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EXCITATION TRANSFER BY CHLOROPHYLL a IN MONOLAYERS AND THE INTERACTION WITH CHLOROPLAST GLYCOLIPIDS*

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ABSTRACT

In mixed monolayers with purified chloroplast glycolipids and other colorless lipids, chlorophyll a fluorescence exhibits a decrease in quantum efficiency with increasing chlorophyll concentration. The fluorescence, which is strongly polarized in dilute films, becomes progressively depolarized as the area fraction of chlorophyll increases, and it is completely depolarized in a pure chlorophyll a monolayer. The observed behavior is consistent with an inductive resonance mechanism of energy transfer among the chlorophyll molecules with a critical transfer distance of 20 to 80 Å, depending on the model chosen for the energy transfer mechanism.

The purified glycolipids--mono- and digalactosyl diglycerides and sulfoquinovodiglyceride--separately form stable, compressible monolayers of the liquid-expanded type on an aqueous subphase and in an atmosphere of nitrogen. At maximum compression the three glycolipids occupy areas of 55, 80, and 47 A^2-molecule^{-1}, respectively, in the monolayer. Mixed monolayers of chlorophyll a with, separately, the monogalactolipid and the sulfolipid behave upon compression as two-dimensional solutions. The fluorescence polarization at high chlorophyll concentrations in mixed monolayers indicates that several of the lipid diluents facilitate local ordering of the pigment molecules.

* * * * *

A photosynthetic unit consists of a number of chlorophyll molecules associated with a rate-limiting dark step in the particular photochemical reaction sequence under investigation. Depending on whether one measures oxygen evolution accompanying CO_2 fixation[1] or the Hill reaction,[2,3] the ratio of chlorophyll to essential components such as manganese or cytochromes present in low concentrations,[4] or minimum requirements of potent inhibitors,[5] the size of the photosynthetic unit varies from 250 to 2500 chlorophyll molecules. Regardless of how the unit is characterized, the chlorophyll contained in it must act cooperatively in absorbing light quanta and in transferring the excitation energy to an associated site of chemical activity. Despite extensive investigation of physical and chemical properties of the photosynthetic apparatus, the mechanism of this energy transfer *in vivo* has not been unequivocally determined.

Since Hughes,[6] Hansen,[7] and Langmuir and Schaefer[8] first made monolayers of mixed chlorophyll *a* and *b* extracts in the 1930's, numerous workers have investigated chlorophyll *a*-containing films. A review of these studies appeared recently.[9] The results suggest that these model systems are well suited to our purpose. The compression behavior and spectroscopic properties of pure chlorophyll *a* monolayers are well characterized.[10,11,12] Mixed films of the pigment with surface active lipids in which it is miscible appear to be ideal two-dimensional solutions.[13] Their absorption spectra have striking similarities to those of biological material. The dependence of chlorophyll *a* fluorescence yield on pigment and/or quencher concentrations in monolayers indicates that energy transfer occurs among pigment molecules in the interface

environment.[14] Although emission from diluted chlorophyll a monolayers has been reported as depolarized, the experimental arrangement used in the study did not permit theoretical analysis.[15]

We have studied the fluorescence properties and electronic energy transfer of chlorophyll a molecules in monolayers at a nitrogen-water interface. Mixed films of chlorophyll together with colorless surface-active lipids undergo increased fluorescence quenching and depolarization with increasing chlorophyll a concentration in the films. The experimental evidence shows that the pigment molecules do not move freely in these environments. Thus, transfer is restricted to a non-radiative mechanism which does not involve transport of mass and does not in general require pigment contact.[16] Two such mechanisms, inductive resonance[17] and exciton migration,[18] have been treated extensively in the literature. Franck and Livingston[19] and Katz[20] suggested a role for these processes in photosynthesis many years ago. Lacking sufficient experimental information, they were unable to evaluate the relevance of the mechanisms. The question of which, if either, of these mechanisms occurs in chloroplast lamellae has not yet been resolved. We have examined the applicability of these two mechanisms to our model systems. Exciton migration appears to be possible only in a random two-dimensional array of chlorophyll a at very high pigment concentrations. Energy transfer by inductive resonance accounts satisfactorily for our experimental results over the full range of concentrations studied.

The diffusion of localized excitons has recently received attention as a possible means of energy transfer in the photosynthetic apparatus.[21,22]

According to Trlifaj's mathematical treatment of this mechanism the energy transfer rate is proportional to the inverse sixth power of the intermolecular distance.[23] As this dependence on separation is also exhibited by inductive resonance energy transfer, the two processes cannot be distinguished by our method of investigation.

THEORY

Fluorescence depolarization by energy transfer

An array of fixed, isolated, randomly-oriented molecules excited by linearly polarized light will exhibit polarized emission. The degree of this polarization measured in the forward direction depends only on the orientation of absorption and emission oscillators. If the molecules rotate while in the excited state, or if they constitute an ensemble of molecules which interact so that excitation energy is transferred among them, the observed fluorescence of the system will become depolarized. In the absence of molecular movement, the extent of fluorescence depolarization is a measure of the extent of energy transfer among differently oriented molecules.

This may be seen by expressing the observed macroscopic polarization as a function of the energy transfer rate. We start with the equation of Weber[24]

$$1/P - 1/3 = (1/P_o - 1/3) \left[\sum_n f_n \left(\frac{3 \cos \theta - 1}{2}\right)^n\right]^{-1} \qquad (1)$$

where P is the observed polarization, P_o the limiting polarization in the absence of energy transfer, f_n the fraction of the fluorescence intensity emitted by the n^{th} molecule to be excited as the energy is transferred in the array, and θ is the average angle between emission oscillators in any pair of molecules. To obtain 1/P in closed form,

f_n should be expressed in the form a x b^n, with a and b independent of n. In the general case of a random array of molecules in which back-transfers occur, a simple expression for f_n is not easily obtained. For this case we have

$$f_n = \frac{1}{\tau} \int_0^\infty \overline{p_n(t)}\, e^{-t/\tau}\, dt$$

where $p_n(t)$ is the probability that the n^{th} excited molecule is excited at time t, and τ is the experimental lifetime of the excited state. The most general expression for $p_n(t)$ is

$$p_n(t) = -1/\tau - \sum_\mu w_{n\mu}\, p(\mu,r_\mu) + \sum_\nu w_{\nu n}\, p_\nu(t) p(n,r_n) \qquad (2)$$

Here, the w_{ij} are pairwise transfer rates from molecule i to molecule j, the form of which depends on the strength of the average molecular interaction, and the p's are partition functions for a random distribution of molecules. The last term is included to describe properly weighted back transfers of energy among identical molecules. This equation does not admit of a simple solution, and we have not obtained a closed form expression for equation (1) in this general case.

If, however, back transfer is ignored and certain assumptions are made as to the arrangement of the molecular array, Weber[14] has shown that equation (1) is easily solved. We shall consider special cases of fluorescence depolarization due to energy transfer, after describing the model system in detail and discussing the energy transfer mechanisms in this context.

Description of the system

First we determine P_o, the limiting degree of polarization in the absence of energy transfer, for a two-dimensional monolayer containing chlorophyll a. The x-y plane is that of the monolayer, which is excited

from the z direction by linearly polarized light, with the electric vector parallel to the y-axis (Fig. 1a). The limiting degree of fluorescence polarization, observed in the z direction, from an ensemble of molecules is then defined as

$$P_o = \frac{\bar{I}_y - \bar{I}_x}{\bar{I}_y + \bar{I}_x} \quad , \tag{3}$$

where $\bar{I}_x$ and $\bar{I}_y$ are the average fluorescence intensities with electric vectors in the x and y directions. The absorption, A, and emission, F, oscillators of a given molecule both lie in the plane of the porphyrin ring of chlorophyll _a_[25] and define an angle α in this plane. The oscillators form angles θ_A and θ_F, respectively, with the z-axis. The projections of the absorption and emission oscillators in the x-y plane form angles ϕ_A and ϕ_F with respect to the x-axis. The fluorescence intensities emitted by this molecule in the x and y directions are

$$\begin{aligned} I_x &= (\sin\theta_A \sin\phi_A)^2(\sin\theta_F \cos\phi_F)^2 \\ I_y &= (\sin\theta_A \sin\phi_A)^2(\sin\theta_F \sin\phi_F)^2 \end{aligned} \tag{4}$$

A rationalization of this coordinate system in terms of the chemical properties of chlorophyll _a_ is shown schematically in Fig. 1b. The placement of the carbonyl and carboxyl groups in the aqueous interface follows the reasoning of Bellamy, _et al._[11]

