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The Development of the Intense Circular-Dichroic Signal during Granum Formation in Greening Etiolated Maize

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In greening maize mesophyll, circular dichroism (c.d.) revealed the early formation of protein–chlorophyll complexes, followed by unorganized chlorophyll. The intense c.d. [Gregory & Raps (1974) *Biochem. J.* **142**, 193–201] appeared later still, whereas membrane–membrane contact (stacking), measured from electron micrographs, appeared much earlier. Isolated grana, which still showed stacking, lost 92% of their original intense c.d.; intense c.d. is not therefore simply dependent on stacking.

The organization of chlorophyll has been studied in the mesophyll chloroplast of maize (*Zea mays* L.), by means of circular dichroism (c.d.). Our results show that it depends on the stage of the process of greening in how far the chlorophyll–protein complexes contribute to the intense c.d. signal characterized by Gregory & Raps (1974). The c.d. of the complexes appears in the first 5h, and is followed by the synthesis of chlorophyll, which has a weak c.d. usually associated with unorganized pigment. The intense c.d. appears later. Membrane–membrane contact (stacking) was measured from electron micrographs, and there was a considerable delay between the development of stacked membranes and the subsequent appearance of the intense c.d. signal.

Mature chloroplasts were disrupted by ultrasound, and grana were separated from stroma lamellae. The grana showed swollen loculi, but apparently preserved their membrane–membrane contact, and the intense c.d. signal was decreased to some 8% of that of the original chloroplasts.

It is concluded that the intense c.d. signal, although clearly associated with the grana, is not simply dependent on the presence of membrane–membrane contact.

Chlorophyll *a* is organized in the chloroplast; for example, there is a well-established division of the pigment between two chlorophyll–protein complexes and free, or less firmly bound, pigment (see, e.g., Thornber, 1975). Circular dichroism (c.d.) has been used to characterize these forms (Gregory *et al.*, 1974; Scott & Gregory, 1975). Examination of whole chloroplasts (containing grana) reveals a c.d. signal which is some ten times greater than that of the isolated complexes. The origin of this intense c.d. signal is not clear, but it is apparently connected in some way with the functioning of the granum (see Gregory, 1975).

In the present paper we investigate the appearance of the c.d. signal in greening etiolated maize and its loss when chloroplasts and grana are disrupted.

Materials and Methods

Seedlings of maize (*Zea mays* L., var. M.V. 861) were grown on moist filter paper at 25°C in complete darkness. Leaves of 9-day-old seedlings were harvested after different periods of illumination (white light, 2500lx). Pieces (0.5cm × 2.0cm) were cut from the first leaves and used for the measurement of absorption and c.d.

Mesophyll chloroplasts were isolated from green leaves grown under ordinary greenhouse conditions in the medium described by Anderson & Boardman (1966), containing 0.3M-sucrose, 0.05M-sodium phosphate and 0.01M-KCl, pH7.2. Samples (1g) were blended twice in a VirTis 45 blender (10s at 20000r.p.m.). The homogenate was filtered through a sieve (pore diam. 30μm), and the chloroplasts were collected by centrifugation at 1500*g* for 10min. Chloroplasts were resuspended in a hyperosmotic medium (Jacobi, 1971) containing 1mM-$MgCl_2$, 1mM-Tricine and 0.3M-NaCl, pH7.8. This medium preserved the granum structure during the ultrasonic treatment (Ohki *et al.*, 1971).

Isolation of grana was performed by using Jacobi's (1971) procedure in an MSE 100 disintegra-

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tor operating at a frequency of 20kHz (amplitude 2μm). The grana were released from the stroma lamellae by four 20s runs at 20s intervals. Unbroken chloroplasts were removed by centrifugation at 1500*g* for 10min, and the grana were collected after spinning at 2500*g* for 10min. Grana were resuspended in the isolation medium. In some experiments the grana were disrupted by digitonin treatment (Anderson & Boardman, 1966).

