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TIME COURSE OF SPECTRAL SHIFTS IN THE RED-ABSORPTION BAND AND LOSS OF PHOTOCHEMICAL OXYGEN-LIBERATING CAPACITY IN ISOLATED ASPIDISTRA CHLOROPLASTS

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SUMMARY

1. Out of the various chlorophyll types, C_b650 and C_a680 are preferently labile in isolated Aspidistra chloroplasts.

2. The time course of a thermally induced change of C_a680 coincides with that of the decay of the capability to perform the Hill reaction, either without added oxidant, or in the presence of 10^{-2} M *p*-benzoquinone.

3. This coincidence suggests that excited C_a680, in addition to C_a670, is involved in the photochemical O_2 evolution. Such a conclusion supports the interpretation of earlier obtained indications.

4. No such correlation occurs upon addition of 2,6-dichlorophenolindophenol as a Hill reagent. It is concluded that in case of the presence of this oxidant only C_a670 operates in the oxidative part of the photochemical system.

INTRODUCTION

The red-absorption band of chlorophyll *in vivo* shows a complex structure, *cf.* refs. 1, 2. Various authors reported on the occurrence of weak shoulders superimposed on this band, and ascribed them to the presence of different chlorophyll *a* forms. In a previous paper[1], mention was made of two spectral shifts in the region of the red-absorption band of isolated Aspidistra chloroplasts at room temperature. The occurrence of these shifts suggested that, under such conditions, two forms of chlorophyll change their absorption characteristics, and, possibly, also other properties, with time.

Chloroplast suspensions are capable of evolving O_2 in the light. This Hill reaction is thermo-labile. In general, the mentioned ability is lost for the major part after a 15-min storage at room temperature in the dark. It might be that the loss of the latter property is related to the change mentioned above in the absorption of one or both labile chlorophyll types.

In the same paper[1], this possibility has been mentioned, and a few preliminary results in this respect have been reported. In the pertaining experiments, photochemical O_2 liberation was measured with the Warburg technique. The disadvantage

Abbreviation: DPIP, 2,6-dichlorophenolindophenol.

of such a procedure consists of the requirement of a relatively long period for the establishment of temperature equilibrium. As the major changes occurred during this adaptation period, only preliminary indications were obtained. In the present study, the former experiments are repeated and supplemented with the much faster potentiometric method for measuring redox changes.

MATERIAL AND METHODS

Chloroplast preparation

Chloroplast suspensions were prepared from fresh Aspidistra leaves as described earlier[1], with the exception that the pH of the 0.02 M phosphate buffer was adjusted to 7.2. The preparation was divided into two portions. One of them was diluted with buffer solution so as to adjust its transmission to about 60% at a 1-cm length of the light path. In some experiments the light transmission was adjusted to 30%. The diluted suspension, in its turn, was divided into two samples. Together with the undiluted portion, one of the diluted samples was stored in the refrigerator ("0° sample"), whereas the remaining diluted sample was kept in the dark at about 23°("23° sample"). The storage period lasted from 1.5–2 h.

Absorption measurements

Difference spectra of the 23° sample *versus* the 0° sample were determined with a Beckman DK2 recording spectrophotometer, using 1-cm cuvettes. The recordings were established every 5, 10, or 15 min.

Redox measurements

The reduction capacity of illuminated chloroplasts was measured potentiometrically. Use was made of a vibrating Pt electrode so as to prevent establishment of local concentration differences and to avoid the formation of small gas bubbles at the surface of the electrode. The Hill-reaction rate was determined every 15 min. For each determination 4 ml of chloroplast suspension was used. Such a determination consisted of the following series of measurements: 1.5 min in the dark, 1.5 min in the light, and, again, 1.5 min in the dark. The difference between the slope of the "light recording" and the mean of those of both "dark recordings" was taken as a measure for the photochemical activity. After each series of measurements, the electrodes were rinsed with glass-distilled water twice.

As a light source a 100-W incandescent lamp was used. The light beam was focussed at the plane of the electrode after passing an orange OG5 Schott glass filter. The application of such a filter was needed because of an electrode response to blue light.

The Hill reaction was measured either without addition of an oxidant, in the presence of 10^{-2} M *p*-benzoquinone, or 10^{-4} M DPIP.

Course of the experiments

The time courses of spectral shifts and rate of the Hill reaction were established simultaneously. With the spectral measurements, the cuvette containing the 23° sample was used as a reference. The other cuvette was provided with the 0° sample. The latter sample was taken from the refrigerator and allowed to equilibrate to room

temperature in the dark for 5 min. Then, the difference-spectrum recordings were started. When at 23°, the spectral change of the 0° sample starts to proceed, and, finally, equals that of the 23° sample. With time, therefore, the shape of the difference spectrum approaches a straight line. The amplitudes of the difference "oscillations", *cf.* A and B in Fig. 1, are taken as a measure for the spectral change.

