ABSTRACT

DETERMINATION OF PORPHYRIN RING ORIENTATION
IN SPINACH CHLOROPLAST EXTRACT CHLOROPHYLL
BLACK LIPID MEMBRANES BY PHOTOVOLTAGE SPECTROSCOPY

By

Herman G. Weller, Jr.

Photovoltage spectroscopy with polarized light was used to investigate the structure of black lipid membranes formed from spinach chloroplast extracts. The photovoltage action spectrum of the chlorophyll black lipid membrane is similar to the absorption spectrum of the membrane-forming solution, with a red and principal blue peak. The magnitudes of these peaks were found to depend on the direction of polarization of the exciting light. This is apparently a direct consequence of the dichroism of the membrane. The polarized light photovoltage data were used to obtain information on the orientation of chlorophyll in the membrane.

The chlorophyll principal blue transition moment was calculated to make an angle of 21 ± 2° with the plane of the membrane; the red transition moment, an angle of 38 ± 2°. From these angles, an angle (averaged over the chlorophyll a and chlorophyll b in the membrane) of 45 ± 5° was calculated for that

between the plane of the porphyrin ring and the plane of the membrane.

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By

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INTRODUCTION

In green algae and higher plants, photosynthesis is the process occurring in the chloroplasts in which (a) electromagnetic energy from incident visible light activates the reduction of nicotinamide adenine ($NADP^+$) to NADPH and the oxidation of water, and (b) carbon dioxide is reduced to $\{CH_2O\}_n$. (Lehninger, 1970)

The grana contain essentially all the photosynthetic pigments of the chloroplast as well as the enzymes required for the primary light-dependent reactions. The paired thylakoid membranes are the sites of the light-trapping systems in the chloroplast structure (Rabinowitch and Govindjee, 1969). The isolated chloroplast lamellae when illuminated perform electron transport from water to ferredoxin, yielding oxygen gas and reduced ferredoxin. Phosphorylation of ADP to ATP accompanies this electron transport (Hill, 1937; Hill, 1965; Arnon et al., 1954).

The correspondence between photochemical action spectra and the light absorption spectra of various green algae and photosynthetic higher plants have led to the conclusion that chlorophyll must be the primary light-trapping molecule in green cells (Clayton, (1971).

Because of the complexity of the photosynthetic system

detailed investigation of the primary physical processes of photosynthesis, involving energy and electron transfer (Clayton, 1965), has been very difficult. A great deal has been inferred from the study of simpler systems. The properties of chlorophyll in solution (Goedheer, 1966), in the solid state (Ke, 1966; Cherry, 1968), and in monolayers (Ke, 1966) have been studied.

The light reactions of photosynthesis and the associated electron transport reactions have been shown to occur within the internal membrane system of chloroplasts, while the CO_2 fixation reactions occur within the stroma regions of the chloroplast (Trebst et al., 1958; Park and Pon, 1961). The thylakoid membrane is composed of about 52% lipid and 48% protein by weight, with about 10% being chlorophyll (Park and Biggins, 1964).

The lamellar structure of the photosynthetic apparatus undoubtedly is important in its function. Thus, studies of chlorophyll in solution or in crystalline form have provided a limited amount of information which is directly relevant to the role of the membrane in photosynthesis. Artificial lipid membranes have been employed to study various components of biological membranes in a bilayer environment.

A great deal of evidence has been accumulated in recent years indicating that the gross structure of many biological membranes is that of a fluid lipid bilayer matrix in which are "dissolved" amphipathic intrinsic membrane proteins, lipoproteins, and glycoproteins. (See, for example, Singer and Nicolson, 1972; Bretscher, 1973. Many additional references are contained therein.)

Electron paramagnetic resonance studies with phospholipid bilayers and rabbit sarcoplasmic reticulum by McConnell and co-workers have indicated that lipids may be very mobile in the plane of the membrane (Kornberg and McConnell, 1971; Scandella et al., 1972), but much less mobile in a direction perpendicular to the plane of the membrane (Kornberg and McConnell, 1971; McNamee and McConnell, 1973).

