

Differential Light-Scattering of Granal and Agranal Chloroplasts and their Fragments

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Intact (class-A) granal and agranal maize chloroplasts and chloroplast fragments were examined for differential scattering of circularly polarized light (measured at 90°) and c.d. (circular dichroism) (measured at 0°) by using a modified spectropolarimeter with a large acceptance angle. Useful c.d. information was obtained by making corrections for scattered light. Chloroplast fragments exhibited a large and characteristic differential scattering of circularly polarized light recognizable in the presence of granal chloroplasts. It is confirmed that agranal chloroplasts do not have the intense 682 nm c.d. peak that is assigned to the presence of grana.

The degrees of organization of chlorophyll in the photosynthetic apparatus have been resolved by the application of c.d. (circular dichroism); specific interactions between chlorophylls and between chlorophylls and protein have been demonstrated in protein-chlorophyll complexes (Gregory *et al.*, 1972; Scott & Gregory, 1975), and a much larger signal has been shown in intact chloroplasts, which has been claimed to be due to grana (Gregory *et al.*, 1972; Faludi-Dániel *et al.*, 1973). C.d. spectra of particle suspensions are subjected to more or less serious scattering distortions due to the unequal scattering of left and right circularly polarized light. Consequently there has been experimental and theoretical interest in the effect of scattering on the c.d. of chloroplasts and fragments (Philipson & Sauer, 1973; Gregory & Raps, 1974).

Gregory & Raps (1974) have studied the spectral distribution and angular dependence of intensity in the differential scattering of circularly polarized light by granal chloroplasts, and have shown that the character of c.d. spectra was changed only slightly by scattering distortions.

In previous work we could show that selective scattering of chloroplasts is largely influenced by ultrastructure and intactness (Bialek *et al.*, 1977). Therefore one might expect that spectral distribution and/or intensity of differential scattering of circularly polarized light are also affected by the structural characteristics of the chloroplast material.

In the present paper we are concerned with the organization of chlorophyll in the chloroplast as

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revealed by c.d. and light-scattering. We examine the differential scattering of right and left circularly polarized light from granal and agranal chloroplasts, and from chloroplast fragments. It is shown that there is differential scattering at an angle of 90°, but that it does not significantly affect measurements of c.d. It is also shown that the 90° differential scattering from agranal chloroplasts and fragments is sufficiently large and characteristic to be investigable even in the presence of granal material.

Materials and Methods

Mesophyll and bundle-sheath chloroplasts were prepared from the first leaf of maize (*Zea mays* L., var. MV 660) seedlings grown in a greenhouse for 9-11 days. Class-A chloroplasts were isolated from suspensions of mesophyll protoplasts and bundle-sheath cells produced by enzymic degradation of the cell walls (Rathman & Edwards, 1976; Bialek *et al.*, 1977). The protoplasts and cells were suspended in the medium described by Anderson & Boardman (1966). The cell suspensions were disrupted by passage through a hypodermic needle of 0.5 mm diameter. Cell debris was sedimented by centrifugation at 400g_{av.} for 1 min and the supernatant was used for taking measurements. Preparations of granal chloroplasts were judged to be up to 90% intact on the basis of the restriction on the entry of K₃Fe(CN)₆ and O₂ evolution (Heber & Santarius, 1970). This test was not applicable to bundle-sheath chloroplasts, which are deficient in O₂-evolving capacity, and therefore electron-microscopic analysis was applied. Under the electron microscope, such

preparations were seen to contain 90% class-A chloroplasts, and cross-contamination did not exceed 5%.

Class-B chloroplasts were prepared by differential grinding of the mesophyll and bundle-sheath tissue (Faludi-Dániel *et al.*, 1973) in the medium used for suspending class-A chloroplasts. Chloroplasts were collected by centrifugation at $1200g_{av}$ for 10 min and suspended in the isolation medium. Preparations of mesophyll chloroplasts obtained by this method contained mainly class-B chloroplasts with 5–10% class A and some class C. Preparations of bundle-sheath chloroplasts contained more (possibly 20–30%) of class-C chloroplasts. Cross-contamination of the samples varied between 5 and 20%.

Fragmentation of chloroplasts was carried out by digitonin treatment (100 mol of digitonin/mol of chlorophyll). After 30 min incubation at 5°C the samples were diluted with 4 vol. of suspending medium to minimize the molecular solubilization of the chloroplast material. Unbroken chloroplasts were sedimented by centrifugation at $1200g_{av}$ for 10 min and the supernatant was used for measurements.

