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The Differential Scattering of Circularly Polarized Light by Chloroplasts and Evaluation of their True Circular Dichroism

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Chloroplasts isolated from pea leaves display an intense circular dichroism in the range 600 to 720nm. Circularly polarized light is also differentially scattered by chloroplasts, and this effect can be confused with circular dichroism. By using an instrumental modification it was possible to distinguish, and record separately, the ellipticities of the transmitted light (circular dichroism) and of the scattered light in the same c.d. instrument. By means of a light-scattering apparatus, the intensity of unpolarized light scattered by chloroplasts was measured as a function of wavelength and of angle. This measurement allowed the aforementioned ellipticities to be corrected for mutual interference. At a concentration of 4μg of chlorophyll/ml (the optimum practical concentration of chloroplasts at which there was no significant interaction of scattering and absorption effects) spectra of true circular dichroism (circular differential absorption) and circular differential scattering were obtained. The former showed maxima, positive at 688nm and negative at 676nm, with an intensity $\Delta\theta = 8.3\,m^\circ \cdot litre \cdot (mg\ of\ chlorophyll)^{-1} \cdot cm^{-1}$. The latter had a maximum at 683nm with an intensity of +47m° with respect to the solvent baseline; this value is independent of the concentration of chloroplasts in dilute suspensions. It is suggested that the intense circular dichroism of chloroplasts reflects specific chlorophyll–chlorophyll interactions in the light-harvesting pigment. The advantages of this method for determining the c.d. of scattering suspensions over those of other investigators are discussed.

As more knowledge about the light reactions of photosynthesis is gained, it becomes evident that a close examination of chlorophyll–chlorophyll and chlorophyll–protein or chlorophyll–lipoprotein interactions has to be made to understand the manner in which light energy is converted into chemical energy. This is especially true of chlorophyll *a*, which has a multiple role in the photosynthesis of green plants. It is the most abundant pigment for the absorption of light, it provides an efficient energy-conduction system between the point of light-absorption and the photosynthetic reaction centres, and it is intimately associated with the operation of the reaction centres themselves.

One of the methods currently available for the examination of the organization of chlorophyll involves the analysis of c.d. (circular-dichroism) spectra of chlorophyll at its various levels of organization, e.g. chlorophyll–protein–detergent complexes, thylakoid fragments, chloroplasts and whole cells. One great advantage of c.d. measurements over the more established methods of fluorescence and absorption spectroscopy is that whereas monomeric chlorophyll has a larger fluorescence than aggregated pigment, and an absorption of the same order, it has a very small intrinsic c.d. compared with the very large c.d. effects obtained from chlorophyll–chlorophyll or chlorophyll–protein interactions. Therefore there is no need for a large subtraction to be made to eliminate the effect of the monomeric material. A second advantage is that the qualitative form of a c.d. spectrum can be analysed to provide *prima facie* evidence of the type of interaction present (dimer, trimer, etc.). These advantages are shared with the related technique of o.r.d. (optical rotatory dispersion). C.d. is preferred to o.r.d., since the curves obtained are qualitatively more simple, and are restricted to narrower wavelength ranges, representing the absorption range of each chiral chromophore.

The c.d. spectra of small thylakoid fragments in the 600 to 720nm region of the spectrum (the chlorophyll region) show that chlorophyll–chlorophyll interactions play a major role in the origin of the c.d. (Dratz *et al.*, 1967). Thylakoid fragments broken down by anionic detergents result in chlorophyll–protein–detergent complexes (Thornber *et al.*, 1967) associated with photosystems I and II (Gregory *et al.*, 1971). These complexes exhibit c.d. Gregory *et al.* (1972) suggest that the c.d. spectra of these complexes, when added together, approximate that of the

thylakoid fragments, and conclude that the chlorophyll of the complexes is the main aggregated chlorophyll of the thylakoid fragments.

It is of interest to compare this data with c.d. spectra of whole chloroplasts and even of whole cells. C.d. spectra of large chloroplast fragments and whole chloroplasts are more intense in relation to chlorophyll concentration than those of the smaller fragments. The c.d. spectra of the two also differ, the former being two-banded and the latter three-banded. However, any conclusions resulting from these spectra have to take into account the contribution of differential circular light-scattering, since scattering is pronounced in chloroplast suspensions, whereas small thylakoid fragments and chlorophyll–protein–detergent complexes are almost clear and scattering is negligible. To carry out a valid study of chlorophyll at various levels of organization, the contribution of differential circular scattering to the c.d. of intact chloroplasts, and to scattering samples in general, has to be evaluated.

In the present paper, an analysis is presented by which the c.d. signal is resolved into two components, one being due to differential scattering, and the other to the differential absorption of right- and left-handed circularly polarized light (true c.d.). Therefore discussions concerning the interactions of chlorophyll molecules in light-harvesting arrays can now include the evidence from scattering samples which are more closely related to living photosynthetic systems.

