Evidence that Circularly Dichroic Chlorophyll Forms *a*-682 and *a*-710 are Oriented at Right Angles to the Thylakoid Membranes of Whole Chloroplasts, and that the Circular Dichroism is Light-Dependent

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Chloroplasts from the pea (*Pisum sativum* L.) suspended in iso-osmotic buffered medium were oriented by flow in specially constructed cuvettes and examined for circular dichroism (c.d.). In one cuvette the flow was transverse to the direction of the light-path, but the other cuvette was designed so that flow and the light-path were coaxial. The induced orientation is such that the chloroplasts appear to move edgewise. C.d. was maximum when the light-path lay in the plane of the chloroplast disc. The intense c.d. of intact chloroplasts ascribed by Gregory & Raps [*Biochem. J.* (1974) 142, 193–201] to bulk chlorophyll *a* was found to contain two components, one the split-exciton type centred at 682 nm and the other a simple maximum at 700–710 nm. The chlorophyll *a*-710 form was distinguished by its greater dependence on chloroplast orientation. The preferred direction of the transition moment in both chlorophyll forms was at right angles to the plane of the chloroplast, that is, at right angles to the plane of the thylakoids. This is in conflict with several reports based on polarization of fluorescence. It is suggested that the present effect is due to thylakoid–thylakoid interaction. Evidence for this is the reversible diminution in the c.d. signal caused by illumination in the presence of electron-transport reagents. It is argued that the c.d. is an indicator of chlorophyll movement, or changes in the thylakoid–thylakoid distance, possibly related to ion movement, affecting energy transfer between photosynthetic units.

Since the concept of the photosynthetic unit was put forward by Emerson & Arnold (1932) it has been part of our understanding of photosynthesis that chlorophyll molecules are arranged in such a way that absorption of light by any one of several hundred chlorophyll molecules is followed by a rapid migration of energy to a common trap. The resolution of the absorption spectrum of chlorophyll *a* *in vivo* into components with differing absorption maxima [see e.g. French *et al.* (1972)] provides a possible potential-energy gradient for the migration, and has stimulated studies of the organization of chlorophyll that makes it possible. Discussions of the relevance of exciton theory to the energy migration, for example, met the difficulty that the chlorophyll matrix should at least approach a crystalline condition and have a degree of common orientation in the bulk of the pigment. Two methods used, dichroism of individual chloroplasts and polarization of fluorescence [see, e.g., Olson (1963)] revealed that the chlorophyll *a* form absorbing at 695 nm or longer wavelengths was oriented with the planes of the chlorin rings parallel with the thylakoids, but that there was little orientation of the bulk chlorophyll (which absorbs at approx. 676 nm). This finding was, however, challenged by the results of studies in which chloroplasts either in cells of

algae such as *Chlorella* (Geacintov *et al.*, 1971) or isolated from spinach (Breton *et al.*, 1973) were oriented by a magnetic field or by mechanical means, and revealed both polarized fluorescence and linear dichroism, indicating substantial orientation of chlorophyll in a major fraction absorbing at 681 nm. Although Geacintov *et al.* (1971) suggested the possibility that the orientation of the chlorophyll might have been caused by the magnetic field used to align the chloroplasts, the data from mechanical orientation reported by Breton *et al.* (1973) (the spreading of chloroplasts on quartz plates) was similar to that from the magnetic study, and hence the orientation of the chlorophyll molecules was not induced by the magnetism. In a study of bacteriochlorophyll in a photosynthetic bacterium by Morita & Miyazaki (1971), linear dichroism was found to be similar both in membrane material oriented by the spreading method and in whole cells oriented in a flow cell, thus showing that the spreading method also did not generate the orientation of the chlorophyll molecules. Breton *et al.* (1973) discussed a number of alternative explanations of the linear dichroism, and came to the conclusion that it was necessary to revise the earlier view and recognize a considerable extent of orientation of chlorophyll

with the planes of the chlorin rings parallel with the plane of the chloroplast. This is accepted for the purposes of the present study; however, the results presented here reveal that there is a specific fraction of chlorophyll, also absorbing at 681–682 nm (chlorophyll *a*-682), which is oriented at right angles to the plane of the chloroplast.

