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# The Polarization of Fluorescence and Energy Transfer in Grana[1]

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## Introduction

The results of several experiments suggest that the first step in green plant photosynthesis is a cooperative act carried out by a few hundred chlorophyll molecules. By using very short flashes of light, spaced far apart so as not to interfere with each other, Emerson and Arnold (1) found that the maximum number of carbon dioxide molecules reduced per flash was some 2500 times less than the number of chlorophyll molecules. Tamiya (2) repeated the experiments using very much longer flashes and found that the maximum amount of carbon dioxide reduced per flash by 1 g. dry weight of algal cells was $7.3 \times 10^{-8}$ moles at 25°C. and $3.7 \times 10^{-8}$ moles at 7°C. The longer flash times used by Tamiya would allow a part of the photosynthetic mechanism to "recycle" during the flash. Since this effect should be less at the lower temperature, the 7°C. measurement and the chlorophyll content of $7.0 \times 10^{-5}$ moles/g. of dried algae, given by Tamiya, will be used to calculate the ratio of chlorophyll to photosynthesis. This ratio is 1900. This large number of chlorophyll molecules [which can be determined by other methods than by flash experiments, as shown by Gaffron and Wohl (3)], is for the reduction of one carbon dioxide molecule, and thus must be divided by the number of quanta used to reduce one carbon dioxide molecule in order to obtain the number of chlorophyll molecules involved in the absorption of one quantum. The exact number of quanta used by the plant to reduce one molecule of carbon dioxide is still uncertain, but it is probably between 4 and 10. Thus the number of chlorophyll molecules per quantum used will be between 200 and 500.

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By measuring the oxygen produced by short flashes of known energy, Kohn (4) determined an absolute absorption cross section for photosynthesis and found a value of $9.7 \times 10^{-14}$ cm.$^{-2}$, which is some 360 times the maximum absorption cross section of chlorophyll in the spectral region used. Thus the number of chlorophyll molecules needed to make the large cross section is larger than 360.

Thomas *et al.* (5) measured the Hill reaction as a function of the size of fragments of spinach grana. They found that, as the size was reduced, the photochemical activity disappeared at a certain critical volume, $10^6$ cu. A., estimated to contain 40–120 chlorophyll molecules.

The three experiments just described show that there are several hundred chlorophyll molecules present for each quantum that is used by the plant, and since any one of the molecules might have made the absorption, and because it is known that at low light intensities the light absorbed is used with an efficiency of 30% or greater, there must be present in the plant some means of transferring energy for a distance comparable to the linear dimensions of a few hundred chlorophyll molecules at the concentration they have in the grana.

In the present paper, some measurements are given of the polarization of the fluorescent light from living plants. The polarization is so low that it is believed that much of the fluorescent light must be emitted by chlorophyll molecules that did not themselves absorb the exciting light; it thus shows the energy transfer just discussed.

## Materials and Methods

The chlorophyll solutions were made by extracting spinach or sugar-beet leaves with a small quantity of methyl alcohol. A part of the extract was then mixed with 100 or more times as much castor oil in order to have the pigments dissolved in practically 100% oil.

The chlorophyll–protein solutions were made by the method of Smith (6) from spinach or sugar-beet leaves.

The living plants used were *Chlorella pyrenoidosa* (Emerson strain) that were grown in Knop's solution in the usual manner.

Measurements were made with a polarizing microscope that had been dismantled and from which all the "optics" had been removed with the exception of the low-aperture condenser lens. The deposition of the apparatus can best be described in terms of a rectangular coordinate system with the $X$-$Y$ plane horizontal. The polarizer and condenser were clamped so that they sent a beam of slightly convergent polarized light along the $Y$ axis toward the origin. The polarizer was rotated so that the electric vector was in the $Z$ direction. The light was furnished by a Farrand monochromator illuminated with a 500-w. projection lamp. A large rectangular cuvette was mounted so that it contained the origin of the coordinate

and was so oriented that one face was normal to the $Y$ axis and one normal to the $X$ axis. This cuvette, as well as the filters to be mentioned, was examined in polarized light to be sure that there were no strains in the glass that might rotate the plane of polarization. The microscope tube containing the analyzer was clamped along the $X$ axis and was provided with a No. 6217 photomultiplier in a light-tight housing at the far end. The filters (Corning Nos. 2403 and 2030) were placed between the cuvette and the analyzer so as to transmit the fluorescent light of chlorophyll to the photomultiplier but not the scattered exciting light of shorter wavelength.