To obtain the macroscopic polarization observed from an ensemble of molecules [Equation (3)], the intensities must be averaged over all possible molecular orientations; _i.e._, over all azimuthal angles and all allowed polar angles, subject to the restriction that α = constant. Performing these operations (see Appendix A), we obtain finally

$$P_o = \frac{\cos^2\alpha - 2\cos\alpha \cos\theta_A \cos\theta_F + \cos^2\theta_A \cos^2\theta_F}{\sin^2\theta_A \sin^2\theta_F} - 1/2 \tag{5}$$

If absorption and emission oscillators are parallel, $\alpha = 0$ and $\theta_A = \theta_F$. The limiting degree of polarization then reduces to

$$P_o = \frac{1 - 2\cos^2\theta + \cos^4\theta}{\sin^4\theta} - 1/2 = 1/2 .$$

When absorption and emission oscillators are perpendicular,

$$P_o = \cot^2\theta_A \cot^2\theta_F - 1/2$$

We emphasize that Equation (5) is obtained using the assumption that a constant average angle $\bar{\beta}$ (Appendix A) determines the orientation of the molecular planes with respect to the plane of the ensemble, which assumption is probably applicable to our experimental model.[11,15] In this case, if $\alpha = 0$, P_o is the same as that observed in random three-dimensional systems.[26] However, when the absorption and emission oscillators are not parallel, the limiting degree of polarization in the two-dimensional model depends on the orientation of the molecule with respect to the surface as well as upon α. In three-dimensional systems, P_o is a function only of the angle between the oscillators.

The error introduced into Equation (5) for P_o if β is not constant may be determined by differentiating Equations A2 and A3 with respect to β. We obtain

$$\frac{d(\cos\theta_A)}{\cos\theta_A} = \frac{d(\cos\theta_F)}{\cos\theta_F} = \cot\beta\, d\beta .$$

For example, a fluctuation of 3° in β for $\bar{\beta} = 155°$ causes an uncertainty in $\cos\theta_A$ and $\cos\theta_F$ of about 4%. These values seem reasonable for highly compressed monolayers containing chlorophyll a.

Energy transfer mechanisms

Many discussions of the criteria for the occurrence of exciton migration and inductive resonance energy transfer have appeared in the literature.[17,18] Recently Förster[27] has presented a unified approach to this

problem, distinguishing three possible cases. Depending on the relative magnitudes of molecular interactions and spectral band widths, energy transfer among like molecules may occur by free or localized exciton migration, or by inductive resonance processes. Förster calculates that the approximate boundary between the latter two possibilities is characterized by

$$u_{vv'} \gtrsim \Delta\varepsilon'/4 \ . \tag{6}$$

Here $u_{vv'}$ is the vibronic interaction matrix element between vibrational levels v and v' of an electronic state, and $\Delta\varepsilon'$ is the vibronic bandwidth; i.e., the interaction energy must be greater than one-quarter of this band width if exciton migration is to occur. In the point dipole approximation, the electrostatic interaction energy between electronic states of two molecules i and j is

$$u_{ij} = \frac{k_{ij}|u|^2}{n^2 r_{ij}^3} \ . \tag{7}$$

n is the refractive index of the medium, r_{ij} the center-to-center separation of the molecules, $|u|$ their transition electric dipole moment, and k the orientation factor in the dipole interaction. The vibronic matrix element in equation (6) may be written

$$u_{vv'} = u_{ij} S^2_{vv'} \ , \tag{8}$$

where the vibrational overlap matrix element $S^2_{vv'} \leq 1.0$.[17]

Equations (6) through (8) may be used to determine which mechanism of energy transfer is likely to occur in chlorophyll-containing monolayers. In general, the vibronic band width, $\Delta\varepsilon'$, is obtained from high resolution absorption spectra; however, vibrational band fine structure is not resolved even in low temperature chlorophyll a spectra, and gas

phase spectra of the pigment are not available. Förster suggests that 30 cm^{-1} is a reasonable estimate for vibronic band widths,[27] but cautions that the existence of localized excitons in a system where vibrational band fine structure cannot be observed is questionable. We calculate $|\mu|$ from the relation of McRae and Kasha,[28] using $f = 0.23$ and $\lambda = 6.7 \times 10^{-5}$ cm, for chlorophyll a in a polar solvent.[29] Then for an average refractive index of 1.17 for the monolayer environment[14] we have

$$u_{vv'} = 1.15 \times 10^5 \frac{k S_{vv'}^2}{r^3} \quad (cm^{-1}), \qquad (9)$$

if r is the average nearest neighbor separation in angstroms. The orientation factor is

$$k_{ij} = \cos \psi_{ij} - 3 \cos \psi_i \cos \psi_j ,$$

where ψ_i and ψ_j are the angles between the i^{th} and j^{th} transition dipoles, respectively, and the line joining them, and ψ_{ij} is the angle between dipoles. An average over all allowed possible orientations of chlorophyll molecules in the monolayer is required to obtain k. For the monolayer geometry, where the line joining any two dipoles is parallel to the monolayer plane, we have

$$k = \sin^2\theta_F (\cos \psi_i \cos \psi_j - 2 \sin \psi_i \sin \psi_j) + \cos^2\theta_F$$

since we have assumed β is constant and hence $\theta_i = \theta_j = \theta_F$. When this expression is averaged over all possible azimuthal angles, we find that k may vary from $\gtrsim 0.1$ for a random array to 1.0 for parallel oscillators.

If equation (9) is now substituted into equation (6), we see that the interaction energy of molecules in a random array exceeds one-quarter of the bandwidth only if the pigment molecules are less than 5 Å apart,

provided in addition that the vibrational overlap integral approaches unity. This spacing is approximately equivalent to that of adjacent chlorophyll a molecules. Thus, in a randomly oriented array, and/or in the case that the vibrational overlap integral is significantly less than unity, the interaction energy is not sufficiently strong that exciton migration is likely to occur. Only in a monolayer containing a high mole fraction of chlorophyll a in an ordered array, in which case fluorescence will not be strongly depolarized, is exciton migration probable. Otherwise, we predict that energy will migrate among pigment molecules by the mechanism of inductive resonance.

Critical distance for transfer by inductive resonance

The above considerations suggest that we consider that energy is transferred by inductive resonance among chlorophyll a molecules in the monolayers. Förster[30] has developed an extensive formalism for the general three-dimensional case. He shows that the rate of pairwise energy transfer is proportional to the square of the interaction energy [Equation (7)] and defines a critical distance for transfer, R_o, as follows:

$$w_{ij} = \frac{4\pi^2 |u_{ij}|^2}{h \Delta\epsilon'} \sum_{v,v'} S^4_{vv'} = \frac{1}{\tau} \left(\frac{R_o}{r_{ij}}\right)^6 \qquad (10)$$

Here, w_{ij} is the pairwise energy transfer rate, τ is the experimental fluorescence lifetime of the pigment molecule, and the other symbols are used as previously defined. R_o, which is that molecular separation at which emission and transfer are equally probable, may be calculated from spectral parameters according to an equation obtained from a classical derivation of the transfer rate,[30]

$$R_o^6 = \frac{9\, k^2 c^4 \ln 10}{128\pi^6\, n^4\, N'} \frac{\tau}{\tau_o} \int_o^\infty \varepsilon(\nu)\, f(\nu)\, \frac{d\nu}{\nu^4} \qquad (11)$$

c is the velocity of light, N' the number of molecules in a millimole, n the refractive index of the medium, τ_o the natural fluorescence lifetime of the molecules, $\varepsilon(\nu)$ the extinction coefficient of the molecules at frequency ν, and $f(\nu)$ the normalized fluorescence spectrum. We will compare a critical distance so computed from chlorophyll a monolayer spectral properties by Tweet, et al.[14] with separations determined from fluorescence polarization characteristics, as described below.

Ideally, equation (10) is to be inserted in equation (2). The general solution to equation (1) is then obtained for the degree of fluorescence polarization as a function of molecular separation and involving the critical distance. Because we have not been able to obtain equation (2) in closed form, we shall instead consider two limiting cases. The first is one discussed by Förster himself. If a single transfer of excitation energy among molecules in a random array is sufficient to depolarize fluorescence, the relative degree of polarization is a direct measure of the fraction of initially excited molecules which fluoresce. This assumption obviously represents the greatest possible decrease in polarization with increasing energy transfer. In this case, Förster defines a critical concentration C_o such that

$$C = C_o \quad \text{when } P = P_o/2 \ . \qquad (12)$$

At this concentration the average separation between interacting molecules is the critical distance R_o. C_o may be obtained from plots of 1/P vs. C. The separation R_o, which in this case of one-step depolarization gives an upper limit for the critical distance, is then calculated directly..