The starting point for the present paper is the intense c.d. (circular dichroism) spectrum obtained between 640 and 740 nm with whole chloroplasts by Gregory & Raps (1974), who showed that the effect of differential circularly polarized light-scattering could be isolated, and removed from the apparent c.d. spectrum. In that paper the origin of the signal was suggested to be a split-exciton interaction of chlorophylls, but there was a difficulty in that the long-wavelength tail of the spectrum differed from the expected form. The deviation was tentatively ascribed to contributions from chlorophyll fractions absorbing at longer wavelengths. Evidence for this interpretation is now given in the present paper. Chloroplasts, being approximately oblate spheroids, orient themselves when flowing in narrow vessels, and the c.d. signal was found to be strongly dependent on the flow direction in relation to the direction of the light beam in the spectropolarimeter.

Circular dichroism is preferred here because it is selective for pigment (almost entirely chlorophyll *a* in this study) in an asymmetric environment, such as in pigment-protein or pigment-pigment interactions. It was previously shown (Gregory *et al.*, 1972) that in the chlorophyll-protein complexes CPI and CPII (Thornber *et al.*, 1967) the c.d. spectra are each characteristic in form and enhanced in magnitude relative to unorganized chlorophyll; the complexes can be recognized by c.d. in the presence of a large quantity of unorganized pigment.

Following Phillips (1963) and others, the results are discussed on the basis that in the chlorophylls the dipoles associated with light-absorption in the red region of the spectrum are in the plane of the chlorin ring.

Materials and Methods

Chloroplasts were prepared as described by Gregory & Raps (1974) and the media, concentrations and method of recording c.d. were as given in that paper, except where otherwise stated. Circular dichroism is measured in terms of ellipticity (θ) in units of millidegrees (m°). This is related to the difference in the extinctions of the sample with respect

Fig. 1. Flow cells used in the spectropolarimeter

(a) Transverse cell (type 48, Starna Ltd., see the Materials and Methods section), quartz, light-path 2 mm. (b) Axial cell, sectional drawing; quartz windows, with other parts of borosilicate glass, sealed by epoxy resin; light-path 20 mm, diameter of flow tube 4 mm.

to right- and left-handed circularly polarized light (E_R , E_L respectively), since 1 m° corresponds to $(E_R - E_L) = 3 \times 10^{-5}$.

Flow system

Chloroplasts were diluted and stored in a vessel in the sample cavity of the spectropolarimeter. From there they were pumped at negative pressure through a flow cell and out to waste via an LKB Perplex pump delivering 4 ml/min. The transverse-flow cell (type 48; quartz) (Fig. 1*a*) was obtained from Starna Ltd., London E13 OPA, U.K. It had a light path length of 2 mm and the width of the internal space was 8 mm, so that at the pumping speed given the linear velocity was 4 mm/s. The chloroplast suspension used in this cell contained 20 μg of chlorophyll/ml, so that the product (concentration $\times$ path length) was 4 $\mu\text{g}/\text{cm}^2$ as specified in Gregory & Raps (1974). The axial-flow cell was designed for this experiment and is illustrated in Fig. 1*b*). The path length was 20 mm and the chloroplast concentration used with it was 2 μg of chlorophyll/ml. The diameter of the flow tube was 4 mm, giving a cross-sectional area of 12 mm². The windows were of quartz, fastened to the flow tube by epoxy resin. Both cells were used with a perforated plate mounted on the cell carrier, so that the ray in the spectropolarimeter was symmetrical about the optical axis.

Illumination system

The chloroplast suspension was kept in the dark in ice outside the spectropolarimeter and pumped into the flow cell via a glass coil which could be illuminated by the focused light from a 100 W tungsten lamp through a red filter [details given in Gregory (1974)]. The transit time in the coil was 80 s. The chloroplast suspension contained $\text{K}_3\text{Fe}(\text{CN})_6$ as a Hill reagent.

In a second experimental arrangement, the same reaction digests were placed in a small (approx. 30 ml) bottle which could be illuminated completely by the lamp system. From the bottle the chloroplast suspensions were pumped through the flow cell. The former system had an advantage in that since the chloroplast reservoir was kept in the dark and in ice the experiments could be continued indefinitely, whereas the small bottle was exhausted in a few minutes. However, the second method overcame the difficulty inherent in the first that when the light was switched off, the change in c.d. could be interpreted either as a relaxation of the light-induced change, or as a replacement of light-treated chloroplasts with others that had received less light or none at all. The main drawback at the present time is that the second method gives kinetics of dubious value, since the temperature is difficult to stabilize. In fact the relaxation kinetics

(but not the light-induced change) can be seen to increase in rate as the mixture warms up, and the c.d. value declines irregularly, owing to deterioration of the chloroplasts from the same cause, after 2–3 min. Results presented below are subject to this qualification.