Philipson & Sauer (1973), in a paper published while the present paper was in preparation, claim that the contribution of differential scatter to chloroplast suspensions is very pronounced and difficult to ascertain (or to separate from the total c.d.). They suggest that this differential scatter is responsible for the observed c.d. pattern of chloroplasts and that, if it were eliminated, the c.d. of intact chloroplasts would approximate to that of fragmented ones. We take issue with their conclusions and offer an alternative explanation of their data.

Materials and Methods

Whole chloroplasts (type B; Hall, 1972) were prepared from the leaves of greenhouse-grown peas (*Pisum sativum*, var. Meteor). Leaves (20g) were homogenized in an MSE Atomix blender at top speed for 10s in 100ml of grinding medium consisting of 8mM-Na_2HPO_4, 8mM-KH_2PO_4 and 0.33M-NaCl (pH6.8). The brei was strained through nylon bolting cloth (25μm mesh; Henry Simon Ltd., Stockport, U.K.) and centrifuged at 4°C for 1min at 500*g*. After decanting the supernatant, the pellet of chloroplasts was suspended, by using cotton wool and a glass rod, in a medium containing 0.33M-sorbitol, 20mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid], 5mM-KCl, 8mM-Na_2HPO_4, 1mM-$MgSO_4,7H_2O$ and 3mM-$Na_2CO_3,10H_2O$, adjusted to pH7.6 with NaOH.

The concentration of chloroplasts used in all of the experiments was equivalent to 4μg of chlorophyll/ml unless otherwise stated. Chlorophyll determinations were done as described by Mackinney (1941).

The Cary 61 CD Spectropolarimeter (Varian Associates, Monrovia, Calif., U.S.A.) was used for all c.d. measurements. It was modified to make light scattering determinations as follows. The sample cavity was cleared, and the cuvette was placed on a blackened metal block so that its image was focused on to the photocathode by the collecting lens. A blackened metal plate, perforated by a 1mm diam. aperture, was placed adjacent to the cuvette, thus decreasing the height of the beam entering the cuvette. This decreased the effect of stray light, and made the emergent light beam and the scattered light effectively radially symmetrical around the optical axis. The emergent beam itself was prevented from reaching the photomultiplier by suspending a blackened metal disc (1.72cm diam.) immediately in front of the collecting lens in the exit aperture of the sample compartment. Light scattered at angles between 2.5° and 4.6° could pass through the annulus formed between the disc and the edge of the collecting lens and be brought to a focus on the phototube. This arrangement is shown in Fig. 1. The disc was easily removed for the determination of direct c.d. (keeping the cuvette and perforated plate in position). Direct c.d. so determined contains a contribution from light scattered at angles between 0° and 4.6°. Dynode

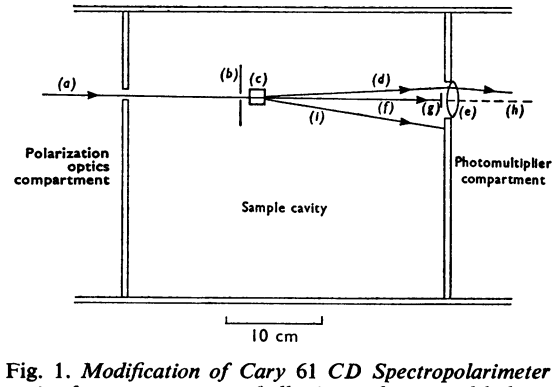

Fig. 1. *Modification of Cary 61 CD Spectropolarimeter optics for measurements of ellipticity of scattered light at angles between 2.5° and 4.6° (plan view)*

(*a*) Monochromatic circularly polarized light beam, (*b*) perforated metal plate, (*c*) cuvette, (*d*) scattered ray focused by (*e*) lens on to photomultiplier, (*f*) direct light beam blocked by (*g*) metal disc, (*h*) optical axis of incident light, (*i*) light scattered beyond collecting angle of lens.

voltages were recorded for chloroplasts and medium alone in both scattering and transmission modes (i.e. with and without the disc respectively), and from these the corresponding intensities of light reaching the photomultiplier were determined according to the handbook supplied with the spectropolarimeter. D-Camphor-10-sulphonic acid (BDH Chemicals Ltd., Poole, Dorset, U.K.) (0.1%) was used as a c.d. calibration standard ($\theta^{0.1}_{291} := 304 m^{\circ} \cdot cm^{-1}$).

