RESOLUTION OF THE FLUORESCENCE BANDS IN GREENING CHLOROPLASTS OF MAIZE

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SUMMARY

Fluorescence spectra were determined at -196° in chloroplasts of greening maize leaves in order to analyse the structural characteristics of the spectra. Digitonin-treated mature chloroplasts were also investigated. Difference spectra calculated from the emission spectra measured at different stages of greening revealed the presence of six fluorescence forms with approximate band positions at 675, 685, 695, 705, 720, and 738 nm respectively. With progressing greening, the minor forms became less discernible, since the spectral composition reached a steady-state. With exception of the form at 705 nm the components reappeared in mature chloroplasts when treated with digitonin.

In the *in vivo* absorption spectra of chloroplasts, mathematical analysis has revealed at least six different forms of chlorophyll-*a* /1,2,3/. The existence of different chlorophyll forms implies the possibility that fluorescence spectra show the bands corresponding to that of the absorption spectra. A tentative identification of the absorption forms with fluorescence forms has been given by Litvin /3/ and more recently by Heath /4/. According to Heath: Ca 670 → F 683[1], Ca 677 → F 693, Ca 683 → F 710, Ca 692 → F 725, Ca 702 → F 738. We may add to these the "quasi-free" chlorophyll-*a*, Ca 663, which would have an emission at around 675-680 nm /F 675/, in analogy with chlorophyll-*a* in hydrated solutions, fluorescing at 674 nm /cf 5/.

In contrast to expectation, low-temperature fluorescence spectra of mature chloroplasts of higher plants exhibit only three bands, at 685, 695, and 735 nm. With algae /cf 6/, with ζ-carotenic leaves /7/, and with etiolated bean leaves in the early stages of green-

[1]Ca abbreviates the corresponding absorption form of chlorophyll-*a*; F denotes fluorescence band positioned at the corresponding wavelength.

ing /8/, the long-wavelength band of fluorescence has been found at around 720 nm. A concomitant occurence of the emission bands F 720 and F 735 has been demonstrated in chloroplast fragments /9/, and in lutein-treated chloroplast lamellae of Chlorella as well /10/. Although the composite character of the fluorescence bands has already been suggested /3,9/, experimental evidence with intact chloroplasts has not been reported.

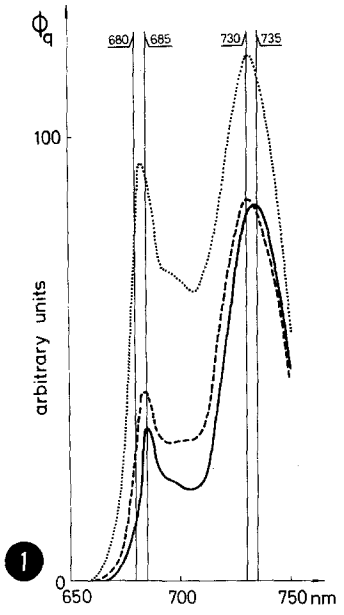
The studies presented here were undertaken to resolve experimentally the fluorescence bands of chloroplasts. Chlorophyll forms fluorescing at 675, 705 and 720 nm were shown to occur in chloroplasts of greening maize leaves. F 675 and F 720 also emerged from mature chloroplasts when treated with digitonin.

MATERIALS AND METHODS

Leaves of 7 day-old seedlings of maize /Zea mays L, var. MV 861/ were harvested after different periods of illumination of 2500 lux white light at 25°. Mesophyll and bundle sheath chloroplasts were isolated by the method earlier described /11/ in the buffer of Anderson and Boardman /12/ containing 0.3 M sucrose, 0.05 M phosphate and 0.01 M KCl, pH 7.4, supplemented by 0.02 M Na-ascorbate and 0.005 M cysteine HCl and 10 mg/l bovine serum albumin /Sigma/. Chloroplasts were collected by a centrifugation at 3000 g for 20 min, and suspended in the isolation medium.

In fragmentation experiments, mature chloroplasts were obtained from plant material grown under greenhouse conditions. Chloroplasts were incubated for 30 min at 5° in the isolation medium containing 0.3 per cent digitonin /molar amounts of digitonin to chlorophyll were adjusted to a ratio of 40:1/. Particle fractions enriched in different photosystems were separated by subsequent centrifugations /12/. The fraction collected at 144000 g /60 min/ could be divided into two subfractions: one cemented to the wall and another which was floating at the bottom of the tube. This floating fraction contained appreciable amounts of the supernatant.

Samples for fluorescence measurements were diluted to an absorbance of 0.01 at the red maximum with a cell thickness of 0.2 mm, and immersed in liquid N_2. Fluorescence was excited at 435 nm with a band width of 5 nm. The light was provided by a 500 W Xenon arc lamp, and transmitted through an ISP-51 monochromator. Emission spectra were determined with a Zeiss SPM 2 grating mo-



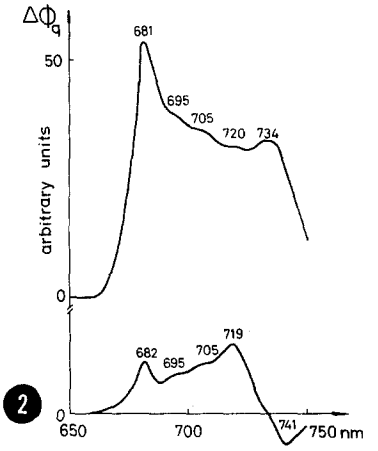


Fig. 1. Emission spectra of mesophyll chloroplasts of maize in different stages of greening determined at -196°. /Illuminated for 9 /.../, 18 /---/, and 48 /——/ hours respectively./

Fig. 2. Difference spectra calculated from the emission spectra of greening chloroplasts. /Upper: calculated between the curves of 9 and 18 hours, lower: between 18 and 48 hours of greening./

nochromator /400 mm, 650 lines/mm, blazed at 570 nm/. The slit was adjusted to a band width of 4 nm. The spectra were detected by an EMI 9558 multiplier and were corrected for the response of the emission monochromator and photomultiplier. Relative intensities were checked with the aid of a plexiglass containing Rhodamine G, which was set in one of the sample positions. Intensities were normalized on chlorophyll-a content, which was separately determined in ethyl ether solution by the two-wavelengths method /13/

Results were obtained from 3-7 independent experiments.

