Title
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Publication Date
1966-12-01

UCRL-17321

University of California

Ernest O. Lawrence Radiation Laboratory

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UCRL-17321

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

AEC Contract No. W-7405-eng-48

CHLOROPHYLL a INTERACTIONS IN LIPID MONOLAYERS

Terry Louise Trosper

(Ph. D. Thesis)

December 1966

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ABSTRACT

In order to elucidate the molecular environment of chlorophyll *a* in chloroplast lamellae and to determine the mechanism of energy transfer among these molecules, compression characteristics and fluorescence properties of simple model membrane systems - lipid monolayers containing chlorophyll *a* - were investigated. Conditions are discussed under which two non-radiative transfer mechanisms, inductive resonance and migration of localized excitons, could occur in the model systems. Fluorescence polarization data for each system are analyzed to determine which of these mechanisms, if either, exists in the models. A monolayer fluorometer was constructed to make the necessary measurements.

A method for purification of small amounts of the lipids was developed. The compression behavior and stability of pure films of these lipids, and of mixed monolayers of chlorophyll *a* with each lipid, are reported. In addition, chloroplast lipid-chlorophyll *a* interactions were studied in carbon tetrachloride

solution. Both monogalactolipid and sulfolipid broke up chlorophyll a dimers in this solvent, by forming one-to-one pigment-lipid complexes.

The degree of polarization of fluorescence from films of chloroplast membrane fragments was also observed.

Experimental results suggest that chlorophyll a is unaggregated and randomly oriented in monogalactolipid, and that energy transfer occurs among pigment molecules by inductive resonance in this environment. In sulfolipid, chlorophyll may be aggregated or partially oriented. In the latter case, exciton migration may occur at high pigment concentrations. It is unlikely that the aggregated portion of the chlorophyll a complement of the photosynthetic apparatus is associated with monogalactolipid, but it may be associated with sulfolipid.

TO *Spinacia oleracea*, countless generations of which have yielded to solvent and homogenizer so that its ecological dependent, *Homo sapiens*, might understand its efficient use of light energy.

ACKNOWLEDGMENTS

I am indebted to Mr. James Hodges for construction of the trough mount and barrier drive mechanism of the monolayer fluorometer, and to Dr. Joseph Apfel for a gift of the dielectric rejection filter.

Professor Melvin Calvin and the staff, visitors, and students in the Biodynamics Laboratory have created a stimulating scientific workshop, of which I have enjoyed being a part. The organic chemists, in particular, made valiant attempts to fill the gaps in my knowledge of their field.

I am grateful to Drs. Alan Portis, Melvin Klein, John Thornley, and Edward Dratz for helpful discussions of the theoretical material.

To my parents goes my deep gratitude for their many hours of patient listening and their unwavering, cheerful encouragement.

Finally, I thank Professors Roderic Park and Kenneth Sauer, who by judicious guidance and excellent example have shown me how exhilarating sound scientific research can be.

I was a recipient of USPHS Biophysics Training Grant #5 T1 6M829 for part of the time this work was undertaken. The research was supported by the U. S. Atomic Energy Commission.

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I. INTRODUCTION

Emerson and Arnold first introduced the concept of a photosynthetic unit to explain the maximum photosynthetic yields they observed in flashing light experiments (1932). The original operational definition of this unit was the number of chlorophyll molecules per rate-limiting step in the reaction series resulting in carbon dioxide reduction or oxygen evolution. Their hypothesis was supported by Gaffron and Wohl's calculations (1936), based on chloroplast size and chemical constitution. Basic to this concept is a requirement for cooperative action of many chlorophyll molecules in absorbing light energy and transferring it efficiently to a site of chemical activity associated with the unit. The unit's existence has received confirmation from research designed to determine its structural and functional aspects in photosynthesizing organisms. Despite extensive investigations, which have yielded a wealth of information on the composition, structure, and chemical reactions of the photosynthetic apparatus, the specific microenvironment of chlorophyll a molecules in chloroplasts remains poorly defined and the mechanism of intermolecular energy transfer not well understood.

Study of energy transfer in a suitably chosen model system of controlled chemical composition should shed light on these phenomena. In the present work, we have investigated energy transfer among chlorophyll a molecules in a lipid matrix. We have observed polarization of fluorescence from pigment-containing lipid monolayers at a gas-liquid interface as a function of chlorophyll concentration in films of various lipids. These systems represent an extreme simplification of the in vivo environment of chlorophyll a molecules. However, properties of the photosynthetic

apparatus and of this two-dimensional model suggest that the latter is a reasonable first approximation to the former. Exercising considerable caution in applying our results, we may then attempt to enlarge upon current understanding of the molecular environment and interactions of chlorophyll a in chloroplasts.

A. Chlorophyll a in Monolayers - Historical Background

The amphiphilic nature of chlorophyll a, the hydrophobic phytol chain and a methyl group attached via hydrophilic ester linkages to the magnesium-containing porphyrin ring (Figure I.1), allows orientation of the molecule at an interface (Hughes, 1936). Mixed chlorophyll a and b extracts were first spread as monolayers on an aqueous subphase in the 1930's. Hughes, reporting pressure-area behaviour and surface potentials, determined that first the phytol chain and then the porphyrin ring are expelled from the surface into the air phase upon compression. He also noted that magnesium is extracted from the pigment molecules when an acidic, pH ≤6, subphase is used. Hanson (1937, 1939) proposed that the porphyrin ring of chlorophyll is planar, after comparison of calculated ring areas with the area occupied per molecule at zero compression of monolayers. Unsuccessful attempts to spread monolayers of methyl chlorophyllides led him to suggest that the phytol moiety is required for surface activity. Langmuir and Schaefer (1937) measured surface viscosity of chlorophyll monolayers as a function of subphase pH and surface pressure. The films behaved as liquid monolayers at pressures below approximately 20 dynes/centimeter, displaying Newtonian viscosities in this region. Immediately after spreading chlorophyll on the surface in a benzene solution, Langmuir and Schaefer observed red pigment fluorescence which disappeared as the spreading solvent evaporated from the monolayer.

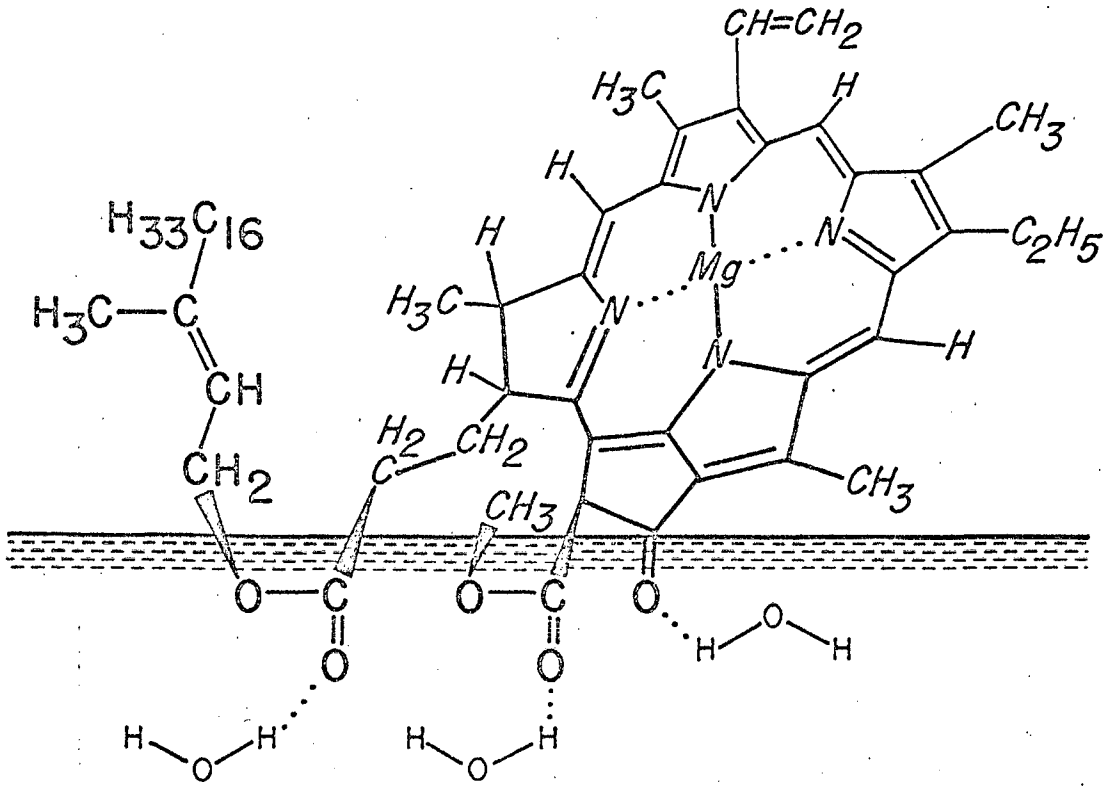

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Figure I.1. Chlorophyll a molecule, showing possible orientation at a gas-aqueous interface in a monolayer under compression.

More recently, properties of pure and diluted chlorophyll a films have been investigated. An excellent review of these studies has just been published (Ke, 1966). The earlier qualitative observations have generally been upheld by this more quantitative work. Colmano (1961), Rosoff and Aron (1965), and Bellamy, et al. (1963) have all studied the chemical stability of chlorophyll a monolayers. They report phaeophytinization on acidic subphases. In addition, Colmano remarked that pigment films were very sensitive to photo-induced bleaching. Bellamy et al. were able to inhibit this process by keeping the monolayers in an inert atmosphere of nitrogen or argon.

Careful pressure-area measurements have shown that the porphyrin rings of chlorophyll a lie approximately in the plane of the subphase surface in a completely expanded film. Upon compression, the rings tilt out of the surface. Monolayers tend to collapse above pressures corresponding to a vertical position of the porphyrin plane (Bellamy et al., 1963).

Mixed monolayers of chlorophyll a and surface active lipids have also been observed (Gaines et al., 1964; Tweet et al., 1964a). Lipids which are miscible with the pigment disperse the latter in an ideal two-dimensional solution, as evidenced by pressure-area characteristics and optical properties of the two-component films.

Differences in absorption spectra of chlorophyll solutions and monolayers may be indicative of molecular associations caused by arranging the molecules in a condensed two-dimensional array. Jacobs et al. (1954, 1957) were the first to investigate this possibility, by comparing the absorption spectra of chlorophyll and chlorophyllide monolayers removed from the aqueous surface to a glass slide with those of chlorophyll and chlorophyllide microcrystals. They found that while the red maximum in

the crystalline spectra showed large shifts, of the order of 70 nm,* compared with the maximum in solution, the monolayer red peaks were shifted only about 15 nm, roughly the same as that observed *in vivo* (Sauer and Park, 1964). Trurnit and Colmano (1959), using improved techniques, observed the same shift. The correlation of the maxima positions with those in spectra of biological materials led Colmano (1962) to try to simulate the spectrum of *Chlorella* with a monolayer of mixed pigments. She found that a mixture of chlorophyll *a*, chlorophyll *b*, and β-carotene in the ratio 6:3:1, when spread on a buffered aqueous subphase and picked up on a glass slide, had an absorption spectrum similar to that of *Chlorella* in both peak positions and peak height ratios. This result suggests that pigment interactions *in vivo* may be essentially two-dimensional.

Ke and Sperling (1966) have carried out time dependent studies of the absorption of chlorophyll *a* monolayers deposited on slides coated with lipid films. Changes in red absorption were noted, optical density decreasing with time. The decrease in the far red was larger than that in the red, indicating that degradation of aggregates occurred preferentially. Since the films were deposited at very high surface pressures, multilayers may have been present.

Recently, Tweet (1963) and Tweet *et al.* (1964a) devised spectrophotometers for measuring monolayer absorption and fluorescence spectra *in situ*. They observed similar shifts in absorption spectra as had been reported earlier, the red absorption maximum being at 683 nm (Bellamy *et al.*, 1963). By diluting chlorophyll *a* films with miscible lipids forming two-dimensional solutions, they shifted the maximum back to 675 nm. They could not accurately measure spectra of monolayers more dilute than 30% chlorophyll *a* by area (Gaines *et al.*, 1964). They also

found much lower fluorescence yields for pure chlorophyll a films or those diluted with an immiscible lipid than for monolayers which were two-dimensional solutions of pigment and lipid. These workers proposed that decreasing fluorescence yields observed as concentration of the fluorescing moiety increased were indicative of increased energy transfer among these molecules. Effects of foreign quencher concentration upon chlorophyll a monolayer fluorescence were studied to test this hypothesis. A decrease in fluorescence yield as quencher concentration increased supported their suggestion (Tweet et al., 1964; Gaines et al, 1965). Tweet et al. (1964b) were unable to detect any polarization of the fluorescence of either pure or diluted chlorophyll a monolayers with their experimental arrangement, which employed oblique illumination of the films. However, with a different optical arrangement, we have succeeded in observing this property.

From this brief review, we propose that chlorophyll a-containing monolayers are reasonable subjects for our investigations. Their spectroscopic properties do not differ grossly from those of biological material. The environment of the pigment molecules may be controlled with relative ease. Two-dimensional monolayer geometry is more simply described than a corresponding three-dimensional system. Lastly, a property of this model system, which we shall relate to energy transfer, namely fluorescence polarization, can be measured with a fair degree of accuracy.

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* nm = nanometer = 10^{-9} meters.

B. Plan of This Investigation

Expressions for the degree of fluorescence polarization applicable to the two-dimensional monolayers are derived in Chapter II. Also in this chapter we discuss the effect on the polarization of non-radiant energy transfer among the fluorescing molecules for varying strengths of pigment interactions.

In order to detect polarized fluorescence from a highly diluted chlorophyll monolayer under controlled conditions, we modified a Langmuir film balance. A description of the monolayer fluorometer, and experimental methods as well as results of the fluorescence polarization studies appear in Chapter III. We used chloroplast structural lipids, in addition to castor oil and oleyl alcohol, as film diluents in these experiments. Some properties of the mixed monolayers of biological materials might be indicative of specific molecular interactions. As these latter would bear upon the interpretation of results and be of particular importance for understanding in vivo molecular relationships, we also studied chlorophyll a - chloroplast lipid interactions in organic solvents (Chapter IV).

Brief investigations of more biological models and current knowledge of the photosynthetic apparatus are considered in Chapter V. A discussion of the implications of the model behaviour for chlorophyll a interactions and microenvironment in vivo concludes the thesis.

II. THEORETICAL CONSIDERATIONS

The rate of energy transfer among chlorophyll a molecules in a monolayer will depend on the strength of the intermolecular interactions in this two-dimensional model system. These interactions are electrostatic, their strength varying with pigment-pigment separation and orientation, and with the dielectric constant of the medium. The medium may further affect electrostatic pigment interactions by complexing with pigment molecules. Thus we can vary energy transfer rates by changing the pigment concentration and using different diluent lipids in the monolayers. We have observed fluorescence polarization of the pigment-containing films, a property which depends on energy transfer, and wish to interpret these data.

In this chapter we examine the published theories of fluorescence polarization and energy transfer, developing expressions appropriate to our model systems where feasible. We must determine the degree of fluorescence polarization as a function of concentration for the applicable energy transfer mechanisms. The equations derived contain molecular parameters which are measured or calculated independently of the fluorescence polarization studies. Thus they permit a comparison of parameters predicted by theories with values observed in the polarization experiments. This comparison may indicate which mechanisms of transfer are operative in the model systems and help us to suggest which could occur in chloroplasts.

A. Degree of Fluorescence Polarization

1. Polarization in the absence of energy transfer or molecular motion

Consider a system of non-interacting molecules irradiated with linearly polarized light. The fluorescence observed is emitted by primarily excited molecules.

If the molecules do not rotate during their excited state lifetime, the polarization of fluorescence from individual molecules depends solely on the mutual orientation of their absorption and emission oscillators. When the molecules are randomly distributed, the observed polarization, which is averaged over all molecular orientations, also depends only on this orientation.

Molecular motion or interactions leading to transfer of excitation energy to molecules oriented differently from the primarily excited ones will result in depolarization. We make the initial approximation that the model systems under investigation are rigid during the excited state lifetime. Then changes in polarization caused by varying the concentration of fluorescing molecules may be attributed to variations in intermolecular interactions and energy transfer with concentration.

Fluorescence polarization in the absence of molecular interactions or rotations is designated the limiting degree of polarization, P_o, of the system. F. Perrin (1929) derived the expression for P_o applicable to a random three dimensional array of molecules excited with linearly polarized light,

$$P_o = \frac{3\cos^2\alpha - 1}{\cos^2\alpha + 3}, \qquad \text{(II.1)}$$

where α is the angle between absorption and emission oscillators. The highest limiting degree of polarization is observed when these oscillators are parallel, the lowest when they are perpendicular, i.e., $-1/3 \leq P_o \leq 1/2$, as α ranges from $\pi/2$ to 0.

We wish to determine the value of P_o when the molecular ensemble is two-dimensional. Restrictions on the orientation of the molecules may be imposed by the nature of the interface and the extent of compression of the monolayer. The derivation of P_o, which is now dependent on the direction of observation of the emitted light, requires a detailed geometrical description of the model.

The x-y plane is that of the monolayer, which is excited by linearly polarized light from the z direction with the electric vector parallel to the y axis. The limiting degree of polarization is then defined as

$$P_o = \frac{\bar{I}_y - \bar{I}_x}{\bar{I}_y + \bar{I}_x} \qquad \text{(II.2)}$$

where $\bar{I}_y$ and $\bar{I}_x$ are the average fluorescence intensities with electric vectors in the x and y directions. Consider the absorption, A, and emission, F, oscillators restricted to a plane of the molecule--a sufficiently accurate assumption for chlorophyll a. The normal to this plane forms an angle β with respect to the surface normal (z-axis, see Figure II.1). For chlorophyll a at an air-water interface, $\pi/2 \leq \beta \leq \pi$. These oscillators form angles θ_A and θ_F with the z-axis, respectively. They are separated by an angle α in the plane of the molecule. The projections of the absorption and emission oscillators in the x-y plane form angles ϕ_A and ϕ_F, respectively, with respect to the x-axis. The fluorescence intensities emitted by this molecule are

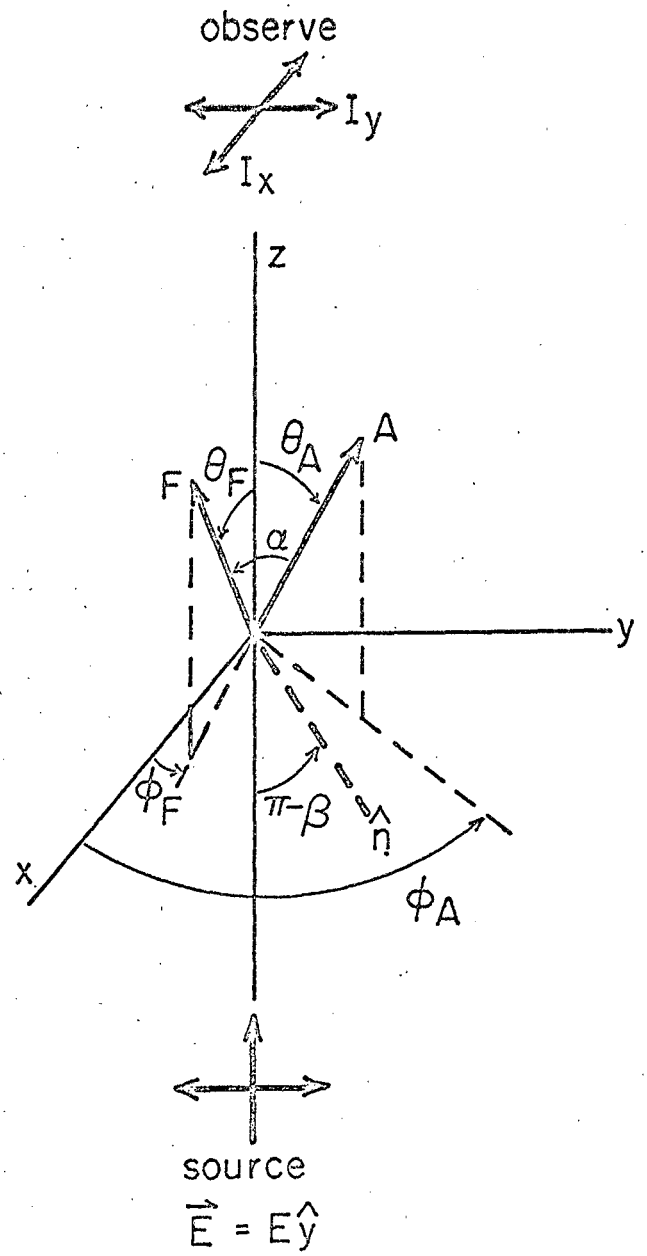

Figure II.1. Orientation of molecular absorption, A, and emission, F, oscillators of chlorophyll a with respect to a monolayer surface (x-y plane. A and F are in the plane of the porphyrin ring. See text for definitions of angles.

$$I_x = (\sin \theta_A \sin \phi_A)^2 (\sin \theta_F \cos \phi_F)^2$$
$$I_y = (\sin \theta_A \sin \phi_A)^2 (\sin \theta_F \sin \phi_F)^2 \qquad \text{(II.3)}$$

To calculate the macroscopic polarization, Equation II.2, these intensities must be averaged over all possible molecular orientations. We accomplish this by averaging over all azimuthal angles and all allowed polar angles, remembering α is constant. ϕ_A and ϕ_F are related by

$$\cos \alpha = \sin \theta_A \sin \theta_F \cos (\phi_A - \phi_F) + \cos \theta_A \cos \theta_F .$$

Rearranging, we obtain

$$\phi_F = \phi_A - \cos^{-1} \left[\frac{\cos \alpha - \cos \theta_A \cos \theta_F}{\sin \theta_A \sin \theta_F} \right] . \qquad \text{(II.4)}$$

Also, θ_A may be defined in terms of θ_F, α and β, as follows:

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

By observing the spatial anisotropy of fluorescence intensity from a monolayer containing chlorophyll a, Tweet et al. (1964b) calculated that

$$\cos \theta_F \sim \sin 20^\circ \cos (\beta - \pi/2) = 0.34 \sin \beta . \qquad \text{(II.5)}$$

We thus obtain

$$\cos \theta_A = \sin \beta (0.34 \cos \alpha \pm 0.94 \sin \alpha) . \qquad \text{(II.6)}$$

According to Equations II.5 and II.6, θ_A and θ_F depend only on α and β. α is fixed in the molecules. Bellamy et al. (1963) and Tweet et al. (1964b) 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 II.4 becomes $\phi_F = \phi_A - \gamma$, where γ is a constant angle. Substituting this expression into Equation II.3 and averaging over all ϕ_A from 0 to 2π, yields upon substitution into Equation II.2:

$$P_o = \frac{\cos^2\alpha - 2\cos\alpha\cos\theta_A\cos\theta_F + \cos^2\theta_A\cos^2\theta_F}{\sin^2\theta_A\sin^2\theta_F} - 1/2 \qquad (II.7)$$

When absorption and emission oscillators are parallel, $\alpha = 0$, and $\theta_A = \theta_F$. Then the limiting degree of polarization reduces to

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

When absorption and emission escillators are perpendicular,

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

We emphasize that Equation II.7 was obtained using the assumption that a constant average angle $\bar{\beta}$ determines the orientation of the molecular planes with respect to the plane of the ensemble, which assumption is applicable to our experimental model. In this case, if $\alpha = 0$, P_o is the same as observed in a random three-dimensional system. However, when the absorption and emission oscillators are not parallel in the two-dimensional model, the limiting degree of polarization 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 of the angle between the oscillators only.

The error introduced into Equation II.7 for P_o by assuming β is constant may be determined by differentiating Equations II.5 and II.6 with respect to β. We obtain

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

For example, a fluctuation of 3° in β for $\overline{\beta} = 155°$ causes an uncertainty in the $\cos\ \theta_A$ and $\cos\ \theta_F$ of about 4%. This situation may not be unreasonable for highly compressed monolayers containing chlorophyll a.

2. Effect of molecular rotation

Perrin has determined the dependence of degree of polarization upon molecular rotation. Assuming that fluorescence intensity decreases according to $I = I_o \cdot fn(time)$ when the ensemble of molecules has been excited instantaneously, and also that the emission oscillators undergo average isotropic rotations described by $\overline{\cos^2 x} = u(t)$, he shows that the polarization is smaller than the limiting value, P_o, according to

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(\frac{2}{3\ u(t)-1}\right). \qquad \text{(II.8)}$$

As the viscosity of the medium is decreased, u(t) will also decrease, resulting in a lower polarization. In a monolayer, molecular rotations in the plane of the surface will be random provided that there is no interaction with surrounding molecules. If we assume, in analogy to the three-dimensional case, that the movement of the molecules in the surface is inversely related to the surface viscosity, the fluorescence polarization of the monolayers will also decrease as the viscosity of the film is lowered.

3. Effect of energy transfer

At infinite dilution, monodisperse solute molecules do not interact and energy transfer is not possible. Thus the experimentally observed

polarization of fluorescence should, in the absence of molecular motion, reach the limiting value, P_o, predicted by Equations II.1 or II.7. As the concentration of the fluorescing species is increased, energy transfer among differently oriented molecules before emission will decrease the polarization. Interactions among the fluorescing molecules cause this transfer. Specific mechanisms of interaction which may be applicable to the model systems under study or to chlorophyll a in the chloroplast will be considered in detail in the next section.

Empirically, the degree of polarization of fluorescent three-dimentional solutions depends on concentration of the fluorescing moiety according to the law first enunciated by Feofilov and Sveshnikov (1941),

$$\frac{1}{P} = \frac{1}{P_o} + Ac\tau \ ,$$

where c is the concentration of the fluorescent molecule and τ is the experimental lifetime of the excited state. A is a constant of the system.

This concentration dependence has been derived theoretically only in special cases. Vavilov (1943) has obtained it for low solute concentrations from a phenomenological consideration of transfer probability which disregards back transfers. By employing an expression for average transfer rate which is applicable to a uniform molecular distribution in which back transfers do not occur, Weber (1954) also arrived at the observed concentration dependence. Although his final equations are not useful to us, we review his formulation of the problem in order to introduce the general case and point out the difficulties which its solution entails.

If a quantum of excitation visits n - 1 different molecules before it is emitted by the nth molecule, the degree of polarization of this emitted radiation will be

$$\frac{1}{P_n} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(\frac{2}{3\cos^2\theta_n - 1}\right)$$

where θ_n is the angle between the initially excited oscillator and the emitting one, and Equation II.8 has been used. If the molecules move during their excited state lifetime, $1/P_o - 1/3$ in this equation is equal to $1/P - 1/3$ obtained from Equation II.8. Henceforth the molecules are assumed not to move, in which case P_o is the limiting polarization of Equation II.1 or II.7. Letting θ be the angle between any pair of emission oscillators in two molecules in a randomly distributed array, Soleillet (1929) shows, using Stokes' parameters, that

$$\frac{3\cos^2\theta_n - 1}{2} = \left(\frac{3\overline{\cos^2\theta} - 1}{2}\right)^n .$$

Weber, with the aid of his addition law (1952), obtains the observed macroscopic polarization

$$\frac{1}{P} - \frac{1}{3} = \left(\sum_{n=o}^{\infty} \frac{I_n}{I} \frac{1}{1/P_n - 1/3}\right)^{-1}$$

where I_n is the fraction of the total intensity I emitted by the nth molecule to be excited as the energy is transferred in the array. With Soleillet's relation, this becomes

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left[\sum_{n} \frac{I_n}{I}\left(\frac{3\overline{\cos^2\theta} - 1}{2}\right)^n\right]^{-1} . \qquad \text{(II.9)}$$

In order to obtain 1/P in closed form, we desire an expression for the fractional intensity emitted by the nth different excited molecule in the transfer sequence of the form $a \cdot b^n$, with a and b independent of n. If $\overline{p_n(t)}$ is the average probability that the nth excited molecule is excited at time t, then

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

where τ is the experimental lifetime of the excited state. $p_n(t)$ depends in a complicated manner on the rate of energy transfer to and from n. We may write

$$p_n(t) = -\frac{1}{\tau} - \sum_\mu \omega_{n\mu}\rho(\mu,r_\mu) + \sum_\nu \omega_{\nu n} \cdot p_\nu(t) \cdot \rho(n,r_n) \quad \text{(II.10)}$$

where the ρ's are partition functions. The form of the transfer rates ω_{ij} will depend on the strength of molecular interactions. The rates are functions of the concentration. The necessity of describing back transfers of energy with the proper weights among randomly distributed identical molecules, and of distinguishing transfer steps, makes the third term in this equation unwieldy.

We do not pursue a mathematical solution of the general transfer case. Rather, we turn to a consideration of possible transfer mechanisms, and special cases in which the dependence of polarization on concentration may be obtained explicitly. We shall use these cases in presenting an approximate evaluation of the data in Chapter III.

B. Energy Transfer Mechanisms

We consider only those mechanisms of energy transfer which could reasonably occur among chlorophyll a molecules in chloroplast membranes or in lipid monolayer models. The conditions necessary for the occurrence of these mechanisms are considered briefly. We present the dependence of fluorescence polarization on the concentration of the fluorescing moiety in two special cases, in terms of a parameter which may be calculated from independently obtained spectral data. This may make possible a confirmation of the existence of a particular transfer mechanism in the model system.