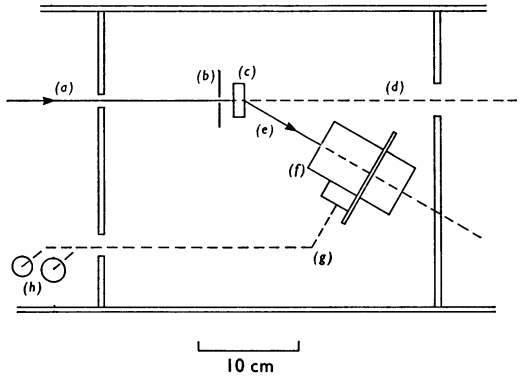

Fig. 2. *Modification of Cary* 61 *CD Spectropolarimeter optics for measurements of ellipticity of scattered light at angles between* 12° *and* 40° (*plan view*)

(*a*) Monochromatic circularly polarized light beam, (*b*) perforated metal plate, (*c*) cuvette, (*d*) optical axis of incident light, (*e*) scattered ray entering (*f*) photomultiplier assembly (with a 1.2cm-diam. aperture) held in a clamp, (*g*) extension leads to (*h*) sockets. The length (*e*) could be varied from 2 to 8cm.

To observe differential scattering at angles greater than 4.6°, a second modification was made (Fig. 2). The photomultiplier assembly was removed from its compartment, mounted on a laboratory stand in the sample cavity, and connected by extension leads to the sockets in the polarization-optics compartment. A collecting lens was not used, the photomultiplier being placed close to the cuvette. The minimum angle that could be accurately investigated without the primary beam interfering was 12°. At angles greater than 40° the intensity was insufficient to actuate the instrument.

In the Results section we present calculations deriving the true c.d. from the apparent c.d. as recorded by the spectropolarimeter in the transmission mode. The principle is that at each wavelength the ellipticity and intensity of the scattered light are measured, and the intensities of right- and left-handed circularly polarized light scattered outside the collecting angle of the detector are calculated and added back to the transmitted beam. Thus light scattered is treated as transmitted, not absorbed. This is justified in the Appendix.

An essential part of determining the total amount of light scattered by a sample involves knowing its dependence on wavelength and angle. For this purpose a Brice–Phoenix Light-Scattering Photometer was used. It was modified by substituting a 250W xenon-arc lamp (Osram XBO 250) as the light source, incorporating a monochromator (Beckman DU) operated with a slit-width of 0.04mm, by using an extended-red S20 photomultiplier (R446HA) and

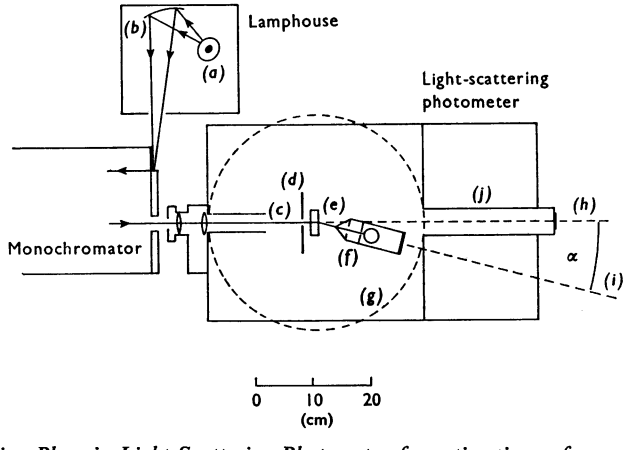

Fig. 3. *Modification of Brice–Phoenix Light-Scattering Photometer for estimations of wavelength and angle dependence of scattering* (*plan view*)

(*a*) 250W xenon-arc lamp, (*b*) focusing mirror, (*c*) incident light beam, (*d*) perforated metal plate, (*e*) cuvette, (*f*) photomultiplier, (*g*) turntable graduated in degrees, (*h*) optical axis of incident beam, (*i*) axis of photomultiplier, (*j*) light-trap tube, (α) scattering angle.

removing the working-standard assembly (Fig. 3). [As a check on the proper functioning of the modified light-scattering photometer determinations were carried out of the molecular weight of solutions of standard polystyrene in benzene, by setting the monochromator to 540nm wavelength and 0.2mm slit-width. The results, when plotted as described by Zimm (1948), were of the expected form and magnitude.] A 1mm-aperture perforated plate, similar to the one described for the c.d. measurements, was used. For angles less than 40° a rectangular cuvette was used, 3cm×4cm in cross section with a 1cm light-path. At greater angles, a cylindrical cuvette with a 3.5cm light-path was used. Light incident on the cuvette was less than 5% polarized. Scattering measurements could be made with accuracy down to 2.5°. Curves were plotted of the intensity of scattered light I_s at angles (α) between 2.5° and 136° (it was impossible to observe at angles greater than 136°) at wavelengths between 600 and 720nm. From the same data, using $I_s \sin\alpha$ to obtain the spherical distribution, the proportion of light scattered between 2.5° and 4.6° to that scattered between 4.6° and 136° was determined. This ratio, called the annular fraction for any wavelength, enables the light scattered in the c.d. instrument at angles greater than 4.6° to be determined from the known intensity of light scattered into the annulus, i.e. from 2.5° to 4.6°.

Results

To analyse the contribution of light-scattering to the c.d. of chloroplast suspensions, it was necessary to determine the dependence of the light scattered on the wavelengths within the range of chromophore absorption. Fig. 4 shows that the wavelength dependence of light scattered by a dilute suspension of chloroplasts was similar at all angles measured. (The use of a logarithmic ordinate scale allows for a better comparison of the shapes of the spectra at the various angles.) The shape of the scattering spectrum is independent of angle.