Studies by Frye and Edidin on intrinsic membrane proteins complexed with fluorescent-labeled specific antibodies in the envelopes of human cells and mouse cells caused to fuse under the influence of Sendai virus, and by Nicolson and Singer on red blood cell intrinsic membrane proteins complexed with specific ferritin-labeled antibodies, have shown that the proteins "dissolved" in the plasma membrane may also be quite mobile laterally. (Frye and Edidin, 1970; Nicolson and Singer, 1971a; Nicolson and Singer, 1971b; Nicolson and Singer, 1971c)

The black lipid membrane has been introduced as a model system for the study of biological membrane components in a bilayer lipid matrix separating two aqueous solutions by Mueller, Rudin, Tien, and Wescott. (Mueller, Rudin, Tien, and Wescott, 1962) The black lipid membrane exhibits many properties which are similar to those of biological membranes, e.g., thickness, resistance, capacitance, and interfacial tension (Tien, 1971).

The chlorophyll black lipid membrane separating two aqueous phases has been proposed as a model system for the study of the primary processes of photosynthesis of green plants. Various properties of black lipid membranes in the dark have been measured,

e.g. water permeability, bifacial tension, thickness, resistance, and dielectric breakdown (Ting et al., 1968). Recently, light-excitable properties such as fluorescence (Alamuti and Lauger, 1970), absorbance (Steinemann et al., 1971; Cherry et al., 1971), and photovoltage effects (Tien, 1968) have been investigated.

It has been found that with Fe^{3+} in one aqueous phase visible light incident on the spinach chloroplast extract chlorophyll-lipid bilayer induces a transmembrane voltage (Van and Tien, 1970). This was to be expected since a "photovoltaic" effect in layers of chlorophyll a, b, a+b, and other pigments applied to a metallic electrode lowered into an electrolyte had been observed and studied earlier by Yevstigneyev, Terenin, and co-workers (Yevstigneyev and Terenin, 1951; Yevstigneyev, 1962; Terenin and Putseiko, 1961; Yevstigneyev and Savkina, 1963; Putseiko, 1963).

More recently, Getov and Jordanova have found that in layers of chlorophyll a and b applied to a semi-transparent gold electrode illumination causes a "photo-emf," the gold electrode always being positive, and the spectral distribution of the photo-emf upon illumination on the electrode almost coincides with the optical absorption spectrum of chlorophyll (Getov and Jordanova, 1972).

The chlorophyll black lipid membrane photo-emf may be comparable to the trans-thylakoid voltage calculated by Witt and co-workers from absorbance changes at 515 nm of chlorophyll b during electron transport and photophosphorylation in spinach chloroplast preparations (Junge and Witt, 1968; Schliephake et al.,

1968; Witt, 1972). This calculation involved assumptions of concomitant trans-thylakoid proton transfer, thickness and dielectric constant of the membrane lipid layer, and the area of thylakoid covered by one electron transport chain. Witt et al. arrived at values of about 50 mV for $1.5(10^{-5})$ sec of "saturating intensity" excitation at 630 - 680 nm, about 200 mV for the maximum voltage upon excitation of longer duration, and in permanent light a steady-state value of about 100 mV.

The light-induced emf of the artificial chlorophyll-lipid membrane has been found to depend on the wavelength of the illuminating light (Van and Tien, 1970). A "photo-emf action spectrum" can be obtained by scanning the visible wavelengths.

The present work concerns the finding that the magnitudes of the peaks of the photo-emf action spectrum depend on the direction of polarization of the exciting light. This appears to be a direct consequence of the absorption properties of the chlorophyll in the artificial membrane, and may be used to determine the orientation of the chlorophyll porphyrin ring in the black lipid membrane. (Weller and Tien, 1973)

The principal blue transition moment was calculated to make an angle of $21 \pm 2^{\circ}$ with the plane of the membrane; the red transition moment, an angle of $38 \pm 2^{\circ}$. From these angles, an angle of $45 \pm 5^{\circ}$ is calculated for that between the plane of the porphyrin ring and the plane of the membrane. These values are averaged over the chlorophyll a and chlorophyll b present in the membrane.