Experimental evidence rules out some transfer processes which have been suggested as operative in biological systems. The trivial mechanism of emission and reabsorption may be disregarded, as Feofilov (1961) has shown that the depolarizing effect of this process cannot account for the observed extent of depolarization. The small effect of reabsorption is differentiated from the total depolarization observed by its dependence on the volume of the sample and its having no effect on the excited state lifetime of the fluorescent molecules. In a monolayer under compression, as well as in chloroplast membranes, the possibility of molecular migration and energy transfer by collisions of excited and unexcited pigment molecules is unlikely. Various workers have proposed semi- or photoconductive energy transfer mechanisms in vivo, and photocurrents have been measured in biological materials. However, the efficiencies observed are not sufficient to account for the high photosynthetic yields reported (Clayton, 1966).

We assume then, that energy is transferred among fluorescent molecules by a non-radiative mechanism that does not involve transport of

mass and does not require pigment contact. Two such mechanisms, inductive resonance and exciton migration, have been treated extensively in the literature. Franck and Livingston (1949) and Katz (1949) both suggested a role for these processes in photosynthesis many years ago. Lacking sufficient and accurate experimental information, they were unable to discuss the problem in depth. The question of which one of these mechanisms occurs in chloroplast lamellae, if either, has not yet been resolved. We will address this problem after discussing experimental results in light of the theoretical considerations presented here.

Electrostatic interactions among molecules are responsible for both these transfer mechanisms. In the point dipole approximation, the pairwise interaction energy is

$$u_{ij} = \frac{k\,|\mu_{ij}|^2}{n^2\, r_i{}^3{}_j} \qquad \text{(II.11)}$$

$|\mu|^2$ is the square of the dipole moment, n the refractive index of the medium and r_{ij} the center to center distance between molecules i and j. When the average interaction energy in the ensemble is computed, the orientation factor,

$$k = \cos \psi_{ij} - 3 \cos \psi_i \cos \psi_j \qquad \text{(II.12)}$$

where ψ_i and ψ_j are the angles between the ith and jth dipoles, respectively, and the line joining them, and ψ_{ij} is the angle between the dipoles, must be averaged over all possible positions of the molecules. In a three-dimensional random array,

$$\cos \psi_{ij} = \sin \psi_i \sin \psi_j \cos \phi_{ij} + \cos \psi_i \cos \psi_j$$

where ϕ_{ij} is the angle between projections of the ith and jth dipoles onto a plane perpendicular to the line joining their centers. Thus in this model,

$$k\ (3D) = \sin \psi_i \sin \psi_j \cos \phi_{ij} - 2 \cos \psi_i \cos \psi_j \qquad (II.13)$$

The angles ψ_i, ψ_j and ϕ_{ij} are independent, and the average value of k may be calculated directly.

We must also determine the average value of k for the monolayer models. In this case, the line joining dipoles i and j in two different molecules is parallel to the plane of the monolayer. Then

$$\cos \psi_i = \sin \theta_i \sin \phi_i$$

$$\cos \psi_j = \sin \theta_j \sin \phi_j$$

$$\cos \psi_{ij} = \sin \theta_i \sin \theta_j (\cos \phi_i \cos \phi_j + \sin \phi_i \sin \phi_j) + \cos \theta_i \cos \theta_j$$

Substitution into Equation II.12 yields

$$k\ (2D) = \sin \theta_i \sin \theta_j (\cos \phi_i \cos \phi_j - 2 \sin \phi_i \sin \phi_j) + \cos \theta_i \cos \theta_j \qquad (II.14)$$

All the angles may be varied independently of each other. ϕ_i and ϕ_j may vary from 0 to 2π, but if we again assume that the normal to the molecular plane in which the oscillators are located forms a constant angle with respect to the surface normal, θ_i and θ_j will be constant. Thus the average angular factor in the two-dimensional models is obtained by averaging Equation II.14 over ϕ_i and ϕ_j only. We will need to use these forms of k in a modification of Equation II.11 when we calculate energy transfer rates appropriate to inductive resonance or exciton migration.

1. Inductive resonance

Forster (1948, 1949, 1951) has developed an extensive formalism for energy transfer by inductive resonance, to describe the phenomena of sensitized fluorescence and fluorescence depolarization in dilute viscous solutions.

The transition oscillator of an excited molecule i induces a dipole field in a second molecule j a distance r_{ij} from it. Both classical and quantum-mechanical treatments, the latter assuming a continuum of excited states, of this dipole-induced dipole problem lead to the same results (Forster, 1951; 1960). The pairwise transfer probability, expressed as a frequency, is given by

$$\omega_{ij} = \frac{4\pi^2 |u_{ij}|^2}{h\Delta\epsilon'} \sum_{v,v'} g_v^* g_{v'} S^4_{vv'} \qquad \text{(II.15)}$$

where h is Planck's constant, u the point dipole interaction energy given by Equation II.11, $\Delta\epsilon'$ the band width of a single vibronic energy level, and $S_{vv'}$ the Franck-Condon overlap integral for the vibronic transition. The g factors are normalizing factors for the populations of the v and v' vibrational levels.

Forster defines a critical distance R_o as that molecular separation at which transfer and emission are equally probable according to

$$\omega_{ij} = \frac{4\pi^2 k^2 |\mu|^4}{h\Delta\epsilon' n^4 (r_{ij})^6} \sum_{vv'} g_v^* g_{v'} S^4_{vv'} = \frac{1}{\tau}\left(\frac{R_o}{r_{ij}}\right)^6 . \qquad \text{(II.16)}$$

τ is the experimental lifetime of the excited state, and Equation II.11 has been used. This transfer rate, summed over all possible pairs of molecules, appears within the summations on the righthand side of

Equation II.10, for the probability $p_n(t)$ that the nth visited molecule is excited at time t.

When R_o is derived from classical electromagnetic theory, Forster finds

$$R_o^6 = \frac{9\ k^2\ \ln 10\ c^4}{128\pi^6\ n^4\ N'} \quad \frac{\tau}{\tau_o} \int_o^\infty \varepsilon(\nu)\ f(\nu)\ \frac{d\nu}{\nu^4} \qquad \text{(II.17)}$$

where c is the velocity of light, N' the number of molecules in a millimole, τ_o the natural fluorescence lifetime of the molecules, $\varepsilon(\nu)$ the extinction coefficient of the molecule at frequency ν and $f(\nu)$ the quantum spectrum of fluorescence. The integral may be obtained from the overlap of absorption and fluorescence spectra. Using Equation II.17, Tweet et al. (1964b) have calculated R_o from their spectral data on monolayers containing chlorophyll a. With appropriate modifications in k and n, they find $R_o = 54 \pm 8$ Å. We will wish to compare this value with one calculated from polarization data.

Since the general solution of Equation II.9 is not available, we must make simplifying restrictions. We will consider two cases, one step depolarization and a uniform array without back transfer.

a. One step depolarization

The first, due to Forster (1951), will yield an upper limit for R_o. If one transfer of excitation energy among molecules in a random array is sufficient to depolarize fluorescence completely, the relative degree of polarization is a direct measure of the fraction of initially excited molecules which fluoresce:

$$\frac{P}{P_o} = \frac{6\ \Phi_A/\Phi_C}{5 + \Phi_A/\Phi_C}\ .$$

Here, Φ_A is the fluorescence yield of the initially excited molecules, and Φ_C the total yield at concentration C. This assumption obviously represents the fastest possible decrease in polarization with increasing energy transfer. Furthermore, Forster expresses the relative yield, Φ_A/Φ_C as a function of the critical concentration C_o, chosen such that

$$\frac{P}{P_o} = \frac{1}{2} \qquad \text{when} \quad C = C_o \tag{II.18}$$

This critical concentration may be read directly from graphs of 1/P vs C, and the critical distance, R_o, calculated from it. We will assume for this calculation, as did Forster, that the average pigment distribution is uniform. We obtain a maximum value for the critical separation with this method.

b. Uniform array without back transfer

In the second case, suggested by Weber (1954), the fluorescence polarization is assumed to be inversely proportional to an average transfer rate. Implicit in Weber's use of this assumption is the omission of back transfers from consideration and the further assumption of a spatially uniform molecular array. These assumptions are embodied in the substitution of

$$\frac{I_n}{I} = \left(\overline{\sum_j \omega_{ij}}\right)^n \left(1 - \left(\overline{\sum_j \omega_{ij}}\right)\right)$$

into Equation II.9. This model overlooks the fact that energy transfer will occur rapidly between two very closely spaced molecules, yet as the number of these transfers increases, polarization of fluorescence emitted by the molecules is not correspondingly decreased. As a result, the average depolarizing effect of the sum of the energy transfers is

overestimated, and the critical distance underestimated.

With this discussion firmly in mind, we proceed to derive the dependence of polarization on concentration in this second case. Application of our final expression will yield a lower limit for the critical distance.

Equation II.9, after rearrangement, now becomes

$$\frac{1}{p} - \frac{1}{3} = \frac{1}{P_o} - \frac{1}{3} \; 1 + \frac{3}{2} \; \overline{\sin^2\theta} \; \frac{\overline{\sum_j \omega_{ij}}}{1 - \overline{\sum_j \omega_{ij}}} \; . \qquad \text{(II.19)}$$

$\overline{\sum_j \omega_{ij}}$ is obtained by averaging, over all angular orientations, the sum of the pairwise transfer rates for all r_{ij}. Because Forster's critical distance R_o includes the angular factor k, we define a new critical distance, R_o' as follows:

$$\omega_{ij} = \frac{1}{\tau} \; k^2 \left(\frac{R_o'}{r_{ij}} \right)^6 \; .$$

Comparison with Equation II.16 shows that (II.20)

$$R_o^6 = k^2 \, (R_o')^6 \; .$$

Then the average transfer rate is, where 2a is the molecular diameter,

$$\overline{\sum_j \omega_{ij}} = \frac{(R_o')^6}{\tau} \left\langle \int_{2a}^{\infty} \frac{\rho(r_{ij}) \; dr_{ij} \; k^2}{(r_{ij})^6} \right\rangle_{\text{angles}} \; .$$

In a three-dimensional uniform array, $\rho(r)dr = 4\pi r^2 C dr$, C is the concentration per unit volume, and k is given by Equation II.13. By substitution and integration we obtain

$$\overline{\sum_j \omega_{ij}} \quad (3D) = \frac{3\pi C \, (R_o')^6}{2 \tau \, (2a)^3} .$$

Now, at low concentrations, in the absence of fluorescence quenching,

$$1 - \overline{\sum_j \omega_{ij}} = \frac{1}{\tau} .$$

If we make these substitutions in Equation II.18, and compute $\overline{\sin^2\theta_{ij}}$, we obtain

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right) \left[1 + \frac{45}{32} \frac{\pi C \, (R_o')^6}{(2a)^3}\right] .$$

Plotting 1/P vs C, we may calculate R_o' from

$$R_o' \, (3D) = \left[\frac{32}{45\pi} \frac{\text{Slope} \, (2a)^3}{\frac{1}{P_o} - \frac{1}{3}}\right]^{\frac{1}{6}} . \qquad \text{(II.21)}$$

Use of Equation II.19 then yields the critical distance R_o which applies to this special case.

A similar treatment of the two-dimensional case with $\rho(r)dr = 2\pi r C' dr$, C' as the concentration per unit area, and Equation II.14 gives

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right) \left[1 + \frac{3\pi C' \, (R_o')^6}{4 \, (2a)^4} \cdot B \left(\cos^2\theta_i \cos^2\theta_j + \frac{5}{4} \sin^2\theta_i \sin^2\theta_j\right)\right] .$$

We have used the simplifying notation

$$B = 1 - \cos^2\theta_i \cos^2\theta_j - \frac{1}{2} \sin^2\theta_i \sin^2\theta_j = \overline{\sin^2\theta_{ij}} .$$

R_o', the critical separation in two dimensions, is again obtained from a plot of 1/P vs C':

$$R_o'(2D) = \left[\frac{4\,(2a)^4\,\text{slope}'}{3\pi \left(\frac{1}{P_o} - \frac{1}{3}\right)' B\,(\cos^2\theta_i \cos^2\theta_j + \frac{5}{4}\sin^2\theta_i \sin^2\theta_j)} \right]^{\frac{1}{2}} \quad \text{(II.22)}$$

Energy transfer by inductive resonance occurs between emission oscillators in different molecules. Thus in the monolayer models where we have assumed θ_F is a constant defined by Equation II.5, $\theta_i = \theta_j = \theta_F$ is to be substituted into Equation II.21 for $R_o'(2D)$.

From polarization data we will calculate the upper and lower limits of the critical molecular separation according to Equations II.18 and II.21 or II.22. If these empirical values compare favorably with those computed from spectral data, we may conclude that fluorescence depolarization observed in the model systems is consistent with energy transfer by inductive resonance among the chlorophyll a molecules in the two-dimensional arrays.

2. Exciton migration

Energy transfer due to exciton migration has been discussed in monographs on molecular excitons (Davydov, 1962; Knox, 1963) as well as in various review articles (McClure, 1960) and symposia (Kasha, 1963). In general, two types of excitons have been considered: 1) strong or free, and 2) weak or localized (Kasha, 1963b). These two cases have been considered to be completely separate from inductive resonance energy transfer.

Recently Forster has used a unified approach to the problem of determining which of the three transfer mechanisms, strong or weak ex-

citons or resonance transfer, occurs in a given system. We shall review briefly the criteria he derives, which are based on a comparison of the electrostatic intermolecular interaction strengths with molecular spectral properties. We shall see that under certain conditions we may disregard one or two of the three alternatives. We use available data for chlorophyll a and Forster's expressions to determine when exciton migration may occur in our model systems. Having decided when we are justified in expecting exciton migration to occur, we discuss experimentally observable properties pertinent to this type of energy transfer in our systems.

a. Criteria for coupling strengths

An exciton exists in a molecular ensemble when the molecular interactions are sufficiently strong that excitation energy undergoes oscillatory transfers among the molecules (Forster, 1960). Excitons have been classified as free or localized, corresponding to strong or weak molecular coupling (Kasha, 1963b). This classification reflects the length of time the excitation remains at any one molecular site, with respect to the relaxational time constants of the system. When the time the excitation stays on one molecule exceeds the vibrational relaxational period of the molecule, the localized exciton picture may be appropriate (Davydov, 1962). Alternatively, the distinction between strong and weak excitons can be made by a comparison of the molecular interaction energy with the spectral band widths of electronic transitions of molecules in the ensemble (Simpson and Peterson, 1957; Kasha, 1963b). In this scheme, if the interaction energy is less than the electronic band width, the exciton is considered weak, or localized.

The weak exciton case is to be distinguished from that of very weak coupling, defined analogously by Forster (1965) as occurring when the interaction energy is much less than an individual vibronic band width. In the very weak coupling situation, which is equivalent to inductive resonance, energy transfer is no longer oscillatory. The excitation remains on a particular molecule longer than the time required for intramolecular vibrational energy to exchange with the lattice. Such an exchange, or "collision" of the molecule with its surroundings, destroys phase relationships between molecules in the system. In this case, energy transfer is diffusive rather than oscillatory.

Forster (1965) derives numerical relationships for the limits between the cases of strong, weak, and very weak coupling. Starting with the assumption that at time $t = 0$, a particular molecule in the ensemble is excited, he calculates the probability that another molecule is excited at a later time, t, from the square of the coefficient of the appropriate term in the time-dependent wave function. In order to integrate equations for this probability over broad electronic energy bands, the time the excitation is at the particular molecule must be very short. For this case, which is equivalent to strong coupling, Forster requires

$$2|u| \gg \Delta E \qquad \text{(II.23)}$$

where u is the total electronic interaction matrix element, and ΔE is the electronic band width. When the coupling is weaker, so that only vibronic energy levels interact, the energy may stay at one molecule for a longer time period. The integration is now performed over a vibronic band of width $\Delta\epsilon'$, so that the restriction on interaction

energy becomes

$$\Delta E > 2|u_{vv'}| >> \Delta\epsilon' \ . \qquad \text{(II.24)}$$

$u_{vv'}$ is the vibronic interaction matrix element between two vibrational levels of an electronic state. This inequality defines the range of interaction energies for which the weak exciton scheme is appropriate.

If the coupling is very small, so that resonance can occur only within a small region of a vibronic band, excitation energy is localized for a time long compared with those in the two cases just discussed. The expression for the probability that a molecule is excited at time t is then evaluated in the limit of large t. This process imposes the inequality

$$|u_{vv'}| << \Delta\epsilon' \qquad \text{(II.25)}$$

on the system. Forster has further determined that $u_{vv'} \simeq \Delta\epsilon'/4$ is the approximate limit between weak exciton coupling and very weak coupling leading to inductive resonance.

b. Application to chlorophyll a

We wish now to evaluate these criteria for chlorophyll a in lipid monolayers. We use Equation II.11 for the interaction energy, setting r_{ij} equal to the average nearest neighbor distance. The dipole moment is obtained from

$$|\vec{\mu}|^2 = \frac{f}{4.7 \times 10^{+29}\bar{\nu}}$$

where f is the oscillator strength, and $\bar{\nu}$ the center of gravity in cm^{-1} of the absorption band of the molecules (McRae and Kasha, 1964). For chlorophyll a in a nonpolar solvent, f = 0.23 and $1/\bar{\nu} = 6.7 \times 10^{-5}$ cm

(Sauer et al., 1966). Due to the extreme thinness of the monolayers compared with the wavelength of the exciting light, the refractive index of the medium is taken as the average of that of the subphase and the nitrogen atmosphere above the film. We thus obtain $n \simeq 1.17$. Substituting these values into Equation II.11, we obtain

$$u = 1.15 \times 10^5 \frac{k}{r^3}$$

where r is the center-to-center nearest neighbor distance in Angstroms. The orientation factor, k, may range from 0.1 for a random molecular distribution to 1.0 for parallel oscillators. Table II.1 lists values of the interaction energy as a function, or r for these two values of k.

Since absorption spectra of chlorophyll a monomers in dilute lipid monolayers are not available (Gaines et al. have published spectra of concentrated films, only) we approximate the band widths ΔE and $\Delta\varepsilon'$ from a three-dimensional solution spectrum of the pigment in a polar solvent. The total electronic absorption band, which is composed of distinct vibrational bands, has a width of approximately $\Delta E \simeq 2000$ cm^{-1}. The 0 - 0 vibrational band is about 600 cm^{-1} wide. Gas phase spectra of chlorophyll a are not available, and low temperature solution spectra do not show further resolution of this vibrational band. Thus, Forster's vibronic band width, which is the width of a component of the vibrational band, cannot be obtained from experimental data. Forster (1965) suggests that 30 cm^{-1} is a reasonable estimate for this quantity at room temperature, but cautions that the existence of weak excitons is questionable when vibrational band fine structure cannot be observed.

We now compare these spectral band widths with the interaction energies listed in Table II.1, to decide when excitons could occur in the pigment systems. Even when chlorophyll a molecules are adjacent,

Table II.1

Interaction energy of chlorophyll a molecules as a function of nearest neighbor distance and molecular orientation

r	k = 0.1	k = 1.0
5 Å	88 cm^{-1}	880 cm^{-1}
10 Å	11.5 cm^{-1}	115 cm^{-1}
20 Å	1.4 cm^{-1}	14 cm^{-1}
25 Å	.75 cm^{-1}	7.5 cm^{-1}

r = 5 Å, and parallel, k = 1, the interaction energy is not high enough for formation of strong excitons. Weak excitons may exist, under conditions of favorable orientation, when the pigment molecules are 20 Å or less apart. However, when the molecules are randomly oriented, excitons probably would not be present unless the average nearest neighbor separation were less than approximately 10 Å. When chlorophyll a molecules are further apart, we expect energy transfers would occur by the mechanism of inductive resonance.

The average nearest neighbor separation of chlorophyll a molecules in the monolayers is calculated from the concentration of pigment in the lipid and the area per molecule at any given pressure. These two quantities are known from experimental conditions. The relative orientations of the molecules in the films are, however, unknown. By considering the effect of exciton transfer on the polarization of fluorescence, we hope to elucidate the extent of pigment orientation.

c. Properties of chlorophyll a-containing monolayers in which excitons occur

In view of the above discussion, we shall be concerned in this section with localized or weak excitons only. Free excitons apparently cannot exist in the mixed monolayers, because the interaction energy is not sufficiently large.

Forster has shown (1960) that the average pairwise transfer rate for localized exciton migration is

$$q_{ij} = \frac{4\,|u_{ij}|}{h} \sum_{vv'} g_v^* g_{v'} S_{vv'}^2 \qquad \text{(II.26)}$$

where the interaction energy u_{ij} is given by Equation II.11, and the other symbols have the same significance as in Equation II.15. Comparison of the above relation with Equation II.15, which is the inductive resonance transfer rate, shows that the localized exciton rate may be considered due to a first order perturbation by the interaction energy, whereas resonance transfer is a second order perturbation phenomenon. Because the interaction energy between two molecules is inversely proportional to the cube of their separation and the summation over Franck-Condon overlap integral is ≤ 1, exciton migration will occur much more rapidly than will resonance transfer between a given pair of molecules.

Now, in a system in which exciton migration is efficient, a pairwise energy transfer rate has little physical significance. However, we may use Equation II.26 to gain insight into the extent of migration of localized excitons in a molecular ensemble. As in Forster's treatment of the inductive resonance case, we set

$$q_{ij} = \frac{4\,k\,|\vec{u}|^2}{n^2 h\, r_{ij}^3} \sum_{v,v'} g_v^* g_{v'} S_{vv'}^2 = \frac{1}{\tau}\left(\frac{R_e}{r_{ij}}\right)^3$$

where

$$R_e^3 = \frac{4\,k\,|\vec{u}|^2}{n^2 h} \sum_{v,v'} g_v^* g_{v'} S_{vv'}^2 . \qquad \text{(II.27)}$$

From spectral data we may calculate the "critical distance," R_e, for exciton transfer defined by this relation. We use the expression (Forster, 1960)

$$\frac{\Phi_o}{\Phi} = \frac{\tau_o}{\tau} = \frac{3\,h\,\lambda_o^3}{64\pi^4 n \tau |\vec{\mu}|^2}$$

where λ_o is the center of gravity of the absorption and emission bands, and Φ/Φ_o the relative fluorescence yield. We make the following approximations. Employing Tweet et al.'s (1964) assumption that the relative fluorescence yield is not appreciably different in films from that in dilute solutions, we set $\Phi/\Phi_o \simeq 0.3$. We again approximate the refractive index, $n \simeq 1.17$. From the published absorption and fluorescence spectra of diluted chlorophyll a monolayers (Bellamy et al., 1963; Gaines et al. 1964) we obtain $\lambda_o = 675 \pm 5$ nm. Lastly, we must evaluate the sum over the Franck-Condon overlap integrals of the vibrational states, weighted by the population factors of those states. As Forster points out (1960), this sum is equivalent to the probability of emission by a transition to the populated vibrational level of the ground state of an unexcited molecule, and is of the order of 0.1 for dye molecules. We approximate this probability from overlap of monolayer absorption

and emission spectra in the region of the 0 - 0 red band, assuming that only the lowest vibrational levels are occupied. Again using Gaines et al.'s spectra (1964), we find $\Sigma = 0.16$. Substituting these quantities into Equation II.27 yields $R_e = 560$ k Å. Possible overestimation of ϕ/ϕ_o and the sum could cause an overestimation of R_e by as much as a factor of 2, so $R_e = (400 \pm 120)$k Å. If the exciton state exists in a random array of adjacent molecules, $k = 0.1$, and $R_e = 40 \pm 12$ Å, which is the same order of magnitude as the critical distance, R_o, for resonance transfer. However, for exciton migration to occur in a dispersed array of chlorophyll, k must approach unity. Then $R_e = 400 \pm 120$ Å.

Rather than interpret the distance R_c as the molecular separation below which energy transfer is more probable than emission, we suggest that it be considered the average radius of the area over which an exciton could migrate in the ensemble. When R_e is large, this area must contain many pigment molecules in a fairly well oriented array in order for an exciton state to exist in the system. Thus, although the excitation will reach a molecule a few hundred Angstroms away from the initially excited one with a probability as great as the emission probability, it may visit many molecules in between these two in the process.

If R_e is to be considered a transfer distance, it might appropriately be equated with the average path length of migration before emission. In either of these two cases, we see that excitation moves, on the average, much further before emission if an exciton state is formed than if the energy is transferred by inductive resonance.

When localized excitons exist in a separated array of chlorophyll a molecules, the fact that many molecules are momentarily excited before emission will not automatically imply that fluorescence is depolarized.

As we have seen, the pigment molecules must be favorably oriented for such an exciton state to exist, whereas random molecular orientations in the ensemble are the cause of depolarization. We therefore predict that the increased extent of energy transfer due to formation of excitons at a given concentration in a system of dispersed molecules will not cause a proportionate decrease in fluorescence polarization, because favorable orientation is required for the formation of this exciton state.

On the other hand, if chlorophyll a molecules are randomly oriented, exciton states are not formed in the system until the pigment concentration has increased to the point where the nearest neighbor distance is ~5 Å. This distance is only slightly larger than the thickness of the porphyrin plane of chlorophyll. Thus the decrease in fluorescence polarization observed as the pigment concentration is increased in a random array is due to energy transfer by inductive resonance only, up to almost 100% chlorophyll. Below this latter point, we would expect no abrupt change in the rate of polarization decrease because there is no change in the energy transfer mechanism.

The character of the polarization vs concentration curve may therefore enable us to determine the extent of orientation and the type of energy transfer occurring at relatively high chlorophyll a concentrations in the monolayers.

3. Diffusion of localized excitons

Some investigators concerned with the mechanism of energy transfer in photosynthesis have considered energy migration via diffusion or random walk of localized excitons (Duysens, 1952; Pearlstein, 1964; 1966). Making certain assumptions about the arrangement of pigment

molecules in the cell, they have shown that calculated transfer rates and critical distances are consistent with some experimentally observed properties of the biological systems. Trlifaj (1958) has determined theoretical transfer probabilities for diffusion of localized excitons in a three-dimensional array. The frequency of transfer is directly proportional to the square of the interaction energy, i.e., to $1/r^6$. Therefore, this mechanism of energy transfer can be treated as was inductive resonance, and it cannot be distinguished from the latter by our investigations.

The theoretical considerations presented here will aid us in evaluating our experimental results for energy transfer mechanisms having $1/r^3$ and $1/r^6$ dependences on molecular separation. We shall calculate critical separations for energy transfer among chlorophyll a molecules by the weak, $1/r^6$, interaction from polarization data for low pigment concentrations. These distances will be compared with other empirical values of the critical distance. Consistency of these numbers would support the hypothesis that inductive resonance energy transfer occurs in the model.

The criteria for strong interactions in these models have been discussed. Indications of energy transfer due to exciton migration in monolayers with high concentrations of chlorophyll a will be sought. After limitations of the theories and accuracy of the data have been considered, we hope to suggest under what conditions these two transfer mechanisms occur in the models and in the chloroplast.

III. LIPID MONOLAYER STUDIES

We have discussed properties of the fluorescence of molecules in a two-dimensional array in terms of the mechanism of energy transfer among them. In order to propose which transfer mechanisms occur under what conditions in the chlorophyll a-containing monolayer models, we require fluorescence polarization data not previously measured. The experiments described in this chapter were undertaken primarily to obtain such data.

In order to observe polarization of chlorophyll a fluorescence as a function of pigment concentration, the monolayers were diluted with miscible surface active lipids. In addition to castor oil and oleyl alcohol, we used isolated chloroplast structural lipids for this purpose. As the surface properties of these latter lipids have not been extensively studied, their monolayer behavior, and that of mixed films of chlorophyll a and structural lipids, is reported in detail.

We discuss the experimental results in terms of the analysis outlined in the last chapter. The data support the hypothesis that energy transfer among chlorophyll a molecules widely dispersed in a two-dimensional lipid environment would proceed by inductive resonance. We note some peculiarities in the behavior of chlorophyll a present at high concentrations in films of plant lipids which may be indicative of stronger intermolecular interactions. The significance of these observations in determining the pigment environment in vivo is discussed later.

A. Experimental Procedures

1. Monolayer Fluorometer

For simultaneous observation of monolayer compression properties and fluorescence polarization, we required a surface film balance with a suitably arranged and very sensitive optical system. A commercial Langmuir type film balance (Central Scientific Co., "Cenco Hydrophil Balance," catalogue #70551) was modified to suit this purpose.

The lacquer coat was scraped from the inner surfaces and edges of the bronze trough, which was then painted black and heavily coated with paraffin ("Parowax", American Oil Company, Chicago). This surface was renewed as necessary. The torsion balance supplied with the instrument, with the platinum foils and mica float lightly paraffined, was used without further alteration. The trough, with torsion balance attached, stood on a mount of Dural aluminum. On the latter, which was equipped with leveling screws, was constructed an automatic barrier drive mechanism similar to that designed by Gaines (1963). This machinery moved the barrier slowly and continuously back and forth along the trough edges.

