680, C<sub>a</sub>695 and C<sub>a</sub>700 is discussed.

## INTRODUCTION

It has been recognized by many authors that the red absorption band of chlorophyll *a* is composed of a number of weak shoulders in addition to the main maximum. Each of them, as well as the main maximum, is suggested to be due to absorption by a particular chlorophyll-carrier complex. Such chlorophyll *a* forms maximally absorbing around 670 and 680 m $\mu$ , termed C<sub>a</sub>670 and C<sub>a</sub>680 respectively, have been observed in all green plants studied, *cf.* BROWN AND FRENCH<sup>1</sup>, whereas C<sub>a</sub>695, in smaller amounts, occurs in a number of species, if not all, *cf.* FREI<sup>2</sup>, in particular in *e.g.* Euglena, *cf.* BROWN AND FRENCH<sup>3</sup>. The occurrence of a chlorophyll *a* form with maximum absorption around 700 m $\mu$ , C<sub>a</sub>700, was deduced from difference spectra by KOK<sup>4</sup>, who termed this type "P700". For a more detailed survey, considering also the concepts about the photochemical functions of these types, reference is made to SMITH AND FRENCH<sup>5</sup>.

In addition to the absorption shoulders due to the mentioned types, THOMAS<sup>6,7</sup> observed the occurrence of some more, rather weak, shoulders mainly at the short-wave side of the red absorption band, namely around 664 and 667 m $\mu$  in *Aspidistra* chloroplasts, and around 660, 663, 667, and, possibly, 694 in addition to 698 m $\mu$  in *Anacystis nidulans* cells. THOMAS AND VAN DER WAL<sup>8</sup> studied the extractability of the types in acetone-buffer mixtures. They found that various chlorophyll *a* forms

are extracted at different rates. However, since treatment with acetone causes a shift of the absorption band, unambiguous identification of types with absorption maxima close to each other was not possible.

SMITH AND FRENCH<sup>5</sup> commented upon the conclusion of the first author<sup>6,7</sup>, holding that the number of chlorophyll *a* forms *in vivo* seems to be larger than originally assumed. They remarked that, according to VANDENBELT AND HENRICH<sup>9</sup>, spectral anomalies can be produced by overlapping of absorption bands. The latter authors showed that, under suitable conditions, the number of absorption maxima and shoulders is increased or decreased due to this effect. What happens depends on the distance between the positions of the absorption maxima of the components, the relative intensities, and the half-width values of the separate bands.

There is an earlier mentioned<sup>6,7</sup> argument in favour of the absence of such anomalies. This argument consists of the fact that no coupling between the frequencies of occurrence of the various absorption shoulders was observed. It was felt, however, that the criticism of SMITH AND FRENCH<sup>5</sup> needed additional study. Moreover, since the exact positions as well as the half-width values of the separate bands of chlorophyll *a* forms are unknown, it is impossible to conclude to the actual number of such forms from mere observation of absorption spectra. The present paper deals with additional experiments concerning the various chlorophyll *a* types.

#### MATERIAL AND METHODS

##### *Chloroplast preparations*

Suspensions of *Aspidistra elatior* chloroplasts were prepared as mentioned earlier<sup>6</sup>, with the exception that the 0.02 M phosphate buffer was adjusted to pH 7.2 instead of 6.9.

##### *Heating*

For the heat-treatment experiments, the chloroplast suspensions were heated in sealed reagent tubes in a water bath. The mentioned temperatures refer to the suspensions, whereas the heating periods regard the duration of the exposure to the temperature in question for the individual chloroplasts or cells. The equilibration time, thus, is left out of consideration.

The influence of heating was studied with intact cells as well as aqueous extracts of *Chlorella vulgaris*.

##### *Redox measurements*

The redox potential of chloroplast suspensions was determined potentiometrically with the equipment described earlier<sup>9</sup>. The Hill reaction was measured in the presence of 0.01 M steam-distilled *p*-benzoquinone. Acetone-treated and non-treated preparations were examined alternately, each of them 4–7 times. The redox potential was recorded for 8 min in the dark followed by 2.5 min in the light.

##### *Chlorophyll determination*

The chloroplast suspensions were provided with 4 parts of acetone, and centrifuged at about  $30000 \times g$  for 1 h. The chlorophyll content of the supernatant was determined according to ARNON<sup>11</sup> in a Hilger spectrophotometer.