There exists a possibility that the acidic bathing

solution employed in these experiments converts some or all of the chlorophyll a and chlorophyll b in the artificial membrane to pheophytin a and pheophytin b, respectively, by the removal of the Mg atom from the center of each porphyrin ring. This pheophytinization reaction would depend on the degree of exposure of the membrane chlorophyll porphyrin rings to the aqueous phases.

The porphyrin ring orientation angle determination in the experiments described herein does not supply enough information to ascertain the availability of the chelated chlorophyll Mg atom to the acidic bathing solutions. Thus there exists the possibility that the porphyrin ring orientation angle obtained may be for pheophytin and chlorophyll.

CHAPTER II

ORIENTATION OF THE CHLOROPHYLL PORPHYRIN RING IN MODEL MEMBRANE SYSTEMS AND IN BIOLOGICAL MEMBRANES: A LITERATURE REVIEW

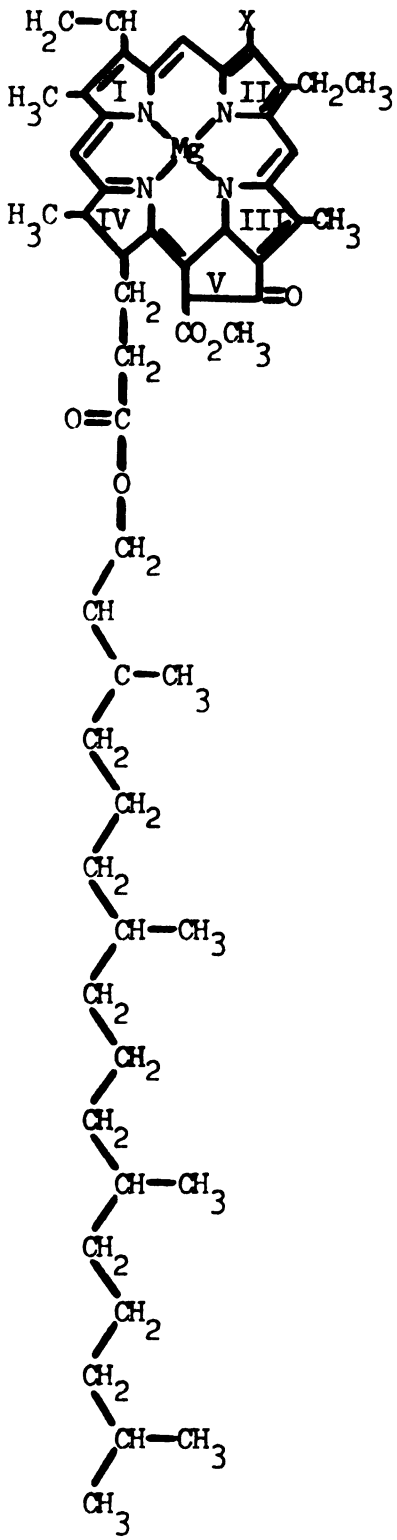
Some Chlorophyll Chemistry

Chlorophyll (Figure 1) is a molecule with an unusual combination of electron donor-acceptor properties (Katz, 1973). The ring V keto C=O group can function as donor, the central Mg atom as acceptor. In the absence of extraneous nucleophiles, donor-acceptor interactions form chlorophyll dimers, (Chl_2), and oligomers, $(Chl_2)_n$. With monofunctional electron donors, monomeric chlorophyll species form. Bifunctional donors may cross-link chlorophylls through Mg atoms to form large polynuclear adducts of colloidal dimensions.