An assessment of the proportion of light scattered into the annulus (2.5°–4.6°) of the c.d. collecting lens to that scattered outside (4.6°–180°) at the appropriate wavelengths was also necessary. Fig. 5 shows the angular dependence of the light scattered at 660nm. The curves obtained at other wavelengths were proportional, within experimental error, indicating that the ratio of light scattered within the annulus to that outside is invariant with wavelength throughout the spectral region of interest (600–720nm). The annular fraction obtained by dividing the area A, indicated in Fig. 5, by B, at all wavelengths was 2.83±0.40. Fig. 6 shows that the amount of light scattered at angles above 40° is negligible.

In both Figs. 4 and 5 the values have been corrected for the effective pathlength seen by the photomulti-

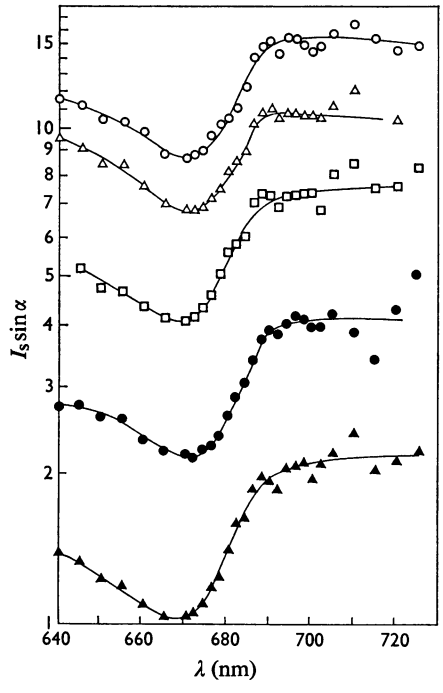

Fig. 4. *Wavelength dependence of light scattered by chloroplasts*

Measurements were made in the modified Brice–Phoenix photometer. The chlorophyll concentration was 4μg/ml, slit-width 0.04mm; 1cm light-path. The shape of the scattering spectrum is shown at five angles to give the characteristic distribution. △, 2.5°; ○, 0.5°; □, 7°; ●, 10°; ▲, 15°.

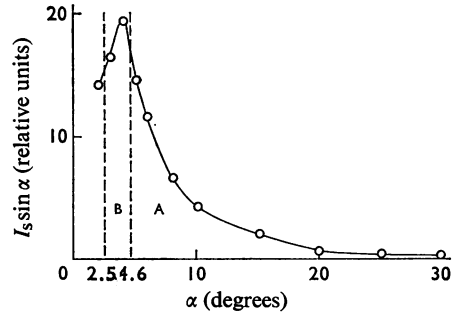

Fig. 5. *Angular dependence of light scattered by chloroplasts at 660nm*

Measurements were made in the modified Brice–Phoenix photometer. The chlorophyll concentration was 4μg/ml, slit-width 0.04mm; a rectangular cuvette with a 1cm light-path was used. The annular fraction was obtained by dividing area A by area B. For further details see the text.

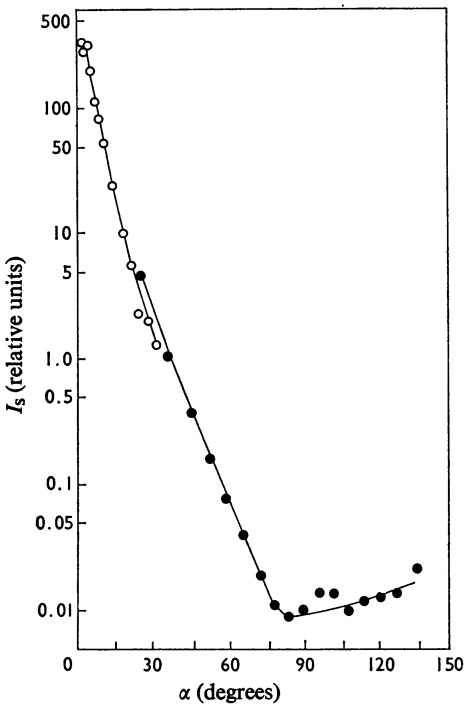

Fig. 6. *Intensity of light scattered by chloroplasts between 2° and 136° at 680 nm*

Measurements were made in the modified Brice–Phoenix photometer. The chlorophyll concentration was 4 μg/ml. ○, Values obtained at low angles by using a rectangular cuvette, 1 cm light-path; slit-width 0.1 mm. Angles are corrected for refraction. ●, Values obtained by using a cylindrical cuvette, 3.5 cm light-path; slit-width 0.7 mm. Note that the two curves have not been combined since errors are greatest in the region of overlap. The ordinate I_s is the photometer signal obtained with chloroplasts minus that obtained with medium alone, multiplied by the transmittance of the chloroplast sample, normalized for constant light intensity.

plier, normalized for constant input light, and multiplied by $\sin\alpha$ so that the intensity was measured over the surface of a sphere as the value of α increased from 0° to 180°. The intensity of scattered light (I_s) increases without any apparent limit as α tends to zero (Fig. 6). In Fig. 5, since I_s is multiplied by $\sin\alpha$ and since $\sin\alpha$ is zero when α is zero, a maximum in the curve is expected. This maximum was repeatably observed to be at approx. 4°, and may be associated with the size of the scattering particles (Kerker, 1969), but is not germane to the arguments presented in this paper.