*Acetone treatment*

The chloroplast suspensions were centrifuged at about 35000  $\times$  *g* at  $-2^{\circ}$  for 20 min. The supernatant was decanted and replaced by the required acetone–buffer mixture. The sediment was resuspended and subsequently centrifuged at the mentioned speed and temperature for 30 min. Next, the supernatant was decanted again and replaced by buffer. The sediment was resuspended, and the suspension filtered over cotton wool. The reference suspension was treated in the same way, except for the fact that buffer solution was added instead of the acetone–buffer mixture. The suspensions were kept in the dark and on ice until use.

*Absorption measurements*

Absorption spectra as well as difference spectra were established with a Beckman DK2 recording spectrophotometer, using 1-cm cuvettes. As a rule, the absorption of the suspensions at 1 cm light path amounted to 60–70 % in the peak region.

## RESULTS

*Addition experiments*

In order to check whether the “addition” of shoulders to absorption spectra of *Aspidistra* chloroplasts might cause the earlier mentioned anomaly effects, the spectra of two, separate, chloroplast preparations of about the same absorbancy but showing different shoulders were established. Next, the suspensions were mixed at a 1:1 ratio, and the spectrum of the mixture was determined. The position of the shoulders in each of the spectra was read. The results are shown in Table I.

TABLE I

POSITION OF SHOULDERS IN ABSORPTION SPECTRA OF SEPARATE AND COMBINED *ASPIDISTRA* CHLOROPLAST SUSPENSIONS

In Expts. 2, 3 and 4 one and the same sample was used for suspension of chlorophyll *a*. (?) indicates that due to the weakness of the shoulder determination of the exact position was difficult.

Experiment	Position of main maximum (*) and shoulders in $m\mu$		
	Suspension <i>a</i>	Suspension <i>b</i>	Suspensions <i>a</i> + <i>b</i>
1	696, 678*, 673, 670, 668, 664, 657	699, 677*, 670, 667	698, 696, 677*, 673, 670, 668, 664, 657(?)
2	693(?), 678*, 670, 667, 664(?)	693(?), 678*, 673, 670, 667, 664(?)	692, 678*, 673, 670, 667, 664
3	693(?), 678*, 670, 667, 664(?)	693, 678*, 674(?), 668, 663	693, 678*, 674, 672(?), 667, 664
4	693(?), 678*, 670, 667, 664(?)	687, 678*, 674, 667, 664	692, 687, 678*, 674, 668, 663

The data suggest that no additional shoulders show up in the spectra of the combined extracts. The position of the shoulders in the combined suspensions is nearly the same as in the individual samples. Differences amounted to 2  $m\mu$  at most. Consequently, the results seem to indicate that absorption anomalies, as discussed by VANDENBELT AND HENRICH<sup>9</sup> are not encountered when working with *Aspidistra* chloroplast suspensions.

*Absorption difference spectra*

*Aspidistra chloroplasts, individual differences.* Considering the fact that the intensity of the individual shoulders may differ for various chloroplast suspensions, it is to be expected that shoulders of different intensities referring to two preparations stand out more clearly in difference spectra than they do in single absorption spectra. Consequently, by studying difference spectra of a number of pairs of chloroplast suspensions, one may envisage the showing up of various chlorophyll *a* forms as maxima or minima. Moreover, if the shoulders observed in the single absorption spectra were due to overlap anomalies, simultaneous occurrence or absence of combinations of maxima or minima should be evident. The results are shown in Table II.

TABLE II

POSITION OF MAXIMA AND MINIMA IN THE RED ABSORPTION REGION OF CHLOROPHYLL *a* IN DIFFERENCE SPECTRA OF PAIRS OF SUSPENSIONS OF ASPIDISTRA CHLOROPLASTS

Densities equalled at main-peak positions. (?) indicates positions of which the interpretation is not certain.

<i>Suspension pair</i>	<i>Position of maxima or minima in m<math>\mu</math></i>
a-b	693, 676, 670, 665(?), 660
c-d	688, 667, 663(?)
e-d	686, 678(?), 666, 663
f-g	684, 670(?), 662
h-g	683, 659
i-g	702, 685, 678

The densities of the suspensions to be compared were adjusted such that the intensities of the main maxima equalled each other as much as possible. Since the scattering conditions varied slightly for the various suspensions, the adjustment could be only approximate. In cases in which the intensities of these maxima did not equal each other, the maximum showed up in the difference spectra around 685 m $\mu$ , that is, at longer wavelengths than it occurs in single spectra. In the latter ones, it is located around 678 m $\mu$ . The reason for this difference is believed to be the same as that to be discussed for the heat-treatment experiments. The absorption at the main maxima amounted to about 70 %.