The baseline for the differential scattering of circularly polarized light was obtained with suspension of bleached chloroplasts and fragments. Bleaching was performed by a procedure based on that of Doucha & Kubin (1976). The suspensions were made up to 0.3% peroxyacetic acid (Persteril; Chemicke Zavody, Czechoslovakia), adjusted to pH 7.2 and illuminated through a 3.0 cm water filter with a 250 W high-pressure mercury lamp. The progress of bleaching was checked in a spectrophotometer by using Shibata's (1959) method. At the end of 20–25 min treatment, the suspensions did not show any appreciable absorbance characteristic of chloroplast pigments, and the A_{750} (due only to light-scattering) was about the same as for untreated materials. The bleached chloroplasts were sedimented at $1200g_{av}$ for 10 min; the bleached fragments at $144000g_{av}$ for 60 min. The supernatants were decanted and discarded and the pellets suspended in appropriate amounts of suspending medium. Under the electron microscope the thylakoid network was seen to be fairly well preserved.

Ellipticity measurements were carried out in a JASCO 40/C spectropolarimeter fitted with a special cell holder, which allowed the collection of light at either 0° or 90° angle, by means of a fibre-optic light guide leading to the photomultiplier (see Fig. 1). The spectropolarimeter detects the difference in the intensity of light reaching the photomultiplier when the sample is illuminated in turn with right and left circularly polarized light. At an angle of 0° this is effectively a measure of the ellipticity of the trans-

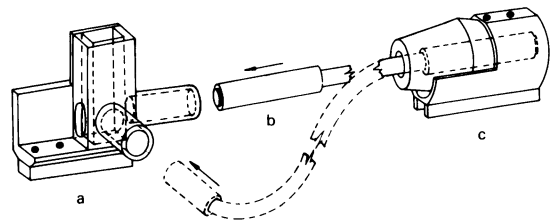


Fig. 1. Cell holder and light guide for measurement of light scattered at 90° in the JASCO 40/C spectrophotometer. The beam travels from left to right. The cell holder (a) is connected to the light-guide (b) in one of two positions, corresponding to 0° and 90° . The guide terminates in a block (c) such that the end of the guide is against the window of the photomultiplier.

mitted light. At 90° , however, the measurement is better described as differential scattering of circularly polarized light, although units of ellipticity are retained for convenience. The light guide attenuated the light by some 50%, but had no effect on the c.d. signal as judged with the tris(ethylene-diamine) cobalt iodide monohydrate standard supplied by JASCO, and with chloroplast suspensions of large (200m°) and weak (2m°) signals. The metal parts were blackened to minimize reflections. The acceptance angle was approximately 36° .

The samples were adjusted to a relative absorbance of 0.7 at 670 nm with respect to an arbitrary zero at 750 nm in a $10\text{mm} \times 10\text{mm}$ fluorescence cell. This high concentration was required to obtain a sufficient intensity of light scattered at 90° . Under these conditions, differential fluorescence could not be detected. Since the intensity of light scattered at right angles by the medium was too low to actuate the instrument, suspensions of bleached material were used to obtain baselines for the ellipticity measurements both at 0° and at 90° . The bandwidth was set at 8 nm, and measurements were made at room temperature.

The intensity of forward scattering by various samples was estimated from the photomultiplier-voltage indication (which had been previously calibrated) with samples at various distances from the photomultiplier. The differences in photomultiplier voltage, expressed in terms of an absorbance value, indicate the relative loss in light-intensity with a decreased acceptance angle, and could be regarded as a measure of the relative extent of forward scattering.

Results and Discussion

Fig. 2 shows the ellipticities measured at 0° and 90° with mesophyll chloroplasts, both when intact and when fragmented with digitonin. The 0° (trans-