For the determination of the photochemical activity, the 0° sample was taken from the refrigerator and also allowed to equilibrate to room temperature in the dark for 5 min. Then, 4 ml of it were pipetted into the measuring vessel and the activity was determined, whereas the remainder of the sample was kept in the dark at 23°. For the next determination, a new aliquot from the dark-stored sample was taken. In this way, an activity decrease due to illumination was eliminated.

RESULTS

Fig. 1 shows an arbitrary example of a difference spectrum. When comparing this figure with the difference spectrum depicted in Fig. 6 of a previous paper[1], three discrepancies are evident. The first one is trivial: the sign of the "oscillation" is reversed due to a mutual replacement of both cuvettes. The second one concerns the fact that the shape of the "oscillation" is more symmetrical in the present paper than in the earlier one. The third discrepancy consists of a difference in spectral location of the "oscillations" in both figures. The "center" of the major shift in the earlier experiments occurred around 673 mμ, whereas presently this location, as a mean out of 10 experiments, is determined at 672 ± 1 mμ.

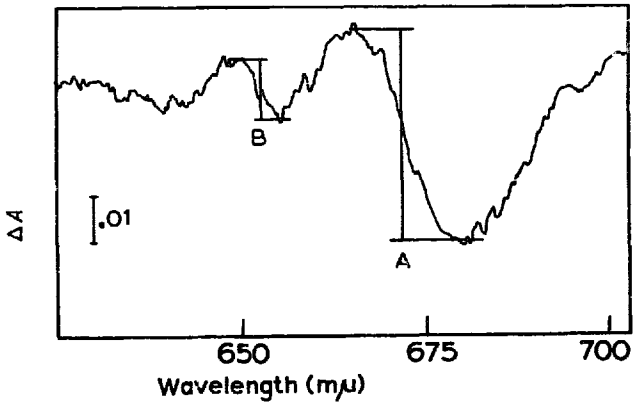

Fig. 1. Difference spectrum of two samples of Aspidistra chloroplast suspensions stored at 0° and 23°, respectively, in the dark for 1.5 h. The 23° sample was placed in the reference position in the spectrophotometer. A, amplitude due to the C_a680 shift; B, amplitude due to the C_b650 shift.

The latter two inconsistencies are to be ascribed to the fact that, formerly, chloroplast suspensions of a 30% light transmission were used, while, as mentioned above, the current experiments are performed with suspensions of about 60% light transmission. These transmission percentages are not corrected for scattering. As in the spectrophotometer the 1-cm cuvettes are adjusted at a large distance, about 12 cm, from the photomultiplier, this apparatus, especially when used for recording of difference spectra, is sensitive towards scattering. The locations of the minor shifts also differ in both studies. Experiments repeated with suspensions of 30% transmission

confirm these conclusions. Moreover, further confirmation is procured by the fact that the time courses of the changes for both suspension concentrations are the same. The present results, therefore, are considered more reliable than the previous ones with regard to the determination of the spectral location for the absorption shifts.

The "phase" of the "oscillation" shows that the shift proceeded towards the blue. The original location of the red-absorption maximum, therefore, should have occurred at a wavelength longer than 672 mμ. In combination with this conclusion, the locations of the maximum and the minimum of the difference spectrum indicate that the original absorption maximum occurred around 680 mμ. The experiments, therefore, suggest that, out of the various chlorophyll *a* types, C_a680 is preferently labile in isolated chloroplasts.

Though in the present experiments the difference spectra are more symmetrical than in the earlier ones, only in some cases true symmetry is encountered. The reason for the occurrence of asymmetrical difference spectra cannot be decided upon with certainty as yet. The asymmetry may be due to three causes: (i) it may be a deformation due to light-scattering, (ii) a fraction of the C_a680 type may be transformed into a derivative absorbing around 670 mμ, whereas another fraction may bleach, and (iii) one and the same fraction of C_a680 may first be transformed in such a derivative, and then bleach. As, with time, the difference spectra approached a straight line, the first reason seems to be the least likely one.

The difference spectra show a minor "oscillation" around 650 mμ. As from measurements with blue-green algae, lacking chlorophyll *b*, no weak shoulders in this region of the red-absorption band could be detected[2], and, moreover, chlorophyll *a* does not markedly absorb in this region even in organic solvents, the minor shift around 650 mμ is tentatively ascribed to chlorophyll *b* (C_b650).