As an example, a difference spectrum, established with the suspension pair a-b, is shown in Fig. 1.

It should be mentioned that a certain displacement might have occurred for shoulders in the upper region of the absorption bands. Therefore, emphasis is laid on the number rather than the exact location of the maxima and minima. As, again, it is evident from Table II that no simultaneous occurrence or absence of two or more maxima or minima is observed, the results may be interpreted as being indicative of the existence of more than 4 chlorophyll *a* forms once more.

*Chlorella vulgaris cells and extracts, influence of heating.* It has been observed earlier<sup>5</sup> that, as evidenced by bleaching, heating affects the C<sub>a</sub>680 form considerably more than the short-wave types. It, therefore, might be profitable to study the effect of heating more closely by establishing difference spectra of heated and non-heated chloroplasts or cells.

Heating effects were studied with *C. vulgaris* cells and aqueous extracts thereof. The absorption band in the red of this organism also shows various shoulders. In Fig. 2 the results with whole cells are shown. Except for a slightly different position of the maxima and minima, the data with aqueous extracts are essentially the same. In the figure, the occurrence of maxima after 5, 10, and 15 min of heating is plotted *versus* the various pretreatment temperatures. The chlorophyll *b* maximum is included. In these experiments only 4 chlorophyll *a* types, namely  $C_a700$ ,  $C_a693$ ,  $C_a683$ , and  $C_a670$  could be traced without doubt. Showing up of the  $C_a673$  type may be possible, but the pertaining evidence is not satisfactory. Most remarkably, a  $C_a693$  form was observed in the difference spectrum only after a 10-min pretreatment with temperatures up to  $85^\circ$ .

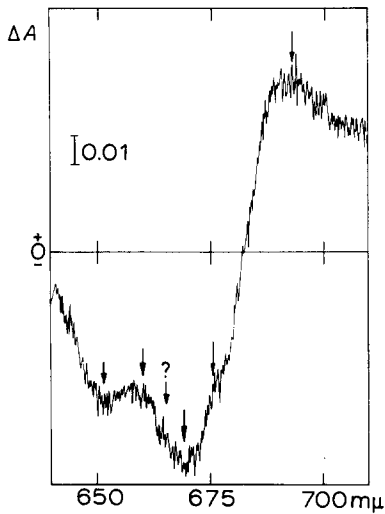


Fig. 1. Difference spectrum of chloroplast suspensions from different *A. elatior* leaves.

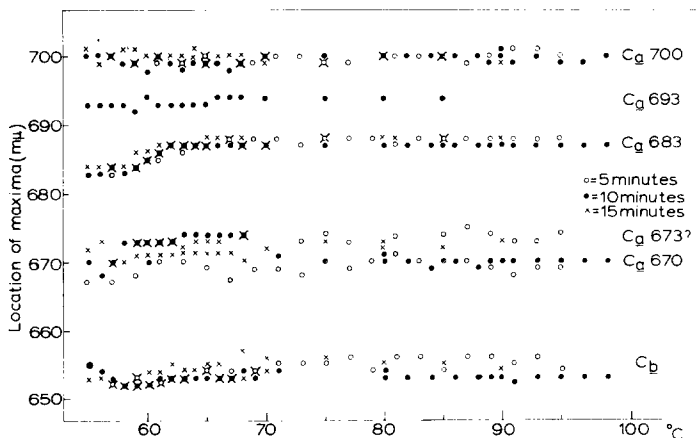


Fig. 2. Effect of heating on the occurrence of absorption shoulders in *C. vulgaris* cells.

Attention is drawn to the fact that the position of the main maximum at 683 m $\mu$  is shifted towards 687 m $\mu$  at temperatures higher than about 60°. A consideration of this phenomenon is postponed till the discussion.