For electron microscopy, small pieces of leaves and granum pellets were immersed in the fixative of Karnovsky (1965) and Millonig's (1961) osmic acid. The samples were dehydrated in alcohol and embedded in Durcupan (Fluka). Thin sections were cut on a Porter–Blum ultramicrotome and stained by using Reynold's (1963) procedure. Electron micrographs obtained by using a J.E.O.L. 100/B electron microscope were analysed for the length of stacked membranes per unit area of chloroplast section.

Absorption spectra of leaves and isolated grana were recorded in a Unicam SP.1800 spectrophotometer in a sample position for turbid materials by using Shibata's (1959) opal-glass method.

C.d. measurements were made in a JASCO 40C spectropolarimeter. The samples were placed close to the multiplier window (distance 12mm). Granum suspensions for c.d. measurements were adjusted to an E_{678} of 0.3 in 1mm cuvettes. The c.d. of greening chloroplasts was measured *in situ*, i.e. in the leaves. This procedure avoided the destruction of the fragile chloroplasts that are characteristic of the early stages of greening, and gave valid results as judged from the perfect match between the c.d. spectra of green leaves and isolated green chloroplasts.

Results and Discussion

Fig. 1 shows absorption and c.d. spectra at four stages of greening in etiolated maize leaves. At 5h of greening the c.d. can be resolved into components representing complexes CP, CPII and free pigment, characterized by Scott & Gregory (1975). During the next interval, the principal change is the accumulation of chlorophyll, a greater proportion of which is in the free-pigment form. At this stage, there is no contribution from any positive component at 684nm; the 684nm signal does, however, appear at 18h. This is at the expense of the free pigments; that is, this pigment fraction acquires or takes part in a new form of organization.

In Fig. 2 the average magnitude of the positive c.d. component normalized for unit absorbance at 684nm is plotted against the time of greening. The membrane stacking in the mesophyll chloroplasts is also shown, expressed as the length of stacked membranes per unit

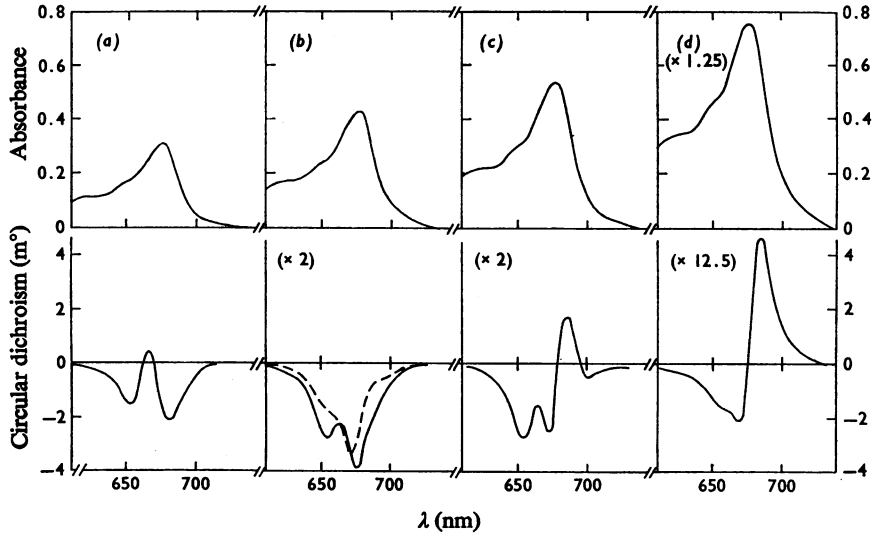

Fig. 1. *Absorption and c.d. spectra of maize leaves at various stages of greening*

C.d. is shown as ellipticity. Measurements were performed on intact leaves grown in darkness and illuminated for (*a*) 5h, (*b*) 12h, (*c*) 18h and (*d*) 42h. ----(*b*, lower part), calculated difference between c.d. spectra of (*b*) and (*a*) normalized to the absorbance of (*b*), and demonstrates the presence of a large amount of free or loosely bound chlorophylls. The ellipticity measured is the value of the ordinate multiplied by the factor shown.