Scattering correction

The correction which removes the effect of differentially scattered circularly polarized light from the observed c.d. spectrum was applied as described by Gregory & Raps (1974). The annular fraction, defined as the ratio of light scattered at angles from 4.6° to 180° to that scattered between 2.5° and 4.6°, was determined for chloroplasts both stationary and flowing in the transverse-flow cell, mounted both vertically and horizontally in the light-scattering apparatus. (The annulus 2.5°–4.6° is the collecting area of the lens of the modified Cary 61 CD spectropolarimeter in the light-scattering mode.) Light-scattering data cannot at present be obtained by using the axial flow cell, and an estimate was made from the other values, on the basis that the mean of the annular fractions for chloroplasts oriented in the three axes should be equal to that for an unoriented chloroplast suspension. The c.d. spectra were then corrected for the effect of scattering by using a computer program devised by Dr. G. Tomalin of the Department of Biochemistry, University of Manchester.

Results

In darkness, chloroplasts showed marked changes in circular dichroism when the pump was switched on and off. Spectra are given in Fig. 2 which show that the changes were of two kinds: a proportional change in the sigmoid curve centred on 682 nm, and a more extensive change at approx. 710 nm. In the transverse-flow cell, the c.d. is diminished during flow; in the axial cell it is increased. Fig. 3 shows transients at the positive and negative extrema induced by stopping and starting the flow in the two cells. At the onset of flow, the transition is more rapid than the response time of the instrument, whereas when flow ceases the signal takes much longer to return to the original position. Fig. 4 shows that the effect saturates at flow rates of approx. 1 ml/min.

It was found that when chloroplasts were oriented in the transverse-flow cell, the intensity of light scattered at angles between 8° and 40° diminished. This effect was more pronounced when the cell was mounted vertically, that is, with the flow at right angles to the plane of the arc scanned by the photomultiplier of the light-scattering apparatus (see Gregory & Raps, 1974). This is consistent with the values obtained for the annular fraction: 2.64 and 2.14

Fig. 2. C.d. spectra, between 720 and 620nm, of whole chloroplasts in (a) the transverse-flow cell and (b) the axial-flow cell

Correction for scattering has been applied. In each set, curves (i) are stationary, curves (ii) are with a flow rate of approx. 4ml/min. Curves (a,i) and (b,i) differ by 10%, possibly owing to uncorrected reflexions in the axial-flow cell. For experimental detail see the Materials and Methods section. C.d. is measured in terms of ellipticity (θ) in units of millidegrees (m°).

for flowing chloroplasts in the transverse-flow cell, horizontally and vertically mounted respectively. The mean value of the annular fraction in the transverse cell was 2.39 flowing, and 3.01 for the static suspension. The value 4.25 was calculated (see the Materials and Methods section) for the annular fraction of a chloroplast suspension flowing in the axial-flow cell. The ellipticity of the scattered light was increased or diminished by flow in the two types of cell in the same sense as the c.d. extremum at 689nm. The corrections for scattering therefore slightly increase positive-going transients and diminish negative-going transients.

By using the transverse flow cell on the stage of a microscope with a magnification of 100, it was possible to observe chloroplasts flowing. Most chloroplasts advance edgewise, with a small rocking movement. It was a subjective estimate that only some 1% of the number were advancing face-on to the direction of flow at a given moment.