The light scattered at angles less than 2.5° could not be measured with any accuracy owing to an intense background scatter. Since angles less than 2.5° (the minimum angle of scattering measured in the c.d. spectropolarimeter) are not required for the calculation below, the absence of valid data in this region is of no consequence. The intensities were corrected for the light scattered by the optical train (there is no significant scattering from the medium at angles greater than 2°) by taking into account attenuation by the chloroplast sample.

The above experiments were carried out by using the modified Brice–Phoenix light-scattering apparatus. Attempts were made to analyse the effects of the angle of scattering on differential scattering-ellipticity measurements in the c.d. spectropolarimeter modified as in Fig. 2. This method was very crude, but did result in scattering ellipticity recordings, which remained relatively constant with varying angle, confirming that the ellipticity of scattered light is not a function of angle. It should be noted that the angles measured were between 12° and 40°. Analysis of scattering at greater angles, with the 3.5 cm cylindrical cuvette, were unsuccessful owing to insufficient light being scattered (when using 4 μg of chlorophyll/ml and 0.08 mm slit-width) to actuate the instrument. The geometry of the components in the sample compartment made it difficult to carry out measurements at angles between 4.6° and 12°. However, the spectra in the range between 12° and 40° were of similar form and magnitude to those determined with the annular optical system (2.5°–4.6°) and we assume the same to hold for the unmeasured angles, especially since measurements of direct light-scattering against wavelength gave similar results for all angles under consideration (Fig. 4).

It was found that the ellipticity of scattered light did not vary with angle provided that the concentration of chlorophyll in the sample did not exceed 5 μg/ml. Below this concentration the proportion of the light that suffers either multiple scattering, or both scattering and absorption, is assumed to be negligible. The magnitude of the ellipticity of the scattered light was found to be independent of the chlorophyll concentration in dilute suspensions, whereas the ellipticity of transmitted light (observed c.d.) was found to be directly proportional to the chlorophyll concentration (Fig. 7) as expected (see the Appendix).

Fig. 1 shows that the photomultiplier of the Cary 61 CD spectropolarimeter accepts transmitted light plus light scattered in a cone up to 4.6° from the axis. This angle can be varied only slightly, by moving the position of the cuvette, because it is necessary that the lens should at all times focus the image of the cuvette on to the photocathode. The intensity of the light that passes through the lens was determined from the dynode-voltage indication (which had been previously calibrated) in terms of an extinction value. Thus the extinction in the transmission mode of the cuvette containing the chloroplast sample and of the medium

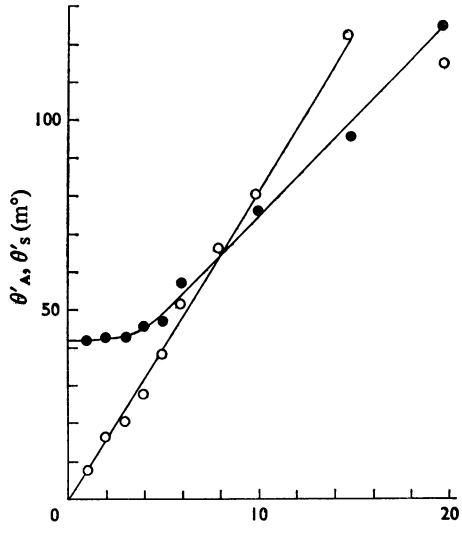

Fig. 7. *Dependence of θ'_s and θ'_A on chlorophyll concentration*

Each point represents the mean of three experiments. ○, θ'_A is the observed transmitted c.d. ●, θ'_s is the observed scattered ellipticity measured in the 2.5° to 4.6° annulus. Slit-width 0.08 mm; 1 cm light-path. For further details see the text.