RESULTS

Greening chloroplasts. Low temperature emission spectra obtained with chloroplasts in different stages of greening, demonstrated a gradual decrease of the fluorescence yield with progressive chloroplast maturation. Spectra of mesophyll chloroplasts prepared from

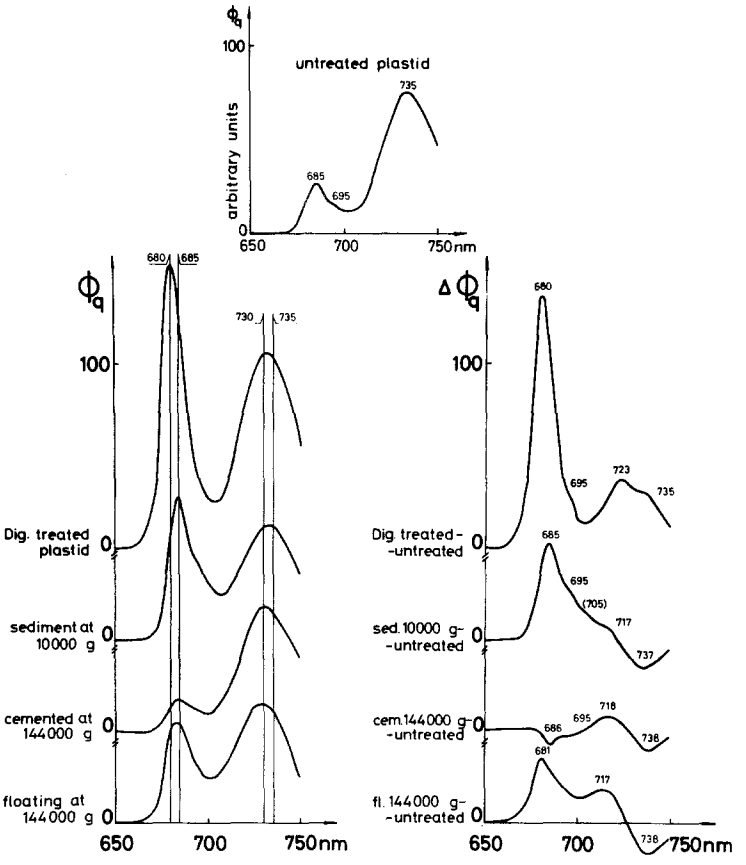


Fig. 3. Emission spectra of intact and digitonin-treated mature chloroplasts isolated from the mesophyll of maize leaves determined at -196°. /Upper: intact chloroplast, left coloumn: emission spectra obtained after digitonin-treatment, right coloumn: difference spectra calculated from the corresponding emission spectra./

the leaves after 9, 18, and 48 hours of illumination are shown in Figure 1. The intensity of the short-wavelength band dropped faster than that of the long-wavelength emission. A concomitant shift in the peak positions /683-685 nm, 731-735 nm/ could also be observed. Difference spectra calculated between the spectra of chloroplasts, illuminated for 9 and 18, as well as for 18 and 48 hours /Fig. 2/, showed a rather composite character. It is evident that the difference spectra contained more bands, than the three main visible components in the emission spectra. In the difference spectra additional bands, F 705 and F 720 were also discernible. The short-wavelength band of the difference spectrum at 681 /682/ nm, being positioned lower than the bands of the emission spectra, indicated

a changing amount of a band below 685 nm /tentatively identified as F 675/ participating in the short-wavelength fluorescence. Similar phenomenon was observed both with mesophyll and bundle sheath chloroplasts obtained from leaves after 6 to 48 hours of greening. With the advance of greening, F 675, F 705, and F 720 decreased in intensity. After 48 hours of greening, when 50-60 % of the maximum chlorophyll content had been synthetized, no further changes of the emission spectra occured suggesting that a constant composition was achieved.

Digitonin-fragmentation. F 675 and F 720 could be reestablished in mature mesophyll chloroplasts when treated with digitonin /Fig. 3/. As a result of the solubilizing and/or disruptive effect of digitonin the emission spectra resembled in yield, shape, and peak position to those obtained with juvenile chloroplasts. The difference spectra showed clearly the band F 720, especially prominent in the particle fractions of 144000 g. High amounts of F 675 resulted in the 2-5 nm shift of the short-wavelength fluorescence maximum of the digitonin-treated chloroplasts and of the small particles contaminated with the supernatant. In digitonin-treated chloroplasts or fractions, F 705 was hardly discernible.

DISCUSSION

Our results show that in greening chloroplasts the presence of six fluorescence forms can be demonstrated. This can be interpreted by the consideration that in earlier stages of greening the distance and orientation of chlorophyll molecules may be less favourable for the transfer of energy between the different chlorophyll forms, and thus the emission at 675, 705, and 720 nm is represented in higher amounts. Consequently the composite character of the main bands can easily be observed.

Digitonin-treatment acts against the structural perfection achieved as a result of the greening process, since it destroys the links between the chlorophyll forms.

In conclusion our results provide experimental evidence in support the ideas postulating the existence of a number of different chlorophyll forms /1,2,3,14/, and suggesting the composite character of the bands in the fluorescence spectra emitted by chloroplasts /9/.

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