*Photochemical activity*

According to THOMAS AND VAN DER WAL<sup>8</sup>, at least some of the chlorophyll *a* forms are extracted in acetone of various concentrations at mutually different rates. As at least two forms, namely C<sub>a</sub>670 and C<sub>a</sub>680 seem to be involved in photochemical oxygen production, *cf.*, *e.g.* R. GOVINDJEE *et al.*<sup>12</sup> and THOMAS *et al.*<sup>10</sup>, it seemed worthwhile to study the influence of acetone pretreatment on the Hill reaction rate, and to look for a possible correlation between preferential extraction of various chlorophyll *a* forms and rate of the photochemical activity. In Fig. 3 the photochemical activity upon pretreatment with acetone is plotted together with extraction data for some chlorophyll *a* forms and auxiliary pigments, redrawn from ref. 8. The positive and negative  $\Delta A$  values indicate that the chlorophyll *a* forms referring to the curves are extracted less or more readily than the C<sub>a</sub>680 type respectively.

When disregarding the "shift" of the photochemical activity curve relative to the extractability curves for a moment, it can be concluded that, up to a pretreatment acetone concentration of about 25 %, the shape of the activity curve coincides with the shape of the short-wave chlorophyll *a* curves rather than with those for C<sub>a</sub>675, chlorophyll *b*, and the variations around 630 m $\mu$ . This means to say that the photochemical activity per unit chlorophyll follows the concentration ratio short-wave C<sub>a</sub> forms/C<sub>a</sub>680. The closest fit occurs between both the activity and C<sub>a</sub>667/C<sub>a</sub>680 curves.

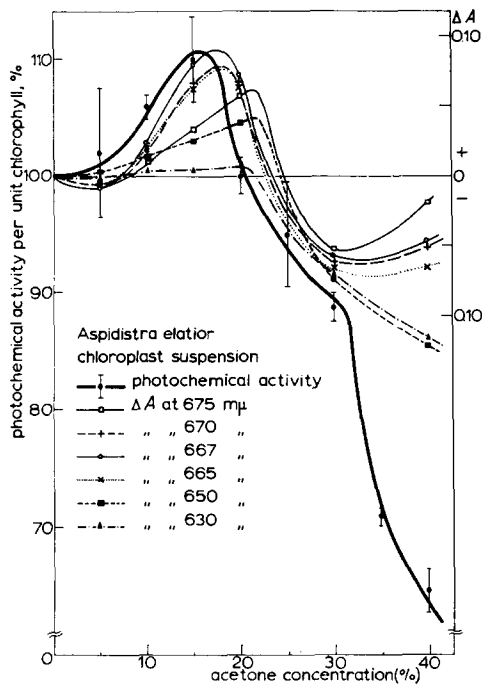


Fig. 3. Effect of acetone on photochemical activity compared with the extractability of various chlorophyll *a* forms in chloroplast suspensions from *A. elatior* in relation to that of C<sub>a</sub>680.

As to the mentioned "shift", disregarded so far, it should be remarked that the chloroplasts stayed in the acetone-containing buffer for 15 more minutes in the Hill experiments than in the earlier absorption measurements<sup>8</sup>. Preliminary experiments convinced us that a 30-min centrifugation in the acetonic medium yielded less scattering activity data than a 15-min centrifugation, as applied with the absorption experiments. This difference may well be responsible for the "shift" in question.

When pretreating with acetone concentrations higher than about 25 %, no correlation between photochemical activity and any short-wave  $C_a/C_a680$  ratio occurred.

This lack of correlation may be due to several reasons, *e.g.* destruction of the involved enzyme systems, or a partial loosening of the chlorophyll-protein bonds. Still, it is remarkable that, after a stay of 30 min in a medium containing as much as 40 % acetone, the photochemical activity did not drop below about 65 %.

#### DISCUSSION

Both the experiments on establishing absorption spectra of separate as well as combined chloroplast preparations from *A. elatior*, and the difference spectra of pairs of non-treated chloroplast suspensions from the same source do not yield evidence for the occurrence of anomalies due to overlap of absorption spectra<sup>9</sup>, as suggested to pertain to the shape of the red chlorophyll *a* band<sup>5</sup>. It is therefore concluded once more that the various weak shoulders on this band actually are suggestive of the presence of an equal number of chlorophyll *a* types. Such would mean that at least 6 chlorophyll *a* forms occur *in vivo*.