Katz has examined the visible absorption spectra by computer deconvolution techniques and found considerable similarity between bulk or antenna chlorophyll in the plant and $(Chl_2)_n$. (Katz, 1973) Electron spin resonance studies have suggested that ESR photo-signal I associated with the photosynthetic reaction center of photosynthetic organisms could arise in a special pair of chlorophyll molecules $(Chl\ H_2O\ Chl)^+$.

Katz pointed out that these structures can be combined to give a structure that possesses both light-gathering properties and photoactivity, $(Chl_2)_n(Chl\ H_2O\ Chl)$. The junction is readily

Figure 1. Schematic representation of the structure of chlorophyll a or b. In chlorophyll a, X is $-CH_3$; in chlorophyll b, X is -CHO (after Lehninger, 1970).



porphyrin ring

phytyl chain

effected by a keto C=O---Mg interaction between the terminal chlorophyll molecule of $(Chl_2)_n$ and the chlorophyll of the special pair that still has an Mg atom available for coordination. (Katz, 1973)

This model accounts for both optical and ESR properties of plant chlorophyll. Such a structure can survive only if access of water to it is strictly limited, otherwise the entire structure will be converted to $(Chl \cdot H_2O)_n$. The electron-transport agents and the enzymes required for the subsequent chemical reactions of photosynthesis presumably would be in the membranes and hydrophilic regions of the chloroplast that surround the chlorophyll. (Katz, 1973)

Theoretical Work

Theoretical work based on experimental observations has suggested that chlorophyll molecules in photosynthetic systems are oriented, and that energy transfer would be much facilitated by suitable chromophore orientation.

For example, the finding that triplet excitation is greatly enhanced, while the quantum yield of fluorescence is diminished many-fold, in chlorophyll aggregates over monomeric chlorophyll suggested to Kasha that a suitable thylakoid concentration of chlorophyll might allow absorbed energy to be transferred in the chloroplast via excitation to a chlorophyll "exciton band," followed by triplet excitation. (Kasha, 1959)

While discussing energy reception and transfer in photosynthesis, Calvin speculated about the orientation of chlorophyll in

the chloroplast lamella and suggested that the porphyrin rings lie in a characteristic pattern, namely at an angle of about 45° to the stacking axis. (Calvin, 1958; Calvin, 1959)

Seely calculated that energy transfer between chlorophyll molecules by a "slow mechanism" (compatible with Forster's inductive resonance theory) would be fastest when the chlorophyll transition moment vectors are in a collinear arrangement and very small when the vectors are parallel but en echelon by an angle of 60° (Seely, 1973a; Seely, 1973b). This suggested that an expeditious use of orientation would be to group as many chlorophylls as possible into collinear files, staggered 60° from each other, so that transfer would be rapid the length of the file but slow from one file to another. The files would lead to the reaction center with as few changes in orientation as possible.

On the basis of X-ray crystallographic studies, Kreutz has postulated that the photosynthetic membrane is composed of three layers: protein, porphyrin ring, and lipid. (Kreutz, 1970; Kreutz, 1972) He felt that the chlorophyll molecules are anchored in the protein layer by means of their phytol chains, and the contact between protein and lipid is established by the porphyrin rings which partially penetrate into the unsaturated fatty acid zones of the lipid layer.

Based on the assumption that chloroplasts in the natural state exhibit a dichroic ratio of $D = 1$ for both the chlorophyll red and principal blue absorption bands (with the exception of chlorophyll-695, for which $D > 1$), Kreutz calculated that the porphyrin ring should make an angle of 54.7° with the lamella plane.

Model Systems

Chlorophyll is very hygroscopic (Ballschmiter and Katz, 1969) and water is necessary to form microcrystalline chlorophyll (Katz et al., 1968). Chlorophyll-water complexes have a similar electron paramagnetic resonance spectrum to photosynthesizing chloroplasts, whereas anhydrous chlorophyll does not. (Katz et al., 1968)

An infrared absorption study of chlorophyll-water aggregates has indicated that the water is hydrogen-bonded both to the ring V ketone carbonyl and to the O-2 ester carbonyl oxygen atoms of the adjacent molecule (Ballschmiter and Katz, 1969).