Two measurements of the intensity of the fluorescent light were made at each wave length. One $I_{\parallel}$ was made with the analyzer rotated so that the electric vector of the light transmitted was in the $Z$ direction, and the other, $I_{\perp}$, was made with the electric vector in the $Y$ direction. The polarization is then given by

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}.$$



## Results

The wavelengths, for which the polarization may justifiably be calculated, were determined by the ratios of scattered light transmitted by the red filters to the fluorescent light. $I_{\parallel}$ has been given (Table I as a function of wavelength for different materials. For chlorophyll solutions, results at any wavelength could be used since the scattered light from the distilled water and the window were very small when compared to the fluorescence from the chlorophyll in castor oil. However, with the *Chlorella* suspension, the scattered light from the yeast sus-

TABLE I

$I_{\parallel}$ *of Distilled Water, Chlorophyll in Castor Oil, a Yeast Suspension and a* Chlorella *Suspension in Arbitrary Units as a Function of Wave Length*

| Wavelength $m\mu$ | Distilled water | Chlorophyll in castor oil | Yeast suspension | *Chlorella* suspension |
|---|---|---|---|---|
| 690 | 0.95 | 120.0 | 560.0 | 230.0 |
| 680 | 0.90 | 160.0 | 460.0 | 90.0 |
| 670 | 0.50 | 114.0 | 280.0 | 32.0 |
| 660 | 0.25 | 79.5 | 118.0 | 21.5 |
| 650 | — | 91.5 | 32.0 | 16.5 |
| 640 | — | 93.5 | 4.5 | 12.5 |
| 630 | — | 93.5 | 0.3 | 10.2 |
| 620 | — | 91.5 | 0.1 | 9.2 |
| 610 | — | 78.0 | — | 8.0 |
| 600 | — | 57.5 | — | 7.2 |

TABLE II

*The Polarization of Chlorophyll in Castor Oil, Chlorophyll-Protein in Water, and* Chlorella *Cells as a Function of Wavelength*

| Wavelength mμ | Chlorophyll in castor oil | Chlorophyll-protein in water | *Chlorella* suspension |
|---|---|---|---|
| 680 | 0.425 | — | — |
| 670 | 0.425 | — | — |
| 660 | 0.414 | — | — |
| 650 | 0.41 | — | — |
| 640 | 0.33 | — | — |
| 630 | 0.24 | 0.159 | 0.033 |
| 620 | 0.24 | 0.155 | 0.031 |
| 610 | 0.264 | 0.151 | 0.031 |
| 600 | 0.195 | 0.10 | 0.028 |
| 590 | 0.07 | — | — |
| 580 | 0.007 | — | — |
| 570 | 0.038 | — | — |
| 560 | 0.12 | — | — |
| 550 | 0.11 | — | — |

pension used as a control was so great at the longer wavelengths that it was necessary to calculate the polarization for only the wavelengths of 630 mμ and smaller.

Since the first chlorophyll–protein solutions made gave very small polarizations, it was thought that they might contain rather large fragments of grana. The preparation was therefore centrifuged at 81,000 × *g* for 1 hr. to remove any large fragments (see *Acknowledgements*). The results shown in Table II were obtained from the supernatant.

In Table II, the polarization of the fluorescent light has been given for a dilute solution of chlorophyll in castor oil, a dilute solution of the chlorophyll–protein complex in water, and a *Chorella* suspension having 0.4 cu. mm./cc. The most important point in the table is the low value of the polarization in the living plant.

The rotation of the plane of polarization by castor oil was measured and found to be 4° in 10 cm. of path. Since a light path of only 2–3 cm. was used in this experiment, a very small error was made by neglecting this rotation. Since light tends to be depolarized as it is scattered, an auxiliary experiment was made with a 10-cm. thickness of a *Chlorella* suspension so dense that only multiple-scattered light was transmitted. Ninety-nine per cent polarization was demonstrated. Therefore, the low value shown by the living plants could not be caused by scattering of light.