Alternatively, we may use the approach suggested by Weber,[24] assuming that fluorescence polarization is inversely proportional to an average transfer rate. This assumption implies that the molecular array is spatially uniform and omits consideration of back transfer. It results in an underestimation of the critical distance for energy transfer, because it overlooks the fact that repeated energy transfers back and forth between two closely spaced molecules occurs with high probability and does not contribute correspondingly to fluorescence depolarization. Using this approximation to obtain a lower limit for the critical separation, we follow Weber in writing

$$f_n = (\overline{\Sigma w_{ij}})^n \quad (1 - \overline{\Sigma w_{ij}}) \tag{13}$$

for the fraction of the total fluorescence intensity emitted after n transfers. Substituting this expression into equation (1), using equations (7) and (10) for the interaction energy and transfer rate, and averaging over all allowed orientations of the pigment molecules in the monolayer geometry, we obtain an expression for the critical transfer distance, R', as a function of the observed polarization and the pigment concentration:

$$R' = \left[\frac{4\,(2a)^4\,S}{3\pi(1/P_o - 1/3)} \quad \frac{1}{B(\cos^4\theta_F + 5/4 \sin^4\theta_F)} \right]^{1/6} \tag{14}$$

S is the slope of a plot of 1/P vs. C, and B is an angular factor (see Appendix B). In calculating this lower limit of the critical separation, we have used a modification of Förster's definition,

$$w_{ij} = \frac{1}{\tau} \left(\frac{R_o}{r_{ij}}\right)^6 = \frac{1}{\tau} k_{ij}^2 \left(\frac{R'}{r_{ij}}\right)^6 \tag{10a}$$

and averaged over all allowed k_{ij} in a random two-dimensional array.

From experimental data we will calculate R_0 [Equation (12)] and R' [Equation (14)] and compare these values of the critical distance for special cases of inductive resonance energy transfer with that value computed by Tweet, et al. from spectral data. This separation, $R_0(s)$, should fall within the range defined by the limiting values obtained from the polarization data, if inductive resonance energy transfer does occur among chlorophyll a molecules in the monolayers.

MATERIALS AND METHODS

Apparatus

The monolayer fluorometer used in these experiments was a modified commercial Langmuir film balance (Central Scientific Company). The trough, painted black and heavily coated with paraffin, was mounted on a base plate on which was constructed an automatic barrier drive mechanism similar to that described by Gaines.(31) Gears were chosen such that the barrier moved at a constant rate of 20 mm/min, corresponding to a change in monolayer surface area of 28 cm^2/min. The trough, torsion balance, and drive mechanism, with the exception of the motor and gears, could be enclosed in a blackened Lucite cover. The torsion balance vernier extended through this cover, which had windows for observation of the float pointer and barrier position indicator. Ports allowed for sweeping the enclosure with inert gas, for spreading the films, and for positioning the photomultiplier.

The optical system, shown schematically in Fig. 2, was designed so that the exciting light was incident along a normal to the surface plane, and the fluorescence emitted was observed in the forward direction. The light source, an air-colled 100-watt mercury lamp (General Electric AH-4),

was mounted horizontally underneath the experimental table. The light passed through a collimating lens, filters I for isolating the 406 nm mercury line (two 406 nm narrow band pass interference filters, Baird-Atomic, Inc., type B-1, with infrared blocking; Corning band pass filter No. 5-58), a polarizer (Polaroid HN 22) which could be rotated exactly 90° in its holder, and a 2" diameter quartz window seated in an opening in the bottom of the trough. A shutter sliding between the trough mount and table had provision for a colored glass filter used as a fluorescence standard. We found that a Corning red cutoff filter, No. 2-63, fluoresced with sufficient intensity in the region of chlorophyll a fluorescence to be useful as a standard. French [32] has reported a similar phenomenon for several cutoff filters.

Filters II for blocking the exciting light (Optical Coating Laboratories, Inc., dielectric rejection filter, OD = 2.07 at 406 nm) and isolating the pigment fluorescence band (two Corning red cutoff filters, No. 2-58 and 2-59) were attached to the lower end of the photomultiplier holder. A separate holder for the analyzer (Polaroid HR), which could be rotated 360°, fit into the photomultiplier holder.

A red-sensitive photomultiplier (RCA 7326 with S-20 response) was used to detect fluorescence. It was operated at 1800 volts from a regulated supply. The photomultiplier was not cooled, but it was sheathed in a mu metal shield connected to the screen on the photomultiplier leads. The photomultiplier output was amplified using a DC microvoltmeter (Keithley, Model 151) and recorder (Moseley, Model 680 Autograf).

A 240 K ohm resistor across the voltmeter input terminals reduced the noise level of the circuit, but lengthened the time constant to approximately 2 sec.

Materials

Chlorophyll a was isolated from spinach chloroplasts by column chromatography according to the method of Anderson and Calvin,[33] and the microcrystalline suspension in isooctane stored in a refrigerator under nitrogen. All operations with the pigment were carried out in dim green light or in the dark.

Castor oil (Baker Castor Oil Company, dB refined grade, MW 928, viscosity 6.8 poise) was taken from freshly opened cans. Oleyl alcohol (9-octadecen-1-ol, Hormel Institute, University of Minnesota), reported to be at least 99% pure, was used directly.

Plant structural lipids, monogalactodilinolenate and sulfoquinovodiglyceride, were extracted from spinach chloroplasts and purified by column and thin-layer chromatography by a modification[34] of the procedures of O'Brien and Benson[35] and Nichols.[36]

Benzene (Baker and Adamson or J. T. Baker, reagent grade) used as the spreading solvent was redistilled from sodium hydride. All monolayers were spread on a subphase of 10^{-3} M aqueous phosphate buffer, pH 7.6 - 7.8, made from reagent grade potassium mono- and dibasic phosphates and distilled, deionized water.

Methods

Standard techniques were used to spread monolayers from benzene solutions.[37] Pure chlorophyll a solutions were prepared by dissolving a known weight of dried pigment in benzene. The concentration was then checked with the absorption spectrum, using the extinction coefficients of Seely and Jensen.[38] If mixed films were to be formed, aliquots of the pigment solution were combined with aliquots of lipid solution,

which had been made up to 1 mg/ml in benzene. The final concentrations of all spreading solutions were adjusted so that 1 to 4 x 10^{16} molecules could be deposited on the surface in 50 to 200 microliter aliquots. Monolayers were formed after the fluorometer had been assembled, the trough filled and covered, and the enclosed space swept with buffer-saturated nitrogen gas. The light reaching the photomultiplier with polarizers crossed and parallel, and the fluorescence of the standard fluorescing filter were recorded before films were spread. After a short time had been allowed for evaporation of the spreading solvent, the monolayers were compressed at a constant rate to a surface pressure of approximately 12 dynes/cm. The films were maintained at this pressure while several measurements of fluorescence intensity with polarizers crossed and parallel were taken. Thirty-sec to 1-min traces were recorded for each polarizer setting to minimize the effects of spurious noise from the mercury arc and photomultiplier. The final barrier position was read from a scale on the cover which was calibrated to actual position on the trough.

We also observed the fluorescence polarization of three-dimensional viscous solutions of chlorophyll a using the monolayer fluorometer. This was accomplished by placing a small covered pyrex petri dish containing solutions, prepared as described by Goedheer,[39] directly over the window in the bottom of the empty trough. The edges of the dish were masked to avoid light scattering. Fluorescence intensities were measured as described above for films. In this case, however, the petri dish filled with solvent was used to obtain blank readings.

The degree of fluorescence polarization was calculated from each pair of readings with polarizers crossed and parallel according to

$$P = \frac{\Delta}{2I_{\perp} + \Delta} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad (15)$$

where $\Delta = I_{\parallel} - I_{\perp}$. The intensity values were corrected for the signal observed in the absence of a film and for variations in source intensity as indicated by the fluorescence standard. Then the average value and standard deviation of P were computed for each sample film or solution. If the chlorophyll a molecules are fixed in the systems, the limiting polarization, P_o, is reached at infinite dilution, when no energy transfer can occur. We determine this degree of polarization in each diluent lipid by extrapolation of a plot of 1/P vs. pigment concentration per unit area (or per unit volume). The critical distances for transfer by inductive resonance [Equations (12) and (14)] are obtained from the slope of the least-squares straight line fits to these data at low concentration.