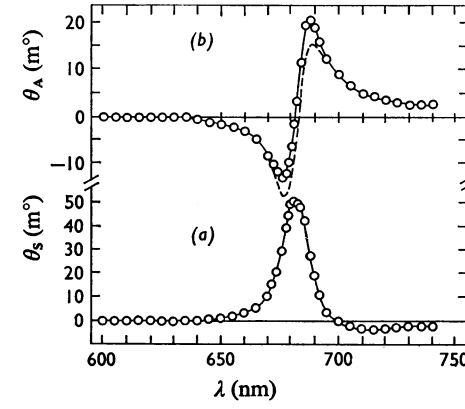

Fig. 8. *Corrected c.d. spectra of intact pea chloroplasts*

The chlorophyll concentration was 4 μg/ml, slit-width 0.08 mm; 1 cm light-path. (*b*) Observed transmitted c.d. spectrum, θ'_A (----), and corrected transmitted c.d. spectrum, θ_A (——). (*a*) Observed (θ'_s) and corrected (θ_s) scattered c.d. spectra. The two spectra are essentially superimposable. For details see the text.

alone in the same cuvette were determined in relation to a common arbitrary baseline (no cuvette in the instrument) and are referred to as E_{TC} and E_{TM} respectively.

In the scattering mode the disc inserted to block the transmitted beam cut out light scattered at angles less than 2.5° from the axis. The intensity of light scattered between 2.5° and 4.6°, passing through the annulus, was determined in the same way as above, giving 'scattering-extinction' values E_{SC} and E_{SM} for chloroplasts and medium respectively. The reference for the 'scattering-extinctions' was the same as that used for the determination of the direct extinctions, so that the difference between any two of the four parameters at any one wavelength could be used to calculate the ratio of the intensities of the light reaching the photomultiplier, eliminating the arbitrary baseline. Thus we calculated the ratio of the intensities of light scattered to that transmitted by chloroplasts:

$$I_{SC}/I_{TC} = \text{antilog}(E_{TC} - E_{SC})$$

and of the light scattered to that transmitted by the medium:

$$I_{SM}/I_{TM} = \text{antilog}(E_{TM} - E_{SM})$$

Referring to the ellipticity of chloroplasts in the scattering mode as recorded by the spectropolarimeter as θ'_S, the correction for the light scattered by the medium is given by:

$$\theta_S = \theta'_S - \theta'_A \left(\frac{I_{SM} I_{TC}}{I_{SC} I_{TM}}\right)$$

where θ_S is the corrected scattering-ellipticity, and θ'_A the recorded c.d. This correction was applied point-by-point to give the spectrum of θ_S as shown in Fig. 8(*a*). The true circular dichroism, θ_A, was then calculated from the recorded c.d. (θ'_A), by using the annular fraction 2.83:

$$\theta_A = \theta'_A + 2.83\theta_S(I_{SC}/I_{TC})$$

This correction was made point-by-point, and the resulting true c.d. spectrum is shown in Fig. 8(*b*). It should be noted that the scattering correction does not greatly alter the overall shape of the c.d. curve, although there is a greater contribution of right-handed scattering than of left-handed scattering. There is a red shift in θ'_A of approx. 2 nm.

In the corrected c.d. and scattering spectra (Fig. 8*a* and 8*b*) a correlation is observed between the wavelengths of maximum light-scattering (683 nm) and the crossover wavelength (683 nm), which is between the negative (676 nm) and positive (688 nm) maxima of the c.d. spectra. The true c.d. spectrum (Fig. 8*b*) gives a peak-to-peak intensity, $\Delta\theta_A$, of 8.3 m°·litre·(mg of chlorophyll)$^{-1}$·cm^{-1} and the differential scattering (Fig. 8*a*) has an ellipticity θ_S of +47 m°, which is independent of the chlorophyll concentration in dilute chloroplast suspensions. The values for the ellipticities thus obtained are similar to those obtained in

separate experiments for observed transmitted and scattered c.d. spectra [$\theta'_A = 8 m^\circ \cdot litre \cdot (mg\ of\ chlorophyll)^{-1} \cdot cm^{-1}$, and $\theta'_S = 42 m^\circ$ in Fig. 7], indicating that the corrections are negligible at low concentrations of chlorophyll.