Examples of the time courses of both spectral variations together with that of the decay of the photochemical activity of a chloroplast preparation without addition of an oxidant are plotted in Fig. 2. With the exception that the photochemical activity was measured in the presence of 10^{-2} M *p*-benzoquinone, the same is plotted in Fig. 3. In order to facilitate comparison, the data referring to the spectral shifts are plotted on a percentual scale. For each of these curves, the 100 % value was extrapolated from the determinations.

From Figs. 2 and 3 it is evident that the time course for the proceeding of both spectral shifts is different. The shift due to a change of C_b650 is established much faster than that due to a change of C_a680. In the various experiments, the C_b650 shift is completed within 20–40 min, the C_a680 change needs 1–2 h for completion.

The decay of the potentiometrically determined photochemical activity follows the same time course as the slow shift. The low-activity values are less accurate: they approach the noise level of the apparatus. However, the conclusion about the coincidence of both time courses is supported by the phenomenon that a relatively slow shift of C_a680 is accompanied by an equally slow activity decay, whereas at a relatively fast proceeding of the C_a680 shift, the activity declines equally fast. The examples, depicted in both figures, are selected to demonstrate extreme cases in this respect. The rates of shift and decay are not determined by the presence or absence of benzoquinone. These rates differ with various samples. As the samples were prepared in as much the same way as possible, it may well be that the rates in question depend on the pre-experimental condition of the chloroplasts.

As shown in Fig. 4, no coincidence of the photochemical activity and the slow shift is observed when using 10^{-4} M DPIP as a Hill reagent. With this oxidant, the activity in question did not decline within at least 3 h.

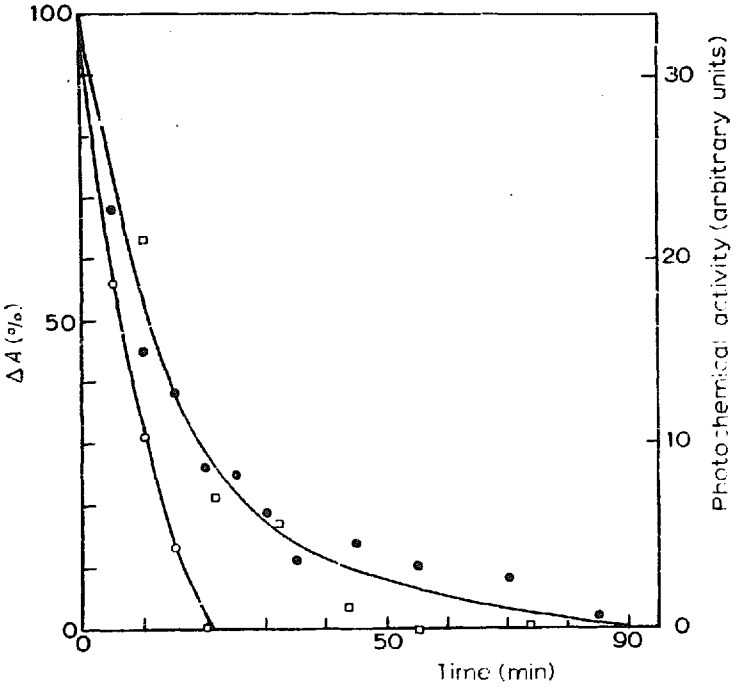

Fig. 2. Time courses of the C_b650 shift (○), the C_a680 shift (●), and the decline of photochemical activity (□) without added oxidant.

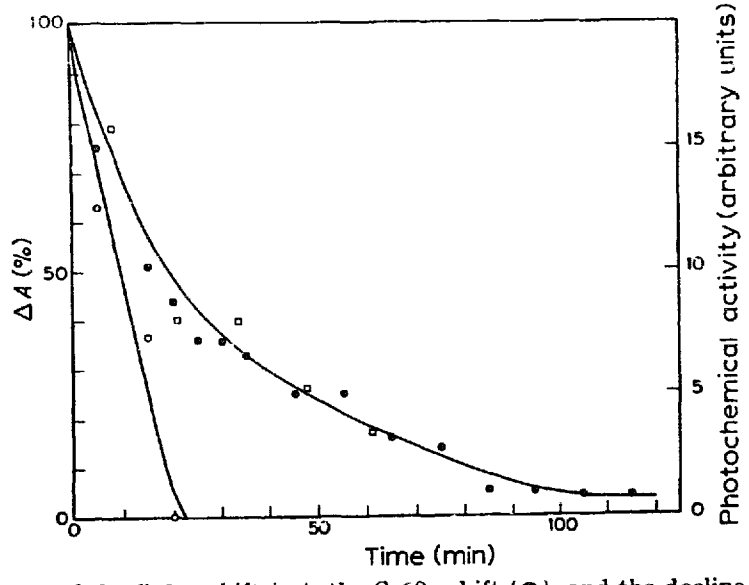

Fig. 3. Time courses of the C_b650 shift (○), the C_a68[illegible] shift (●), and the decline of photochemica activity (□) in the presence of 10^{-2} M *p*-benzoquinone.