Pea chloroplasts were found to have the common form of dishes in which the planes of the thylakoid system are closely parallel to the principal plane of the dish. From these observations it is concluded that in the axial-flow cell during flow the light travels in the plane of the chloroplast dish, which is also the plane of the thylakoid membranes. In the transverse cell there is a higher proportion of chloroplasts in which the light is incident perpendicularly to the thylakoids. The c.d. is less in the transverse cell and greater in the axial cell during flow, therefore the c.d. features both at 682 and at 710nm are more strongly associated with the direction of light along the plane of the thylakoid. Since the electric vector of the light is transverse to the direction of propagation, the transition moment of the molecules responsible for the c.d. must have a preferred orientation at right angles to the plane of the thylakoid. The transition moment of the principal red absorption band in chlorophyll *a* is known (see Phillips, 1963) to lie in the plane of the chlorin ring in the direction of rings I and III. Therefore the preferred orientation of the chlorophyll *a* responsible for the intense c.d. of the whole chloroplast is such that the chlorin rings are on edge with respect to the thylakoid, and that the axis defined by rings I and III is at right angles to the thylakoid plane. A quantitative form of the argument is given in the Discussion section.

When the chloroplasts were illuminated before reaching the flow cell, the c.d. was observed to be diminished, irrespective of whichever type of flow cell was used. This effect required fresh chloroplasts (not more than 2 h old) and the presence of an electron-transport cofactor. $K_3Fe(CN)_6$ was used as a Hill reagent in the experiments shown in Fig. 5, which show that the effect of illumination appeared at both the positive and negative extrema of the c.d. curve. Fig. 6 shows transients obtained by switching the

Fig. 3. Transients in the c.d. signal induced by stopping and starting the flow

(a) 689 nm and (b) 676 nm in the transverse cell, and (c) 676 nm and (d) 689 nm in the axial cell. The spectropolarimeter was used with a period of 10 s. F, suspension flowing; N, suspension not flowing (i.e. stationary).

Fig. 4. Effect of varying flow speed on the c.d. signal of whole chloroplasts at 689 nm in the transverse flow cell

Chloroplasts (concn. = 20 μ g of chlorophyll/ml) were pumped through the flow cell (path length 2 mm) in the spectropolarimeter by a variable-speed pump. The reservoir of chloroplasts was kept cold (0°C) and dark.

lamp on and off; the diminution of the c.d. signal takes some 50 s to develop, and a longer time to relax. The relaxation time approaches the time taken for the chloroplasts to traverse the illumination coil, and this makes the reversibility of the light effect uncertain; the relaxation might be due to the arrival in the flow cell of chloroplasts that had not been fully illuminated. Accordingly the experiment was performed in which the chloroplast reservoir was illuminated until the light effect was fully developed, then darkened; Fig. 7 shows the c.d. transients obtained. Therefore the light effect is fully reversible. Confirmation of this is shown in Fig. 8 in which the flow was stopped when the light effect was fully developed. The flow effect relaxes first and is followed by a slower relaxation which can be attributed to the reversing of the light effect.

It is concluded from the above experiments that the organization and orientation of the chlorophyll responsible for the c.d. feature at 682 nm is to some extent destroyed by light, and re-formed in darkness.

The light effect does not appear unless a reagent such as $K_3Fe(CN)_6$ is present, nor does it appear in the presence of the Hill reagent and the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea nor in the presence of an uncoupling agent such as 5 μ M-carbonyl cyanide *m*-chlorophenylhydrazine. This suggests strongly that the light effect is dependent on the conservation of energy resulting from electron transport and its associated ion movements. The slow kinetics of the light effect make it likely that the effect is due to ion movements rather than to the membrane potentials that have been shown by Witt

Fig. 5. C.d. spectra of chloroplasts flowing in the transverse cell after passing through the coil, (A) in darkness and (B) having been subjected to illumination for 1 min with bright red light

The medium contained 2 mM- $K_3Fe(CN)_6$. For other details see the Materials and Methods section.

and co-workers (reviewed by Witt, 1971) to arise with sub-millisecond kinetics.

Discussion

The two components of circularly dichroic chlorophyll

The sigmoid c.d. spectra published by Gregory & Raps (1974) were interpreted as a split-exciton effect of the type often found with dimers (see Dratz *et al.*, 1967). These spectra appear in this paper in Figs. 2(a)(i) and 2(b)(i). The lack of positive-negative symmetry can be ascribed to a contribution from non-dimer chlorophyll centred at the same wavelength. The long-wavelength tail, however, deviates severely from the expected form in that it declines too slowly as the wavelength increases. Under flow conditions in the transverse cell this deviation is decreased and curve (ii) of Fig. 2(a) reaches the abscissa at 710 nm. That part of the c.d. spectrum (in non-flow conditions), between 700 and 740 nm, which is too great in magnitude to be accounted for by the split-exciton hypothesis, is now seen to be at least in part due to an independent effect, centred at 700–710 nm, differentiated from the split-exciton chlorophyll by its greater dependence on the orientation of the chloroplast. The split-exciton interpretation is thereby strengthened, and an additional form of chlorophyll *a* distinguished.