Discussion

A number of workers have recently become aware of the problem of differential light-scattering in the analyses of the c.d. of particulate systems. This is especially true in the elucidation of biological membrane structure, where attempts are being made to determine the α-helical content of constituent proteins by comparing them with clear solutions of standard proteins. A diminished intensity of c.d. in the u.v. and a bathochromic shift of the c.d. maxima have been ascribed to the differential scatter of left- and right-circularly polarized light (Urry & Krivacic, 1970). Wrigglesworth & Packer (1968*a*,*b*) tried to resolve the problem by using 90% glycerol to decrease the effects of light-scattering in their studies of molecular conformational changes in mitochondria. The effectiveness of the glycerol in eliminating differential light-scattering is in question (Glaser & Singer, 1971; Schneider *et al.*, 1970). Urry & Krivacic (1970) showed that the differential scattering artifact could be calculated from the known dispersion curve in the case of poly-L-glutamic acid-model-system particles, and that similar significant corrections could be applied to the c.d. spectra of membranes (Urry *et al.*, 1971). In agreement with Urry *et al.* (1971), Glaser & Singer (1971) attributed anomalies in their c.d. spectra of intact erythrocyte membranes, when compared with fragmented ones, to differential light-scattering. However, theoretical analysis of the optical artifacts in suspensions of large particles [taking into account the Duysens (1956) absorption-flattening effect] led them to conclude that the correction is small and does not significantly alter their estimation of the average helicity of erythrocyte proteins. They state that perhaps in the case of larger particulate systems, a scattering correction would be necessary. Their results differ markedly from those obtained by Urry *et al.* (1971), who found perceptible distortions in the apparent c.d. of erythrocyte membranes, which they corrected by eliminating the differential light-scattering contributions, as in the case of poly-L-glutamic acid particles. Dorman & Maestre (1973) tried to resolve the light-scattering problem by collecting as much as possible of the light scattered by a bacteriophage suspension in the photocathode of a large-diameter photomultiplier which could be moved close to the sample cuvette to increase the solid angle of collection. Geometry-sensitive c.d. spectra were shown to be due to differential light-scattering within the sample, which in turn was claimed to indicate an ordered asymmetry of the particles being examined. Other workers (Gordon, 1972; Gordon & Holzwarth, 1971), attribute differential light-scattering to the particulate nature of the sample, rather than its ordered asymmetry. By making use of Mie theory the latter were able to obtain valid c.d. spectra for large spherical particles in suspension. The limitation of Mie theory scattering is that it is valid only with large spherical particles giving highly symmetric scattering patterns (Gordon, 1972; Philipson & Sauer, 1973). Philipson & Sauer (1973), working with suspensions of spinach chloroplasts, state that differential scattering is an inherent part of whole chloroplasts, being due to the ordered arrangement of the internal membranes of the chloroplast. They obtained different spectra as a result of varying the distance between the chloroplast suspension and the photomultiplier, whereas the c.d. of sonicated chloroplasts, which are non-scattering, remained the same. They based their conclusions on the work of Dorman & Maestre (1973), but argue that because of the complex ordered structure, no c.d. spectra of whole higher-plant chloroplasts are valid. They back up their argument by demonstrating that the c.d. of prokaryotic photosynthetic systems is similar before and after fragmentation and is independent of detector geometry. They also point out that the photosynthetic membranes of prokaryotes are not extensively aggregated or appressed in the manner characteristic of granulated chloroplast thylakoids found in higher plants.

We agree that differential light-scattering is a contributing factor to the c.d. spectrum of particulate systems. The extent and significance of its contribution is still in controversy. In our attempts to resolve the extent and form of the contribution of differential scattering to c.d. spectra of whole chloroplasts, we have undertaken a different approach to the problem. Although our conclusions are valid only for chloroplasts, the method may readily be adopted for use on other systems. The differential light scattered by a chloroplast suspension has been separated from the normal transmitted c.d. by using small-hole optics and a disc in front of the light-collecting lens which allows only scattered light to enter the photomultiplier. The relatively large size of the chloroplast (5–10μm diam.) leads to scattering being virtually confined to a narrow forward cone, so that the determination of the total light scattered is that much more precise. [Classical Mie scattering theory for spherical particles does not hold because chloroplasts have a complex internal structural organization and they are not truly spherical (Philipson & Sauer, 1973).] The light-scattering measurements show that a negligible amount of light is scattered outside an angle of approx. 40°. Our overall approach to obtaining a corrected c.d. spectrum from a particulate sample is similar to that of other investigators (Dorman &

Maestre, 1973), in that we collect all of the scattered light and treat it as transmitted. Our use of the light-scattering photometer and the application of the calculated correction avoid the problems of varying the detector geometry and of using large photocathode surface areas. Also we are working at wavelengths in the visible range of the spectrum in which high-intensity monochromatic light can be obtained in a glass optical system.

It should be noted that there is a red-shift in the observed transmitted c.d. spectrum (θ'_A) that is revealed when the scattering correction is made (Fig. 8*b*). This is in agreement with the observations of Urry & Krivacic (1970), who demonstrated a red-shift that was due to scattering.

We have established a concentration range in which scattering and absorption effects are distinct. The upper practical limit appears to be 4–5 μg of chlorophyll/ml (Fig. 7). This condition does not appear to have been fulfilled in the work of Philipson & Sauer (1973). Above this range the relative intensity of scattered light is no longer simply dependent on the path-length, concentration and the scattering coefficient (see the Appendix), its ellipticity is no longer independent of path-length and concentration, and non-additive interactions between differential scattering and c.d. complicate analysis by any method. In addition, mutual shadowing by particles is decreased at low concentrations (Duysens, 1956).