The drive mechanism (Figure III.1) consists of a lead screw to which are attached forks for holding the barrier flush against the trough edges, ways for guiding the movement of the forks, a reversible Bodine speed reducer motor, type NSY 12R, 60 rpm, and Boston reduction gears. Two 12-inch stainless steel lead screws, 1/2 inch in diameter, 1.0 ± 0.01 mm/turn, were joined to obtain the required 19-1/4 inch threaded length. At the far end of the assembly, the screw is mounted in the trough support block in a ball bearing washer. The unthreaded

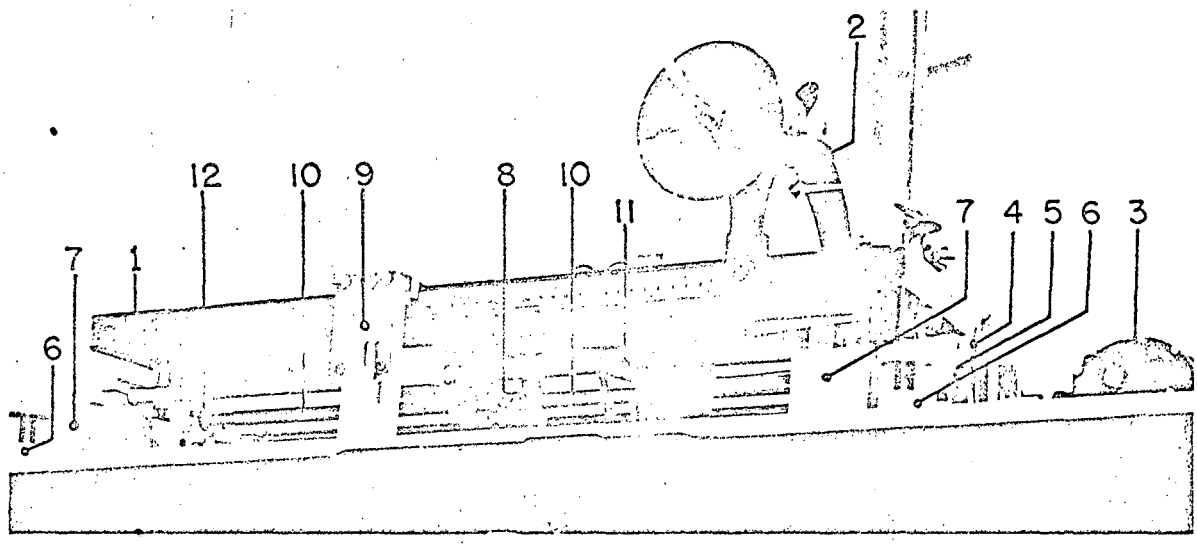

Figure III.1. Monolayer fluorometer automatic barrier drive and trough mount.

1) Trough
2) Torsion balance
3) Motor
4) Gears
5) Teflon washer
6) Leveling screws
7) Support block
8) Collar
9) Barrier fork
10) Lead screw
11) Barrier fork ways
12) Ball-bearing washer

end of the screw passes through the other support block near the motor and is joined to the gear shaft via a teflon washer and spring arrangement. A gear reduction ratio of 3:1 was used, so the barrier moved at a rate of 20 mm/minute. Because the edges of the trough had not been milled evenly, it was necessary to spring load the barrier in its forks, as well as spring loading the forks to the bronze collar which rode on the lead screw, to prevent strain on the latter.

Since chlorophyll a monolayers and plant lipids tend to bleach and oxidize, respectively, in air (Bellamy, et al., 1963; O'Brien and Benson, 1964), the trough must be kept in a controlled atmosphere in the dark during experiments. A 1/4-inch Lucite cover, painted black, accomplishes this. It encloses the trough, torsion head, and barrier drive mechanism, with the exception of the motor and gears. To allow monitoring of film pressure and area, windows permit observation of the float pointer and barrier position indicator. The cover is constructed so that the vernier scale on the torsion wire is also outside, and may be adjusted without disturbing the film or exposing it to air. Ports are provided in the cover for sweeping the enclosure with gas, for use of a siphon if desired, for spreading the film, and for inserting the photomultiplier (see below).

It is desirable, when studying fluorescence polarization, to have the exciting light impinge on the monolayer normal to the plane of the film, and to observe the fluorescence emitted in the same direction. Although this geometry presents optical difficulties, it has been used (Figure III.2). The light source, an air-cooled GE A-H4 100 watt mercury

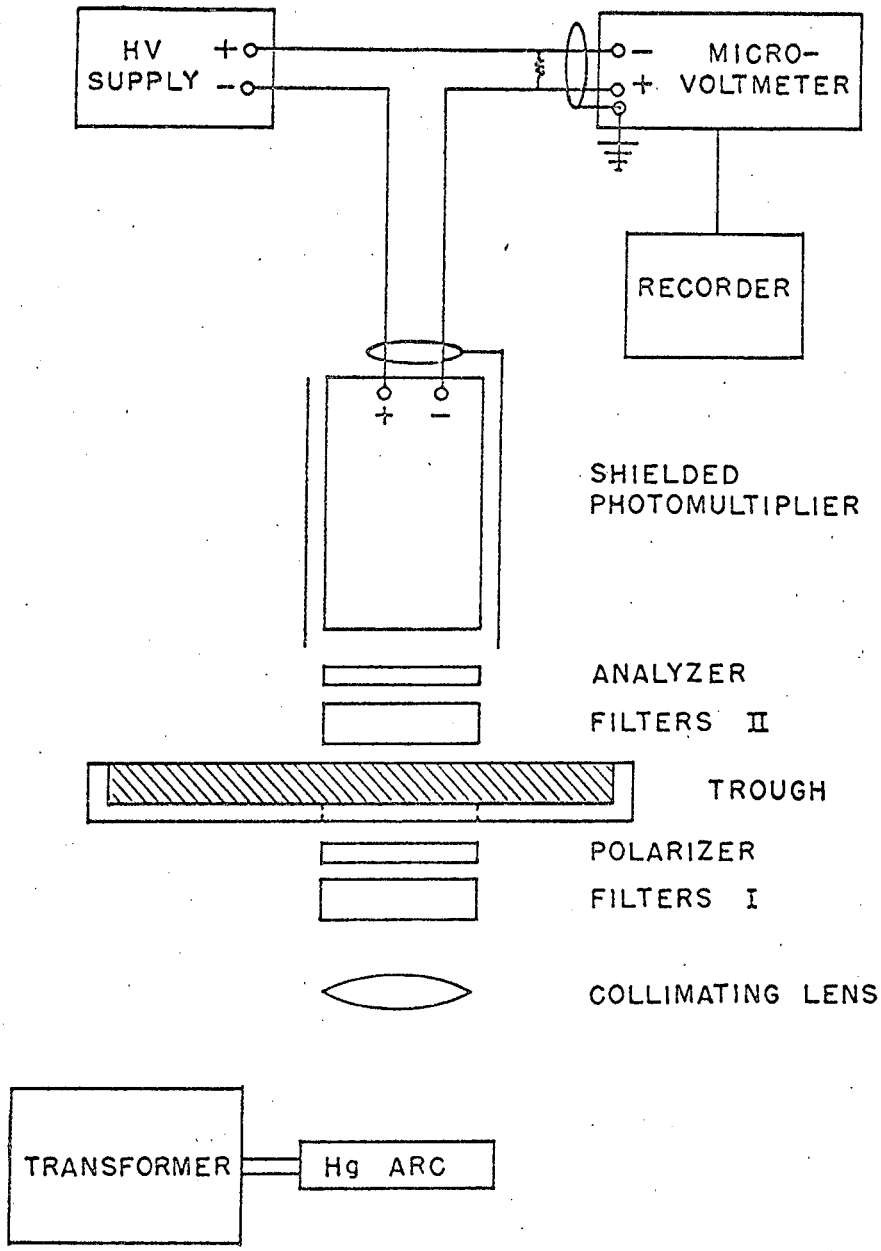

Figure III.2. Monolayer fluorometer electronic and optical schematic diagram. See text for details.

arc lamp, is mounted in a horizontal position on a separate bench under the experimental table, where vibrations from the cooling fan do not disturb the films. The light passes through a collimating lens, filters for isolating the chosen mercury line (Filters I), and a polarizer, Polaroid, HN 22, which can be rotated exactly 90° in its holder. After passing through a hole in the table, a blackened Lucite tube, and a window in the bottom of the trough, the exciting light impinges on the monolayer from below. The window, a 2-inch diameter 1/8-inch thick polished quartz plate, is seated with O-rings to prevent leakage. It is held in place by a stainless steel collar clamped with set screws in a tube welded to the bottom of the trough. A shutter which slides between the trough mount and table has provision for a removable standard fluorescing filter. We found that a Corning red cutoff filter, #2-63, when placed before the trough window, fluoresced with sufficient intensity that it could be used as the fluorescent standard. French (1965) had noted this phenomenon, and has since reported the fluorescence spectra of several cutoff filters. When illuminated with a high intensity visible source, #2-63 fluoresces strongly in the region of chlorophyll a fluorescence, which makes it a particularly useful reference for our work.

The photomultiplier holder fits in the trough cover directly over the window. It, too, is made of blackened Lucite. Attached to its lower end is a holder for filters to block the exciting light and isolate the desired fluorescence band (Filters II). This filter holder extends down into the enclosed area to within an inch of the

water surface when the trough is filled. A separate holder for the analyzer, Polaroid HR, fits into the photomultiplier holder. It can be rotated 360°.

Except where noted, the filter combinations used in all experiments were as follows:

Filters I: Two 406 nm narrow band pass interference filters (Baird-Atomic, Inc., type B-1, with IR blocking)
Corning band pass filter, #5-58

Filters II: Optical Coatings Laboratories, Inc., dielectric rejection filter, OD = 2.7 at 406 nm
Two Corning red cutoff filters, #2-58 and #2-59

Figure III.3 shows the apparatus with the cover and photomultiplier in place, ready for an experiment.

The polarization of the exciting light reaching the monolayer was measured by replacing Filters II with neutral density screens and filters. With the polarizer in either position the exciting beam was greater than 96% polarized.

For the detection of chlorophyll a fluorescence, we used a red-sensitive photomultiplier, RCA #7326, operated at 1800 volts from a Pedersen Electronics 3KV, 10 ma regulated supply. The photomultiplier was not cooled, but was sheathed in a mu metal shield connected to an extra shield on the photomultiplier leads. This latter shield was grounded at the chassis of the DC microvoltmeter, Keithley Model #151, used for signal amplification. The amplified signal could be recorded on a Moseley Model 680 Autograf recorder. A resistor, 0.24×10^6 ohm, was placed across the input terminals of the voltmeter to reduce the noise level. A schematic diagram of the electronic circuit is also shown in Figure III.2.

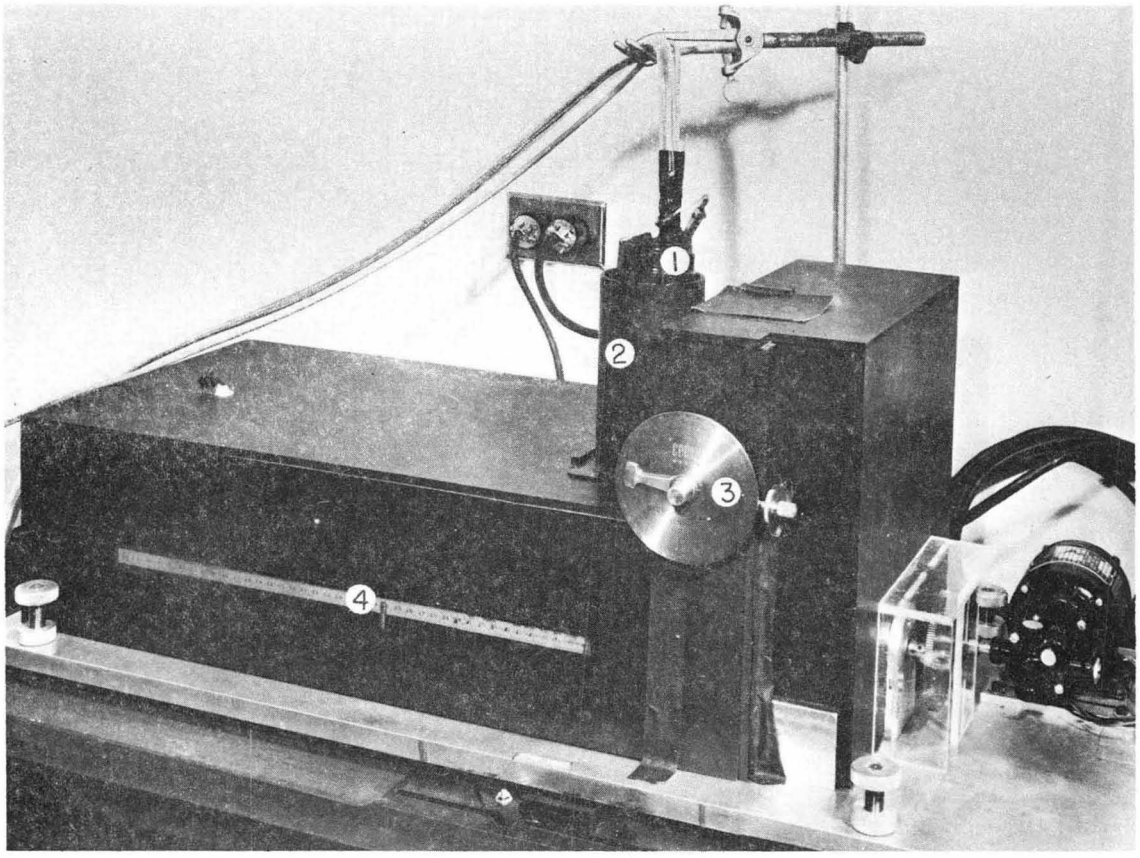

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Fig. III.3. Monolayer fluorometer prepared for experiment.

1) Photomultiplier socket
2) Photomultiplier holder
3) Torsion balance vernier
4) Barrier position indicator

Using this monolayer fluorometer, we could excite a film with approximately monochromatic linearly polarized light absorbed by chlorophyll a and observe the polarization of the entire red fluorescence band. The sensitivity of the photomultiplier served as the upper cutoff for this band. The time constant of the DC detection system was of the order of a few seconds, precluding kinetic studies. Detection systems with faster time constants were not sufficiently sensitive for our purpose. Thus, we could neither detect the presence of, nor determine the polarization of, any delayed fluorescence or phosphorescence which might have emanated from the films. However, a weak phosphorescence or afterglow from chlorophyll a *in vitro* has been reported only when the pigment was carefully dried (Fernandez and Becker, 1959), or at wavelengths longer than these to which the photomultiplier is sensitive (Singh and Becker, 1960), or when chemical changes occur in the pigment (Goedheer and Vegt, 1962). In view of this evidence, we do not make any corrections for possible effects of delayed light.

2. Materials

Chlorophyll a was isolated from spinach chloroplast fragments by chromatography on columns of powdered polyethylene (Dow Chemical Co., melt index < 2) and powdered sugar (C & H) (according to the method of Anderson and Calvin, 1962). The eluate from the sugar column was stored in a refrigerator in the dark under nitrogen. To prepare spreading solutions, aliquots of this microcrystalline suspension of the pigment in isooctane were evaporated to dryness on a rotary evaporator and weighed into a small volumetric flask. After making up the solution to known volume in the appropriate spreading solvent, we checked the

concentration from the absorption spectrum, which was recorded on a Cary 14 spectrophotometer, using extinction coefficients reported by Bellamy et al. (1963) or Seely and Jensen (1965). When later in the investigations, disagreement in molarities calculated by weight and from optical density indicated that some of the chlorophyll had degraded, it was rechromatographed on powdered sugar in small lots with freshly distilled isooctane. The pigment was dried on the column, the broad chlorophyll a band cut out, and the chlorophyll eluted with ether or acetone to avoid contamination by colorless impurities soluble in the isooctane. The repurified pigment was used immediately. All operations on the chlorophyll were carried out in dim green light or in the dark.

Castor oil, dB refined grade, MW 928, viscosity 6.8 poise, was obtained from the Baker Castor Oil Company, Bayonne, N. J. Aliquots of the oil were taken from freshly opened cans, as it tended to oxidize with time when exposed to air.

We used oleyl alcohol (9-octadecen-1-ol), > 99% purity, from the Hormel Institute, University of Minnesota. The viscosity of this compound is not available in the literature. We determined the viscosity of our sample relative to glycerol (Baker and Adamson, reagent grade) using a modified Ostwald viscometer. A Kimax viscometer tube, #300, immersed in an oil temperature bath, was maintained at 25.0 ± 0.1°C during the measurement. With a total liquid volume of 5 milliliters for each material, we took several readings of flow time through the viscometer capillary. Using standard procedure for the calculation (Daniels et al., 1956), we found $\eta = 0.57 \pm 0.06$ poise for oleyl alcohol at this temperature.

Plant strucutral lipids (Figure III.4) were obtained from spinach chloroplasts prepared by the method of Park and Pon (1961), except that versenol was omitted from the grinding buffer. O'Brien and Benson (1964) have described a method for isolation of the sulfo- and galactolipids from plant leaves and algae. We isolated these lipids from chloroplasts by a modified procedure described in the next section.

Chloroform, methanol, and acetic acid solvents (Baker and Adamson or J. T. Baker, reagent grade) used for the lipid preparations were all distilled prior to use. Nitrogen was bubbled through each solvent before it was put in contact with the lipids. 2,2-Dimethoxypropane (acetone-dimethylacetal) was obtained from K & K Laboratories, or from Dow Chemical Co.

Benzene, and hexane containing 1% pyridine by volume were used as spreading solvents. Baker and Adamson or J. T. Baker reagent grade hexane and pyridine from Brothers Chemical Co. were used without further purification. Benzene from the same sources was redistilled from sodium hydride and kept in a tightly stoppered bottle.

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 water which had passed through a deionizing column of mixed Dowex 50 and 8 resins, 100 - 150 mesh.

3. Isolation and Purification of Plant Lipids

Chloroplasts prepared by the method of Park and Pon (1961) from approximately 1 kilogram of freshly washed spinach leaves were homogenized in about 10 volumes of chloroform - methanol, 2:1 by volume, under nitrogen. The extract was filtered, also under nitrogen. The

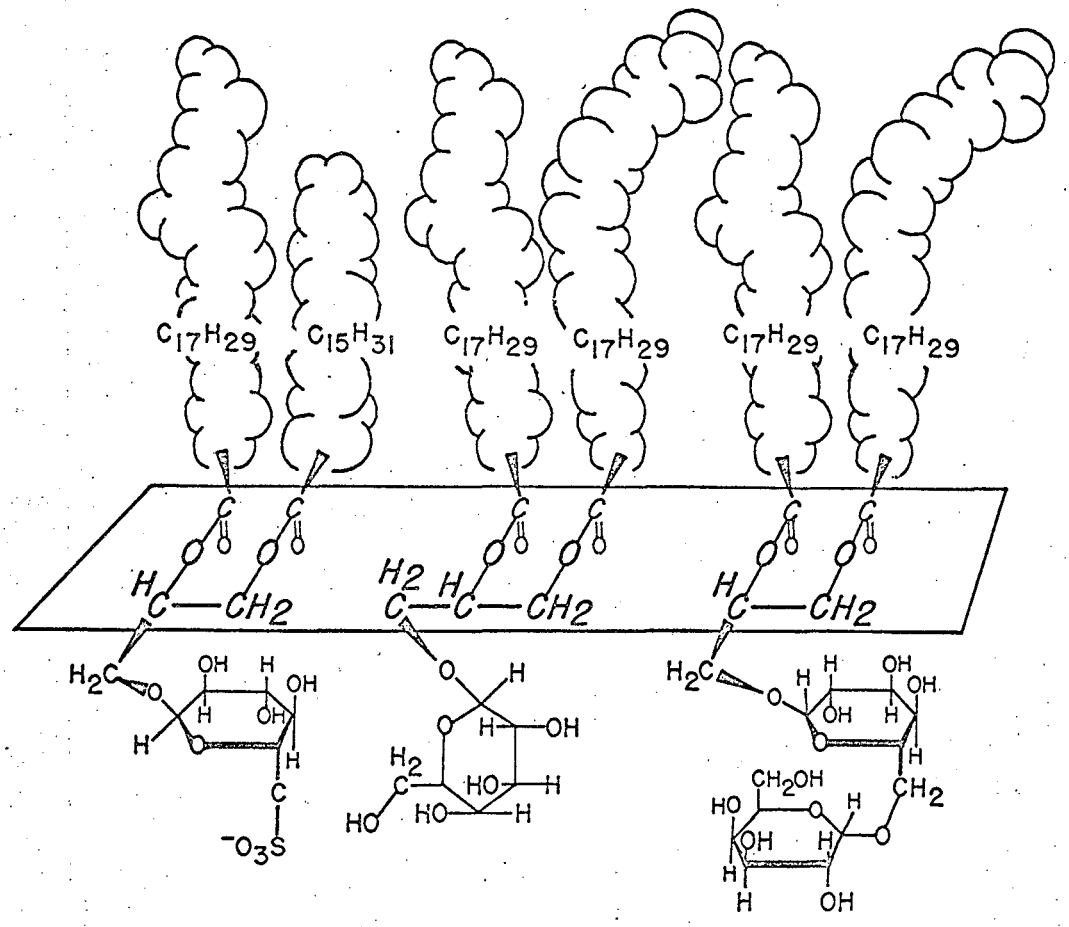

Figure III.4. Chloroplast structural lipids, according to Benson (1964), showing possible orientations at a gas-aqueous interface. Note polar sugar substituents and hydrophobic fatty acid groups.

residue was reextracted in the same solvent until the extract was only slightly colored and the residue was pinkish- or yellowish-brown.

Sulfolipid was isolated from the combined extracts by Florisil and DEAE cellulose column chromatography according to O'Brien and Benson's procedure. All operations were carried out under nitrogen. The effluent from the first column could be put directly onto the top of the next via a three-way stopcock and jointed glass tubing. Eluents were also introduced onto the columns under nitrogen, by use of slight positive pressure to force them slowly out of large reservoirs. The final eluate from the DEAE cellulose column, containing the sulfolipid, was evaporated to dryness on a rotary evaporator. The resulting residue, resuspended in approximately 60 ml of chloroform-methanol, 4:1, was dialyzed in tubing prepared according to O'Brien, et al. (1964) against 1600 mls. of deionized water. The dialysis was carried out in a nitrogen box for 30 - 40 hours, with several water changes. The sulfolipid formed a fluffy white phase at the interface between the chloroform and the water-methanol phases in the dialysis bag. It was also partially dissolved in both phases, as evidenced by their slight yellowish color. At the end of the dialysis, the contents of the bag were evaporated to dryness on a rotary evaporator and weighed. The lipid was then resuspended to a concentration of 1 milligram/ml in redistilled benzene or chloroform-methanol, 2:1, and stored in a stoppered flask under nitrogen.

We found that some monogalactolipid was eluted from the Florisil column by the chloroform-methanol, 9:1, solvent when we followed O'Brien and Benson's procedure. Also, some pigment passed through the DEAE column in the chloroform-methanol, 2:1, eluate with the rest of the

galactolipids. Thus we resorted to preparative thin-layer chromatography to purify mono- and digalactolipids.

Plates were coated with Silica Gel G (Research Specialties Co., Richmond, Calif.) following the techniques of Lepage (1964) and activated 20 minutes at 110°C just prior to use. The last third of the chloroform-methanol, 9:1, eluate from the Florisil column, which eluate had been concentrated by evaporation, was streaked onto three plates under nitorgen. A fourth plate was spotted and run simultaneously. The plates were developed with chloroform-methanol, 9:1, for 50 minutes in the dark and then allowed to dry in a nitrogen box. All pigmented spots on the fourth plate were marked before the plate was sprayed with 50% H_2SO_4 and charred at 200°C for 15 minutes. This procedure brought out a large spot which ran directly behind the slowest running pigmented compound visible before charring. This compound had previously been identified by its behavior in various solvent systems as monogalactolipid (Nichols, 1964). The corresponding areas on the streaked plates were then scraped off into a sintered glass funnel. The lipid was eluted from the support with chloroform-methanol, 9:1, still keeping the material under nitorgen. The eluate was evaporated to dryness on a rotary evaporator and weighed. Finally the lipid was resuspended to a concentration of 1 milligram/ml in redistilled benzene and stored in a stoppered flask under nitrogen. This material was checked for purity by thin-layer chromatography on plates prepared as described above, using chloroform-methanol, 9:2, as a developing solvent. The eluate was spotted beside it for comparative purposes. In this more polar solvent, any pigments and quinone impurities would run closer to the

solvent front, and the polar lipids would be more widely separated. After spraying with 50% H_2SO_4 and charring, we could detect only one spot in the purified material, corresponding to that which had been tentatively identified at monogalactolipid in the eluate. A laboratory record of analytical thin-layer chromatograms was kept by outlining spots with a fine hypodermic needle and Xeroxing the plates.

The digalactolipid was obtained similarly from the DEAE column chloroform-methanol, 2:1, eluate. However, a developing solvent of chloroform-methanol, 4:1, gave the best separation of this more polar lipid. Despite rechromatographing we were unable to free it completely of chlorophyll-like contaminants, which remained at a relative concentration of approximately 0.01 mole percent. Therefore, we did not use this lipid for fluorescence polarization or chlorophyll a-lipid interaction studies.

The purity of sulfolipid preparations was also ascertained by thin-layer chromatography. A solvent system of chloroform, methanol, acetic acid, and water, 85:15:12:1, was used to obtain sufficient migration of this very polar lipid. One sample was found to contain about the same amount of pigment contaminant as the digalactolipid. The pigment concentrations were calculated from optical densities of solutions of the lipids at 665 nm. Spectra were recorded on a Cary 14 spectrophotometer. The identity of the lipids was further confirmed by performing an anthrone test for sugars on the products of acid hydrolysis. Anthrone reagent gives a green color with galactose, maximum absorption of the rather broad band occurring at 625 nm (Weenink, 1962). The product formed in the presence of sulfoquinovose, however, absorbs further in the blue, having a maximum at 592 nm (Figure III.5).

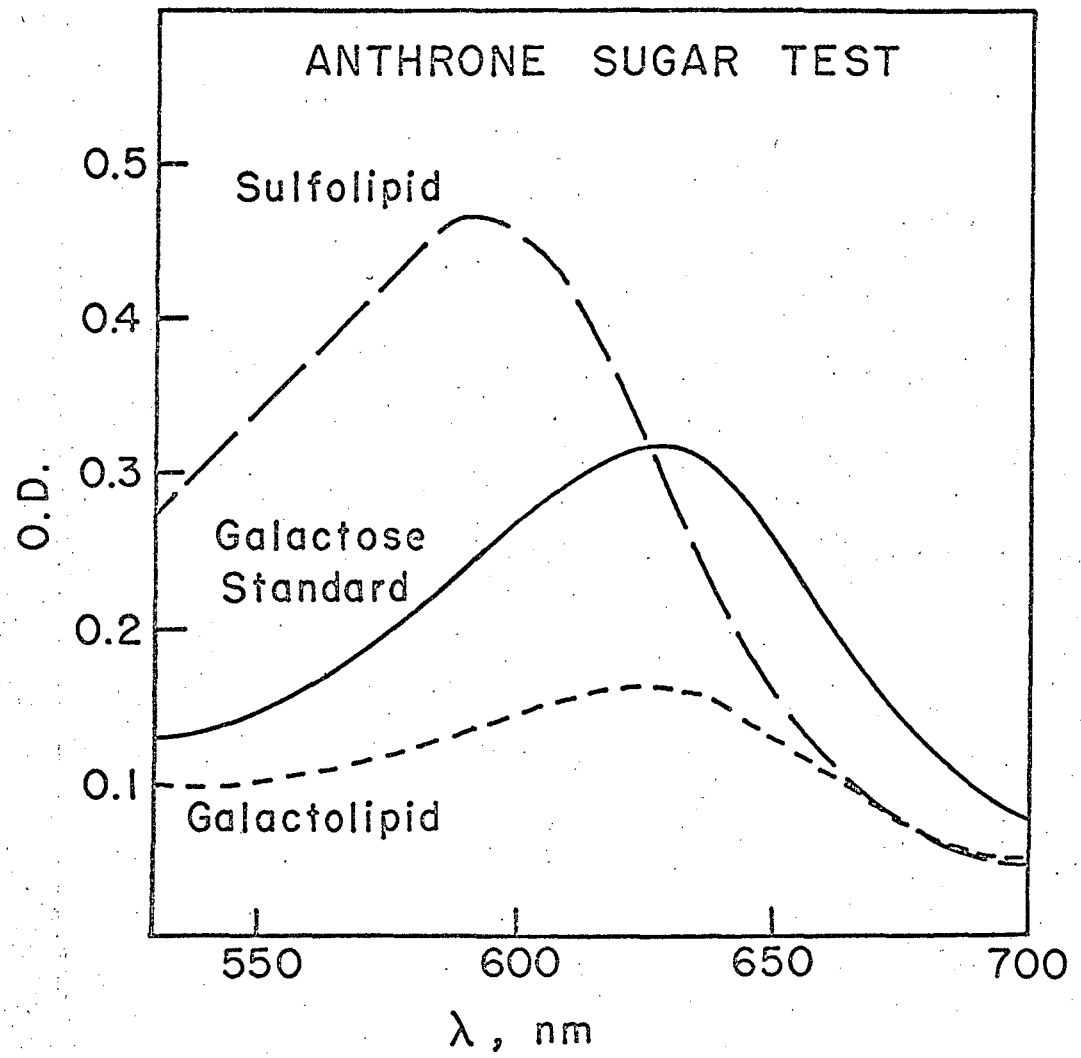

Figure III.5. Anthrone color reaction with galactose (——), hydrolyzed galactolipid (----), and hydrolyzed sulfolipid (— —). Relative optical densities have not been normalized to unit weight of sugar.