In the experiments on heat sensitivity of the various types, however, only 4 chlorophyll *a* forms showed up. There may be various reasons for this discrepancy. For instance, it might be that the types which could not be traced in the difference spectra are relatively insensitive to heat. Another reason, which seems to be more likely, may be due to the considerably increased scattering which forced to use wider slits. It might also be that heat treatment deformed the shape of the absorption spectrum to such an extent that recognition of smaller maxima and minima became obscured. Though, therefore, these experiments are not revealing with regard to the main theme of this paper, an earlier indicated observation may be briefly considered here. It was mentioned that, at temperatures higher than about 60°, a shift of the main maximum from 683 to 687  $m\mu$  occurred. At these temperatures, a considerable amount of the  $C_a680$  form is bleached, whereas the  $C_a670$  absorption is distinctly less reduced. Since the location of the red absorption maximum in the single spectrum is mainly determined by the overlapping red bands of both  $C_a670$  and  $C_a683$  types, whereas in the difference spectra in question the  $C_a670$  influence is greatly cancelled out due to the considerable reduction of the  $C_a680$  form, the true location of the absorption maximum due to the latter form may be better approached with this type of difference spectra than with single spectra. Therefore, the absorption maximum of the main chlorophyll form, usually indicated  $C_a680$ , is likely to be actually located at a wavelength at least 4  $m\mu$  longer than that measured in single absorption spectra.

The question arises whether the various chlorophyll *a* forms exert different functions in the photosynthetic process. GOVINDJEE AND RABINOWITCH<sup>13</sup> first mentioned that at least two chlorophyll *a* forms are responsible for different photochemical reactions. Various authors, *e.g.* DUYSSENS *et al.*<sup>14</sup>, WITT *et al.*<sup>15</sup>, LOSADA *et al.*<sup>16</sup>, ALLEN

AND MURCHIO<sup>17</sup>, and GOEDHEER<sup>18</sup>, considered the different functions of C<sub>a</sub>670 and C<sub>a</sub>680. One of these forms is shown to induce cytochrome oxidation, whereas the other one causes cytochrome reduction upon excitation. KOK<sup>19</sup>, *cf.* also KOK AND HOCH<sup>20</sup>, noticed the oxidation of P700 (C<sub>a</sub>700) upon illumination, whereas KOK AND BEINERT<sup>21</sup> suggested that the oxidized form of C<sub>a</sub>700 is responsible for the production of one of the light-induced electron-spin-resonance signals.

YAKUSHIJI *et al.*<sup>22</sup> and TAKAMIYA *et al.*<sup>23</sup> isolated a water-soluble, photosensitive, chlorophyll-protein complex from *Chenopodium album*. The red absorption band of this complex occurred at 667 or 668 m $\mu$ . One may wonder whether the position of the maximum is slightly changed due to the isolation procedure, and might originally have occurred at 670 m $\mu$ , or not. In this respect, it may be worth while mentioning that, in the suspensions of *Aspidistra* chloroplasts, studied in the present experiments, the absorption shoulder referring to C<sub>a</sub>667 is recognizable nearly as distinctly as that due to C<sub>a</sub>670. The fact that, often, these shoulders occur simultaneously indicates that they are indicative of different forms of chlorophyll *a*.

It was mentioned above that the rate of the Hill reaction with *p*-benzoquinone as an oxidant as well as the ratio of the amounts short-wave C<sub>a</sub>/C<sub>a</sub>680 are affected by acetone at relatively low concentrations in a similar way. The pertaining curves in Fig. 3 indicate that the closest fit occurs for those of the ratio C<sub>a</sub>667/C<sub>a</sub>680 and the Hill reaction rate. Due to the scattering of the data for photochemical activity, it is not possible to conclude that C<sub>a</sub>667 rather than C<sub>a</sub>670, or C<sub>a</sub>665, is active in the studied reaction. However, it seems allowed to state that the data suggest that C<sub>a</sub>667 is likely to function in this Hill reaction at least as efficiently as C<sub>a</sub>670.

Whether the remaining chlorophyll *a* types are essential for the proceeding of different photochemical reactions remains to be seen. It may quite well be that part of these chlorophyll forms is not essential at all with regard to the photosynthetic processes. But even then, a study of these types may be worth while as it might provide information about the various states of the chlorophyll molecules *in vivo* which, in turn, might improve the understanding of the structure of the photo-synthetic apparatus.

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