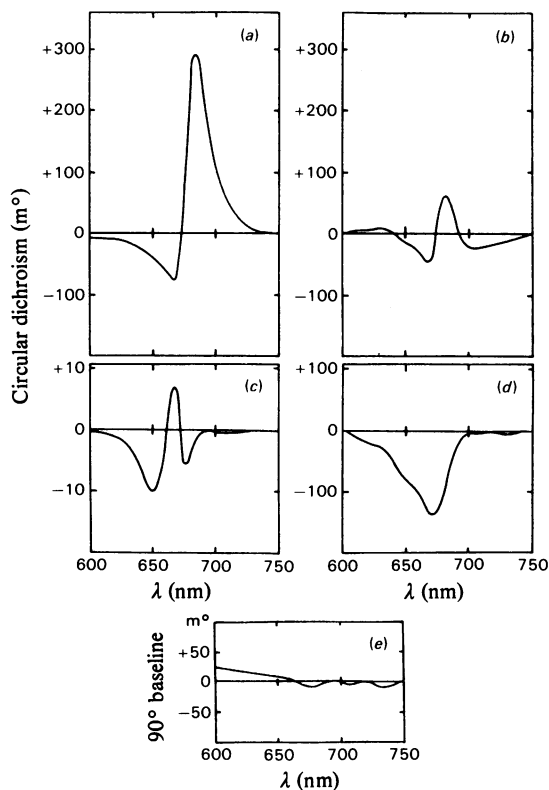


Fig. 2. Ellipticities of transmitted and scattered light from intact maize mesophyll chloroplasts and fragments. Intact chloroplasts: (a) 0° (transmitted); (b) 90° (scattered). Digitonin-prepared fragments: (c) 0°; (d) 90°. (e) Scattering of bleached chloroplasts at 90° used as a reference baseline in (b) and (d). Scattering of bleached fragments was the same.

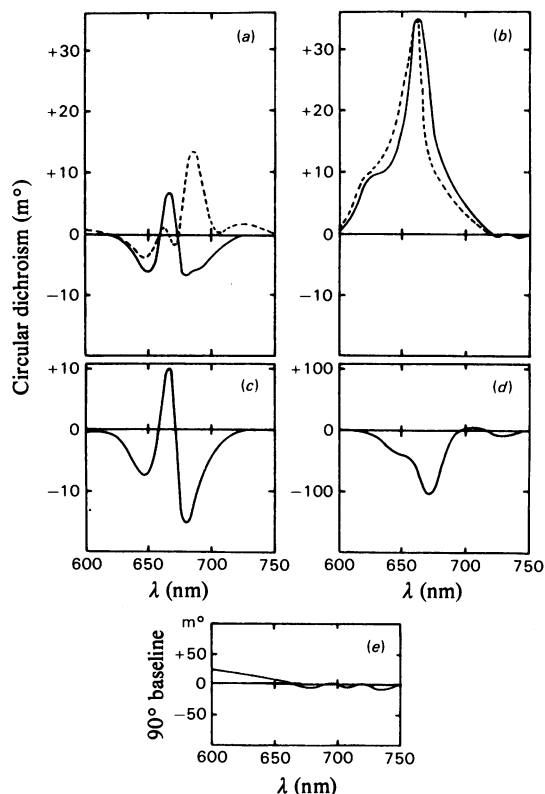


Fig. 3. Ellipticities of transmitted and scattered light from intact bundle-sheath chloroplasts and fragments. (a) 0° (transmitted): (i) dashed curve, class-A chloroplasts; (ii) solid curve, class-B chloroplasts. (b) 90° (scattered): (i) dashed curve, class-A chloroplasts; (ii) solid curve, class-B chloroplasts. (c) and (d) fragments prepared by digitonin from class-B chloroplasts; 0° and 90° respectively. (e) the scattering of bleached chloroplasts (initially class A), measured at 90° and used as a reference baseline in (b) and (d).

mission) c.d. (Fig. 2a) is in agreement with previously published work; it should, however, be noted that for transmission c.d. at these concentrations there is an error due to the obscuring effect of light-scattering (the A_{obs} of Urry & Krivacic, 1970). The error discussed by Gregory & Raps (1974) is less serious here, because the photomultiplier of the JASCO J-40C instrument collects a wider angle of scattered light than does the Cary 61 CD.

On disrupting the lamellae with digitonin (Fig. 2c), we found the characteristic c.d. signal reported by Gregory *et al.* (1972) and a new scattering effect shown in Fig. 2(d), a large negatively elliptic scattering at 670 nm.

The scattering at 90° from intact mesophyll chloroplasts (Fig. 2b) can be interpreted as a peak with positive ellipticity at 682 nm superimposed on the negative peak at 670 nm described above. The positively scattering peak is presumed to be the

same phenomenon as the low-angle scattering peak reported by Gregory & Raps (1974). In the present batch of maize the ellipticities are higher than previously observed, and much more than have been observed in peas.