The anthrone reaction is very sensitive to the conditions of the test. Excess anthrone reacts with the colored product, resulting in decoloration. Overheating will bleach the product. The color intensity increases with time in the presence of sulfoquinovose, but apparently not of galactose. The anthrone reagent itself is very light sensitive and ages rather quickly. In addition, we wished to detect small quantities of sugars, of the order of 50 - 100 micrograms. After considering these difficulties and studying various procedures reported in the literature (Radin, et al., 1955; Bailey, 1958; Weenink, 1963), we chose the following conditions to suit our requirements. They proved to be sufficiently sensitive and fairly reproducible.

A stock anthrone solution, 10 mg/ml in concentrated H_2SO_4, was aged for 4 hours in the dark, and then stored in the refrigerator. No stock solution was kept more than two days. Two hundred to 500 microgram aliquots of glycolipids and 50 - 250 microgram galactose standards were hydrolyzed in 2 ml reagent grade H_3PO_4 (85%) for 15 minutes at 90 - 95°C, and then cooled 5 minutes in ice. Then 5 ml of freshly prepared anthrone reagent (1 ml anthrone stock solution in 24 ml H_2SO_4/H_2O, 2:1) were added and the solution stirred vigorously. The mixture was heated for 12 minutes at 90 - 95°C, and then cooled in ice in the dark for 30 minutes to allow full color development. The optical density of the solutions from 520 to 700 nm was then recorded on a Cary 14 spectrophotometer, using the control solution (2 ml H_3PO_4 + 5 ml anthrone reagent heated as were the samples) in the reference compartment. The 0.1 slidewire of the instrument was used for recording the absorbance of dilute solutions. Galactose standards gave an optical density ratio at the two

wavelengths of interest, 592/625, of 0.75 under these test conditions. Due to the presence of other lipid hydrolysis products, this ratio was slightly higher for the galactolipids. Sulfolipid hydrolysis products produced a ratio of 1.3. Ratios of the order of one, which were obtained from earlier lipid samples, which were not purified by thin-layer chromatography, indicated cross-contamination of sulfo- and galactolipids. The sugar concentrations of the samples were calculated from the absorbances of the known galactose standards. From this data we could determine the original amount of lipid in the samples and use this as a further criterion of purity. Molecular weights computed from the structures given by Benson (1964) and shown in Figure III.4 were used in these calculations.

4. Monolayer Techniques

All experiments were performed in a small closed room maintained at 18 ± 2°C. The room could be darkened completely, and was also equipped with a dim green safe light. Before each film was spread, the trough, movable barrier, and float were washed copiously with distilled and then deionized water, to remove dust and traces of chemical contaminants. After leveling the mount with the trough in place, the torsion balance was screwed in position. The torsion balance supports did not fit flush against the trough edges when the latter were coated with paraffin. To prevent leakage of the films at these points, it was necessary to place small, pliable L-shaped polyethylene washers between the trough edges and the torsion balance supports. The trough was then filled to the brim with the subphase buffer described in section 2. Approximately 1640 ml of buffer filled the trough sufficiently

to produce a slight convex meniscus above the edges. The subphase surface was then swept clean ofdust and lint, before the movable barrier was put in place. We found that nylon sewing thread (Brook's "Crystal" Thread) was excellent for this purpose. After noting the position of the barrier on the trough, we placed the cover carefully over the apparatus. The enclosure was flushed with nitrogen gas saturated with subphase buffer at slight positive pressure throughout an experiment.

If the compression properties of the film were to be observed, an aliquot of spreading solution was next deposited dropwise onto the subphase surface with a micropipette which fit through a small hole in the cover. Spreading solution concentrations were adjusted so that $1 - 4 \times 10^{16}$ molecules could be deposited on the surface in 50 - 200 microliter aliquots. Slow spreading permitted evaporation of the spreading solvent as each drop expanded on the surface. This was necessary to prevent the spreading solvent from reaching the paraffined trough edges and barriers in large enough quantity to dissolve some of the paraffin. Reproducibility of pressure vs. area behavior indicated that this difficulty was successfully avoided. After a few minutes had been allowed for complete evaporation of the solvent and formation of the lipid monolayer, the torsion balance was zeroed and the film compressed by movement of the barrier at a constant rate. The torsion balance was adjusted to null position and the surface pressure read every 30 seconds. Compression was stopped when the barrier reached the light path, or when film collapse was indicated by a leveling off or decrease in surface pressure. If we stopped compression before collapse, the stability of the monolayer at the final pressure was observed.

In all cases, the films were reexpanded and the zero-point compression checked to ascertain that the lipid did not leak past the float or barrier during the experiment.

When monolayer fluorescence polarization was studied, the room was darkened after the trough was covered, and the photomultiplier assembly installed in the cover. After the mercury arc had warmed up and the photomultiplier dark current stabilized, the light reaching the phototube with polarizers crossed and parallel was recorded. A small signal, the order of a few tenths of a millivolt, was always detectable in the absence of a film. This was due to incomplete blocking of the exciting light by the rejection filter and weak fluorescence from the red cutoff filters. After recording the signal from the fluorescing filter used as a standard, we spread and compressed the monolayer as described above. Compression was stopped at a pressure of approximately 12 dynes/centimeter, at which pressure the films were still stable. The monolayer was maintained at this pressure while several measurements of fluorescence intensity with polarizers crossed and parallel were taken. Thirty-second to one-minute 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 along 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 of solution directly over the window in the bottom of the empty trough. The edges of the dish were masked to avoid light scattering. Sample

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.

B. Results of the Monolayer Studies

1. Compression Characteristics

A monolayer of a pure substance is generally characterized by the pressure-area curve obtained as the film is compressed slowly and uniformly (e.g., Adam, 1941). This curve is a plot of the force per unit length, or surface pressure, on the float connected to the torsion balance, as a function of the average area occupied per molecule in the monolayer. The pressure and area at which a film collapses, the slope of the pressure-area curve, and the area extrapolated to zero pressure should all be reproducible quantities characteristic of the molecules spread. These remarks apply equally well to a film of ideally mixed substances which form a two-dimensional solution. In this case the pressure-area plots should be additive and collapse should occur reproducibly at a pressure intermediate between those of the pure materials (Crisp, 1949). At pressures below the collapse pressure, the monolayers should be stable. The predicted ideal behavior of a mixed film is calculated from the pressure-area data of the pure substances according to

$$A(\pi) = n_1 A_1(\pi) + n_2 A_2(\pi) \qquad \text{(III.1)}$$

where n_1 and n_2 are the mole fractions of substances 1 and 2. Monolayers of components which are immiscible or which interact chemically generally have anomalous and irreproducible compression characteristics, although the pressure-area curves may be additive. Behavior of a mixed

film upon compression thus serves as a fairly sensitive indication of its stability and uniformity.

We have observed the compression characteristics of the pure and mixed monolayers used in fluorescence polarization studies. These properties will aid us in determining the state of the chlorophyll a molecules in the films, as well as indicating monolayer instability.

a. Chlorophyll a. Bellamy, et al. (1963) have studied the compression behavior of pure chlorophyll a monolayers thoroughly. Our data agree with theirs within an experimental spreading error of approximately 5% (Figure III.6). The solid line is the average of data from six samples, spread on 10^{-3} M phosphate buffer, pH 7.8, which were compressed at rates from 10 to 13 $Å^2$/molecule/minute. These rates are within the range where Bellamy, et al. observed reproducible behavior.

b. Chlorophyll a + castor oil. The pressure-area behavior was within 10% of that predicted by an ideal mixture, at as high a chlorophyll a concentration as 0.5 mole fraction (Figure III.7). At chlorophyll a concentrations below approximately 0.1 mole fraction, the films behaved essentially as did those of pure castor oil. Monolayers of pure castor oil have been investigated by several workers (e.g., Adam, 1941). Collapse pressures in the range of chlorophyll a concentrations between 0.1 and 0.9 mole fraction depended on the relative amount of chlorophyll in the film, and exceeded the collapse pressure of a pure castor oil monolayer.

c. Chlorophyll a + oleyl alcohol. This monolayer system has been thoroughly studied by Gaines, et al. (1964). We used chlorophyll a concentrations less than 0.05 mole fraction in the films, in which range Gaines, et al. observed ideal behavior.

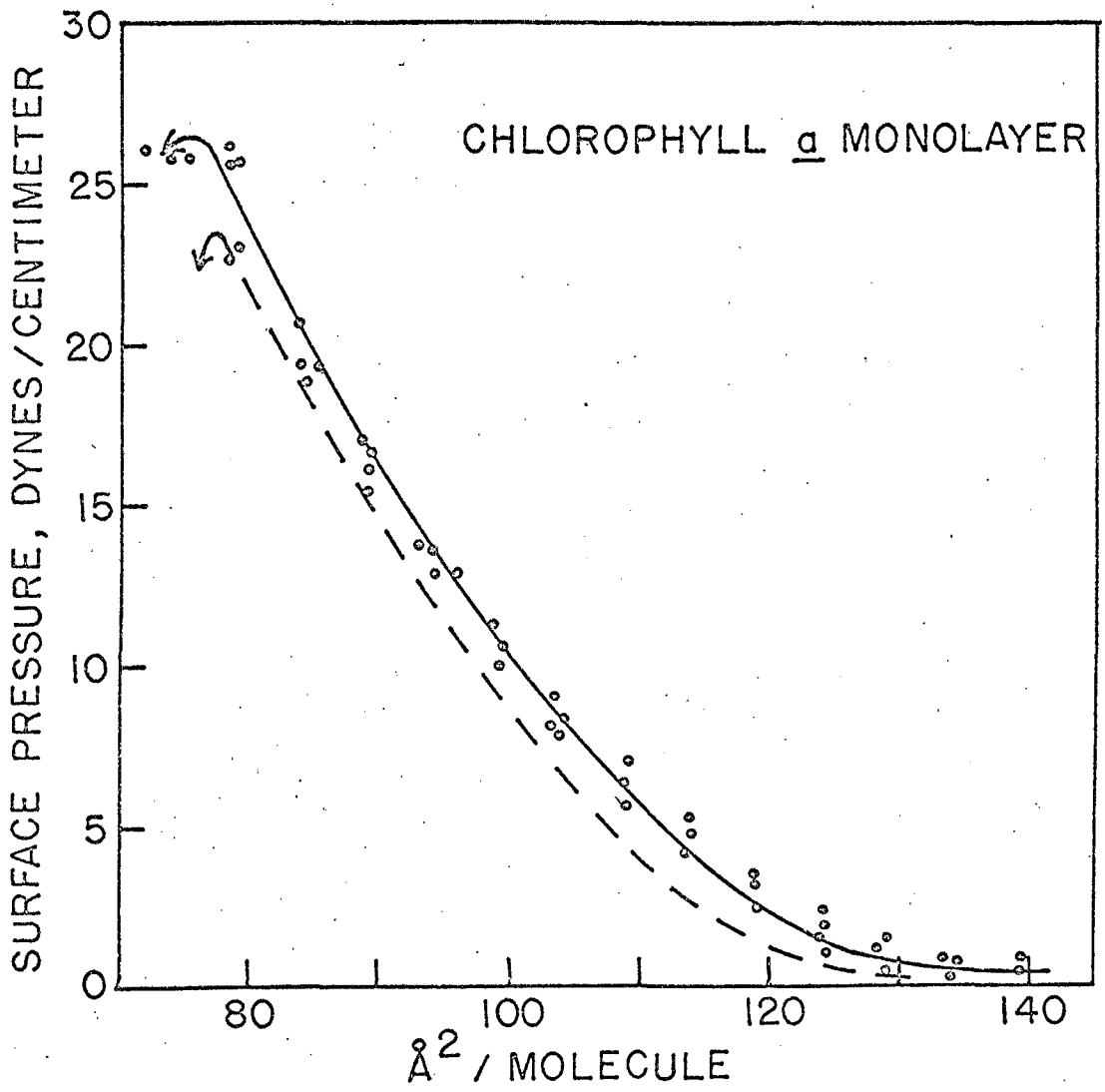

Figure III.6. Chlorophyll a monolayer pressure - area behavior. Solid curve, this work, spread on 10^{-5} M phosphate buffer, pH 7.8, compressed 10 $Å^2$ per molecule per minute. Dashed curve, Bellamy, et al (1963), pH 8.0, other conditions similar.

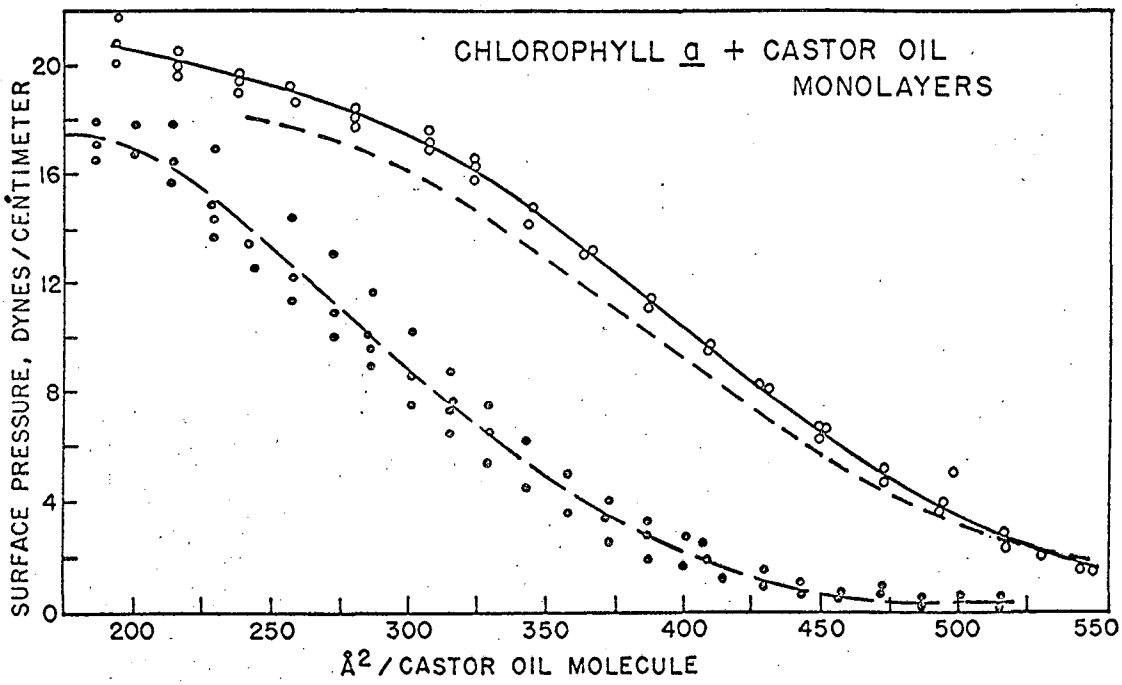

Figure III.7. Pressure - area characteristics of pure castor oil monolayer (— —); and castor oil + chlorophyll a, 1:1 area fraction (——) average of three samples; (----) calculated, assuming ideal mixing.

d. Plant structural lipids. Monogalactodiglyceride, digalactodiglyceride, and sulfoquinovodiglyceride make up approximately 45% of the total lipid complement of chloroplast membrane fragments (Lichtenthaler and Park, 1963). Highly surface active due to their amphiphilic nature (Figure III.4), they are logical candidates for chlorophyll a monolayer diluents. These structural lipids form stable compressible monolayers of the liquid expanded type (Adam, 1941) on 10^{-3} M phosphate buffer, pH 7.6, in a nitrogen atmosphere. The pressure-area curves (Figure III.8) are reproducible when freshly isolated material is used. However, the galactolipids seem to undergo changes after a few days, despite storage in benzene in the dark under nitrogen. Extrapolation of the pressure-area graphs to zero pressure yields the maximum area occupied per molecule by a uniform monolayer. Sulfoquinovodiglyceride, having the most polar hydrophilic group of the three lipids, has a maximum packing area of slightly less than 47 Å^2 ± 10%. This is approximately the area of the glyceryl moiety of the molecule, if the sulfoquinovose extends down below the water surface. That the extrapolated areas of the mono- and digalactolipids, 55 and 80 Å^2 respectively, are larger than that of the sulfolipid is expected. However, uncertainties in spreading and in the state of the lipid molecules make attempts at a quantitative description of packing in these monolayers ambiguous. The collapse pressures of the monolayers were between 36 and 41 dynes/centimeter, and were reproducible for each lipid.

As the plant lipids are partially water-soluble, we checked the stability of the monolayers with time at pressures used in the fluorescence polarization measurements. Figure III.9 is a plot of the surface

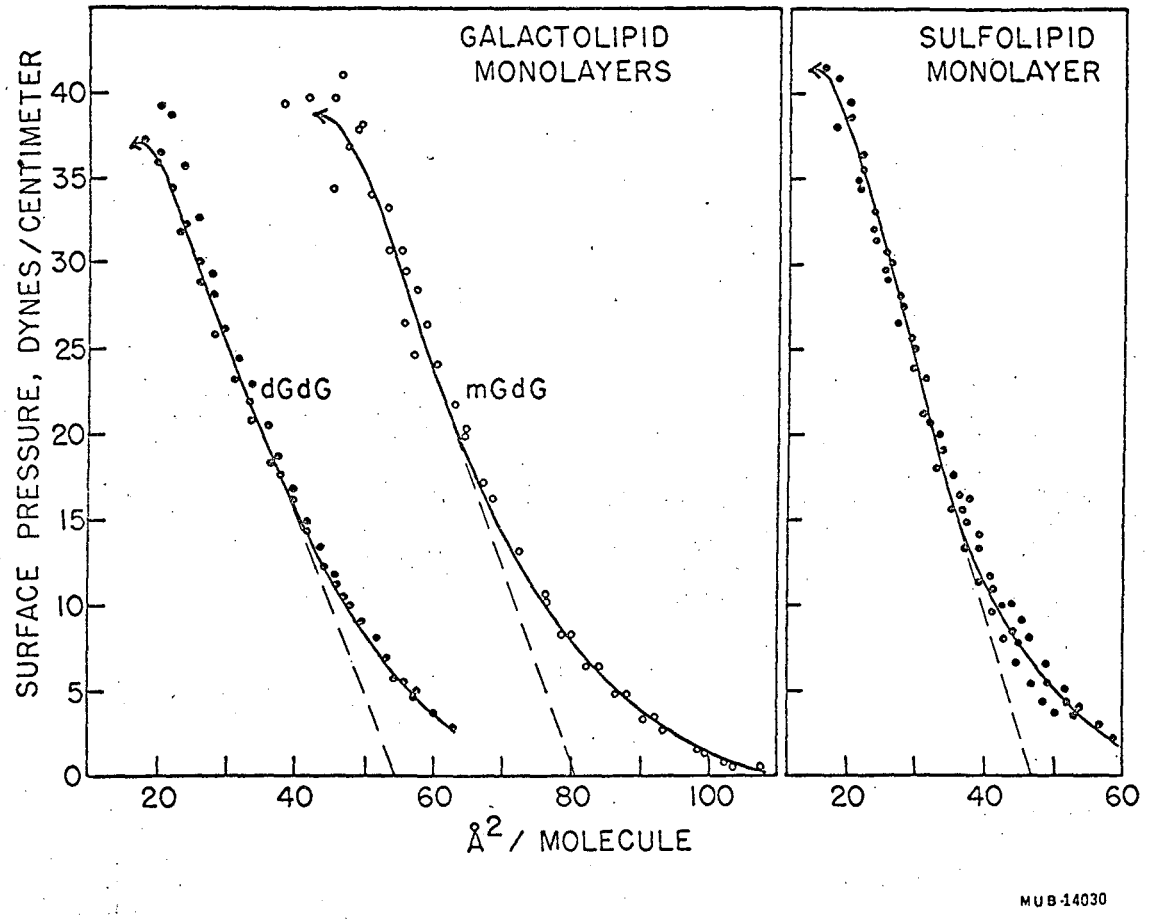

Figure III.8. Pressure - area characteristics of chloroplast glycolipids. mGdG = monogalactodiglyceride; dGdG = gigalactodiglyceride. Each curve is the average of four or more samples. See text for discussion of extrapolated values.

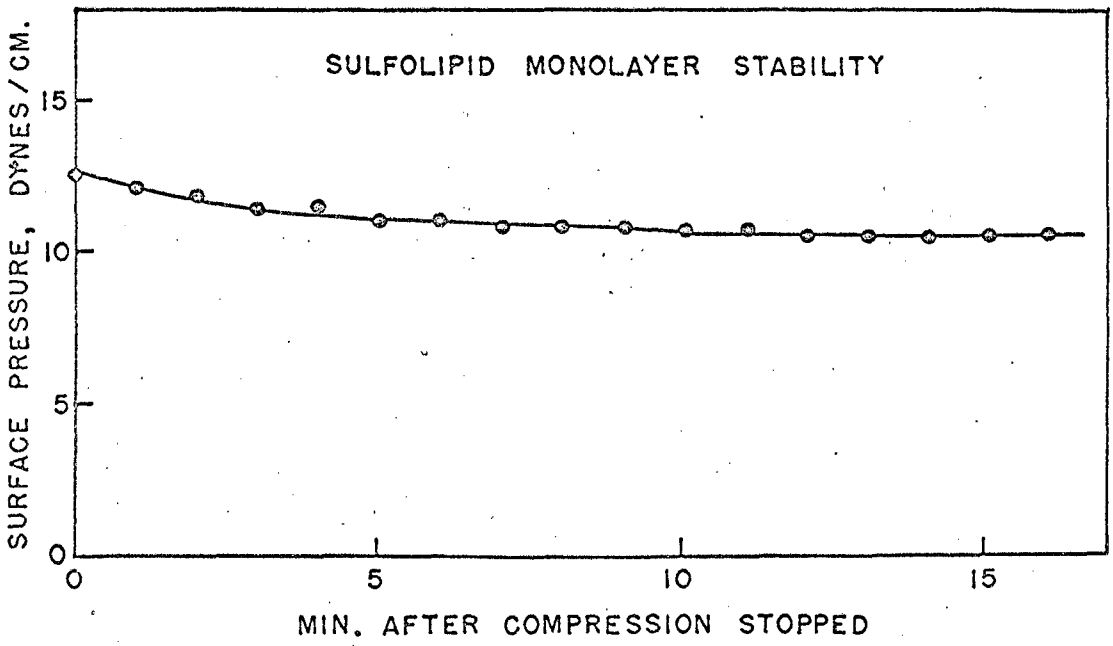

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Figure III.9. Sulfolipid monolayer maintained at constant area, 33 $Å^2$ per molecule, under nitrogen, after compression at a rate of 4 $Å^2$ per molecule per minute.

pressure of a sulfolipid film maintained at constant area. As may be seen, the pressure fell slightly at first and then remained constant for over a quarter of an hour. Monogalactolipid films at constant area remained at constant pressure for at least 20 minutes, which was the length of time required to complete fluorescence polarization measurements.

e. Chlorophyll a + plant lipids. At low mole fractions of chlorophyll a, mixed films of pigment and sulfolipid or of pigment and monogalactolipid behaved essentially as did the pure lipid films. Pressure-area plots of mixed monolayers containing larger amounts of chlorophyll a are reproduced in Figures III.10 and III.11. Within experimental error, the observed curves, which are averages of three samples each, agree with the theoretically predicted ones. The collapse points also depend on the amount of chlorophyll a in the film, approaching the collapse pressure of a pure pigment monolayer when the mole fraction of chlorophyll exceeds 0.1.

Mixed monolayers of monogalactolipid and chlorophyll a were stable with time at pressures of 15 dynes/centimeter and below, at all concentration ratios used for fluorescence polarization measurements. However, when the chlorophyll a mole fraction exceeded about 0.04 in sulfolipid films, the monolayers were not reproducibly stable with time at pressures above approximately 10 dynes/centimeter.

f. Discussion. The pressure-area curves for the mixed films were within experimental error of the weighted sums of the plots for the two components. This agreement will be observed either for an ideally mixed film, or for a film of immiscible molecules. The variation in collapse pressure with amount of pigment in films of all four

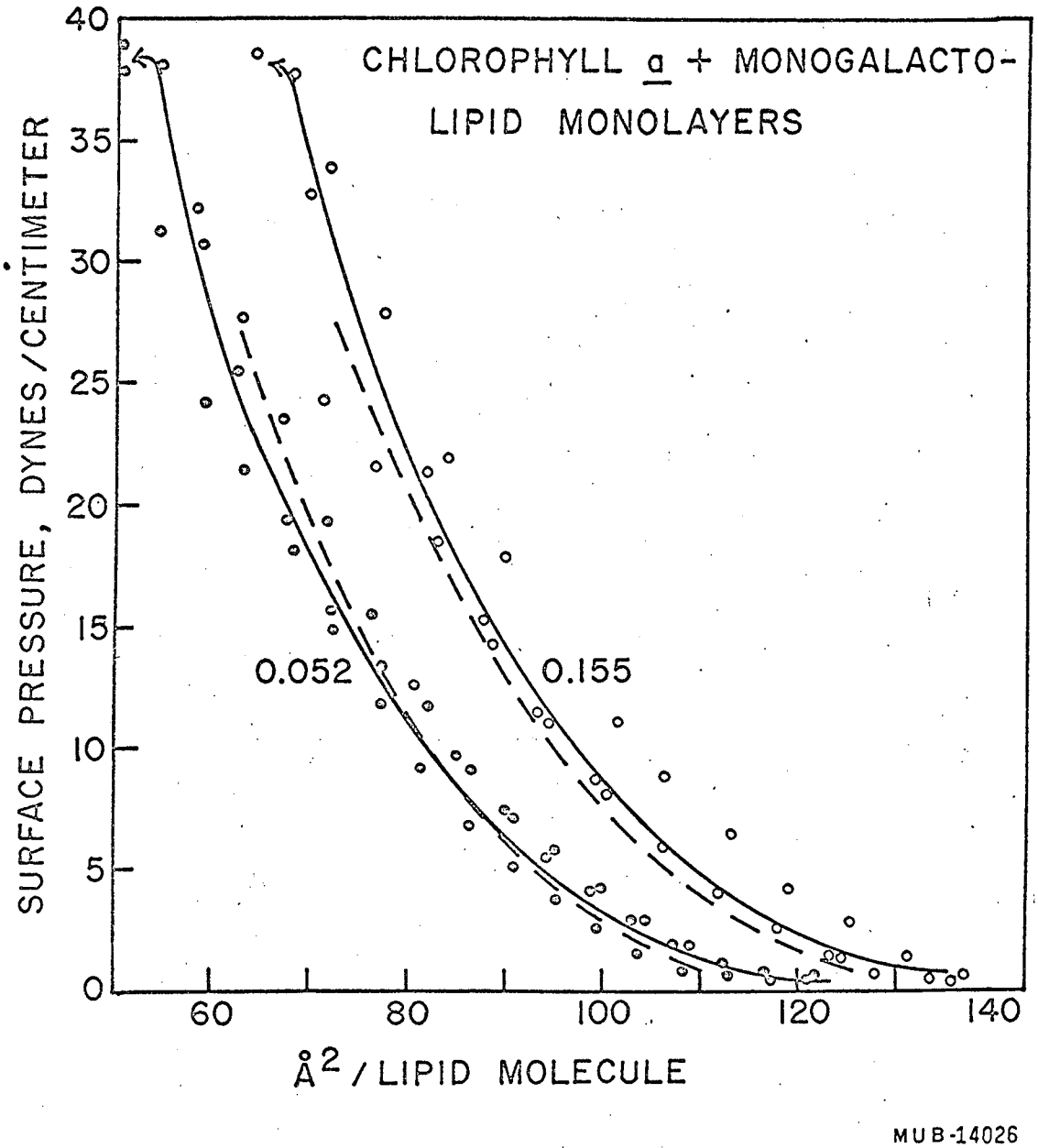

Figure III.10. Mixed monolayers of chlorophyll a and monogalactolipid. (——) average of three samples; (----) theoretical pressure - area behavior of ideal two-dimensional solutions. Mole fractions of pigment indicated on curves.