RESULTS AND DISCUSSION

Viscous solutions of chlorophyll a

Several workers[39,40,41] have reported limiting fluorescence polarizations of $P_o = 0.2 \pm 0.04$ for chlorophyll a in castor oil, excited by 406 nm light and observed with conventional apparatus. Extrapolation of our data for this system yielded a maximum polarization of 0.214 ± 0.008, in agreement with the published values. For chlorophyll a dissolved in oleyl alcohol, we obtained a maximum polarization of 0.109 ± 0.025. A lower limiting polarization is expected in this solvent, owing to the lower viscosity of the alcohol.

The maximum polarization possible in the absence of energy transfer or rapid molecular rotation in a random three-dimensional array of

molecules is 0.5. This value is reached when the absorption and emission oscillators are parallel.[16] The published value of 0.42 for the polarization in the long wavelength region shows that chlorophyll a in castor oil solution undergoes little, if any, rotational depolarization.[40] The absorption band near 406 nm has been assigned to the B_y transition, which is approximately parallel to the oscillator responsible for emission.[40,42] The low observed polarization at 406 nm, which is about half the value expected for a parallel transition, probably results from a partial overlap with the B_x band centered near 435 nm together with a somewhat non-parallel orientation of the B_y and the emission oscillators. Calculation of transition moment directions using a point monopole expansion for chlorophyll a supports the latter conclusion.[43]

Extrapolation of our polarization measurements to infinite viscosity using a double reciprocal plot[26] yields a maximum polarization of 0.24, which is still well below the theoretical limit. Using the equation of Perrin[26] for the limiting polarization in three dimensions and our value, 0.24, for P_0, we find the angle between the effective 406 nm absorption and red emission oscillators to be about 35°.

Pure chlorophyll a monolayers

The fluorescence of several pure chlorophyll a monolayers was recorded at a surface pressure of 12 dynes/cm. In no film did the degree of polarization calculated from equation (15) exceed 0.008. In most cases it was less than this. The relatively weak fluorescence did not limit the precision of the measurement, which was ± 0.004, even for large, highly polarized signals.

In a monolayer of pure chlorophyll a the pigment molecules are sufficiently close to each other that the occurrence of an exciton state is a possibility. However, even in the absence of exciton migration, energy transfer would be expected to proceed very rapidly among adjacent molecules. If the chlorophyll a molecules were randomly oriented with respect to the normal to the film surface, the fluorescence should be depolarized regardless of the energy trnasfer mechanism. We observed unpolarized fluorescence, which suggests that the pigment molecules are unordered in a pure chlorophyll a monolayer on an aqueous subphase.

Chlorophyll a in lipid monolayers

Figs. 3 and 4 show the observed degree of polarization as a function of the fractional area occupied by chlorophyll a in monolayers of the four diluent lipids used. Areas are calculated from pressure-area data for pure lipid and for pure pigment films, using the assumption that all chlorophyll a molecules are tilted out of the monolayer plane to the same extent. As discussed above, the error so introduced is probably small. The dependence of relative fluorescence yield per unit area occupied by chlorophyll, Φ/Φ_0, upon pigment concentration is also shown in the figures.

The fluorescence yield and degree of polarization of the pigment decreases as chlorophyll concentration in monolayers of all four diluent lipids increases. On the basis of results for pure chlorophyll monolayers we would expect the polarization to decrease to less than 0.01 as the pigment concentration approaches unity. However, in films of castor oil, oleyl alcohol, and sulfolipid the fluorescence polarization

does not appear to fall to zero. This phenomenon was checked carefully in films of chlorophyll a and sulfolipid.

Feofilov and Sveshnikov[44] have observed that fluorescence polarization of some dyes fails to approach zero at high concentrations in viscous solution. As in the present case, the phenomenon occurs in the region of strong fluorescence quenching, where fluorescence lifetimes are considerably shortened. It has been attributed[16] to a substantial reduction of the number of energy transfers possible during the shortened lifetime of the excited state. We question whether this explanation is completely satisfactory for our pigment-containing monolayers, because the polarization falls smoothly to zero as chlorophyll a concentration is increased in monogalactolipid films (Fig. 4A). If lifetime shortening were due simply to increasing concentration, we would expect residual fluorescence polarization at high pigment concentrations in this lipid also. Instead, the diluting lipid seems to determine the polarization behavior of the pigment in the monolayers.

The observed results may be accounted for if chlorophyll a is randomly dispersed in films of galactolipid, but is partially oriented in the other lipid monolayers. In these latter films, increasing pigment concentration would not cause a corresponding decrease in polarization, because the concommitant increase in energy transfers would be occurring among partially aligned molecules. This explanation is applicable regardless of the extent of pigment aggregation. On the other hand, pressure-area behavior of the mixed films indicates that chlorophyll a is indeed miscible with these lipids.[13,34]

In mixed monolayers in which the pigment molecules are oriented at the higher concentrations, the value of k in equation (9) would approach unity and exciton migration could possibly occur. Fluorescence polarization measurements might not reflect this change in energy transfer mechanism upon increasing concentration, because the degree of polarization should reach a constant minimum value owing to the molecular alignment. Exciton interaction may therefore be effected by the diluent lipid, insofar as the lipid determines the extent of pigment orientation at close order.

Recently Sperling and Ke[45] presented evidence for the existence of pigment aggregates in pure and mixed monolayers of chlorophyll a and arachidic acid. The extent of this aggregation changed with time, and the aggregates, initially containing some degree of order, appeared to become disordered with time when removed from an aqueous to a lipid surface. We detected no systematic decrease in in situ monolayer fluorescence polarization with time up to the point of substantial fall in fluorescence yield of the samples. (Measurements were not made beyond this time (ca. 45 min), as we assumed that the decrease in yield reflected chemical changes of the pigment from prolonged exposure to light and water.) This finding indicates that the changes in molecular order observed by Sperling and Ke may have been due to the treatment their films received.

The polarization data at low chlorophyll concentrations in mixed films is plotted as 1/P vs. C in order to determine the limiting polarization, P_0, in the absence of energy transfer for each system (Figs. 5 and 6). These values of P_0, Table I, are well below those calculated

from equation (5), assuming either that the emission and absorption oscillators are parallel ($\alpha = 0°$, $P_0 = 0.5$) or that they form an angle ($\alpha = 35°$, $P_0 = 0.36 \pm 0.09$). This result suggests that the monolayers under compression were not rigid systems, and further, that monolayer viscosities are lower than the corresponding bulk viscosities. Although molecular movement is no doubt restricted in monolayers under pressure, it is unlikely that it is completely prevented in these liquid-expanded films. By permitting molecular motion, media of finite viscosities would lower the observed values of the limiting polarization.

We can use the same fluorescence polarization data to calculate critical distances for energy transfer by inductive resonance. The slopes of the 1/P vs. C plots are obtained from the least-squares straight line fits to the data. From these values and the limiting polarizations, we can compute R_0 [Equation (12)] and R' (Equation (14)], the upper and lower limits, respectively, of the critical separation.

For the calculation of R', it was necessary to assign a value to the molecular diameter, 2a, and to determine θ_F. We followed Bellamy, et al.[11] in computing the angle β from the average area per pigment molecule at the surface pressure at which fluorescence measurements were made. Then, from equation (A2), $\theta_F = 72 \pm 4°$. We assume that the effective molecular diameter is equivalent to the distance of closest approach of two pigment molecules in the film, which separation may vary from the thickness of a porphyrin ring, about 5 Å, to the length of its side, 15 Å, i.e., $2a = 10 \pm 5$ Å.

The results are summarized in Table I. The assumption that one transfer of energy is sufficient to depolarize emission yields a critical

transfer distance three to four times as large as the alternative case involving repeated transfers among fluorescing molecules in a uniform array, as mentioned previously.

The experimentally determined limiting values, R_o, for the critical separation for inductive resonance energy transfer are to be compared with the quantity $R_o(s)$ calculated from chlorophyll a monolayer spectral properties by Tweet, et al.[14] Starting with Förster's relation [Equation (11)], they used an average value of the orientation factor appropriate to the monolayer geometry and approximate values for the refractive index of the medium, the monolayer molar extinction coefficient, and the experimental fluorescence lifetime. They found $R_o(s) = 54 \pm 8$ Å, allowing for the shift of the red absorption maximum of the pigment in the film. This value falls between the upper and lower limits of the critical distances listed in Table I for each of our experimental systems.