## Discussion

In a very interesting paper on the fluorescence of solutions, Perrin (7) calculated the effect of Brownian movement on polarization according to the theory developed by Smoluchowski and Einstein. He derived the equation

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{RT}{\eta V}\tau\right)$$

where $P$ is equal to the polarization observed and $P_0$ is the fundamental polarization or that which is to be expected in the absence of any rotation, $R$ = gas constant, $T$ = absolute temperature, $\tau$ = lifetime of the excited state, $\eta$ = viscosity, and $V$ = molecular volume of the pigment.

Perrin measured the polarization of the fluorescence of chlorophyll in a number of solvents having different viscosities. He used the above equation to determine $P_0$, and found the value 0.42 in the red absorption band. Using the molecular volume of 2600, he estimated $\tau$ to be $3 \times 10^{-8}$ sec. The data for chlorophyll in castor oil given in Table II are in agreement with the findings of Perrin in that: (*a*) a polarization of 0.425 was found in the red band; (*b*) as a function of wavelength, the polarization drops to very nearly zero at 580 m$\mu$ and then becomes larger at shorter wavelengths; and (*c*) there is no negative polarization at any wavelength.

If the values given by Perrin are used, it is found that for the castor oil solutions the term

$$\frac{RT}{\eta V}\tau = \frac{8.3 \times 10^7 \times 9 \times 10^{-6}}{8.32 \times 2.6 \times 10^3} = 0.035.$$

For the chlorophyll–protein solution this term will be about 800 times as large owing to the change in viscosity from castor oil to water. Since Smith (6) estimated the chlorophyll–protein to have a molecular weight in excess of 70,000, the term will be smaller, because of the change in molecular volume, by the ratio of 27. The intensity of the fluorescence from the chlorophyll–protein is apparently comparable to that of the living plant and thus 0.01–0.10 of that from a chlorophyll solution, making $\tau$ smaller by the factor 0.01–0.10. When all three factors are combined, the term might be expected to be

$$\frac{RT}{\eta V}\tau = 0.01\text{–}0.10.$$

If these numbers are placed in Perrin's equation, a higher polarization is predicted than is found for the chlorophyll–protein solution (see Table II). Possibly each protein molecule is combined with more than one chlorophyll molecule and the mechanism of depolarization to be discussed is important.

Because the viscosity of the chlorophyll–protein complex embedded in the solid grana must be essentially infinite, and because the low intensity of the fluorescent light implies a short lifetime $\tau$, the polarization for chlorophyll in the living plant would be expected to be larger than for the chlorophyll–castor oil solution. Table II shows that this is not so.

Since the idea of molecular rotation fails completely in explaining the low polarization shown by the living plants, it is fortunate that there is another known mechanism of depolarizing fluorescent light. Perrin discussed a type of self-depolarization found at higher concentrations of pigments due to the transfer of energy between molecules and to the random orientation of those molecules to which the excitation energy is transferred by resonance. Treatments of this subject have been given by several authors [see Pringsheim (8)]; however, here an attempt will be made to calculate only the number of transfers needed to explain the low polarization.

Let

$N_t$ = total number of chlorophyll molecules,

$\sigma$ = absorption cross section for one chlorophyll molecule in square centimeters,

$I$ = exciting light intensity expressed as quanta per square centimeter per second,

$N_0$ = number of excited chlorophyll molecules that absorbed the light themselves,

$N_1$ = number of excited chlorophyll molecules where there has been only one transfer of excitation energy,

$N_2$ = number of excited chlorophyll molecules where there have been two transfers of excitation energy,

and so on.

Let

$\alpha$ = rate at which excited chlorophyll uses the energy for photosynthesis or wastes it as heat,

$\beta$ = rate at which excited chlorophyll fluoresces,

$\gamma$ = rate at which excitation energy is transferred from one chlorophyll molecule to another.