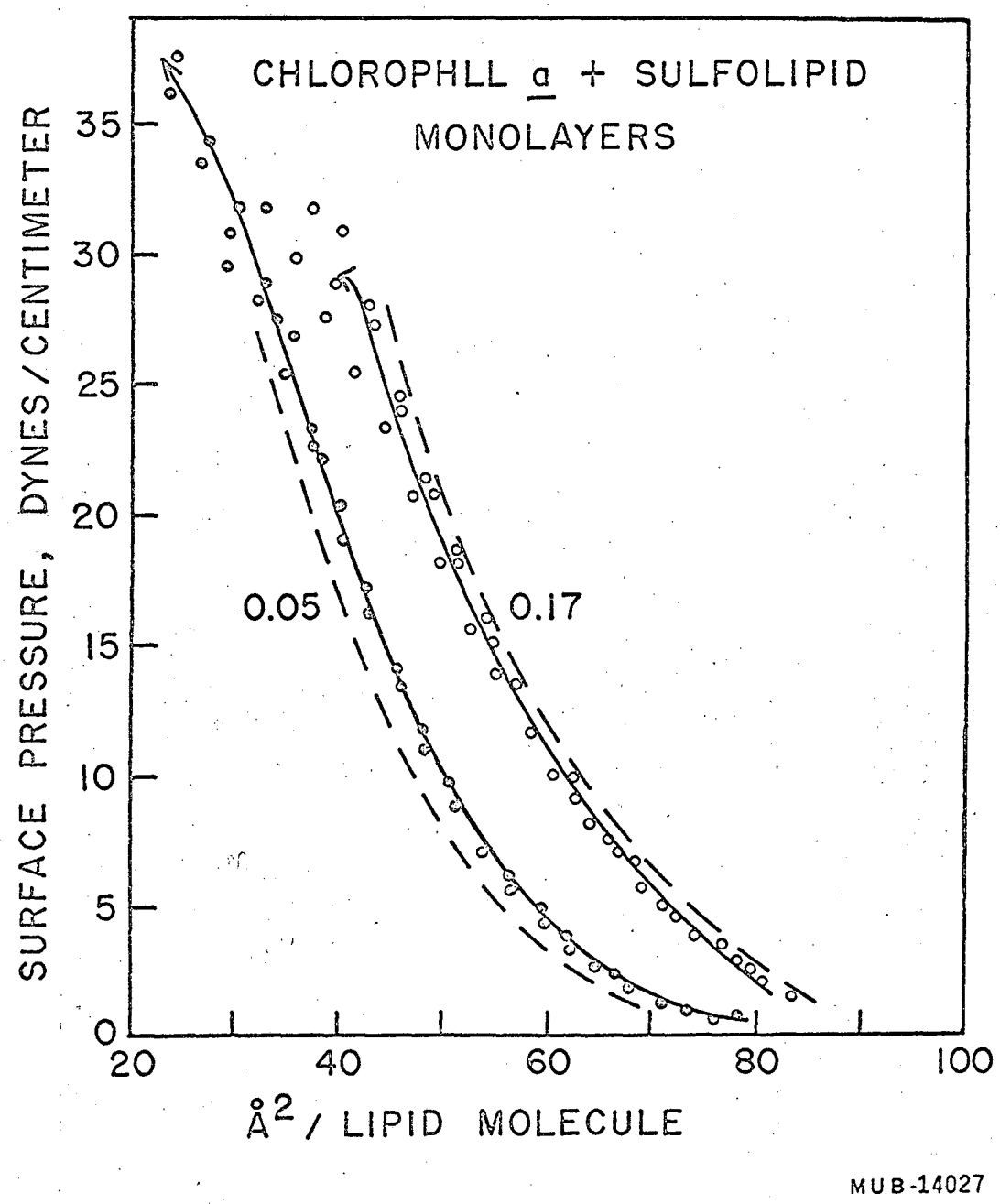

Figure III.11. Mixed monolayers of chlorophyll a and sulfolipid. (____) average of three samples; (----) theoretical behavior for ideal two-dimensional solutions. Mole fractions of pigment indicated on curves.

lipis, and the increase in fluorescence yield observed upon chlorophyll a dilution (section B.2) suggest that the mixed monolayers are actually two-dimensional solutions.

The instability of sulfolipid films containing chlorophyll a is contrary to this interpretation. This phenomenon could be due to pigment molecules enhancing the solubility of sulfolipid in the aqueous subphase, or to the formation of specific chlorophyll-lipid complexes which are partially water soluble and occupy the same area in the interface as the sum of the two component molecular areas, or to slow phase changes in filmsunder pressure. With the exception of a phase separation, these possibilities are consistent with the proposed dispersion of pigment molecules in the sulfolipid monolayer. Evidence suggesting that a phase separation of the pigment does not occur is obtained from fluorescence polarization data discussed below. Lipid-chlorophyll complexing will be considered later (Chapter IV).

2. Fluorescence Polarization of Chlorophyll a in Monolayers

We assume that a monolayer containing chlorophyll a in a lipid with which it is miscible is a random two-dimensional pigment array. In light of the results just presented, this is not an unreasonable assumption to make at low chlorophyll a concentrations. Then the expressions developed for inductive resonance transfer in Chapter II, Section B.1, may be applied to the fluorescence observed from the chlorophyll a molecules in such a film. The exciting light impinges on the monolayer from below, traveling in the z-direction (refer to Figure II.1). It is linearly polarized, with the electric vector parallel to the plane of the film. The fluorescence in the z-direction is observed from above

the monolayer after passing through an analyzer, the transmission axis of which is parallel or perpendicular to that of the polarizer. The analyzer is not moved during an experiment; the polarizer is rotated by exactly 90° in order to complete the measurements. Preliminary measurements showed that moving the polarizer instead of the analyzer did not introduce a measurable error into the results. We expected this on the basis of Tweet, et al.s (1964) observation that there is no preferred azimuthal orientation taken by chlorophyll a molecules in a monolayer.

From a trace of the intensity reaching the photomultiplier when the polarizers are parallel and then crossed, the degree of polarization of the fluorescence is calculated as follows:

$$P = \Delta'/(2I'_{\perp} + \Delta') = (I'_{//} - I'_{\perp})/(I'_{//} + I'_{\perp}) \qquad \text{(III.2)}$$

where $\Delta' = I'_{//} - I'_{\perp}$, and the prime denotes intensity values corrected for the signal observed in the absence of a film, and for variations in source intensity as indicated by the fluorescent standard. The average value and standard deviation of P were calculated from several $I_{\perp}, \Delta$ pairs for each film or solution. We express the concentration of chlorophyll a in the monolayer as the fractional area occupied by the pigment. Then from plots of 1/P vs. C for each diluting lipid, we obtain the critical distance for energy transfer among the pigment moelcules at low concentrations by inductive resonance. This critical separation is also determined by Forster's method. The experimental and empirical values of R_o are compared, to test the hypothesis that resonance transfer of energy occurs in these dilute pigment systems.

The characteristics of the dependence of polarization on concentration at higher chlorophyll concentrations are carefully observed. They may indicate the presence or absence of exciton states, as discussed in Chapter II.

a. Viscous solutions of chlorophyll a. We studied these well documented three-dimensional systems not only to check the performance of the monolayer fluorometer, but also to be able to compare results from three- and two-dimensional model systems of the same materials. Goedheer (1957) and Gouterman and Stryer (1962) have determined the action spectrum for chlorophyll a fluorescence polarization in castor oil solutions. For very dilute solutions of the pigment in this viscous oil, in which energy transfer is highly unlikely, they report polarizations of 0.2 ± 0.04 when fluorescence is excited by 406 nm light. Stupp (1952), whose published polarization action spectrum does not agree with those of the above workers, also obtained a value of 0.21 at this exciting wavelength. Extrapolation of our data for this system (Figure III.12) to zero concentration yields a maximum polarization of 0.214 ± 0.008, in agreement with the published values. We conclude that the monolayer fluorometer functions satisfactorily.

Using the slope and intercept of the straight line in Figure III.12 in equation II.21, we find the lower limit of the critical distance, $R_0^{\circ}(3D)$, at which transfer probability equals emission probability to be 42 ± 3 Å. This distance corresponds to a spacing of 37 ± 3 Å for R_0. If the critical distance is determined by Forster's method, equation II.18, we find $R_0 = 82 \pm 5$ Å as an upper limit to this parameter. These values of R_0 should be compared with the value of 76 Å calculated by

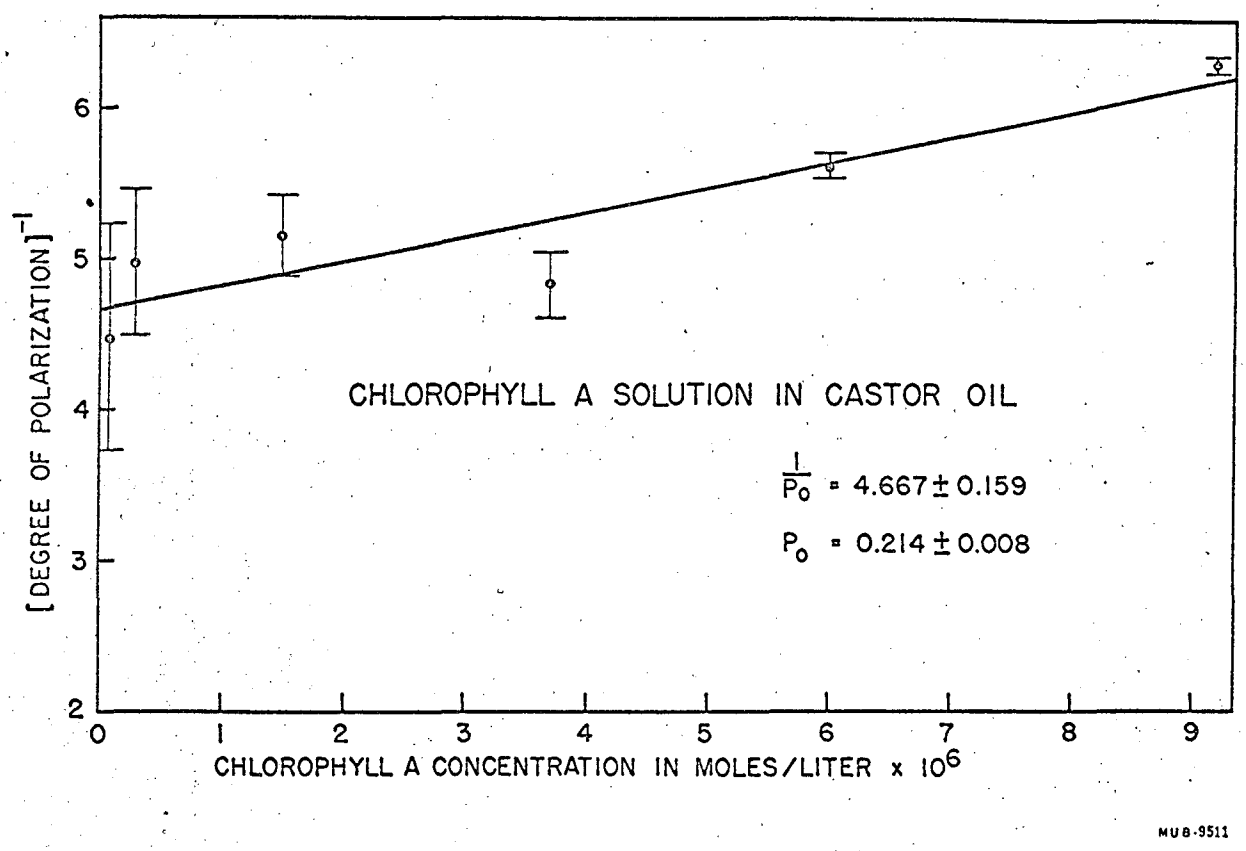

Figure III.12. Inverse dependence of fluorescence polarization upon concentration of chlorophyll a in castor oil solutions at low concentrations. Extrapolation of best straight line fit of data to zero concentration to obtain limiting degree of polarization.

Goedheer (1957) using Forster's method, equation II.17

The critical distance computed from spectral data does indeed fall between the two limiting values obtained from the polarization results. Near agreement with the separation obtained by assuming that one transfer is sufficient to cause depolarization suggests that this approximation is not unreasonable for the viscous, three-dimensional dilute solution.

When we analyzed our data for the system chlorophyll a in oleyl alcohol solution in the same manner, we obtained a maximum polarization of 0.109 ± 0.025. The critical distance, R_{ir} = 37.5 ± 5.5 Å, is within experimental error of that for the castor oil solvent. The same considerations apply to energy transfer in this solvent as to that in castor oil.

The maximum polarization expected in a random three-dimensional system in the absence of energy transfer, if the absorption and emission oscillators are parallel, is 0.5 (equation II.1). It has been proposed that the oscillator absorbing 406 nm light in chlorophyll a is parallel to the fluorescent oscillator. Now, experimental values lower than 0.5 indicate that the molecules move during their excited state lifetime, or that some absorption is due to a transition oscillator which is not parallel to the emission oscillator. Perrin (1939) has shown by specific substitution into equation II.8 that in the absence of energy transfer, $1/P \propto 1/n$, and that $P = P_0$ when $1/n = \infty$. If we plot P vs. 1/n for our two solvents, castor oil and oleyl alcohol, we see that the extrapolation to infinite viscosity is very short (Figure III.13). Then the admittedly bad assumption that the two experimental points do indeed fall on a straight line does not introduce too large an error in the maximum

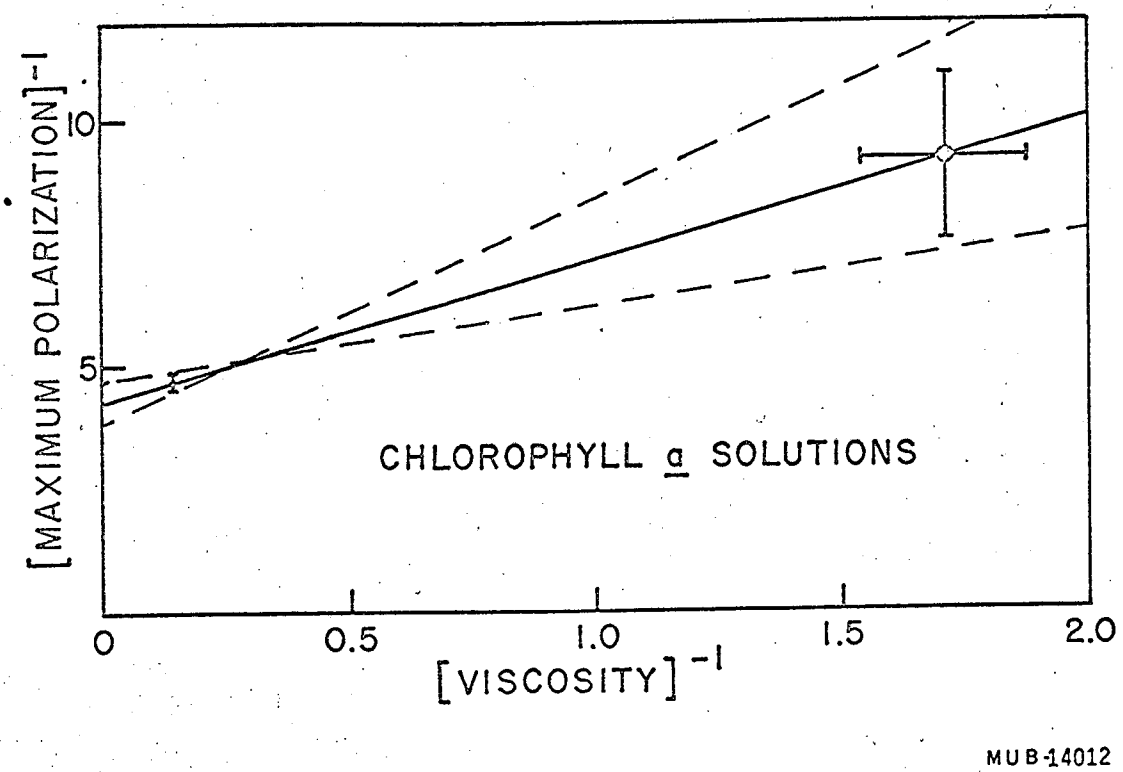

Figure III.13. Inverse dependence of limiting degree of polarization, P, upon inverse solvent viscosity. Extrapolation to infinite viscosity. See text for discussion of points.

polarization obtained from the extrapolation. In performing this extrapolation, we consider the error in the intercept due to the largest possible experimental errors in the point representing the data for oleyl alcohol solvent. The intercept, $P_o = 0.24 \pm 0.03$, is still only approximately one-half the theoretical maximum.

If failure to exhibit the maximum degree of polarization is due, in this system, to overlap of absorption bands of two perpendicular oscillators at the excitation wavelength, as proposed by Stupp (1952), approximately 30% of the absorption at 406 nm must be due to the oscillator oriented perpendicularly to the y-polarized emission transition. By straightforward decomposition we divided the absorption spectrum between 350 and 470 nm of a chlorophyll a solution in a non-polar solvent into three symmetrical Guassian curves centered at 434, 416 and 380 nm (Figure III.14). We see that essentially none of the absorption at 406 nm is due to the x-polarized band at 434 nm. Approximately 40% of the 406 nm absorption, however, is due to the higher energy transition centered at about 380 nm. This transition is thought also to have y-character, in which case it would not contribute to the lowering of the fluorescence polarization. However, current work in this laboratory (Sauer, private communication) using a point monopole expansion to describe the transition moments, suggests that, due to configuration interaction, the transitions corresponding to the blue satellite absorption band have considerable x-character. This situation would be equivalent to $0 < \alpha < \pi/2$ in equation II.1. If this interpretation is correct, substitution of $P_o = 0.24$ in equation II.1 yields $\alpha \cong 35°$.

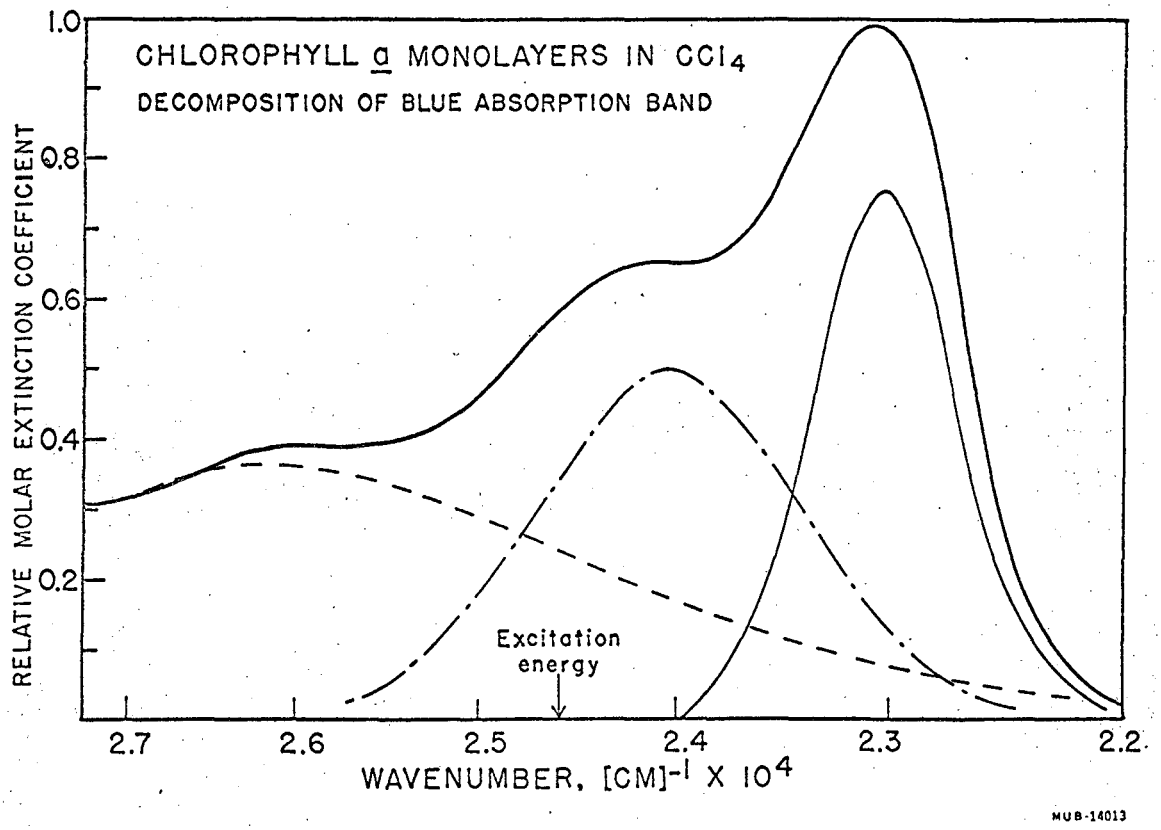

Figure III.14. Decomposition of the blue absorption band of chlorophyll a monomers in non-polar solvent into three Gaussian curves, in an attempt to determine the polarization of the oscillators absorbing at 406 nm. See text for discussion.

The present investigation was not designed to resolve this difficulty. When we evaluate the behavior of the two-dimensional solutions of chlorophyll a in lipid monolayers, we will consider the possible effects of non-parallel absorption and emission oscillators on the maximum polarization observed and thus on the critical distances we calculate. Because of the nature of the model, this ambiguity has only a quantitative, not a qualitative effect on the interpretation of results.

b. Pure chlorophyll a monolayers. The fluorescence of several chlorophyll a monolayers was recorded at a surface pressure of 12 dynes/centimeter. In no film did the degree of polarization calculated from equation III.2 exceed 0.008 ± 0.005. In most cases it was less than this. The best precision obtained with the instrument used as described in section A.4 was ± 0.004, even for large, highly polarized signals. In this case, therefore, the relatively weak fluorescence was not limiting the accuracy of the measurement.

In a monolayer of pure chlorophyll a the pigment molecules are sufficiently close to each other than an exciton state may exist in the film. However, even in the absence of exciton migration, energy transfer may be expected to proceed very rapidly among adjacent molecules. If the chlorophyll a molecules are randomly oriented with respect to the normal to the film surface, the fluorescence should be depolarized regardless of the energy transfer mechanism. We observe unpolarized fluorescence in accord with this prediction.

c. Chlorophyll a in lipid monolayers

The relative fluorescence yield and degree of polarization are shown as a function of chlorophyll a concentration in films of castor oil, oleyl alcohol, monogalactolipid and sulfolipid in Figure III.15, a - d. The pigment concentration is expressed as the fraction of the total area of the monolayer occupied by pigment molecules at the pressure at which the measurements were made. These values of C were obtained from pressure-area curves of the pure substances. We assumed that all chlorophyll molecules were tilted out of the surface to the same extent. As discussed in Chapter II, the error introduced by this approximation is small.

Both fluorescence yield and degree of polarization increase as the chlorophyll a concentration in the film decreases. This behavior is a logical consequence of the fact that intermolecular interaction energy is lowered as the molecular separation is increased. We shall analyze these fluorescence polarization data for evidence of energy transfer by inductive resonance or exciton migration as outlined in Chapter II.

At high concentrations of chlorophyll a in monolayers of castor oil, oleyl alcohol, and sulfolipid, the fluorescence polarization does not appear to fall to zero. This phenomenon was checked carefully with films of chlorophyll a in sulfolipid. The rate of decrease in polarization with increasing concentration appears to lessen at about 0.06 area fraction of pigment. This concentration corresponds to an average pigment separation of the order of 25 Å at the monolayer pressure at

which the measurements were made. The polarization remained approximately constant at 0.015 ± 0.004 up to 30% area fraction of pigment in the film.

Similar behavior has been observed for fluorescent dyes at high concentration in viscous solution (Feofilov and Sveshnikov, 1940). Since this occurs in the region of strong fluorescence quenching, where the fluorescence lifetime is considerably shortened, Feofilov (1961) suggests that the number of energy transfers possible before emission has been reduced sufficiently to prevent further depolarization. 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 (Figure III.15c). 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.

We suggest the following alternative explanation for the observed results. First, chlorophyll a may be randomly dispersed and oriented in galactolipid monolayers, but not in sulfolipid films. In this latter model system, the pigment may be either aggregated or partially oriented. If it is aggregated, lifetime shortening would account for the observed residual polarization. But this possibility is inconsistent with the observation that fluorescence of pure chlorophyll a monolayers is unpolarized (vide infra), in which system the lifetime of the pigment should be as short or shorter than it is in the mixed films. As mentioned above, the alternative to lifetime shortening is partial

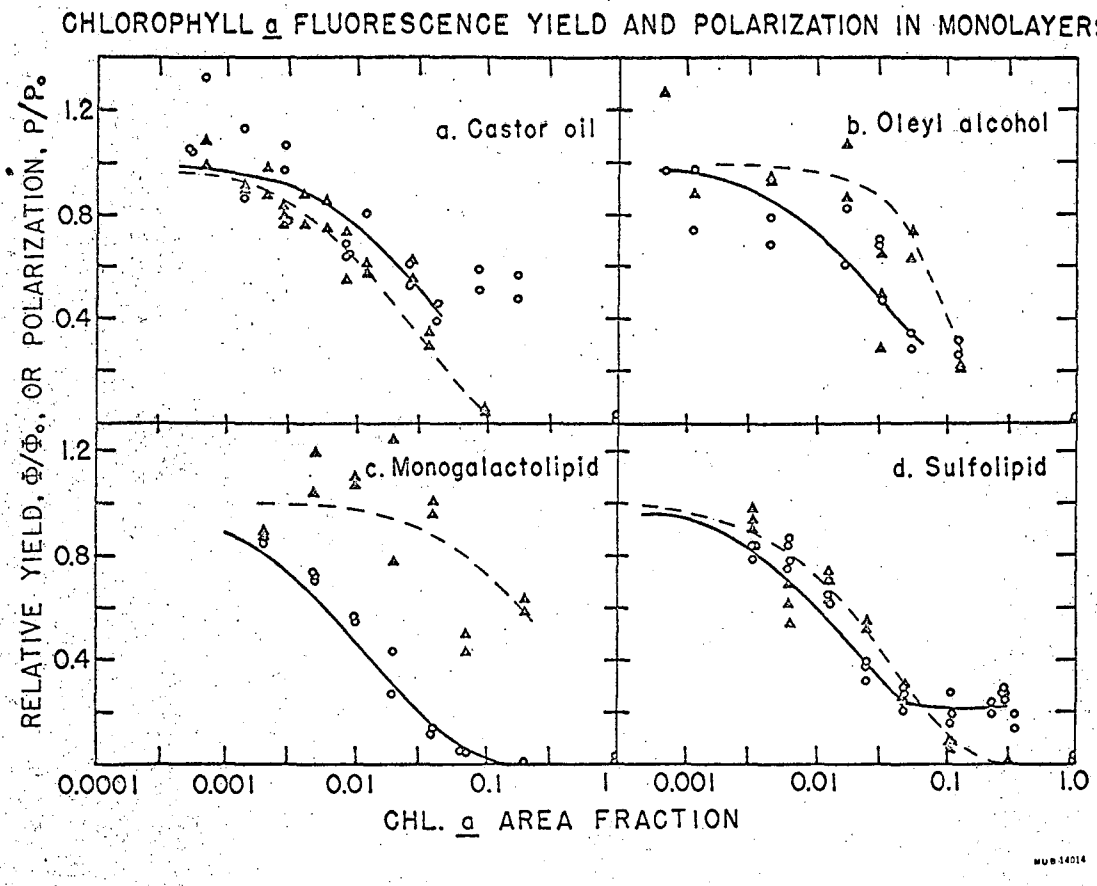

Figure III.15. Dependence of relative fluorescence yield (--▲--) and polarization (__o__) of chlorophyll a in mixed monolayers upon concentration of pigment in the films. Curves are calculated least squares fits to the data. P_o and Φ_o have been obtained from extrapolation of the data to zero concentration of pigment in each diluent.

orientation of the pigment molecules by the sulfolipid. If such alignment were present, increasing the extent of energy transfer by increasing concentration would not result in further depolarization.

If the hypothesis of pigment orientation at high concentrations in sulfolipid is correct, conditions in this system are suitable to exciton formation. From Table II.1, we see that the maximum pigment separation at which excitons may exist in a chlorophyll a array is approximately 20 Å. Thus exciton migration may occur among pigment molecules in a two-dimensional sulfolipid matrix at the same concentrations at which residual polarization was observed.

We do not predict exciton formation in pigment-containing galactolipid monolayers, because chlorophyll a molecules are apparently unoriented in this lipid.

We next examine our results at low chlorophyll a concentrations for evidence of inductive resonance energy transfer. The polarization data of each system have been plotted as 1/P vs. C for low pigment concentrations, and the best straight line fit to the data obtained by the method of least squares (Figure III.16 a - d). We note that the extrapolated values for maximum fluorescence polarization of chlorophyll a in monolayers (Figure III.16 and Table III.1) are well below the value, 0.5, predicted for parallel absorption and emission oscillators (equation II.7) in this geometry. This phenomenon may be due to non-parallel oscillators or to finite viscosities of the lipid monolayers, or both.