The different c.d. spectra of whole chloroplasts and fragmented ones have been ascribed by Philipson & Sauer (1973) solely to an intrinsic differential light-scattering associated with whole chloroplasts, but our results are not in agreement with this conclusion. We suggest instead that the difference in c.d. between whole and fragmented chloroplasts is due to the aggregated light-harvesting bulk chlorophyll *a* being primarily responsible for the c.d. of the chloroplast. Any disturbance of the intact chloroplast results in a breakdown in the highly ordered array of this bulk chlorophyll, revealing the less-intense c.d. of the chlorophyll involved in the chlorophyll–protein complexes (Gregory *et al.*, 1972). We base this interpretation on the fact that concentrated solutions of chlorophyll *a* in carbon tetrachloride (considered to be 85% dimer chlorophyll *a*; Dratz *et al.*, 1967) have a split-exciton spectral form similar to that of chloroplast c.d. spectra. Thus dimeric chlorophyll *a* interactions might explain the predominant c.d. of whole chloroplasts. Any loosening of this closely packed array would be expected to disrupt the dimer or higher aggregation and allow the more detailed c.d. of the chlorophyll–protein complexes to show up, whereas it was previously masked. It may be noted that the c.d. of whole chloroplasts (Fig. 8) differs from that of fragments (Gregory *et al.*, 1972) only at wavelengths ascribed to chlorophyll *a* (it is ten times greater in whole chloroplasts). At 655 nm (wavelength ascribed to chlorophyll *b*) the ellipticities are very similar [0.5 and 0.44 $m^\circ \cdot$ litre $\cdot$ (mg of chlorophyll)$^{-1} \cdot cm^{-1}$ in whole and fragmented chloroplasts respectively]. This would not be expected if the c.d. of whole chloroplasts was primarily a function of the highly ordered array of thylakoids, and it provides additional evidence for the distinct involvement of chlorophyll *a* aggregates in the origin of the c.d. of whole chloroplasts.

It has been well documented that energy transfer between the light-harvesting chlorophyll molecules is by means of resonance transfer (Clayton, 1965). This necessitates a very close approximation of the chlorophyll molecules, preferably with their transition moments oriented parallel to each other. Since most of the transition moments in thylakoid membranes have no preferred orientation, a very close three-dimensional spatial arrangement of the chlorophyll molecules would be expected for resonance transfer to be as efficient as it is (Menke, 1966). We believe that c.d. measurements of whole chloroplasts are uniquely suitable for investigating the type of close interaction between chlorophyll molecules on which the energy-conservation process of photosynthesis depends.

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APPENDIX

Theoretical Rationale for the Scattering Correction of Circular-Dichroism Spectra in Dilute Suspensions

Consider a beam of light passing through a scattering and absorbing medium. Let its intensity be I_x, where x is the distance into the medium from the entry window. Each infinitesimal segment, dx, scatters light of intensity dI_s, and:

$$dI_s = I_x \sigma c \, dx \tag{1}$$

where c is the concentration and σ is the scattering coefficient. It is assumed that the geometry is such that an insignificant quantity of scattered light is absorbed or re-scattered before escaping from the cuvette. The scattering adds to the effect of absorption in attenuating the beam, so that, if ε is the absorption coefficient, then:

$$I_x = I_0 \cdot e^{-(\sigma+\varepsilon)cx} \tag{2}$$

Substituting eqn. (1) into eqn. (2), and integrating:

$$I_s = \frac{I_0 \sigma}{\sigma+\varepsilon}(1-e^{-(\sigma+\varepsilon)cx})$$

Expansion of the exponential allows the approximation

$$I_s/I_0 \approx \sigma cx \tag{3}$$

where the product cx is sufficiently small to justify neglecting higher powers.

When discussing circularly polarized light, both σ and ε have two components, σ_R, σ_L and ε_R, ε_L for right- and left-handed polarization respectively. By definition, ellipticity (θ) is related to the intensities of right- and left-handed circularly polarized light by $\theta = 33\log(I_R/I_L)$ (expressing θ in degrees).

From eqn. (3):

$$\theta_s = 33\log\left(\frac{I_{s\,(R)}}{I_{s\,(L)}}\right) = 33\log\left(\frac{\sigma_R}{\sigma_L}\right) \tag{4}$$

and from eqn. (2):

$$\theta'_A = 33\log\left(\frac{I_{x\,(R)}}{I_{x\,(L)}}\right)$$

$$= 33\times 0.434[(\sigma_L-\sigma_R)+(\varepsilon_L-\varepsilon_R)]cx \tag{5}$$

(where the factor 0.434 converts σ and ε to $\log_{10}$ normally used). Since, at the low ellipticities studied:

$$\log\left(\frac{\sigma_R}{\sigma_L}\right) \approx 0.434\left[\frac{\sigma_R-\sigma_L}{\sigma}\right]$$

it follows that:

$$33\times 0.434(\sigma_R-\sigma_L) = \theta_s \sigma \tag{6}$$

Therefore from eqns. (5) and (6) if:

$$\theta_A = 33\times 0.434(\varepsilon_L-\varepsilon_R)cx$$

then:

$$\theta_A = \theta'_A + \theta_s \sigma cx = \theta'_A + \theta_s I_s/I_x$$

Therefore provided that the concentration-path-length product cx is small enough to justify the given assumptions, the true c.d. (θ_A) can be evaluated by adding to the apparent c.d. (θ'_A) the product of the scattering ellipticity and the proportion of light scattered to light transmitted by the sample.