In Fig. 3, results for bundle-sheath chloroplasts are presented. The transmitted c.d. of digitonin-produced fragments is similar in the two types of chloroplasts (Figs. 2c and 3c). This three-banded spectrum is also found in intact bundle-sheath chloroplasts [Fig. 3a(i)], often together with a fourth, small (682 nm) positive component [Fig. 3a(ii)] as has been reported previously (Faludi-Daniel *et al.*, 1973). However, the use of this result to support the hypothesis that the high magnitude, positive c.d. signal at 682 nm was only found associated with grana was criticized on the basis that

the preparative method might have damaged the agranal chloroplasts, with loss of c.d. There is some precedent for lability in chloroplast structure in that the chlorophyll *b*⁻ mutant of barley (*Hordeum vulgare*) (Highkin, 1950) requires much more gentle rupture of its cells than the wild-type if the intense c.d. signal is to be observed (Gregory & Raps, 1975). The prior isolation of bundle-sheath cells by methods which are mechanically mild, followed by a mild rupturing process, gives a preparation of chloroplasts of which 90% are intact as judged by electron microscopy. In fact there is a positive 682nm c.d. signal present in these chloroplasts [Fig. 3a(i)], but to an extent only 4% of that of mesophyll chloroplasts. While it is possible that this represents an intrinsic c.d. of bundle-sheath chloroplasts, perhaps due to the small amount of stacking that has been shown to exist (two or three thylakoids at a time), it is more likely that most of it is caused by mesophyll contamination (3±2% as shown by the electron microscope).

The 682nm positive c.d. signal in intact bundle-sheath chloroplasts is small enough to show that the genuine characteristic of single lamellae is a three-banded spectrum. The fourth band (682nm) indicates the presence and the degree of membrane stacking. In bundle-sheath chloroplasts the c.d. spectrum is relatively unchanged on disintegration of the membranes with digitonin (Fig. 3c), except for the freeing of the negative 679nm band from the effect of the 682nm positive signal, and a general intensification, probably due to the virtual elimination of the obscuring effect of light scattering.

The 90° scattering from intact bundle-sheath chloroplasts shows a peak of positive ellipticity at 660nm [Fig. 3b(i)]. This cannot be explained readily by reference to the 682nm positive signal, or the 670nm negative signal (Figs. 2d and 3d). There is little contribution from the 682nm peak; the spectra of Figs. 3b(i) and 3b(ii) are similar.

The fragments obtained by means of digitonin show the same sharp negative differential scattering of circularly polarized light at 670nm in both types of chloroplasts (Figs. 2d and 3d). This scattering pattern is very recognizable at 90° even in the presence of grana, or cellulosic contamination from bundle elements, either of which cause too much scattering at low angles for observation of the 670nm effect by the method of Gregory & Raps (1974).

Urry & Krivacic (1970) showed that a differential scattering spectrum was closely related to the difference in the refractive indices for right and left circularly polarized light, that is, the optical rotatory dispersion (o.r.d.). It is, however, clear from comparison of the differential scattering spectra of fragmented chloroplasts (Figs. 2d and 3d) with o.r.d. spectra of chloroplast fragments obtained by,

Table 1. Intensity losses from the measuring light due to scattering by granal and agranal chloroplasts and their fragments

The acceptance angle was changed from 4.2° to 0.7° by varying the distance between the sample and the photomultiplier. *I*, intensity of light reaching the photomultiplier; $\Delta E = \log(I_{4.2^\circ}/I_{0.7^\circ})$.

	$\frac{I_{4.2^\circ}}{I_{0.7^\circ}}$	ΔE
The suspension contained:		
Granal chloroplasts	4.0	0.6
Agranal chloroplasts	4.0	0.6
Fragmented granal chloroplasts	1.0	0.0
Fragmented agranal chloroplasts	1.0	0.0

for example, Ke (1965) and others, that the expected agreement does not apply. We offer no explanation at this time.

Gregory & Raps (1974) have shown that scattering of granal chloroplasts is virtually confined to a narrow forward cone. A similar profile was found with agranal chloroplasts when intensity losses at various acceptance angles were studied (Table 1). These indicate that, with the large acceptance angle used in the measurement of c.d. spectra, most of the scattered light was collected. Consequently, the c.d. spectra of both kinds of chloroplasts were practically free of scattering artifacts (Bohren, 1977).

With chloroplast fragments the scattering was too weak to be detected by the method applied. The photomultiplier voltages recorded by the spectropolarimeter were used to calculate the intensity of the light scattered in relation to that transmitted. It was found that the scattered light was only about 1% of the transmitted beam. Therefore the differential scattering of circularly polarized light, although in itself a large signal, results in a negligible alteration in the ellipticity of the transmitted beam.

As shown in the present work, characteristic differences between the c.d. of intact granal and agranal chloroplasts are not to be attributed to differential scattering. Hence the large c.d. signal characteristic to grana can be interpreted as intrinsic c.d. indicating extensive, chiral, chlorophyll-chlorophyll and chlorophyll-protein interactions.

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