We have calculated the expected value of the limiting polarization, P_o, from equation II.7, using $\alpha = 35°$ for the angle between absorption and emission oscillators. This angle was chosen on the

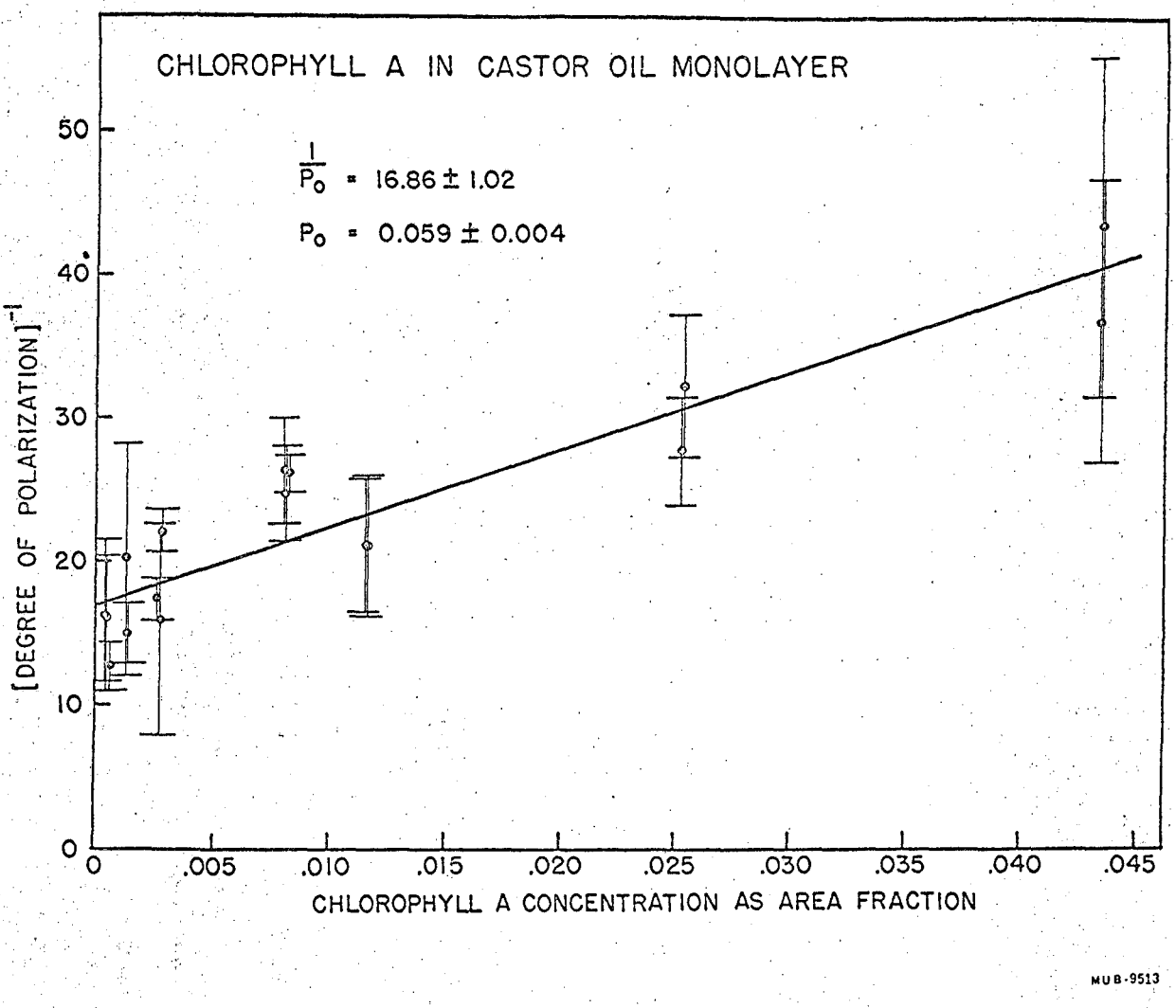

Figure III.16. Inverse dependence of fluorescence polarization on concentration of chlorophyll a in films of various lipid diluents. The least squares straight line fit to the data has been extrapolated to zero concentration to obtain the limiting degree of polarization in each lipid.

a. Castor oil diluent

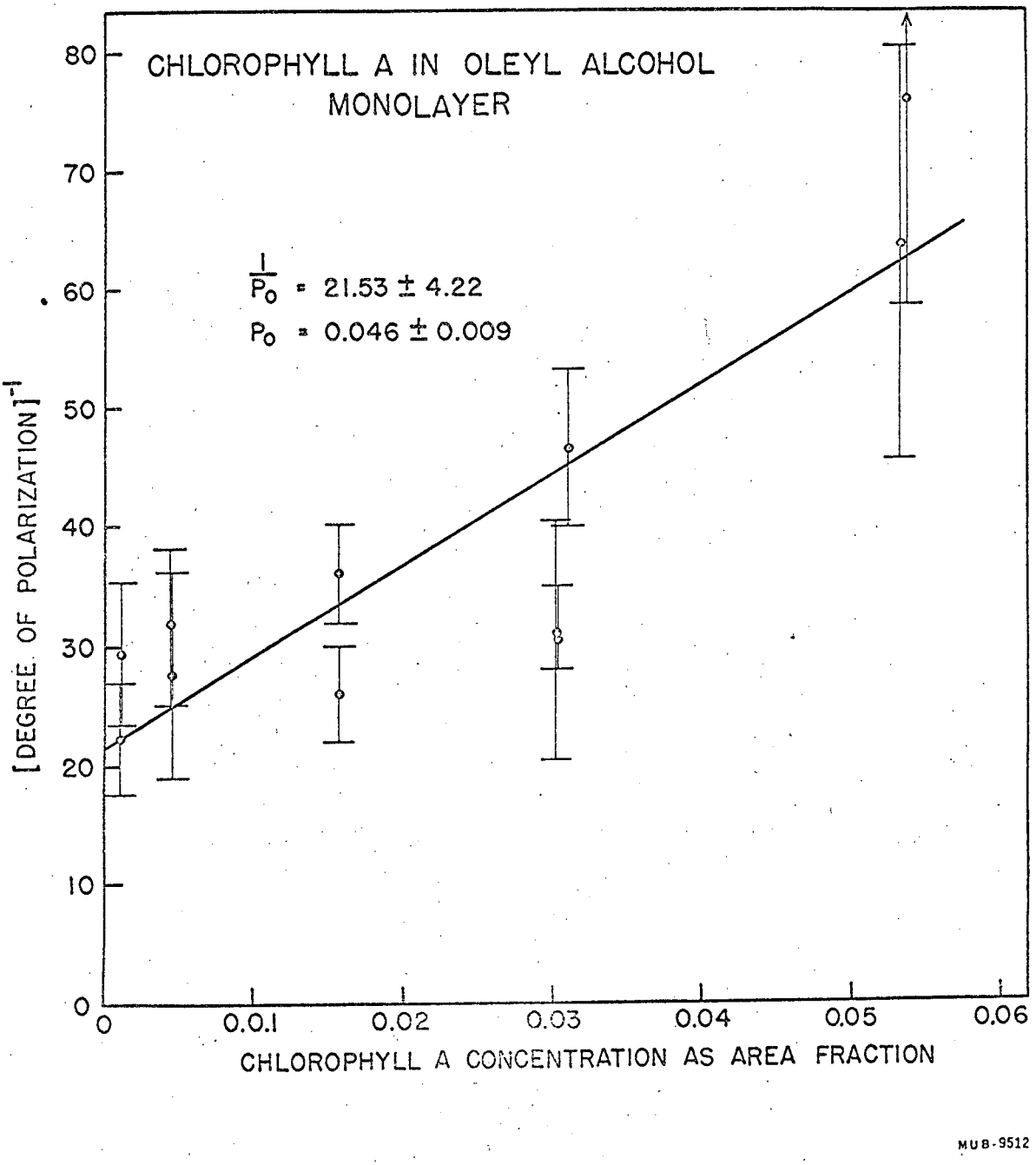

Figure III.16 continued. b. Oleyl alcohol diluent.

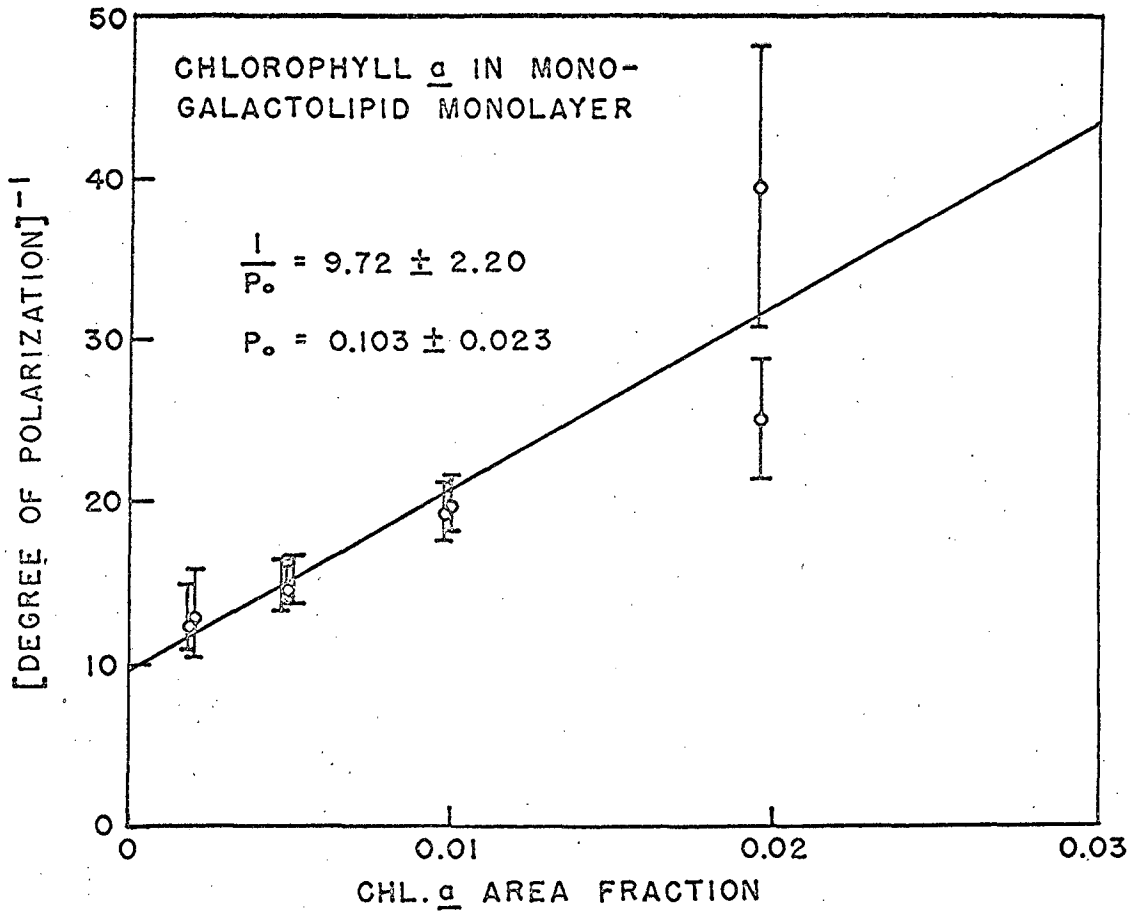

Figure III.16 continued. c. Monogalactolipid diluent.

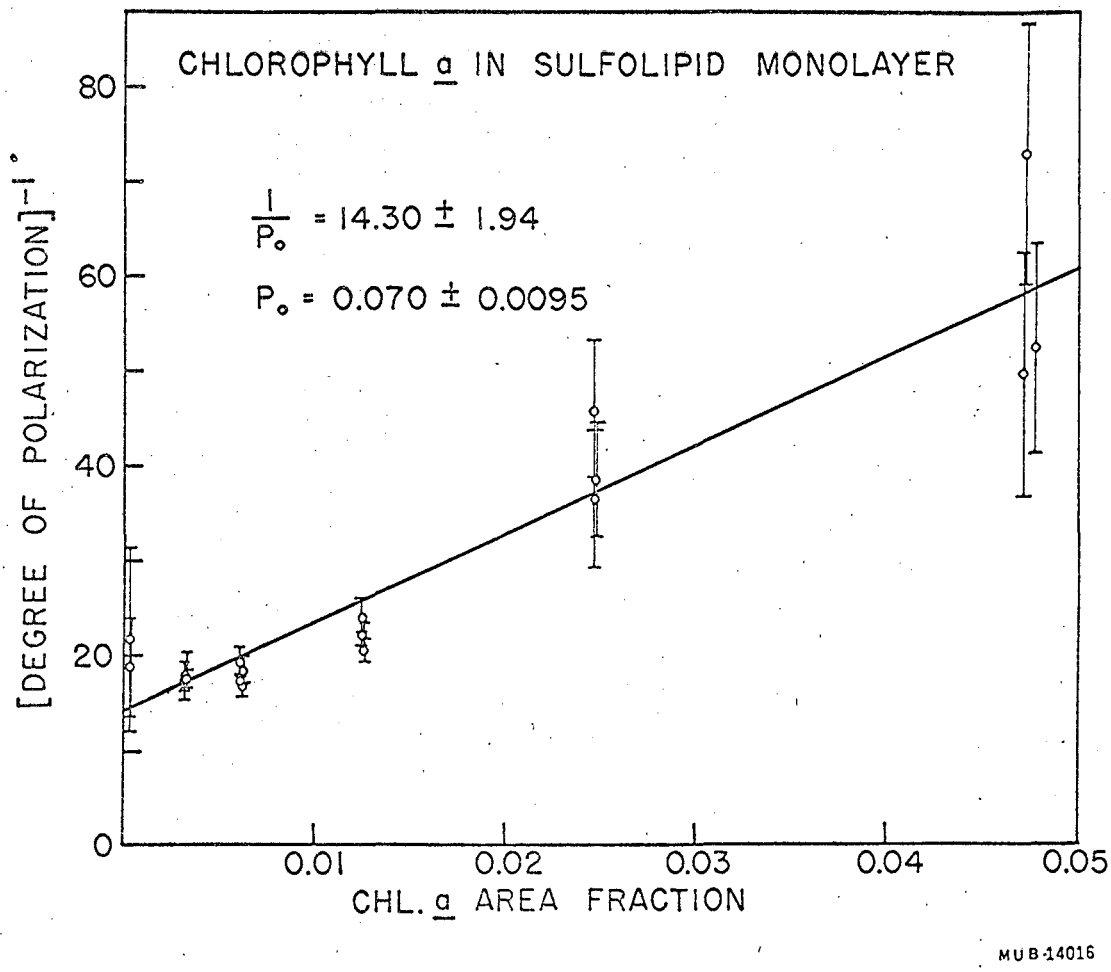

Figure III.16 continued. d. Sulfolipid diluent.

basis of polarization observations made on three-dimensional viscous solutions of pigment and on Sauer's calculations (vide infra). We obtain $P_o = 0.36 \pm 0.09$, the limits including the phase ambiguity in equation II.6 as well as uncertainties in α and β. This value of P_o is still considerable higher than the extrapolated values, suggesting that the monolayers under compression are not rigid systems, and that monolayer viscosities are lower than bulk viscosities for given materials.

We did not have the apparatus necessary for measuring surface viscosity, so could not verify this suggestion experimentally. However, a comparison of available surface viscosities of fatty acids and alcohols (e.g., Boyd and Harkins, 1939) with their bulk viscosities indicates that the former are at least an order of magnitude lower for films at compressions which we used. Although molecular movement is no doubt restricted in monolayers under pressure, it is unlikely that it is prevented in these liquid-expanded films. The media of non-infinite viscosities would lower the observed values of the limiting polarization.

From the slope and intercept of the 1/P vs. C curves, we have calculated the critical distance $R_o(2D)$ for inductive resonance transfer, using equations II.22 and II.20. To perform these calculations, we must also assign a value to the effective molecular diameter, 2a, and calculate θ_p from equation II.5.

First, in choosing the effective-molecular-diameter, we consider the distance of closest approach of two pigment molecules in the film. Since the area per chlorophyll a molecule in the monolayers under pressure is approximately 100 Å^2, we take $2a = (100)^{1/2} = 10$ Å. The smallest possible separation is of the order of the thickness of a pig-

Table III.1

CRITICAL DISTANCE FOR ENERGY TRANSFER IN DILUTE CHLOROPHYLL a MONOLAYERS

Lipid Diluent	Slope[a]	$1/P_o - 1/3$[a]	R_{ir}[b]	$\overline{R_o}$[c]
Castor oil	$5.4 \pm 0.57 \times 10^2$	16.55 ± 1.03	17.5 ± 6 Å	57 ± 4 Å
Oleyl alcohol	$7.58 \pm 1.46 \times 10^2$	21.19 ± 4.21	17.5 ± 7 Å	57 ± 11 Å
Monogalactolipid	$11.37 \pm 2.08 \times 10^2$	9.39 ± 2.21	21.5 ± 8 Å	88 ± 22 Å
Sulfolipid	$9.31 \pm 0.86 \times 10^2$	13.97 ± 1.94	19.5 ± 7 Å	78 ± 9 Å

(a) from Figure III.15 (b) Equations II.22 and II.20 (c) Equation II.18

ment molecule, about 5 Å, and the largest molecular "diameter" the length of a side of the porphyrin ring, about 15 Å. The resulting 50% uncertainty in 2a introduces an uncertainty of 33% in R_o. This error has been addéd to those due to deviations of the slope and intercept of the 1/P vs. C curves, in calculating the range of the experimental critical distance.

All polarization measurements were carried out on monolayers maintained at constant surface pressures between 10.5 and 13 dynes/centimeter. From Figure III.6 we see that the area per chlorophyll a molecule in the film varied less than 5% in these experiments. Following Bellamy, et al (1963), we find that for chlorophyll a monolayers in this pressure range, $\overline{\beta} - \pi/2 = 26.5°$. From equation II.5 we have

$$\theta_F = \cos^{-1}[\sin 20° \cos 26.5°] = 72 \pm 4°.$$

The results of the calculations of the critical distance for each lipid diluent are presented in column 4 of Table III.1. We also list in column 5 of the table the molecular separation, $\overline{R_o}$, at the critical concentration C_o obtained from polarization data by Forster's method (equation II.18).

We wish to compare these critical distances with that predicted by Tweet, et al (1964) from spectral data. As discussed in chapter II, we expect Forster's method of data evaluation to yield an upper limit, and Weber's approach (equation II.19) a lower limit, for the critical separation. Indeed, the separation computed by Tweet, et al, 54 ± 8 Å, falls between the distances calculated from the polarization data. This result is consistent with our hypothesis that inductive resonance energy

transfer among the chlorophyll a molecules causes the observed fluorescence depolarization at low pigment concentrations.

Forster's assumption of complete depolarization after an average of only one transfer of energy appears to be sufficient for the description of the pigment in castor and oleyl alcohol monolayers. It is, however, a poor approximation for the chloroplast lipid systems. The data suggest that in these latter monolayers, more than one intermolecular energy transfer is required to depolarize fluorescence. This requirement would be necessary if pigment orientations were not completely random in the system. As more than one transfer is not necessary for depolarization in castor oil and oleyl alcohol films, a non-random chlorophyll a arrangement is apparently not imposed by confinement of the molecules to an interface, but rather perhaps, by interaction with the diluting lipid.

Compression characteristics of the mixed monolayers do not rule out the occurrence of molecular interactions. Spectral data might give us an indication of the presence of such complexes. Unable to study pigment - lipid complexing by absorption spectroscopy of the monolayers, we have investigated this phenomenon briefly in solutions.

IV. INTERACTIONS BETWEEN CHLOROPHYLL A AND PLANT LIPIDS IN SOLUTION

The observed increase in fluorescence yield and polarization as chlorophyll a concentration was decreased in the mixed lipid monolayers suggests that these lipids disperse chlorophyll a aggregates effectively. That the pigment and lipid molecules interact specifically cannot be determined from the data. Such interaction is indicated, however, at least in the monogalactolipid-chlorophyll a model system, where fluorescence polarization falls rapidly to zero, and yields remain high at fairly high pigment concentrations. The experiments described here were undertaken to investigate this proposed interaction by studying the effect of the plant lipids on chlorophyll a aggregates in solution.

Infrared and nuclear magnetic resonance studies by Katz et al (1963) of deuterated chlorophyll a in carbon tetrachloride established the fact that chlorophyll a exists largely as a dimer in this solvent. Anderson (1963) and Anderson and Calvin (1964), performing similar experiments, came to the same conclusion, and also documented changes in the visible absorption spectrum as the concentration of chlorophyll a was varied. Sauer et al (1966) have made a thorough spectral study of pigment dimer formation in the same solvent. They determined the pure monomer and dimer spectra from difference absorption spectrophotometry of solutions of varying concentrations. From spectrophotometric titration data for samples of known total pigment concentration, they computed the monomer and dimer extinction coefficients and the dimerization constant.

The chlorophyll a dimers may be broken up by addition of polar solvents or Lewis bases to the carbon tetrachloride solutions (Katz et al, 1963). Pyridine and ethanol are the most effective agents investigated so far. Water will also compete as a complexing agent with dimerization (Ku and Sauer, private communication).

The optical rotatory dispersion (Sauer, 1965) and, most recently, the circular dichroism (Dratz et al, 1966) of chlorophyll a dimers have been investigated. A double Cotton effect in the region of the red absorption band of the dimer is characteristic of these spectra. Polar solvents such as ethanol cause attenuation and disappearance of this effect at sufficiently high concentrations.

We propose that the plant structural lipids, monogalactodiglyceride and sulfoquinovodiglyceride, may behave as Lewis bases, interacting specifically with aggregated forms of chlorophyll a--thereby causing its dispersion in solutions or in monolayers. If this does happen, we should be able to follow spectral changes due to a shift in the monomer-dimer equilibrium as we add lipid to a solution of the pigment in carbon tetrachloride. We observed this effect qualitatively in optical rotatory dispersion spectra, and quantitatively by absorption difference spectrophotometry, the latter technique proving more sensitive to changes induced by small amounts of lipid.

The plant lipids do break up the pigment dimers effectively. We calculate the free energy of chlorophyll a-lipid complex formation relative to the free energy of dimerization. Monogalactolipid appears to be as strong a complexing agent as ethanol.

A. Materials and Methods

We used chlorophyll a and plant lipids from stock material prepared as described in Chapter III. A weighed amount of chlorophyll a was evaporated to dryness on a rotary evaporator and stored overnight in the dark in a nitrogen box through which gas was circulating slowly. The pigment was dissolved in carbon tetrachloride from a freshly opened bottle, Baker and Adamson, reagent grade, which had been flushed with nitrogen. This stock chlorophyll a solution, 1.16×10^{-4} M, was stored in the dark in a well stoppered flask in a nitrogen box.

Sample solutions of pigment and monogalactolipid were prepared by resuspending an aliquot of lipid, which had been evaporated to dryness, in a measured amount of stock chlorophyll solution. The sulfolipid, however, was not sufficiently soluble in the carbon tetrachloride pigment solution to permit use of this procedure. Instead, a stock solution of sulfolipid in carbon tetrachloride was prepared, and aliquots of this added to a measured amount of the stock chlorophyll a solution. This method proved satisfactory, but we could not use as large excesses of lipid as were attainable in the experiments with the galactolipid.

The visible spectrum of each sample was recorded on a Cary 14 spectrophotometer, using a 0.1 centimeter light path with a chlorophyll a solution of the same concentration as that in the sample in the reference compartment. The 0.1 slidewire was used when absorption differences were very small. Fresh solutions were prepared for the reference compartment for each sample observed, because the solutions tended to change slowly with time in the light beam.

The optical rotatory dispersion (ORD) of similarly prepared samples was recorded on a Cary 60 spectrophotometer. We also recorded the ORD of pure lipid solution, to ascertain that the lipids themselves were not optically active in the visible region of interest. The ORD curves of the mixed solutions were compared with those of a solution of pure chlorophyll a in carbon tetrachloride at the same concentration.

B. Results and Discussion

1. Optical rotatory dispersion

The ORD between 620 and 720 nm of solutions of lipids and of 1.6×10^{-4}M chlorophyll a with and without an excess of lipid is shown in Figure IV.1. The pure lipids had no optical activity whatever in this region of the spectrum. The characteristic positive Cotton effect exhibited by chlorophyll a dimers was decreased and changed somewhat in shape upon addition of an excess of lipid. This result indicates that the lipids interact in some undetermined manner with the chlorophyll dimer configuration.

We did not pursue the study further, as the analysis and interpretation of the complex ORD spectra was uncertain. Also, it did not lend itself directly to determination of equilibrium constants for complexing.

2. Absorption difference

Upon addition of lipid to the chlorophyll a solutions, the optical density showed a maximum decrease at 682 nm. The long wavelength shoulder of the dimer red absorption band is located very near this wavelength. This shoulder is absent from the monomer spectrum, so a decrease in absorption at this wavelength is indicative of a decrease

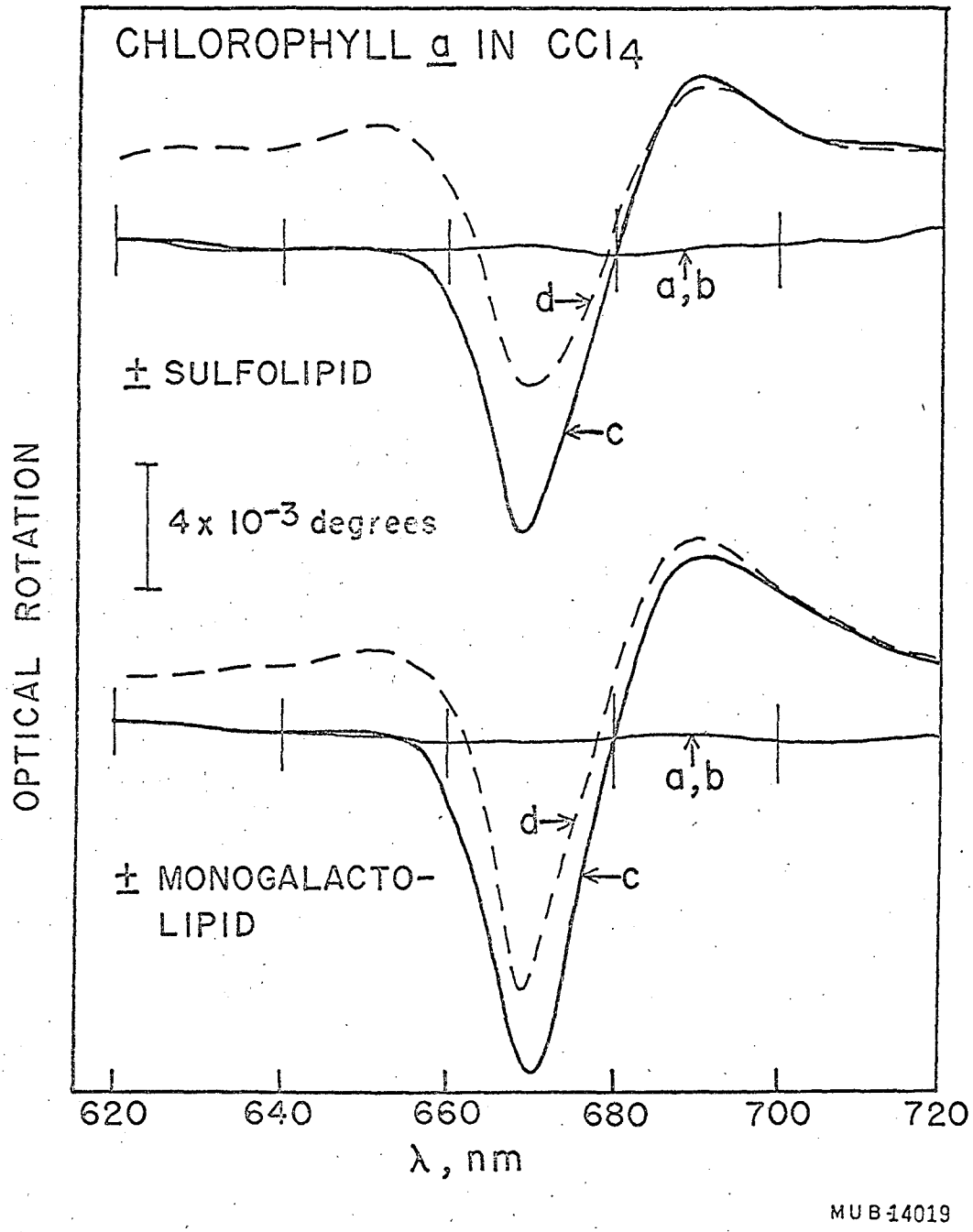

Figure IV.1. ORD spectra of solutions in carbon tetrachloride.

a. Solvent
b. Lipid solution, approximately 10^{-3}M
c. Chlorophyll a, 1.6×10^{-4}M
d. Sample (c) with approximately 5-fold excess of lipid added.

in dimer concentration. The concomittant increase in absorption at 663 nm indicated that the concentration of monomer is increased at the expense of dimer. The magnitude of the changes depends on the amount of lipid added. Figure IV.2, which is a reproduction of some of the data obtained for mixed solutions of chlorophyll *a* and galactolipid, illustrates these points.

A plot of the relative change in optical density at 682 nm, $\Delta OD/OD_{ref}$, vs the relative concentration of lipid added is given in Figure IV.3. The theoretical curve is obtained from an assumed equilibrium constant of complex formation of 8×10^3 liters/mole. This constant and the calculated optical density change curve were obtained in the following manner.