DISCUSSION

From the present results three conclusions can be drawn. (1) In isolated chloroplasts two chlorophyll types, indicated as C_b650 and C_a680, change with time at 23°, as evidenced by spectral shifts. (2) The photochemical activity of such chloroplasts,

measured potentiometrically, either with or without addition of 10^{-2} M *p*-benzoquinone declines at the same rate as that of the C_a680 change. (3) No such a correlation occurs in the presence of 10^{-4} M DPIP, instead of quinone, as an oxidant.

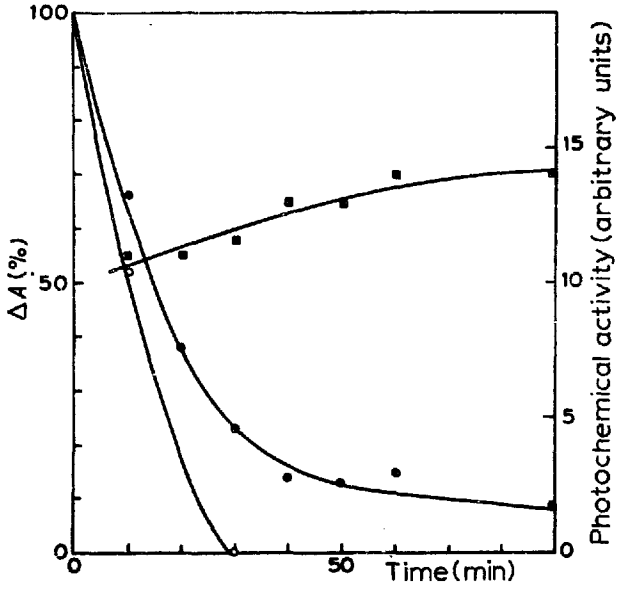

Fig. 4. Time courses of the C_b650 shift (○), the C_a680 shift (●), and of the photochemical activity (□) in the presence of 10^{-4} M DPIP.

The earlier mentioned indication[1] that the Hill-reaction activity in the presence of 10^{-2} M benzoquinone decreases simultaneously with some change in one of the chlorophyll *a* types is confirmed in the present study.

The nature of the observed absorption shifts is not yet elucidated. Nor is it clear why the lability of C_b650 and C_a680 shows up only in isolated chloroplasts. The fact that C_a680 is more labile than the second major chlorophyll *a* type, C_a670, parallels the observations of other investigators, *cf.* SAUER AND CALVIN[3].

The coincidence between the time course of the C_a680 shift and that of the activity decline of isolated chloroplasts in the absence of added oxidants or in the presence of 10^{-2} M quinone suggests that the chlorophyll *a*-type C_a680 is involved in an efficient O_2-liberating mechanism in these chloroplasts.

On the other hand, the lack of such a coincidence in the presence of 10^{-4} M DPIP suggests that a chlorophyll type which did not change during the course of the experiments is primarily responsible for the photochemical activity in the latter case. In terms of the scheme for photosynthesis, proposed by GOEDHEER[4], this type is involved in his "p" system, activated by light of wavelengths shorter than 680 mμ.

Such a conclusion is in keeping with the finding of R. GOVINDJEE *et al.*[5] that the excitation of C_a670, either directly by light absorption or indirectly by transfer of electronic excitation energy from auxiliary pigments, is required for establishment of a fully efficient Hill reaction in the presence of *p*-benzoquinone.

The EMERSON enhancement effect, *cf.* EMERSON *et al.*[6], demonstrates that for maximum rate photosynthesis two pigment systems should be excited. GOVINDJEE AND RABINOWITCH[7] concluded that one of these systems channels the excitation energy into the dark chemical reaction chain via C_a670, whereas the second system operates

via a chlorophyll *a* type absorbing at longer wavelengths. According to these considerations, the present results suggest that both the C_a680 and C_a670 systems are involved in the photochemical activity of isolated chloroplasts in the presence of a natural oxidant, as well as upon addition of 10^{-2} M quinone. However, in the presence of 10^{-4} M DPIP only the C_a670 system seems to function.

The experiments furthermore indicate a way to separate the "short-wave system", *cf.* MYERS AND GRAHAM[8], from the "long-wave system" in a functional respect, namely by storing chloroplast suspensions in the dark at room temperature for about 2 h.

In conclusion it is suggested that in any of the presently studied cases a short-wave component of chlorophyll *a* is involved in the potentiometrically measured photochemical activity of isolated Aspidistra chloroplasts.

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