Fig. 6. Transients in the c.d. signal at 689 nm of chloroplasts flowing in (a) the transverse and (b) the axial cell, induced by light (L) and darkness (D)

The instrument period was 10s in (a) and 3s in (b). Other experimental details were as for Fig. 5.

Fig. 7. Transients in the c.d. signal of whole chloroplasts at 689 nm induced by light and darkness

Illumination was applied to the reservoir containing the chloroplast suspension. The axial cell was used. Other details as for Fig. 5.

Validity of c.d. of oriented systems

Oriented pigment systems, whether they consist of asymmetric chromophores or not, can give rise to spurious c.d. effects [see, e.g., Disch & Sverdlik (1969)

Fig. 8. C.d. transients at 689nm obtained in the transverse flow cell

At zero time transients were induced by: (a) terminating the illumination; (b) stopping the flow of chloroplasts illuminated by Method 1; this has the effect of terminating the illumination as well; and (c) stopping the flow of chloroplasts in the dark. The slow relaxation of curve (a) as well as the fast relaxation of curve (c) can be seen in curve (b).

and Breton & Roux (1971)]. One such effect is that of differential scattering of circularly polarized light; this, however, is corrected for by the procedure of Gregory & Raps (1974). A second problem is that the circularly polarized light-beam is degraded by the sample: if the beam is considered to be resolvable into two plane-polarized beams, polarized at right angles to each other, and 90° out of phase, then the different refractive indices will tend to alter the phase difference, resulting in some loss of circular polarization. The difference in absorption coefficients, also, will tend to remove one component, and in both cases elliptically polarized light remains. The instrument would therefore under-read any c.d. present in the sample. In the present study this effect may be discounted for two reasons: first, the use of the transverse- and axial-flow cells results in decrease and increase in the c.d. signal respectively, and secondly the concentration of chloroplasts may be considered to be too small for such an effect to be observed.

Identification of the sense of the oriented pigment

In a circularly polarized light-beam at any random point and instant, the electric vector is located at random as a radius of a circle in a plane at right angles to the direction of propagation of the beam. The probability of absorption by a given pigment molecule is proportional to the square of the cosine of the angle made by the direction of its absorption dipole and the electric vector; Fig. 9 shows how the average absorption by a randomly oriented population of pigment molecules is calculated to be one-third that of an ideally oriented sample. This is taken to apply to the

Fig. 9. Determination of the average value of $\cos^2 \alpha$, where α is the angle between two vectors, oriented at random

Each value of $\cos^2 \alpha$ is weighted with the area element on the surface of a unit sphere, $2\pi \sin \alpha$. The integral

$$\int_0^{\pi/2} \sin \alpha \cdot \cos^2 \alpha \, d\alpha = \frac{1}{3}$$

is the required average after division by the area of a hemisphere, 2π .

Fig. 10. Diagrammatic representation of the restrictions on the orientation of chloroplasts in the flow cells

In each arrangement illustrated, the left-hand circle represents the plane to which the electric vector of circularly polarized light is confined. The right-hand circle indicates the orientation of the chloroplasts (the thylakoid plane) and of the dipole direction underlying the c.d. effect; the pair of drawings indicates the direction in which rotation is allowed. In the left column the dipole is assumed to lie in the thylakoid plane, whereas in the right it is assumed to be perpendicular to it. In each arrangement an average value of $F = \cos^2 \alpha$ has been calculated, where α is the angle between any direction of the electric vector and any direction of the absorption dipole.