First we must determine the dimerization constant for the reference solution. In the absence of any lipids, the dimerization constant for the equilibrium

$$2\ M \underset{\rightleftarrows}{\overset{K_d}{\rightleftarrows}} D$$

is obtained from the three equations:

$$OD_{682} = \epsilon_m C_m + \epsilon_d C_d \ ; \quad C_t = C_m + C_d \ ; \quad K_d = \frac{C_d}{(C_m)^2} \qquad \text{(IV.1-3)}$$

where C_t is the total chlorophyll *a* concentration in the solution, C_m and C_d are the concentration of monomer and dimer, respectively, and ϵ_m and ϵ_d are the corresponding extinction coefficients at 682 nm. Using the extinction coefficients of Sauer *et al* (1966), we find $K_d = 4.4 \pm 1.1 \times 10^4$ liters/mole. This is much higher than the value they report, $1.0 \pm 0.4 \times 10^4$ liters/mole, possibly because we stored the dry pigment under streaming dry nitrogen gas. This process may

have removed some complexing water molecules normally present, and thus enhanced dimer formation.

Next, we wish to determine the equilibrium constant for complex formation between chlorophyll and lipid. We assume that a one-to-one complex only is formed. Then

$$L + M \overset{K_c}{\rightleftarrows} L \cdot M$$

and

$$K_c = \frac{C_c}{(C_l - C_c)C_m} \qquad \text{(IV.4)}$$

where C_c and C_l are the concentrations of complex and total lipid, respectively. Now, the optical density decreases at 682 nm may be saturated, i.e., at sufficiently high lipid/chlorophyll ratios, further increase in the lipid concentration will not cause any additional change in the absorption of the mixed solution, because all dimers originally present are broken up. At that point in the titration where the optical density change has reached one-half of its maximum value, we assume dimer is present in the solution at one-half of its concentration in the absence of lipid. At these particular concentrations,

$$(C_d)_{\frac{1}{2}} = \frac{1}{2} C_d \qquad \text{(IV.5)}$$

$$(C_m)_{\frac{1}{2}} = \left[\frac{(C_d)_{\frac{1}{2}}}{K_d}\right]^{\frac{1}{2}} = \left(\frac{C_d}{2K_d}\right)^{\frac{1}{2}} \qquad \text{(IV.6)}$$

The total lipid concentration at this point may be read from Figure IV.3. This value, together with the concentrations obtained from Equations IV.5 and IV.6 may then be substituted into Equation IV.4. From our data,

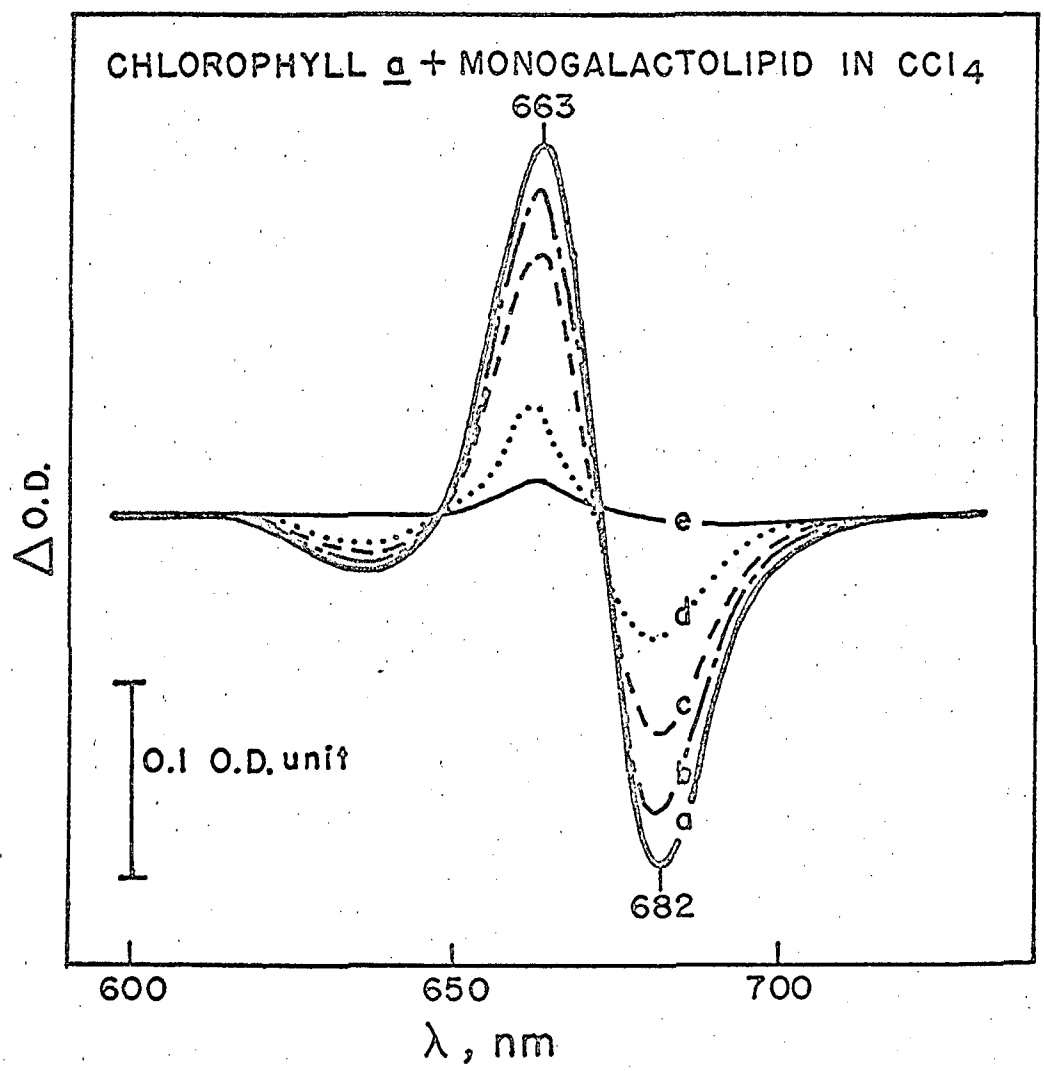

Figure IV.2. Absorption difference spectra. Reference solution, 1.6×10^{-4}M chlorophyll a in carbon tetrachloride; sample solution, same pigment concentration with varying amounts of monogalactolipid.

a. Lipid/chlorophyll = 8.2
b. " 5.2
c. " 3.1
d. " 2.0
e. Baseline

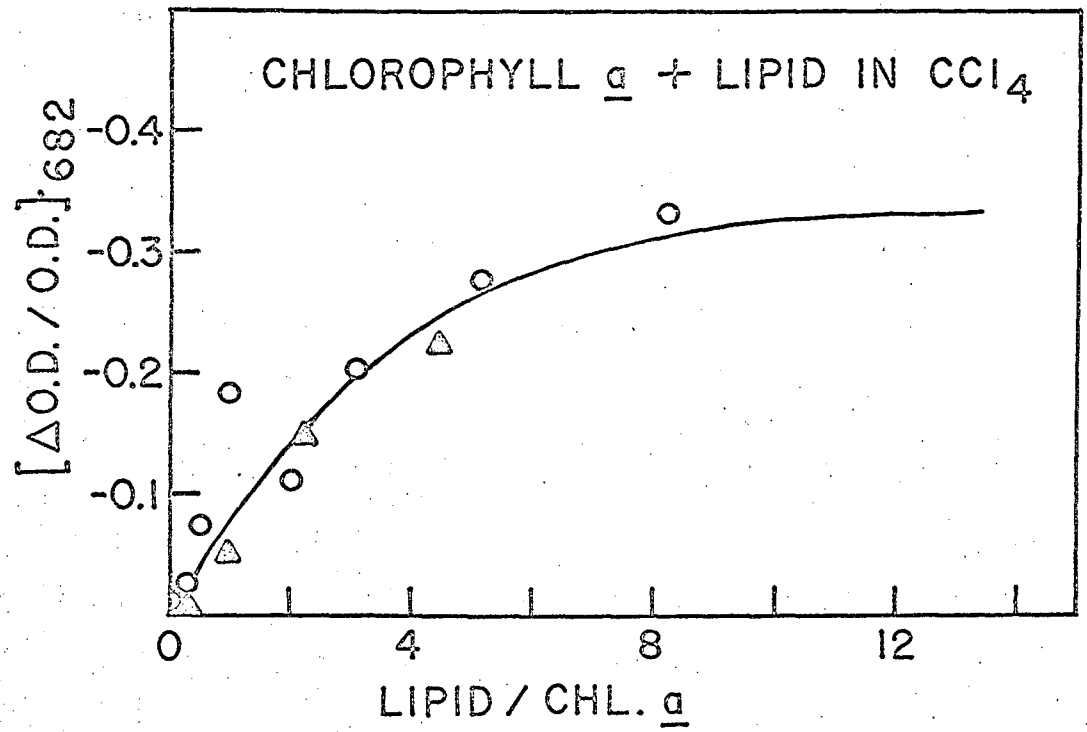

Figure IV.3. Change in optical density at 682 nm as a function of the lipid/chlorophyll a ratio in solutions of carbon tetrachloride. Experimental data for monogalactolipid (o) and sulfolipid (Δ). Theoretical curve calculated for formation of a 1:1 complex with equilibrium constant K_c = 8 x 10^5 liters/mole.

approximating $(\Delta OD/OD)_{1/2} = -0.18$, we find $K_c = 8 \pm 2 \times 10^3$ liters/mole.

In the presence of a lipid which forms only a one-to-one complex with chlorophyll a

$$\frac{\Delta OD}{OD} = \frac{\epsilon_m C_m' + \epsilon_d C_d' + \epsilon_c C_c'}{\epsilon_m C_m + \epsilon_d C_d} - 1 \qquad \text{(IV.7)}$$

where

$$C_t = C_m' + C_d' + C_c' \qquad \text{(IV.8)}$$

If we make this substitution, as well as using Equation IV.3, we obtain a quadratic equation in C_m' as a function of $\Delta OD/OD$ and constants which we can calculate. The extinction coefficient of the complex is also determined from Equation IV.7, using the concentrations and optical density change calculated for the half-titration point. Using our values, we compute $\epsilon_c = 2.3 \pm 0.7 \times 10^4$ liters/mole-centimeter. C_m' obtained from Equation IV.7 for any chosen optical density change is then used to calculate C_l from Equation IV.4. The smooth curve in Figure IV.3 is a plot of $\Delta OD/OD$ vs C_l/C_t. Within error, it is a very good fit to the experimental data for the galactolipid.

The results of this calculation suggest that galactolipid interacts with chlorophyll a dimers in carbon tetrachloride by forming one-to-one chlorophyll-lipid complexes with an equilibrium constant of $8 \pm 2 \times 10^3$ liters/mole. The sulfolipid also appears to form one-to-one complexes. We were unable to obtain data at high enough lipid concentrations to determine the equilibrium constant for their formation quantitatively. However, results at lower sulfolipid concentrations indicate that the equilibrium constant is of the same order of magnitude.

It is of interest to compare the strength of the pigment-pigment and the pigment-lipid interactions. If we consider the free energies of bond formation,

$$\Delta F = -RT \ln K_{eq} \qquad \text{(IV.9)}$$

where R is the gas constant and T the temperature in degrees Kelvin, we find

$$F_d = -6.3 \pm 0.4 \text{ Kcal/mole}, \quad \text{and} \quad F_c = 5.3 \pm 0.3 \text{ Kcal/mole},$$

i.e., the forces giving rise to the chlorophyll-galactolipid complex are approximately 80% as strong as the interaction between pigment molecules. Ku and Sauer (private communication) have noted that ethanol is a complexing agent of similar strength, while the chlorophyll a - water complex is caused by forces of about 65% the strength of the pigment-pigment interaction.

We conclude that galactolipid, and probably also sulfolipid, forms strong complexes with chlorophyll a, and will compete more effectively than water for the pigment. Thus, in the presence of excess lipid, galactolipid-chlorophyll a complexes will be formed at the expense of chlorophyll a aggregation. This result is in agreement with the proposed interpretation of the fact that fluorescence yields remain high at rather high chlorophyll concentrations in galactolipid monolayers.

V. BRIEF INVESTIGATIONS OF BIOLOGICAL MODELS

The subjects of experimental investigations discussed so far have been model systems composed of purified biological materials in an abiological environment. The behavior of more biological models should aid in determining the significance of the results of these studies for understanding the surroundings of chlorophyll a in the cell.

We consider here two studies of complex films composed of chlorophyll a and substances with which it is associated in the plant cell. First, measurements were made on monolayers of lipid extracts to determine the space required by this material, and thus possible modes of packing, in vivo. We also observed fluorescence polarization of films of chloroplast subunits, which data may be compared with that presented in Chapter III and with that for whole chloroplasts reported in the literature.

Before these experiments are discussed, a brief survey of the present understanding of the nature of the photosynthetic apparatus is in order.

A. The Environment of Chlorophyll a in vivo

In higher plants, the photosynthetic apparatus is contained wholly in chloroplasts. As revealed by light and electron microscopy (Park, 1966), these subcellular particles contain lamellae, organized into stacks in some species, embedded in an aqueous stroma matrix. Close inspection shows that the lamellae consist of flattened membrane sacs called thylakoids (Menke, 1960). High resolution electron microscopy of chloroplasts (Weier, et al., 1965) and of membrane fragments prepared by sonication and copious washing of lysed chloroplasts (Park

and Pon, 1963) has demonstrated the highly complex particulate nature of these lamellae. Park and Pon (1961) suggested that the 185 x 155 x 100 Å particles, or quantasomes, observed in the membranes be equated with the functional photosynthetic unit.

Chloroplast membrane preparations have been analyzed chemically, and found to contain approximately 50% protein and 50% lipid by weight. (Lichtenthaler and Park, 1963). The protein fraction contains cytochromes b_6 and f, as well as a large non-heme component (Biggins and Park, 1965). It is apparently largely hydrophobic, as high concentrations of detergent are required to prevent its aggregation in aqueous solution (Biggins, 1965; Criddle and Park, 1964).

Chemical analysis (Lichtenthaler and Park, 1963), fluorescence microscopy (Spencer and Wildman, 1962; Lintilhac and Park, 1966), and absorption spectroscopy (Park, 1965) have all served to locate chlorophyll a and accessory pigments in the lamellae. The visible absorption spectrum of suspensions of quantasome aggregates is very similar to that of whole chloroplasts (Sauer and Park, 1964). The absorption maximum in the red region, where only the chlorophylls absorb, is shifted several nanometers to longer wavelengths in these spectra compared with the maximum of chlorophyll a monomers in several organic solvents (Seely and Jensen, 1965). This red shift, as well as complex optical rotatory dispersion spectra (Sauer, 1965) and a large double circular dichroism component (Dratz, et al., 1966), is indicative of chlorophyll-chlorophyll interactions in vivo. This proposed pigment aggregation is further supported by low chlorophyll a fluorescence yields and fluorescence polarization, relative to dilute solutions of pigment, reported for biological materials (Clayton, 1966; Goedheer, 1957).

Low temperature fluorescence spectroscopy (Goedheer, 1964; Govindjee and Yang, 1966) of leaves and chloroplast fragments gives evidence that chlorophyll a exists in more than one form in vivo. Sauer (1965) and Sauer and Calvin (1962) observed flow and electric dichroism of quantasome aggregates. A large dichroic ratio occurring only at 695 mμ, on the long wavelength side of chlorophyll a absorption, led them to suggest that a small percentage of the chlorophyll a molecules in this material is aggregated in a highly ordered array. Butler, et al. (1964) have detected a polarized component of fluorescence from algae chloroplasts using various wavelengths of exciting light, and shown that this is due to a portion of the bulk chlorophyll in the chloroplasts which is oriented parallel to the lamellae. The phenomenon is observed in cells lacking the small complement of chlorophyll absorbing at 705 nm and believed by some workers to be the only highly oriented pigment in the system. The experimental evidence suggests, therefore, that chlorophyll a may be present in several degrees of orientation and states of aggregation in the plant. The bulk of the pigment molecules is probably in one of three forms: a small, aggregated, but poorly oriented, fraction; a larger, more weakly interacting fraction that is fairly well oriented in the lamellar planes; and a large, unaggregated and unoriented fraction. The optical studies indicate that, at room temperature, some energy may be transferred among these different forms. However, investigations of photosynthetic efficiencies as a function of the wavelength of the exciting light (e.g., Sauer and Park, 1965; Kelly and Sauer, 1965) suggest that a physiological distinction may exist within the pigment complement of chloroplasts which prevents energy exchange between two pigment systems.

Lamellar lipid composition has been reviewed recently (Benson, 1964). Three surface active glycolipids, mono- and digalactodilinolenate, and sulfoquinovodiglyceride, make up approximately 45% by weight of the lipid fraction. If membrane fragments are specifically attacked by galactolipase, the remaining material is still particulate (Bamberger and Park, 1966). Extraction of pigments and most other lipids from these preparations also leaves a particulate residue (Park, 1965). This evidence suggests that the lamellae consist of pigments and lipids coating a protein framework. The amphiphilic nature of the glycolipids (Benson, 1964; O'Brien and Benson, 1964; this investigation, Chapter III) and the hydrophobic properties of the membrane protein further support the hypotheses that chloroplast lamellae are more complex than the classical unit membranes of Robertson (1964).

Calculations based on chlorophyll content and lamellar area of chloroplasts showed that all the chlorophyll molecules could fit in a monomolecular layer in the membranes (Thomas, et al., 1956). Birefringence measurements, intrinsic birefringence being negligible (Goedheer, 1957), as well as the dichroism studies, indicate that the pigments are present in layered structures, in an imperfectly ordered arrangement on a macroscopic level.

In summary, we find chlorophyll a in the plant associated with amphiphilic lipids and hydrophobic proteins in membranous structures. If protein units form the cores of the particulate lamellae, the chlorophyll will be confined to lipid surface layers no more than a few molecules--possibly only one molecule--thick. The structure of chlorophyll a suits the molecule to orientation at hydrophobic-hydrophilic interfaces (see Figure I.1), such as may exist on the

surface of chloroplast membranes. One simple model for this possible *in vivo* arrangement is the chlorophyll-containing lipid monolayer on an aqueous subphase which we have used in this investigation. We now consider more complex two-dimensional systems of chloroplast material.

B. Studies on Quantasome Lipid Extract

Gorter and Grendel (1925, 1928) extracted lipids of red blood cell ghosts and determined the area that a known amount of extract occupied when spread as a monolayer. From this data, they were able to propose a model of the lipid arrangement in the cell membrane. This work and the calculations of Thomas *et al.* (1956) prompted us to carry out a similar investigation of the lipid components of chloroplast membrane fragments. By spreading the lipid extracted from a known weight of quantasomes on the modified Langmuir film balance, the area of a monolayer of the lipid molecules at zero pressure could be determined. A comparison of this value with the surface area and volume of the quantasomes extracted may indicate the arrangement of the lipid molecules in the lamellae.

1. Methods and materials

Quantasome aggregates prepared from leaves of *Spinacia oleracea* according to the method of Park and Pon (1963) were lyophilized and a weighed amount of the lyophilized material extracted as follows:

50 microliters of pyridine were added to the quantasomes, and the mixture diluted with enough n-hexane to make the final suspension 1% in pyridine by volume. The mixed suspension was spun in a clinical centrifuge for 5 minutes and the supernatant decanted. The residue was re-extracted by the same procedure five times.

The combined extracts were evaporated under nitrogen, and both extract and residue were dried overnight in a vacuum desiccator. Weighings of the dried residue and extract indicated that 30.1% of the total weight of the material, equivalent to 58.3% of the total lipid, had been extracted. These figures are based on analyses showing 51.6% of the membrane weight to be lipid (Lichtenthaler and Park, 1963).

The extract was redissolved in n-hexane containing 1% pyridine by volume to a final concentration by weight of 0.256 mg/ml. The chlorophyll a concentration in the sample was determined by the method of McKinney (1940). A known aliquot of extract was evaporated and, under nitrogen, resuspended in 80% acetone by volume. The absorption spectrum of this solution was recorded on a Cary 14 spectrophotometer. The chlorophyll a concentration in the extract was calculated to be 27.6% by weight. Chlorophyll a constitutes only 14.5% by weight of the total chloroplast membrane lipid. Extracting the lipid with the spreading solvent to avoid unknown losses of material upon changing solvents made necessary our working with this extract of unrepresentative composition.

2. Results and discussion

Techniques described in Chapter III A.4 were used to determine the pressure-area behavior of four 200-microliter samples, each containing 0.0512 mg extract. A curve of average pressure vs. area behavior, with area expressed at total film area in square centimeters, is shown in Figure V.1.

The area covered by the 0.0512 mg sample at zero pressure is found by extrapolation of the plot to be 422 cm^2. We assume that the

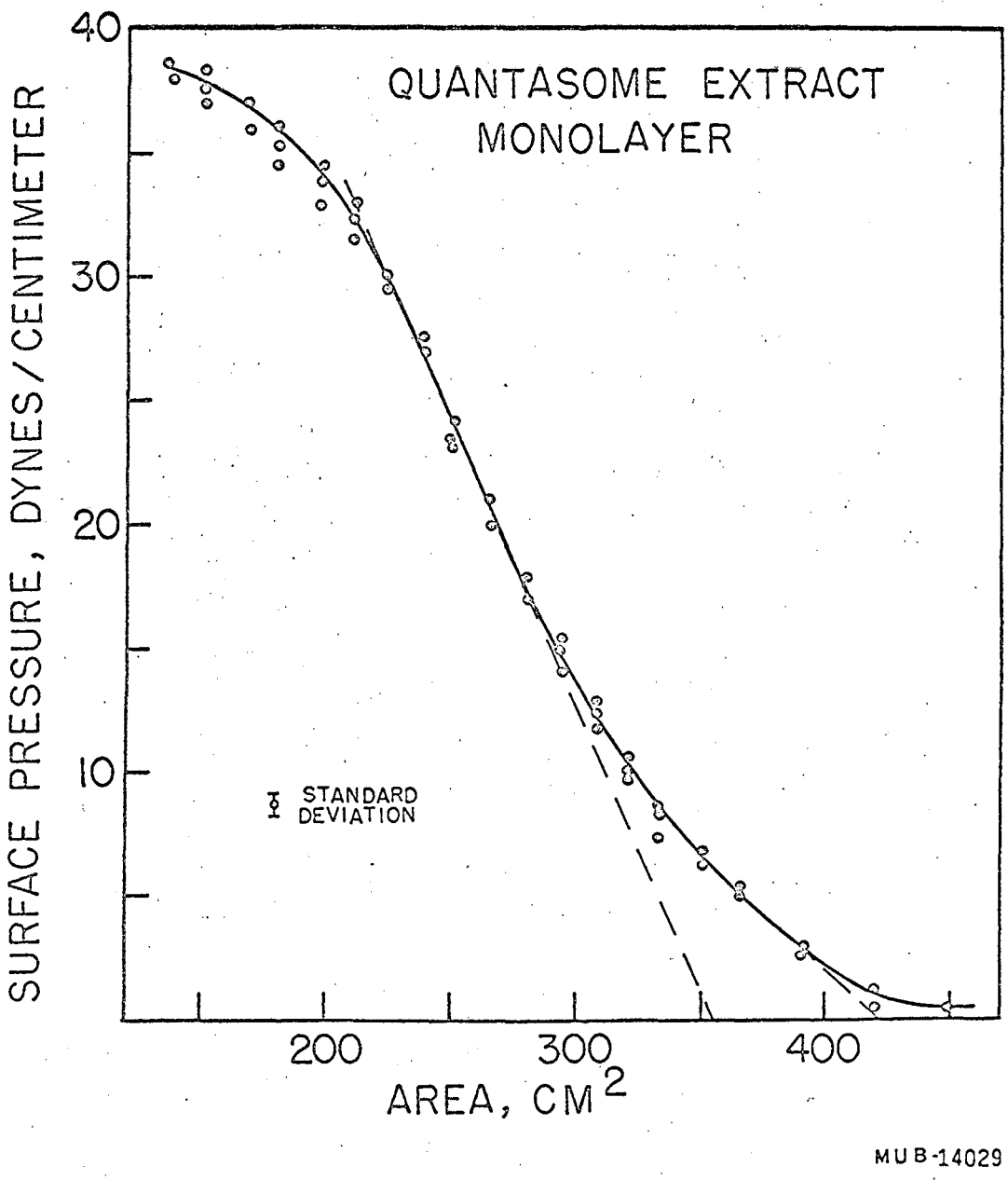

Figure V.1. Pressure - area behavior of a monolayer of quantasome lipid extract, abscissa expressed as total film area per 0.0512 mg. material. See text for discussion of extrapolated values.

area occupied by the extract is approximately the same as that which would be needed by a representative chloroplast lamellar lipid sample of the same weight. If we consider the basic subunit of chloroplast lamellae to be the quantasome of MW 2×10^6 characterized by Park and Pon (1961), and later by Park and Biggins (1964), the 0.0512 mg of lipid material represents 30.1% by weight of 5.3×10^{13} quantasomes. Then, with the above assumptions, the total lipid of one quantasome should occupy an area of 136,000 $Å^2$.

If the quantasome is an ellipsoid with axes 185 x 155 x 100 Å (Park and Biggins, 1964), it has a surface area of approximately 56,000 $Å^2$. A bilayer of lipid coating this particle would thus cover an area of 112,000 Å when spread as a monolayer, if the molecules were similarly oriented in both cases. The 136,000 Å required by the molecules on the Langmuir trough suggests that the lipids in these subunits may not form a unit bilayer membrane about the particles, with the hydrophilic groups of the molecules oriented in the plane of the surface. The results of this experiment do not allow us to distinguish among several other possibilities: 1) that a more highly compressed lipid bilayer is present on the quantasome surface than in the monolayer; 2) that the lipid forms a non-uniform coating on the particles partly thicker than a bilayer; 3) that the lipid molecules are dispersed throughout the subunits; 4) that the extracted lipid does not occupy the same surface area as the same amount of total lipid extract would.

We discard possibility 3) on the basis of the differential extraction experiments (Bamberger and Park, 1966) discussed in section A. In support of the first suggestion, we note that if the extrapolation is

made from the steepest straight segment of the pressure-area curve, the area at zero pressure is 352 cm^2. This corresponds to a surface area per quantasome of 115,000 $Å^2$, which agrees closely with the calculated area of a surface double layer per subunit. As the last explanation is applicable, according to the chlorophyll content, and the second cannot be ruled out, we regard this agreement as fortuitous. These results do, however, lend support to the premise that lamellar lipids and pigments are located on the surfaces of the chloroplast membranes, in thin layers.

The fluorescence properties of these films were not studied quantitatively. Several accessory pigments, as well as chlorophyll a, absorbed the exciting light, and made interpretations in terms of chlorophyll-chlorophyll energy transfer impractical. The fluorescence yield was of the same order of magnitude as that expected from a chlorophyll a-lipid film of the same pigment concentration.

C. Quantasome Supernatant Films

Various workers have measured the polarization of fluorescence of chloroplast fragments in dilute viscous solutions. Arnold and Meek (1956) reported a degree of polarization of 0.15 - 0.16, compared with 0.24 for chlorophyll a in viscous solution, when chlorophyll-protein extracts prepared by the method of Smith (1930) and suspended in water were excited with polarized 600 - 630 nm light. This surprisingly high polarization implies that the fluorescing chlorophyll molecules remained fixed during their excited state lifetime, and that either there was little energy transfer among them or they were highly ordered. This could only be possible in aqueous solution, if the pigment molecules

were well separated in very large protein aggregates, or if the chlorophyll-chlorophyll interactions were so strong that the excited state lifetime was shortened by more than an order of magnitude. In this latter case, a large red shift of the absorption spectrum might be expected, but it is not reported. As the nature of the extract is not well understood, we will not comment further on these observations. Exciting an aqueous Aspidistra suspension with polarized light at wavelengths below 590 nm, Goedheer (1957) was unable to detect polarized fluorescence. If he used red light absorbed by chlorophyll, he observed a polarization of 0.025. Sauer (private communication), reporting fluorescence polarization ratios, found $\rho_u \approx 0.09 \pm 0.06$ for suspensions of quantasomes prepared by the method of Park and Pon (1963) and illuminated with 500 nm light. This corresponds roughly to the degree of polarization observed from whole algae samples.

The fluorescence polarization observed by Goedheer and by Sauer from chloroplast fragments appears to be no larger than that detected from whole cells. We would expect to find no greater polarization from a film of small quantasome aggregates, unless we had succeeded in preparing lamellar fragments containing only oriented chlorophyll, or pigment molecules sufficiently separated that energy transfer among them was impaired. The experiment described below gave no evidence that chlorophyll a in the small quantasome aggregates had a unique orientation.

1. Materials and methods

Chloroplasts were prepared by the method of Park and Pon (1961) from 225 grams of commercially grown spinach leaves, and washed once in dilute buffer. They were then resuspended in 25 ml deionized water and

sonicated approximately 100 seconds at 9 Kc. in a Raytheon sonicator. The sonicate was fractioned as shown in Figure V.2 to give samples Nos. 1, 2, and 3 for use in the experiment. Fluorescence polarization of suspensions of these samples was measured by the technique described for viscous chlorophyll a solutions in Chapter III.