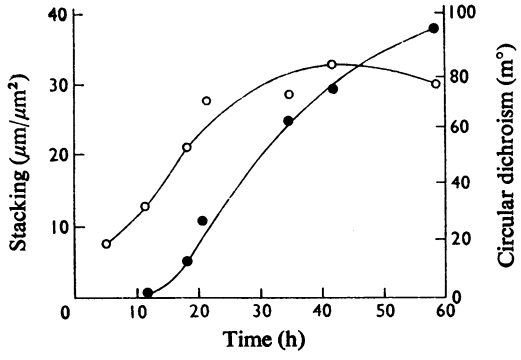

Fig. 2. *Changes in the magnitude of the intense c.d. and membrane stacking in the course of greening*

Intense c.d. characterized by the ellipticity at 684nm and normalized to unit absorbance determined at 678nm are shown. Membrane stacking was measured as the length of stacked membranes per unit area of chloroplasts sections. The points show the average of the values obtained from four to seven chloroplasts characteristic for the respective stage of greening.

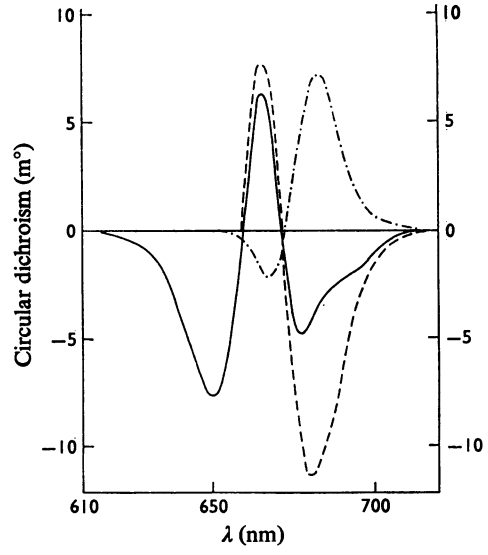

Fig. 3. *C.d. spectra of grana and granum fragments isolated from mesophyll chloroplasts*

—— (*a*), Preparation obtained with ultrasonic treatment; ----- (*b*), the same as (*a*) after digitonin fragmentation; ·-·-· (*c*), (*a*) minus (*b*). Ellipticity values were normalized to unit absorbance measured at 678nm. For other details, see the Materials and Methods section.

area of sections. Some stacking is present at the earliest times examined, whereas the intense signal develops with a relative delay of some 10h. This relationship is not influenced by the demonstration by Horváth *et al.* (1975), that the greening of bundle-sheath cells precedes that of the mesophyll.

It was established by Faludi-Dániel *et al.* (1973) that mature bundle-sheath chloroplasts have a three-banded c.d. signal, despite their possession of rudimentary grana. Therefore the intense signal requires more than just membrane–membrane contact.

Mature mesophyll chloroplasts from leaves grown normally were subjected to brief treatment with ultrasound, and separated into fractions containing isolated grana, and stroma lamellae with fragments of grana. Electron micrographs showed that the grana possessed swollen loculi, but apparently retained their membrane–membrane contact. On further disruption with digitonin (or by further sonication, which was less satisfactory owing to chlorophyll destruction) there was a loss of a positive c.d. component at 684nm, interpreted as a remnant of the intense signal in the grana (some 8% of the original chloroplasts), as shown in Fig. 3.

This observation is a further indication that membrane–membrane contact is only part of the origin of the intense c.d. signal.

Additional factors in the granum which could be responsible for the intense c.d. signal are (i) the multiplicity of thylakoids (8–12 at maximum development), (ii) the local concentration of chlorophyll per unit area of membrane, and (iii) the presence of CPII, which is specific to grana, if the results of Machold (1975) are taken as refuting the claim that CPII is absent from the chlorophyll *b* mutant of barley (Thornber & Highkin, 1974).

The c.d. signal may indicate a chlorophyll *a*–chlorophyll *a* interaction responsible for efficient energy harvesting in the granum. When we understand the origin of the intense c.d. signal, we shall be nearer to understanding the purpose of grana, which are such a striking and widespread feature of plants.

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