We see that Förster's assumption that one energy transfer is sufficient to effect depolarization is approximately valid for castor oil and oleyl alcohol diluents, but apparently not for the chloroplast lipids. In the latter mixed monolayers, more than one transfer of the excitation energy is apparently required. This may, in the case of sulfolipid where the degree of polarization never approaches zero, be due to a partial orientation of chlorophyll by the lipid molecules.

The values of R' in Table I, which are calculated assuming that the pigment system is a uniform array in which back transfers do not occur, significantly underestimate the critical distance. This suggests that back transfers, or energy transfer to similarly oriented molecules,

are frequent, and also that the chlorophyll molecules are probably not uniformly distributed in the lipids.

CONCLUSIONS

The energy requirements for the existence of exciton states in a lipid monolayer containing chlorophyll a indicate that such states are unlikely to occur in a random array of pigment molecules separated by diluent. On the other hand, in a highly ordered array the transfer of excitation energy by exciton migration among non-adjacent chlorophyll molecules is a possibility.

When the interaction energy is not sufficiently strong that exciton states exist, energy transfer can occur by inductive resonance. Such transfer may be characterized by a critical distance, originally defined by Förster.[30] An upper limit and an underestimation of the critical separation, R_0 and R' respectively, are calculated from fluorescence polarization data for several monolayer systems, and compared with the value, $R_0(s)$, obtained from spectral parameters by Tweet, et al.[14] In view of the assumptions made in these calculations, inductive resonance energy transfer appears adequate to account for the observed polarization behavior. These assumptions, however, are not rigorously applicable to the chlorophyll-chloroplast lipid monolayers. The pigment molecules are probably not uniformly dispersed in these lipids. With sulfolipid films, in addition, residual polarization at high pigment concentrations suggests that the chlorophyll a in these monolayers is partially oriented. Thus the mechanism of energy transfer among chlorophyll a molecules in a monomolecular layer depends not only on the pigment concentration, but also on molecular orientation, which may be under the influence of the lipid environment.

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Appendix A

In the model system for chlorophyll a at an air-water interface, the azimuthal angles ϕ_A and ϕ_F are related by

$$\phi_F = \phi_A - \cos^{-1}\left[\frac{\cos\alpha - \cos\theta_A \cos\theta_F}{\sin\theta_A \sin\theta_F}\right] . \qquad (A1)$$

The porphyrin plane is assumed to be tilted with respect to the monolayer plane, the angle of tilt depending on the extent of compression.[11] We specify this orientation by the angle β formed by the normal to the porphyrin plane (the positive direction into the subphase) and the surface normal (z-axis). Then θ_A may be defined in terms of θ_F, α, and β according to

$$\cos\theta_A = \cos\alpha \cos\theta_F \pm \sin\alpha (\sin^2\beta - \cos^2\theta_F)^{1/2} .$$

By observing the spatial anisotropy of fluorescence intensity from a monolayer containing chlorophyll a, Tweet, et al.[15] calculated that

$$\cos\theta_F \approx \sin 20^\circ \cos(\beta - \pi/2) = 0.34 \sin\beta \qquad (A2)$$

We obtain thus

$$\cos\theta_A = \sin\beta (0.34 \cos\alpha \pm 0.94 \sin\alpha) . \qquad (A3)$$

θ_A and θ_F depend only on α and β. α is fixed in the molecules, but is a function of the wavelength of illumination. Bellamy, et al.[11] and Tweet, et al.[13] present evidence suggesting that for a chlorophyll a monolayer under constant compression, the average value of β is a constant determined by the compression. If we assume that $\bar{\beta}$ is indeed constant, equation (A1) may be written $\phi_F = \phi_A - \gamma$, where γ is a constant angle. Substituting this expression into equations (4) and averaging over all ϕ_A from 0 to 2π, yields upon substitution into equation (3)

$$P_o = \frac{\cos^2\alpha - 2\cos\alpha \cos\theta_A \cos\theta_F + \cos^2\theta_A \cos^2\theta_F}{\sin^2\theta_A \sin^2\theta_F} - 1/2$$

Appendix B

Substitution of equation (13) into equation (1) yields, upon expansion of the sum,[24]

$$1/P - 1/3 = \left(1/P_o - 1/3\right)\left(1 + \frac{3\sin^2\theta}{2}\,\frac{\Sigma w_{ij}}{1 - \Sigma w_{ij}}\right) \qquad \text{(B1)}$$

Now, we have from Förster[17]

$$w_{ij} = \frac{1}{\tau}\left(\frac{R_o}{r_{ij}}\right)^6 = \frac{1}{\tau}\,k^2\left(\frac{R'}{r_{ij}}\right)^6 \qquad \text{(B2)}$$

Then the average transfer rate is given by

$$w_{ij} = \frac{(R')^6}{\tau}\left\langle\int_{2a}^{\infty}\frac{\rho(r_{ij})\,dr_{ij}\,k^2}{(r_{ij})^6}\right\rangle_{\text{angles}} ;$$

if 2a is the molecular diameter, and $\rho(r_{ij})$ is the radial distribution function for the molecules. The average is to be taken over all allowed angles in the "random" array. For the model two-dimensional system at hand, this is equivalent to integration over ϕ_i and ϕ_j from 0 to 2π, and substitution of $\theta_i = \theta_j = \theta_F$. The two-dimensional density function is $2\pi rC\,dr$, where C is the concentration per unit area. Performing the integrations indicated in equation (B3), and substitution into and rearrangement of equation (B1) then gives

$$R' = \left[\frac{4(2a)^4\ \text{slope}}{3\pi(1/P_o - 1/3)}\,\frac{1}{B(\cos^4\theta_F + 5/4\,\sin^4\theta_F)}\right]^{1/6}$$

Table I. CRITICAL DISTANCE FOR ENERGY TRANSFER IN DILUTE CHLOROPHYLL a MONOLAYERS

Lipid diluent	Slope*	$1/P_o - 1/3$*	R_o†	R'††
Castor oil	$(5.4 \pm 0.6) \times 10^2$	16.5 ± 1.0	57 ± 4 Å	17.5 ± 6 Å
Oleyl alcohol	$(7.6 \pm 1.5) \times 10^2$	21.2 ± 4.2	57 ± 11 Å	17.5 ± 11 Å
Monogalactolipid	$(11.4 \pm 2.1) \times 10^2$	9.4 ± 2.2	88 ± 22 Å	21.5 ± 8 Å
Sulfolipid	$(9.3 \pm 0.9) \times 10^2$	14.0 ± 1.9	78 ± 9 Å	19.5 ± 7 Å

*From Figs. 5 and 6

†Equation (12)

††Equation (14)

FIGURE CAPTIONS

Fig. 1a. Orientation of molecular absorption, A, and emission, F, oscillators of chlorophyll *a* with respect to a monolayer surface (xy plane). A and F are in the plane of the porphyrin ring. See text for definitions of angles.

(MUB-14008)

b. Proposed orientation of a chlorophyll *a* molecule at an aqueous interface.[11]

(MUB-14007)

Fig. 2. Monolayer fluorometer optical and electronic schematic diagram. See text for details.

(MUB-14006)

Fig. 3. Concentration dependence of relative fluorescence intensity (--Δ--) and fluorescence polarization (—o—) of chlorophyll *a* in mixed monolayers. Curves are calculated from least-squares fits to the data.