By careful dropwise deposition of an aliquot of sample No. 3 on 10^{-3} M phosphate buffer, pH 7.6, we were able to form films of this material. Aliquots of samples Nos. 1 and 2 appeared to sink into the subphase, probably due to their larger particle size. Pressure-area and fluorescence polarization measurements on films of sample No. 3 material were carried out as detailed in Chapter III. After fluorescence intensities had been recorded, the cover of the instrument was removed, the surface swept clean, and a blank reading taken. In this way, any fluorescence due to dissolved material was detected, and data could be corrected for it.

Absorption spectra of the samples were recorded on a Cary 14 spectrophotometer equipped with a scattered transmission attachment. The lipid and protein content of the material was determined by extraction of a dried and weighed aliquot three times each with reagent grade benzene, then acetone, and finally methanol. Both combined extracts and residues were dired overnight in a vacuum dessicator before weighing.

2. Results and discussion

The visible and near ultraviolet absorption spectrum of sample No. 3, which is the supernatant from the quantasome precipitation (Figure V.2) was very similar to that of the quantasome fraction in

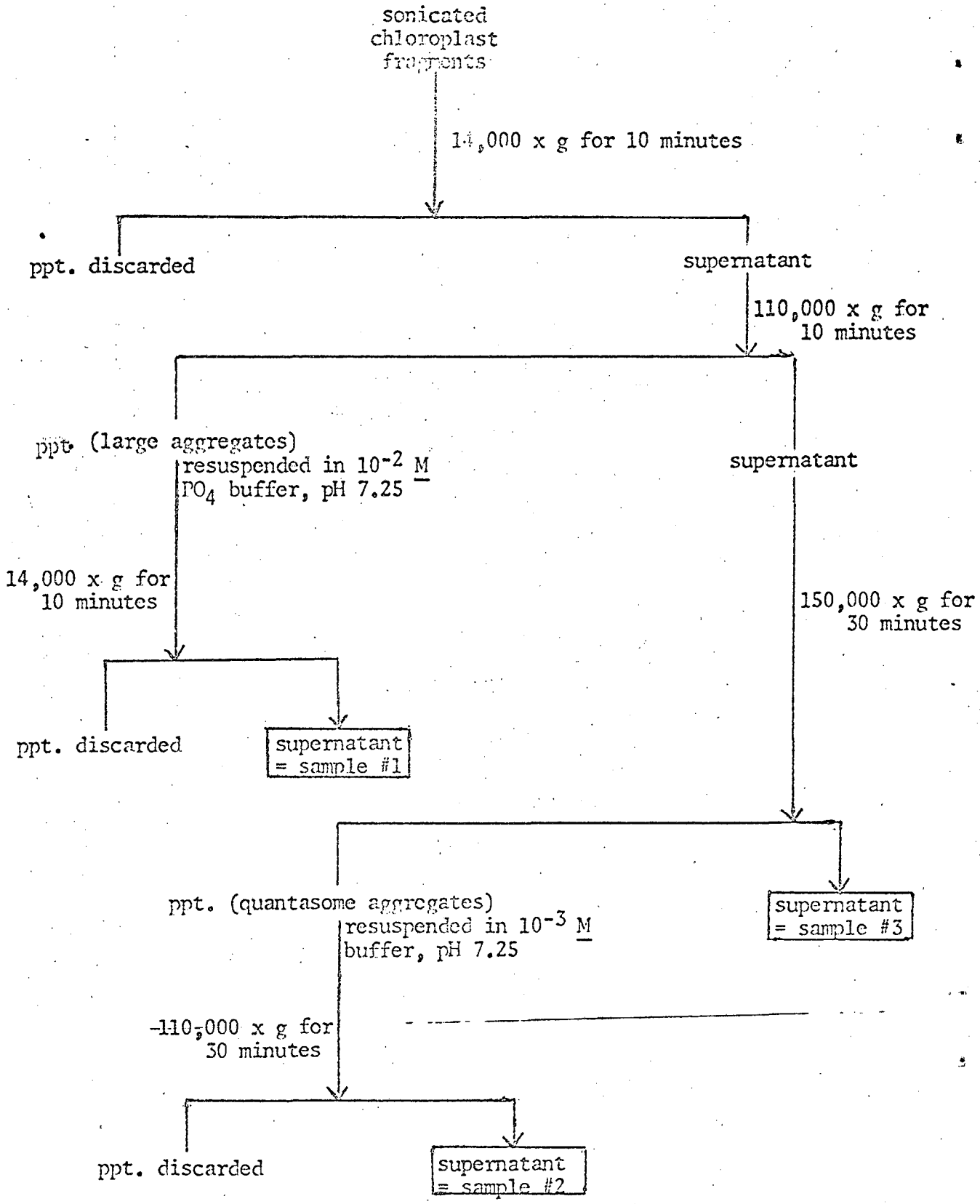
sonicated chloroplast fragments
14,000 x g for 10 minutes
ppt. discarded
supernatant
110,000 x g for 10 minutes
ppt. (large aggregates) resuspended in 10^{-2} M PO_4 buffer, pH 7.25
supernatant
14,000 x g for 10 minutes
150,000 x g for 30 minutes
ppt. discarded
supernatant = sample #1
ppt. (quantasome aggregates) resuspended in 10^{-3} M buffer, pH 7.25
supernatant = sample #3
110,000 x g for 30 minutes
ppt. discarded
supernatant = sample #2

Figure V.2

the red region. However, the supernatant fraction had a higher optical density in the blue and ultraviolet regions where carotene and quinones absorb strongly, indicating that this material was enriched in these lipids. The analysis bore out this prediction. The sample was approximately 70% lipid by weight, compared with 50% lipid in quantasome aggregates. The extra lipid probably enhanced the film-forming capacity of this sample.

The results of fluorescence polarization measurements on the suspensions are listed in Table V.1. We see that fluorescence of these quantasome aggregate preparations is essentially unpolarized.

Table V.1

A. Suspensions

sample	solvent	P
1	10^{-2} M phosphate buffer, pH 7.25	~0
2	" " " "	~0
3	" " " "	~0
2	100% glycerol	~0.001 ± 0.004

B. Films

sample	preparation	P
2	dried film	~0.001 ± 0.004
3	monolayer	0.008 ± 0.006

The films formed by sample #3 were compressible. The pressure-area behavior was not very reproducible, varying as much as 25% (Figure V.3). This was undoubtedly due to partial dissolution of material in

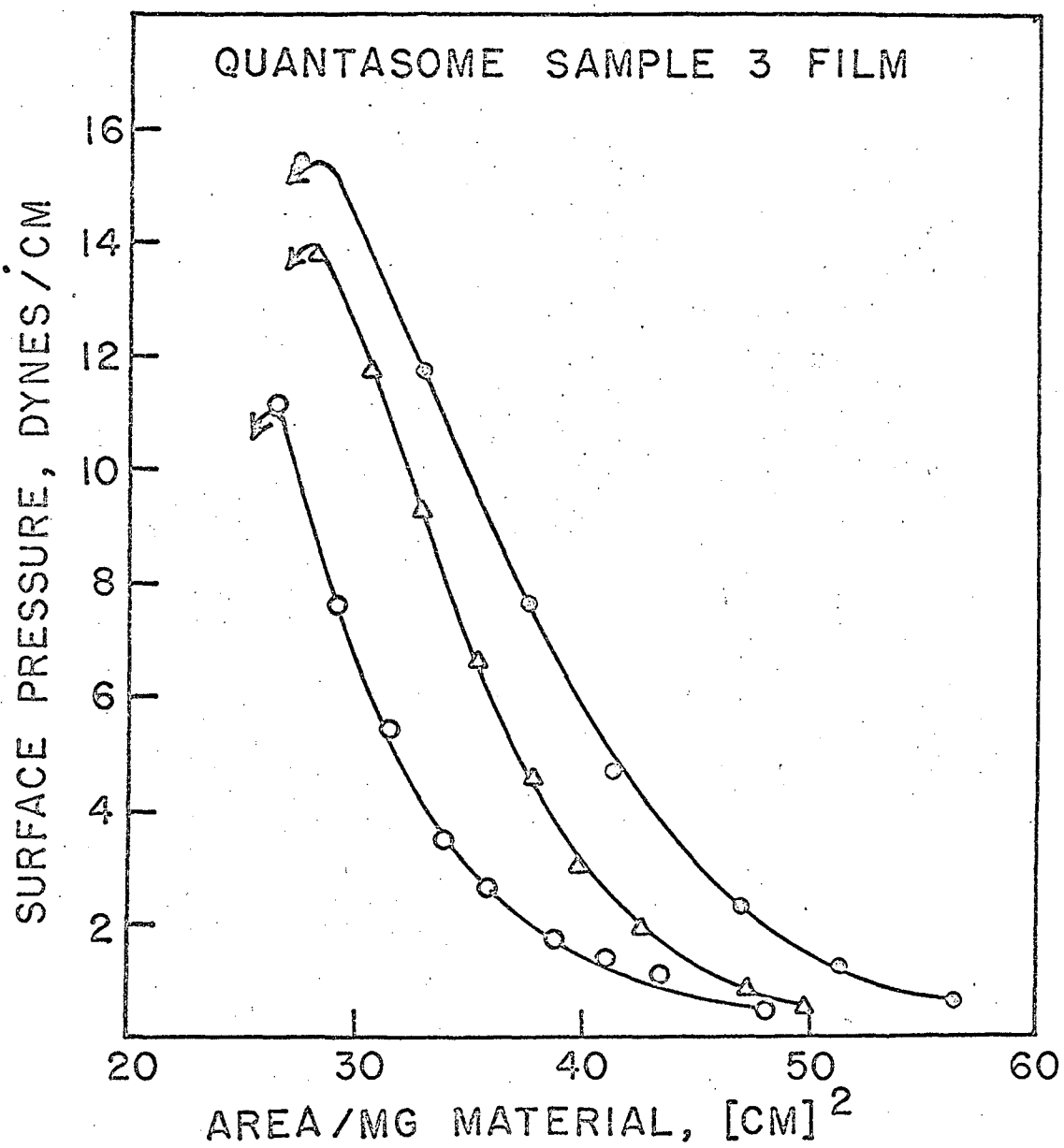

Figure V.3. Compression behaviour of sample #3 as a function area occupied by 0.2 milligrams of material. (o), (Δ), and (⊙), three different films.

the subphase upon spreading. Once a stable film was formed, however, less than 10% change in area occurred in times longer than necessary to perform polarization measurements. The average fluorescence polarization from films of sample #3 calculated according to equation III.2 was 0.008 ± 0.006, after corrections had been applied for the slight film instability. This value is somewhat lower than those reported by Goedheer (1957) and Sauer (private communication) for excitation of chloroplast fragments with red light. However, we illuminated the films with 406 nm light, which is as strongly absorbed by accessory pigments as by chlorophyll a (Rabinowitch, 1951). The former transfer energy efficiently to chlorophyll a. Thus the emission observed came from chlorophyll molecules whose orientation with respect to the primarily excited molecules is not known, and which may be random. In this situation, we would not expect, nor do we find, a degree of polarization as large as that observed when only chlorophyll a molecules are excited.

We conclude that sonication of ruptured chloroplasts does not break up the lamellae into subunits with similarly oriented chlorophyll a molecules, and that energy transfer among pigments in the quantasome aggregates obtained by this treatment is essentially unimpaired. Since each quantasome contains over 200 chlorophylls and a complete complement of accessory pigments, and also because fragments obtained by sonication contain at least several quantasomes, this result is not surprising. Investigation of films of the special chlorophyll-protein complexes reported in the literature (Boardman and Anderson, 1964; Cederstrand and Govindjee, 1966; Olson, 1966; Goedheer,

1966) might indicate assymmetries in these subunits, if they exist.

It is of interest to determine the concentration of chlorophyll a in the monolayers of biological lipids at which the fluorescence polarization is the same as that observed for films of quantasome material. This latter value, 0.008 (Table V.1B), is reached by chlorophyll a in monogalactolipid monolayers at a concentration of 0.09 mole fraction. The fluorescence polarization of the pigment in sulfolipid films does not appear to fall as low as 0.008. Now, the chlorophyll a concentration *in vivo* is estimated as 0.06 to 0.2 mole fraction (Rabinowitch, 1945). We see that the pigment concentration in the galactolipid monolayer, at which polarization equals that observed from quantasome films, falls in this range. This agreement may indicate that fluorescent chlorophyll a molecules in the cell are associated with galactolipid. Likewise, we may suggest that this pigment component is not dispersed in sulfolipid.

VI. GENERAL DISCUSSION AND CONCLUSIONS

In order to determine the mechanism of energy transfer in model membrane systems of chlorophyll a and surface active lipids, we formulated the general problem of fluorescence depolarization as a function of energy transfer rate. The mechanism, and therefore the rate, of energy transfer depend on the strength of intermolecular electrostatic interactions. The observed degree of polarization from a random ensemble of molecules becomes a very complicated function of transfer rate when back transfer is considered. Lacking a solution to this general case, we have investigated polarization behavior under certain specified conditions.

In the chlorophyll a concentration range where we predict very weak coupling, and thus transfer by inductive resonance, polarization data are consistent with our hypothesis. Because of this agreement, we are able to suggest that energy is transferred among well dispersed pigment molecules at a rate proportional to the square of the interaction energy. Apparently energy migration is, in this case, a second order perturbation phenomenon.

At high chlorophyll a concentrations in the model systems, the mechanism of energy transfer appears to depend on the mutual orientations of the pigment molecules. This orientation is determined, in part, by the lipid matrix in which the chlorophyll is embedded. We have proposed that pigment may be randomly dispersed and oriented in monogalactolipid monolayers, and that inductive resonance may remain the dominant mode of energy transfer at all chlorophyll a concentrations

in this lipid. However, an abrupt change in the decrease of fluorescence polarization with increasing pigment concentrations in the sulfolipid films has led us to suggest that chlorophyll a molecules may be partially oriented by the sulfolipid. Such an arrangement would facilitate the formation of localized excitons, the presence of which would result in an energy transfer rate proportional to the interaction energy. This first order perturbation phenomenon would not decrease fluorescence polarization, because the molecules in the system are oriented. Observed behavior coincides with this prediction.

Alternative explanations of our data at high pigment concentrations are possible. However, those based on the assumption of varying extents of aggregation and different fluorescent lifetimes in the different lipid matrices are not consistent with the observed unpolarized fluorescence from the most aggregated model, the pure chlorophyll a monolayer. Had kinetic studies of fluorescence polarization been possible with our apparatus, these other proposals could have been investigated further.

In support of our original suggestions, we do know that the chloroplast structural lipids interact with chlorophyll a dimers in solution, effectively causing dispersal of the aggregated pigment. Monogalactodilinolenate appears to form a very strong one-to-one complex with chlorophyll a, the free energy of complex formation being greater than 80% of that for pigment dimerization. Plant sulfolipid also serves as a strong complexing agent for chlorophyll a. The free energy of one-to-one complex formation appears to be of the same order of magnitude as that for monogalactolipid-pigment complexes.

The extension of these results to biological material is somewhat tenuous, because the liquid-gas interface environment of the model may

be a poor approximation to chloroplast lamellar surface interfaces. Also, the presence of many other molecular species, some with energy levels close to those of chlorophyll a, may affect the chlorophyll-chlorophyll and chlorophyll-lipid interactions studied here. If we assume that these differences in model and biolcgical chlorophyll a environments do not greatly affect pigment interactions, we may make some predictions about chlorophyll in vivo.

The fluorescence properties of chlorophyll a in biological material suggest that absorbed light energy is transferred efficiently among these molecules. Calculations based on energy transfer rates proportional to the square of the strength of the dipole interaction (Duysens, 1952; Takeyama, 1962; Pearlstein, 1966) show that this interaction mechanism may adequately account for the observed properties of the biological material. In addition, we have observed that the second order perturbation phenomenon of inductive resonance is sufficient to account for the extent of chlorophyll a fluorescence depolarization observed in vivo, if the pigment molecules are randomly oriented. In fact, we would not expect exciton migration, a rate process proportional to the interaction energy, to occur in this arrangement. However, exciton migration may occur in the oriented, closely spaced chlorophyll complement of the photosynthetic apparatus, as suggested by the results of sulfolipid-pigment monolayer experiments.

In light of the morphological and spectroscopic evidence, we propose that the dispersed fraction of the "bulk" chlorophyll a may be associated with galactolipid on lamellar surfaces in the chloroplast. Further, if the photosynthetic energy trap is a morphological entity consisting at least in part of aggregated or oriented chlorophyll a, this trap is probably not associated with or embedded in galactolipid.

The failure of fluorescence polarization to fall to zero at high chlorophyll concentrations in sulfolipid could be interpreted as indicating that the pigment molecules are partially aggregated or oriented in the presence of this lipid at an interface. The occurrence of sulfolipid in conjunction with chlorophyll appearance and disappearance (Shibuya and Hase, 1965) and fluorescence polarization changes (Goedheer and Smith, 1959) measured in greening experiments also suggests that this lipid may be involved in pigment aggregation in vivo.

A more sophisticated monolayer fluorometer, with provision for measurement of surface viscosity and potential as well as fluorescence polarization spectra, would allow a search for evidence of such specific molecular arrangements in films. Abrupt changes in the dependence of surface viscosity or surface potential on pigment concentration would indicate phase changes occurring in the film, which may be caused by stoichiometric complex formation. Changes in the state of chlorophyll aggregation would be reflected in spectral changes. Information gained from these experiments would clarify the molecular description of the models. We could then be more precise in comparing the behavior of the models with that of the photosynthetic apparatus.

In this investigation, we have dealt with the simplest possible two-dimensional, two-component model of the chloroplast lamella. An obvious extension of this problem to a more biological level would be the inclusion of accessory pigments and other lipids in the monolayers. However, interpretation of spectroscopic studies would then be feasible only if monochromators were built into the apparatus. When the properties of chloroplast lamellar protein become well understood, making it a

tractable chemical substance, the properties of models containing this material should be studied. We do not yet have any direct evidence that lamellar lipids and pigments will form a surface layer on chloroplast proteins. Anomalous behavior of monolayers containing all three components would provide this information. Finally, variations in the ionic strength and composition of the subphase may induce changes in monolayer properties which could be related to biological membrane activity. In future, we hope that studies such as these will be complemented by those involving liquid-liquid interface and bilayer techniques.

Finally, we note that a monolayer fluorometer capable of measuring polarization spectra may be a useful analytical tool for studying interactions in any system--e.g., energy transfer among dye moieties bound to large surface active molecules--where the description of the model is simplified in two dimensions.

BIBLIOGRAPHY

Adam, N. K. (1933). Trans. Farad. Soc. 29:103.

Adam, N. K. (1941). The Physics and Chemistry of Surfaces, 3rd edition, Oxford University Press.

Anderson, A. F. H. and M. Calvin (1962). Nature 194:285.

Apostol, T. M. (1961). Calculus I, Blaisdell, p. 420.

Arnold, W. and E. S. Meek (1956). Arch. Biochem. Biophys. 60:82.

Bailey, R. W. (1958). Biochem. J. 68:660.

Bamberger, E. S. and R. B. Park (1966). Plant Physiol., in press.

Bellamy, W. D., G. L. Gaines and A. G. Tweet (1963). J. Chem. Phys. 39:2528.

Benson, A. A. (1964). Ann. Rev. Plant Physiol. 15:1.

Boardman, N. K. and J. M. Anderson (1964). Nature 203:166.

Boyd, E. and W. D. Harkins (1939). J. Am. Chem. Soc. 61:1188.

Butler, W. L., R. A. Olson and W. H. Jennings (1964). Biochim. Biophys. Acta 88:651.

Cederstrand, C. N. and Govindjee (1966). Biochim. Biophys. Acta 120:177.

Clayton, R. K. (1966). In The Chlorophylls, ed. L. P. Vernon and G. R. Seely, Academic Press, New York, p. 620.

Colmano, G. (1961). Biochim. Biophys. Acta 47:454.

Colmano, G. (1962). Nature 193:1287.

Crisp, D. J. (1949). In Surface Chemistry, Research Suppl. 23, p. 17, London.

Daniels, F., J. H. Mathews, J. W. Williams, P. Bender and R. A. Alberty (1956). Experimental Physical Chemistry, 5th ed., McGraw-Hill, New York, p. 60.

Davydov, A. S. (1962). Theory of Molecular Excitons, trans. M. Kasha and M. Oppenheimer, Jr., McGraw-Hill, New York.

Dratz, E. A., A. J. Schultz and K. Sauer (1966). Brook. Symp. Biol. 19, in press.

Duysens, L. M. N. (1952). Thesis, University of Utrecht.

Emerson, R. and W. Arnold (1932). J. Gen. Physiol. 16:191.

Feofilov, P. P. and B. Ya Sveshnikov (1940). J. Exptl. Theoret. Phys. (U.S.S.R.) 10:1372.

Feofilov, P. P. and B. Ya Sveshnikov (1941). J. Phys. (U.S.S.R.) 3:493.

Feofilov, P. P. (1961). Physical Basis of Polarized Emission, Consultants Bureau, New York.

Fernandez, J. and R. Becker (1959). J. Chem. Phys. 31:467.

Forster, Th. (1948). Ann. d. Physik (6)2:55.

Forster, Th. (1949). Z. Naturforsch. 4a:321.

Forster, Th. (1951). Fluoreszenz Organischer Verbindungen, Van den Hoecht & Rupprecht, Gottingen.

Forster, Th. (1960). In Comp. Effects of Radiation, ed. M. Burton, J. S. Kirby-Smith and J. L. Magee, John Wiley & Sons, Inc., p. 300.

Forster, Th. (1965). In Modern Quantum Chemistry, ed. O. Sinanoglu, Academic Press, New York, Vol. III, p. 93.

Franck, J. and R. Livingston (1949). Rev. Mod. Phys. 21:505.

French, C. S. (1965). Appl. Optics 4:514.

Gaffron, H. and K. Wohl (1936). Naturwiss. 24:81.

Gaines, G. L. (1963). Gen. Elec. Res. Report 63-RL-3206 c.

Gaines, G. L., W. D. Bellamy and A. G. Tweet (1964). J. Chem. Phys. 41:538.

Gaines, G. L., A. G. Tweet and W. D. Bellamy (1965). J. Chem. Phys. 42:2193.

Goedheer, J. C. (1957). Thesis, University of Utrecht.

Goedheer, J. C. and J. H. C. Smith (1959). Carnegie Inst. of Washington, Yrbk. 1958-1959, p. 334.

Goedheer, J. C. and G. R. Vegt (1962). Nature 193:875.

Goedheer, J. C. (1964). Biochim. Biophys. Acta 88:304.

Goedheer, J. C. (1966). The Chlorophylls, ed. L. P. Vernon and G. R. Seely, Academic Press, New York, Ch. 13, Part I.

Gorter, E. and F. Grendel (1925). J. Exp. Med. 41: 439.

Gorter, E. and F. Grendel (1928). Biochem. Z. 192:431.

Gouterman, M. and C. Stryer (1962). J. Chem. Phys. 37:2260.

Govindjee and C. Yang (1966). J. Gen. Physiol. 49:763.

Hanson, E. A. (1937). K. Akad. van Weten., Amsterdam, 40:281.

Hanson, E. A. (1939). Rec. d. trav. Botan. Neerl. 36:180.

Hochstrasser, R. M. and M. Kasha (1964). J. Chem. Phys. 41:538.

Hughes, A. (1936). Proc. Roy. Soc. A155:710.

Jacobs, E. E., A. S. Holt and E. Rabinowitch (1954). J. Chem. Phys. 22:142.

Jacobs, E. E., A. S. Holt, R. Kromhout and E. Rabinowitch (1957). Arch. Biochem. Biophys. 72:495.

Kasha, M. (1963). Rad. Res. 20:51.

Kasha, M. (1963b). Rad. Res. 20:55.

Katz, E. (1949). In Photosynthesis in Plants, ed. J. Franck and W. E. Loomis, Iowa State College Press, p. 287.

Katz, J. J., G. L. Closs, F. C. Pennington, M. R. Thomas and H. H. Strain (1965). J. Am. Chem. Soc. 85:3801.

Ke, B. (1966). In The Chlorophylls, ed. L. P. Vernon and G. R. Seely, Academic Press, New York, p. 253.

Ke, B. and R. Sperling (1966). Brookhaven Symp. Biol. 19, in press.

Kelly, J. and K. Sauer (1965). Biochemistry 4:2798.

Knox, R. S. (1963). Theory of Excitons, Academic Press, New York.

Langmuir, I. and V. J. Schaefer (1937). J. Am. Chem. Soc. 59:2075.

Lepage, M. (1964). J. Chromatog. 13:99.

Lichtenthaler, H. K. and R. B. Park (1963). Nature 198:1070.

Lintilhac, P. M. and R. B. Park (1966). J. Cell Biol. 28:582.

McClure, D. S. (1960). Solid State Phys. 8:1.

McKinney, C. C. (1941). J. Biol. Chem. 140:315.

McRae, E. G. and M. Kasha (1964). In Physical Processes in Radiation Biology, ed. L. Augenstein, R. Mason and B. Rosenberg, Academic Press, New York, p. 23.

Menke, W. (1960). Experientia 16:537.

Nichols, B. W. (1964). New Biochemical Separations, ed. A. T. James and L. J. Morris, D. van Nostrand Company, Ltd., London, Ch. 15.

O'Brien, J. S. and A. A. Benson (1964). J. Lipid Res. 5:432.

O'Brien, J. S., D. C. Fillerup and J. F. Mead (1964). J. Lipid Res. 5:109.

Olson, J. M. (1966). In The Chlorophylls, ed. L. P. Vernon and G. R. Seely, Academic Press, New York, Ch. 13, Part II.

Park, R. B. and N. G. Pon (1961). J. Mol. Biol. 3:1.

Park, R. B. and N. G. Pon (1963). J. Mol. Biol. 6:105.

Park, R. B. and J. Biggins (1964). Science 144:1009.

Park, R. B. (1965). J. Cell Biol. 27:151.

Park, R. B. (1966). In The Chlorophylls, ed. L. P. Vernon and G. R. Seely, Academic Press, New York, p. 283.

Pearlstein, R. M. (1964). Proc. Nat. Acad. Sci. U.S.A. 52:824.

Pearlstein, R. M. (1966). Thesis, University of Maryland.

Perrin, F. (1929). Ann. Physique 12:169.

Rabinowitch, E. (1945). Photosynthesis I, Interscience, New York, p. 412.

Rabinowitch, E. (1951). Photosynthesis II, Interscience, New York, Ch. 22.

Radin, N. S., S. B. Lavin and J. R. Brown (1955). J. Biol. Chem. 217:789.

Robertson, J. D. (1964). In Cellular Membranes in Development, ed. M. Locke, Academic Press, New York, p. 1.

Rosoff, M. and C. Aron (1965). J. Phys. Chem. 69:21.

Sauer, K. and R. B. Park (1964). Biochim. Biophys. Acta 79:476.

Sauer, K. (1965). Proc. Nat. Acad. Sci. U.S.A. 53:716.

Sauer, K. and R. B. Park (1965). Biochemistry 4:2791.

Sauer, K., J. R. Lindsay Smith and A. J. Schultz (1966). J. Am. Chem. Soc. 88:2681.

Seely, G. R. and R. G. Jensen (1965). Spectrochim. Acta 21:1835.

Shibuya, I. and E. Hase (1965). Plant and Cell Physiol. 6:267.

Simpson, W. T. and D. L. Peterson (1957). J. Chem. Phys. 26:588.

Singh, I. S. and R. Becker (1960). J. Am. Chem. Soc. 82:2083.

Smith, E. L. (1930). Science 88:170.

Soleillet (1929). Ann. Physique 12:23.

Spencer, D. and S. G. Wildman (1962). Austral. J. Biol. Sci. 15:599.

Stupp, R. (1952). Dissertation, University of Basil.

Trlifaj, M. (1958). Czech. J. Physics 8:510 (in English).

Trurnit, H. J. and G. Colmano (1959). Biochim. Biophys. Acta 31:434.

Tweet, A. G. (1963). G. E. Res. Report #63-RL-3434 G, September 1963.

Tweet, A. G., G. L. Gaines and W. D. Bellamy (1964a). J. Chem. Phys. 40:2596.

Tweet, A. G., G. L. Gaines and W. D. Bellamy (1964b). J. Chem. Phys. 41:1008.

Tweet, A. G., W. D. Bellamy and G. L. Gaines (1964). J. Chem. Phys. 41:2068.

Vavilov, S. I. (1943). J. Phys. Rad. 7:141.

Weber, G. (1952). Biochem. J. 51:145.

Weber, G. (1954). Trans. Farad. Soc. 60:551.

Weber, G. (1960). In Comp. Biochem. Photoreactive Systems, ed. M. B. Allen, Academic Press, New York, p. 325.

Weenink, R. O. (1963). Nature 197:62.

Weier, T. E., A. H. P. Engelbrecht, A. Harrison and E. B. Rigley (1965). J. Ultrastruct. Res. 13:92.