If these definitions are used, and it is remembered that the low intensity of the fluorescent light from the living plants means that $\beta$ can be neglected in comparison with $\alpha$, the differential equations governing the excited chlorophyll may be written as

$$\frac{dN_0}{dt} = \sigma I N_t - (\alpha + \gamma)N_0,$$

$$\frac{dN_1}{dt} = \gamma N_0 - (\alpha + \gamma)N_1,$$

$$\frac{dN_2}{dt} = \gamma N_1 - (\alpha + \gamma)N_2, \qquad \text{etc.}$$

$$\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots$$

In the steady-state condition each of these equations will be equal to zero and we have

$$N_0 = \frac{\sigma I N_t}{\alpha + \gamma} = \frac{\sigma I N_t}{\gamma}\left(\frac{\gamma}{\alpha + \gamma}\right)$$

$$N_1 = \frac{\gamma N_0}{\alpha + \gamma} = \frac{\sigma I N_t}{\gamma}\left(\frac{\gamma}{\alpha + \gamma}\right)^2$$

$$N_2 = \frac{\gamma N_1}{\alpha + \gamma} = \frac{\sigma I N_t}{\gamma}\left(\frac{\gamma}{\alpha + \gamma}\right)^3$$

$$\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots$$

Upon addition of all the equations, an expression is derived for the total excited chlorophyll $N_{ex}$

$$\sigma I N_t = \alpha N_0 + \alpha N_1 + \alpha N_2 + \cdots$$

$$\sigma I N_t = \alpha N_{ex}.$$

Therefore,

$$N_{ex} = \frac{\sigma I N_t}{\alpha}.$$

The polarization of the fluorescent light will be given by the average of all the different polarizations weighted by the number of excited molecules of each kind. The $N_0$ chlorophylls will have the polarization $P_0$. The $N_1$ chlorophylls will emit light having the polarization $P_0^2$ as if they were being excited with partially polarized light. The $N_2$ will have the polarization $P_0^3$, etc. Therefore, the polarization will be given by

$$P = \frac{\frac{\sigma I N_t}{\gamma}\sum\left(P_0\frac{\gamma}{\alpha+\gamma}\right)+\left(P_0\frac{\gamma}{\alpha+\gamma}\right)^2+\left(P_0\frac{\gamma}{\alpha+\gamma}\right)^3}{\frac{\sigma I N_t}{\alpha}}+\cdots$$

making the summation and canceling

$$P = \frac{\alpha P_0}{\alpha + \gamma - P_0\gamma}$$

which can be written as

$$\frac{\gamma}{\alpha} = \frac{P_0 - P}{P(1 - P_0)}. \tag{1}$$

Equation (1), with the values from Table II, shows that the excitation energy is transferred from one chlorophyll molecule to another at a rate some 15–20 times as fast as the energy is being used for photosynthesis and heat production.

Three criticisms of the derivation of Eq. (1) should be made. First, the arguments used would be legitimate if it was known that the energy was transferred by resonance, and that all transfers took place in the $Y$ direction. However, the transfers can take place in any direction which means that the polarization is lost track of much faster than is implied by the equation. If it is assumed that all polarization is lost after the first transfer, only the first term is used in the summation and

$$\frac{\gamma}{\alpha} = \frac{P_0 - P}{P} \tag{2}$$

and there will still be at least 8–12 transfers before the energy is used.

Secondly, modern research on the structure of the grana by Wolken and Schwertz (9) and Wolken and Palade (10) suggests that there may be a considerable degree of orientation of the chlorophyll molecules, whereas, in this derivation, it has been assumed that the chlorophyll is arranged at random. It is clear that the polarization will not be reduced if excitation energy is transferred to a chlorophyll molecule having the same orientation as the one that made the absorption. Thus if the chlorophyll is arranged in some regular manner, Eqs. (1) and (2) are lower limits to the rate of transfers.

Finally, the energy could be moved through the grana by the diffusion of some high-energy compound made by the excited chlorophyll until it is either used, or again forms an excited chlorophyll, or the excited chlo-

rophyll could move an electron from a filled band to an empty conduction band, as has been suggested by Szent-Györgyi (11) and by Katz (12), the energy conduction being carried out by these high-energy electrons. However, in expressing the differential equations for the formation of excited chlorophyll by light, for the formation of the carrier by excited chlorophyll, and for the formation of excited chlorophyll by the carriers, it is found that the various rate constants can be so combined as to lead again to Eq. (2).

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## Summary

1. The large ratio between chlorophyll and the amount of photosynthesis produced by a short flash of light, together with the high efficiency of photosynthesis at lower light intensities, suggests that the living plant must have some mechanisms for transferring energy through the grana.

2. The low polarization of the fluorescent light from living plants must be due to much of the fluorescent light being emitted by chlorophyll molecules that did not themselves absorb the exciting light. Thus the low polarization demonstrates an energy transfer between chlorophyll molecules in the living plant.

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