(XBL 676-1136)

Fig. 4. Ditto

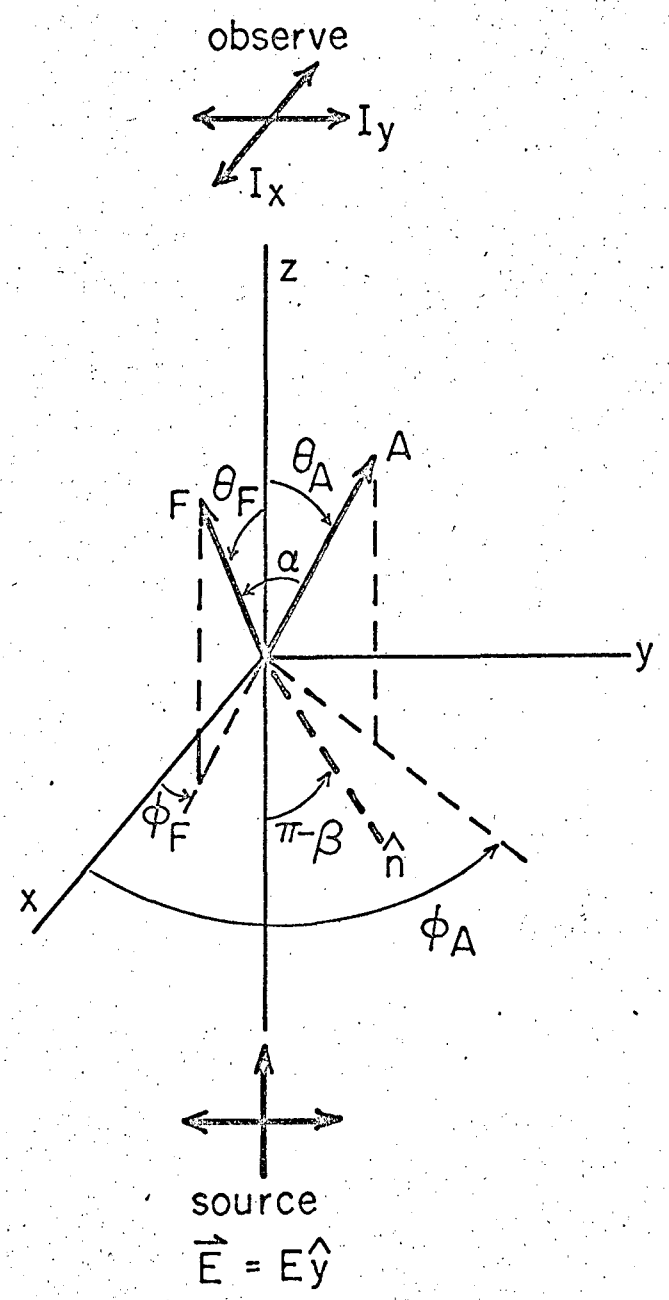
(XBL 676-1135)

Fig. 5. Dependence of the reciprocal fluorescence polarization on chlorophyll *a* concentration in mixed monolayers. The least-squares straight line has been extrapolated to zero concentration to obtain the limiting degree of polarization, P_o, in each case.

(XBL 676-1138)

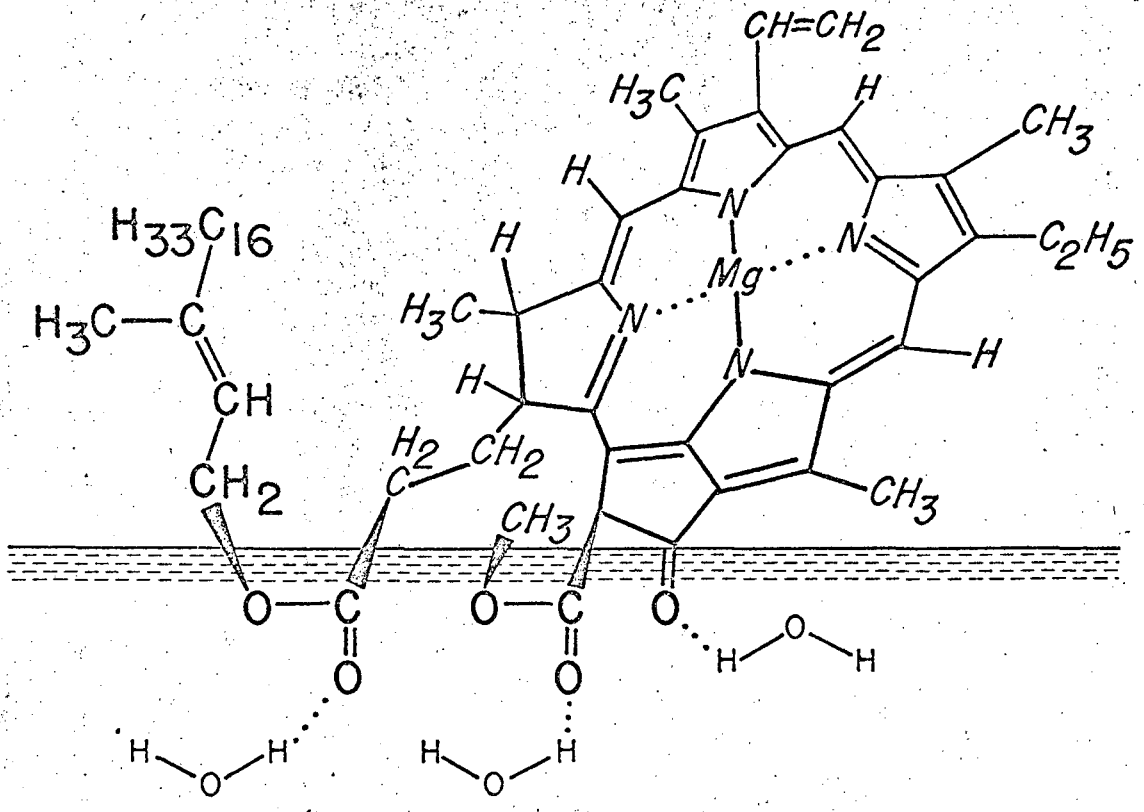
Fig. 6. Ditto

(XBL 676-1137)