suspensions of chloroplasts in the flow cells when there is no flow. J. Breton (unpublished work) has shown that there is no detectable orientation of chloroplasts in static suspensions (as might be induced by gravitational settling or by convection). Fig. 10 shows the basis of further calculations in which the orientations of the chloroplasts are restricted by the flow effect so that there is an absence of chloroplasts advancing face-on. It is necessary to idealize the chloroplast structure as a flat disc. In all cases the distribution of the angle between the electric vector and absorption dipole is calculated, and the square of the cosine integrated with respect to the distribution of each vector. The results given in Fig. 10 show that if the chlorophyll responsible for the c.d. effect is oriented in the plane of the chloroplast then the magnitude of the c.d. transient ($\theta_{689} - \theta_{676}$) (Figs. 2 and 3) would be expected to be decreased by 25% in the axial-flow cell, and unchanged in the transverse-flow cell. If, on the other hand, the chlorophyll is perpendicular to the plane of the chloroplast, then the expected values for the flow/no-flow transient would

be +33% and -25% respectively. The observed values (from Figs. 2 and 3) are +35% and -57% respectively, supporting the conclusion reached in the Results section that the orientation is indeed perpendicular to the chloroplast plane. No explanation is offered, however, for the finding that the transient in the transverse cell is twice as great as predicted. The scattering ellipticity also shows transients of the same order: +27% and -49%.

Nothing in the foregoing is intended to imply that the dipoles of chlorophyll pairs responsible for the split-exciton c.d. signal are exactly parallel; this point has been discussed by Dratz *et al.* (1967) (who applied a simple point-dipole theory) in relation to the split-exciton c.d. features shown by small fragments of thylakoids [interpreted by Gregory *et al.* (1972) as being due to the chlorophyll-protein complex CPI]. However, in the present study it is clear that there is a preferred direction in which the c.d. signal is greatest, and that this direction is nearer to the perpendicular to the thylakoid plane than parallel to it. Since the linear-dichroism studies (see the intro-

duction) have shown that the bulk of the chlorophyll is oriented parallel to the thylakoid plane, the perpendicular fraction must be a minority. This fraction could be responsible for such an abnormally intense c.d. by virtue of interactions between adjacent thylakoids of grana stacks. Philipson & Sauer (1973) and Raps & Gregory (1975) have drawn attention to the apparent correlation between the 682 nm c.d. signal and the presence of thylakoid-thylakoid contact in higher plants and algae; these studies are, however, as yet in a preliminary stage.

The light effect

Both direct and delayed fluorescence have been known for a considerable time to show adaptation behaviour. Thus Lavorel (1959) found that the amount of fluorescence after the onset of illumination rose [as the primary acceptor of electrons from system II, the quencher 'Q' for Duysens & Sweers (1963), became reduced] but then fell, over a period of minutes, a fall not explicable on the basis of changes in Q alone. In their measurements of delayed fluorescence, Bertsch *et al.* (1969) described a routine of adaptation to the onset of the flashing illumination in which the delayed light emission only became stable after a period of minutes. Both prompt and delayed fluorescence have been found to be related to the 'energy level' (capacity for forming ATP in the dark), and Papageorgiou & Govindjee (1968) and de Kouchkovsky (1972) showed that such changes could be also induced by salt and pH treatments that were interpreted as causing structural changes in the thylakoid and hence in the pigment system. This view depends on the theory of Mitchell (1966), supported by evidence initially from Jagendorf & Hind (1963), and amply extended later that during electron transport the thylakoids accumulate H^+ ions, setting up a pH gradient; this pH gradient, together with a slower-forming membrane potential brought about by cation movement, is a store of energy which can drive the formation of ATP. Krause (1974) and Barber *et al.* (1974) have presented data which show that Mg^{2+} -ion movements induced by electron transport in illuminated chloroplasts can explain the above observations and argue that relatively major changes in the structure of the thylakoid membrane system, and of the thylakoid-thylakoid stacking, would be a reasonable inference.

In the present study it is shown that the 682 nm c.d. effect is diminished by light. In the experimental arrangement used, which is not thought to be the best possible, this decrease was of the order of 10% and depended on electron transport taking place under energy-conserving conditions. Although it has yet to be shown experimentally, the conditions under which the light-induced decrease of the 682 nm c.d. was observed were those in which the expulsion of

Mg^{2+} ions should have taken place, and the time-scale of the effect is appropriate. Separation of the thylakoids of a grana stack caused by electrostatic repulsion resulting from such ion movements would provide a unifying hypothesis for the light-induced changes in fluorescence and for the c.d. changes reported in this paper. Further, a possible biological advantage may be envisaged where, in dim light, energy can migrate from photosynthetic units in one thylakoid to those in an adjacent one, thus avoiding wastage of quanta absorbed by a unit during the time when the trap is closed.

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