MUB-14008

Fig. 1



MUB-14007

Fig. 2

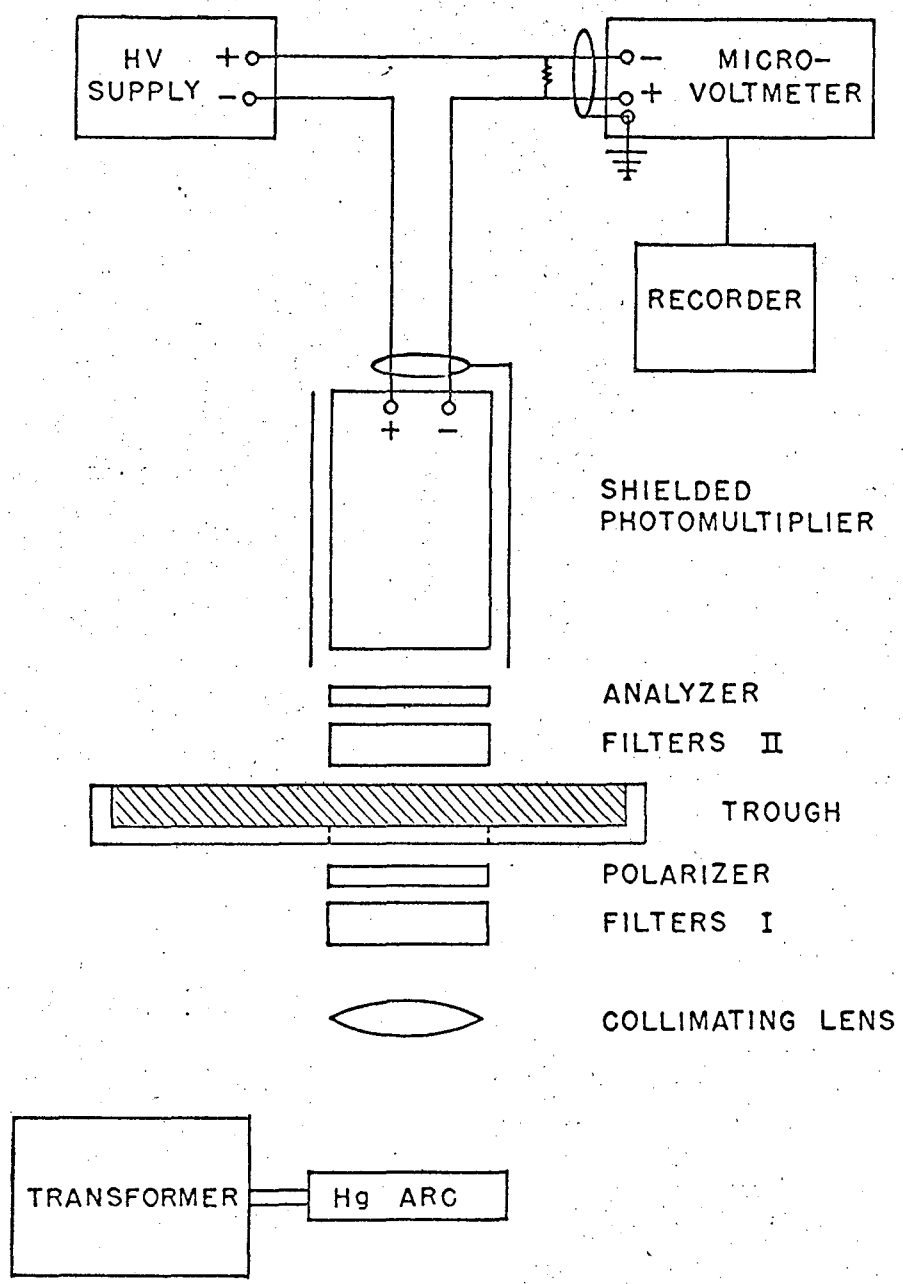
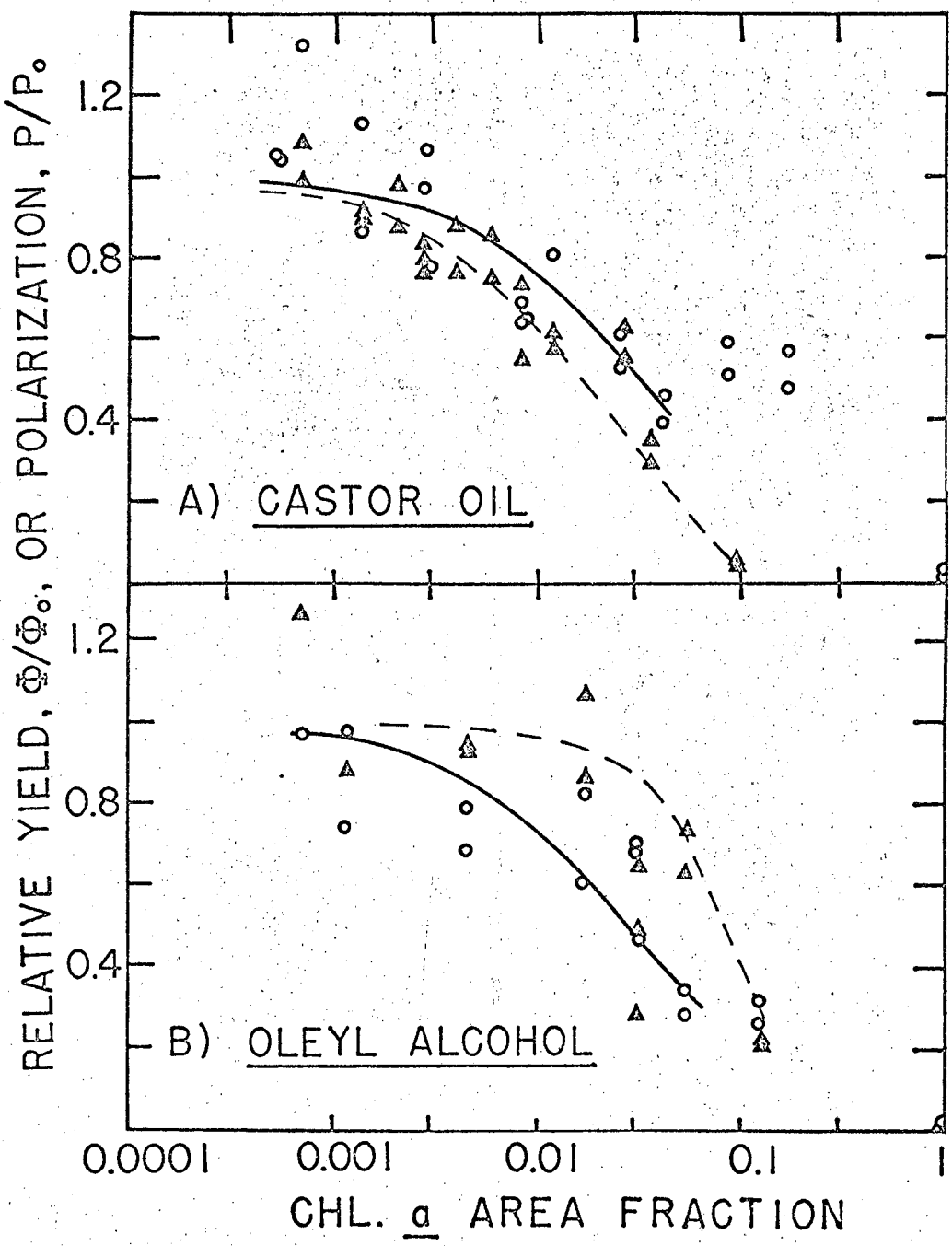

HV SUPPLY
+
−
MICRO-VOLTMETER
RECORDER
SHIELDED PHOTOMULTIPLIER
ANALYZER
FILTERS II
TROUGH
POLARIZER
FILTERS I
COLLIMATING LENS
TRANSFORMER
Hg ARC
MUB-14006

Fig. 3



XBL 676-1136

Fig. 4

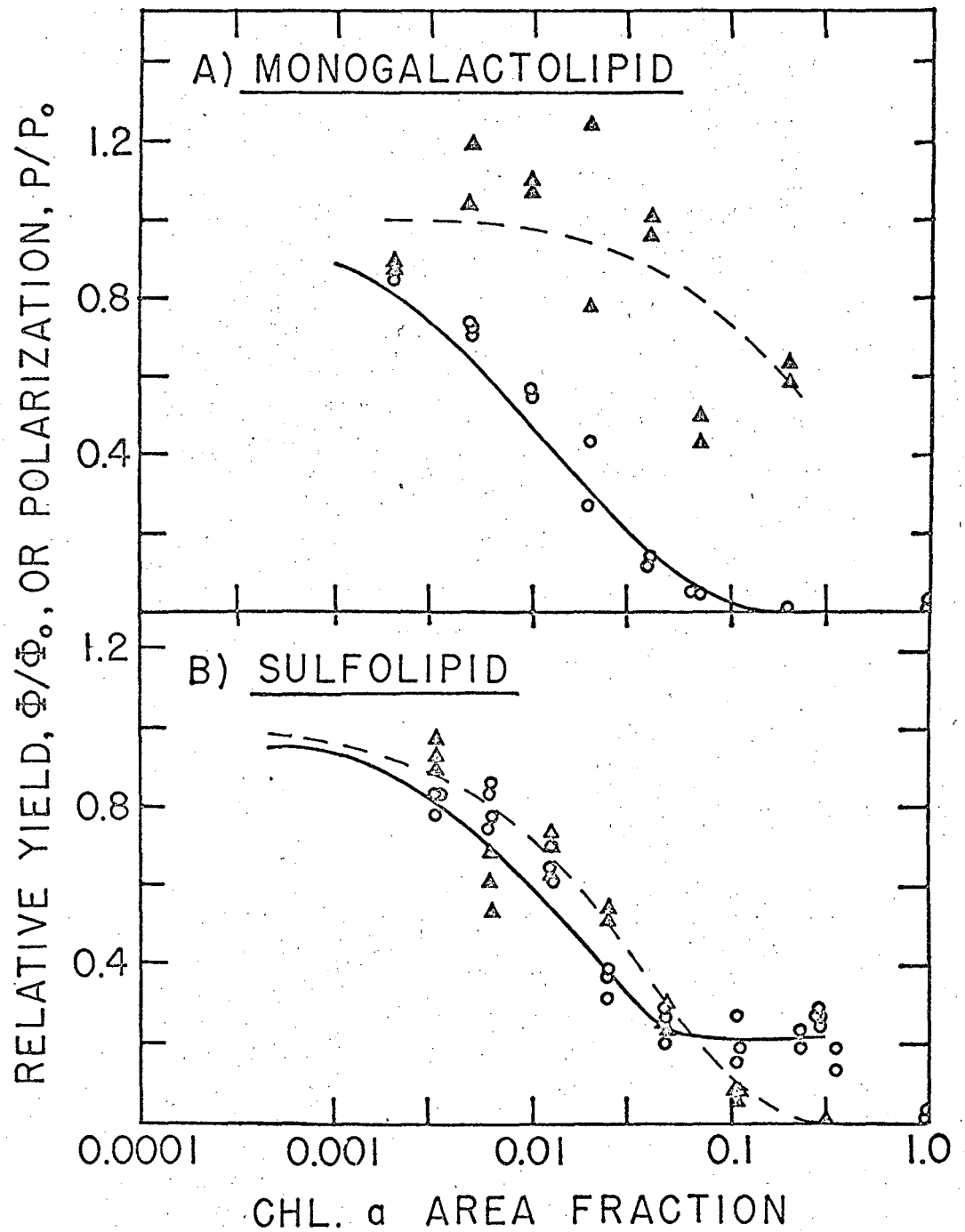

A) MONOGALACTOLIPID
B) SULFOLIPID
RELATIVE YIELD, Φ/Φ₀, OR POLARIZATION, P/P₀
1.2
0.8
0.4
0.0001
0.001
0.01
0.1
1.0
CHL. a AREA FRACTION
XBL676-1135

Fig. 5

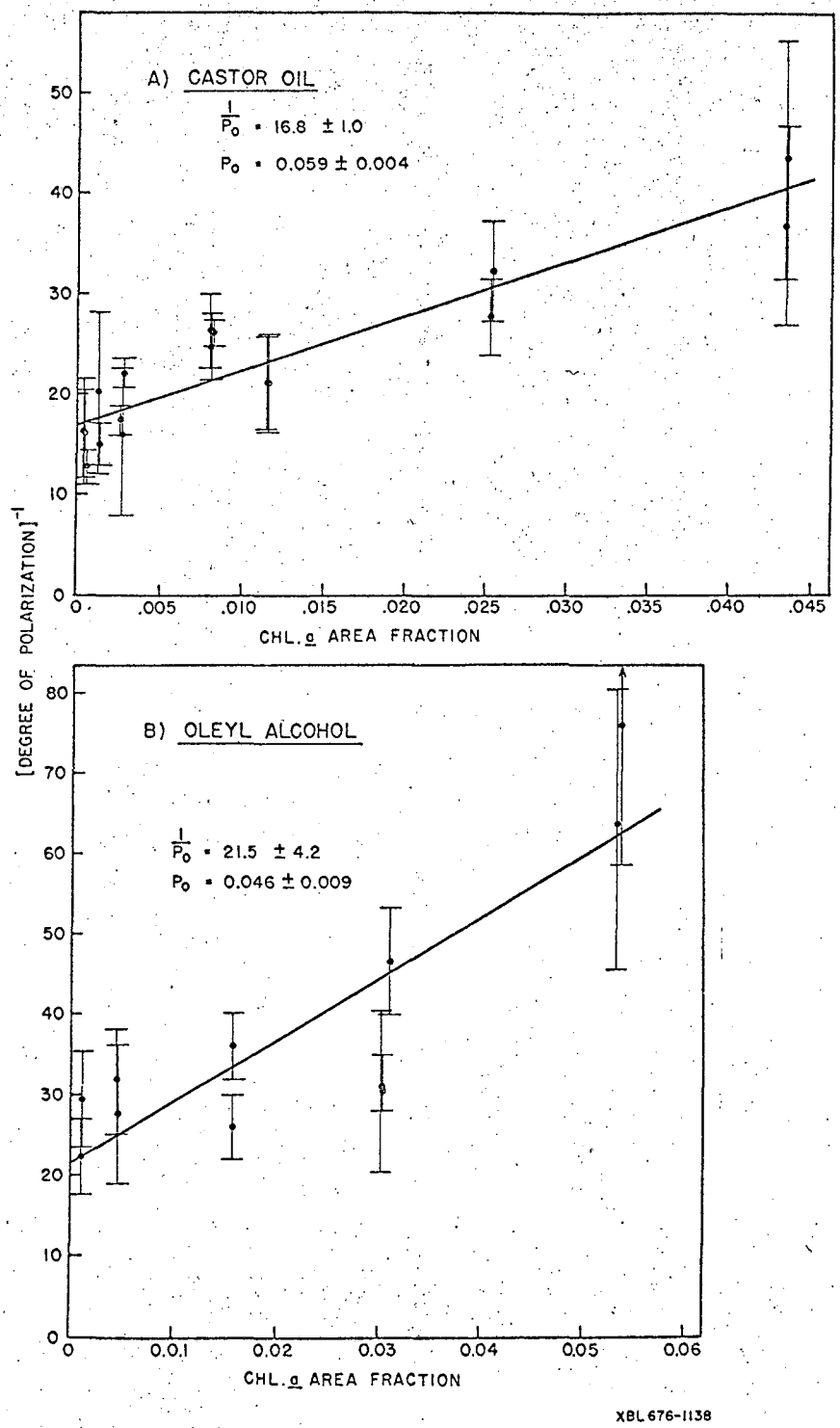

A) CASTOR OIL
$\frac{1}{P_o}$ = 16.8 ± 1.0
P_o = 0.059 ± 0.004
[DEGREE OF POLARIZATION]$^{-1}$
CHL. a AREA FRACTION
B) OLEYL ALCOHOL
$\frac{1}{P_o}$ = 21.5 ± 4.2
P_o = 0.046 ± 0.009
CHL. a AREA FRACTION
XBL 676-1138

Fig. 6

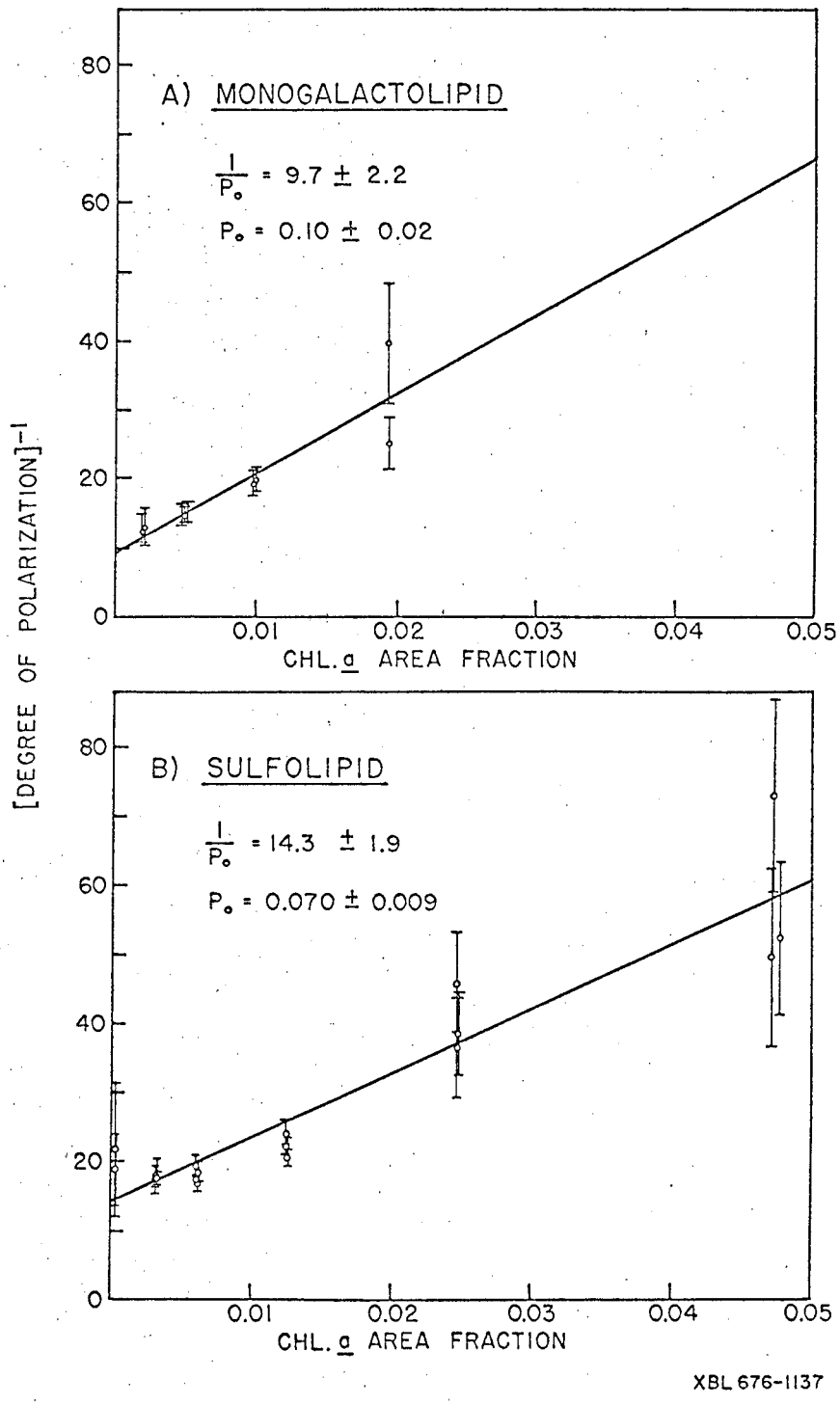

A) MONOGALACTOLIPID
$\frac{1}{P_o}$ = 9.7 ± 2.2
P_o = 0.10 ± 0.02
B) SULFOLIPID
$\frac{1}{P_o}$ = 14.3 ± 1.9
P_o = 0.070 ± 0.009
[DEGREE OF POLARIZATION]$^{-1}$
CHL. a AREA FRACTION
0
20
40
60
80
0.01
0.02
0.03
0.04
0.05
XBL 676-1137

Fig. 7