From X-ray diffraction determination of the structure of MgTPP·H_2) (Timkovitch and Tulinsky, 1969), MgPc·H_2O·$2C_5H_5N$ (Fischer et al., 1971), and methyl pheophorbide a (Fischer et al., 1972), Fischer and co-workers proposed a model of chlorophyll which has dimensions similar to methyl pheophorbide a with the Mg atom 0.50 Å out of the plane and a water molecule 2.02 Å above the Mg atom (Fischer et al., 1972). Hydrogen bonds connect the water molecule to the next chlorophyll. Repetition by simple translations would lead to molecular crowding, but repetition by a 2_1 screw axis would permit a satisfactory fit.

Hanson reported that chlorophyllide can form a monolayer consisting of close-packed porphyrin rings, and he assumed that the porphyrin planes are tilted at a 55° angle with the plane as in crystals. (Hanson, 1939)

From fluorescence polarization study on chlorophyll a-lipid monolayers at an air-water interface, Trosper and co-workers

concluded that in pure chlorophyll a monolayers the pigment molecules are unordered, in chlorophyll a-monogalactolipid monolayers the chlorophyll molecules are randomly dispersed, and in chlorophyll a-"lipid" monolayers (with sulfolipid, oleyl alcohol, or castor oil as "lipid") the chlorophyll molecules are partially oriented (the porphyrin rings making an angle of from 0° to 50° with the interface plane depending on the surface pressure). (Trosper, 1968)

Brody investigated monolayers of chlorophyll a "complexed" with various electron donors and acceptors at an air-water interface (Brody, 1971). From the surface area/chlorophyll molecule in each case, he calculated the angle between the porphyrin plane and the water surface. He found, for example, angles of 39°, 37°, 49°, 46°, and 49° for chlorophyll a "complexed" with phenazine methosulfate (PMS), PMS + ascorbate, benzyl viologen (BV), ascorbate, and dehydroascorbic acid, respectively.

Hoff incorporated chlorophyll a, chlorophyll b, and bacteriochlorophyll a in an oriented phospholipid multilayer and measured the orientation of the chlorophyll molecules by polarization absorbance spectroscopy (Hoff, 1974). The multilayer contained several hundred monolayers, with one chlorophyll molecule per 200 phospholipid molecules. He found angles of $55.4 \pm 1.1^{\circ}$, $51.6 \pm 0.6^{\circ}$, and $51.7 \pm 0.2^{\circ}$ between the porphyrin rings and the plane of the multilayer for chlorophyll a, chlorophyll b, and bacteriochlorophyll a, respectively.

From polarized absorption spectroscopy on an artificial

chlorophyll black lipid membrane with a chlorophyll concentration up to $2.5(10^{13})$ molecules/cm^2, Steinemann et al. found values of $23 \pm 2^{\circ}$, $27 \pm 2^{\circ}$, $29 \pm 3^{\circ}$, and $29 \pm 2^{\circ}$ for the angle between the principal blue transition moment and the membrane plane for chlorophyll a-phosphatidyl ethanolamine, chlorophyll a-dioleoyl-phosphatidyl choline, chlorophyll a-phosphatidyl serine, and chlorophyll b-dioleoyl-phosphatidyl choline membranes, respectively (Steinemann et al., 1972). They found angles of $35 \pm 1^{\circ}$, $34 \pm 1^{\circ}$, $36 \pm 2^{\circ}$, and $28 \pm 2^{\circ}$ for the red transition moments in the same membranes (in the same order).

From these angles, they calculated values of $44 \pm 3^{\circ}$, $46 \pm 3^{\circ}$, $49 \pm 5^{\circ}$, and $42 \pm 4^{\circ}$ for the angle between the porphyrin ring and the plane of the membrane for the above membranes (in the same order as above).

From polarized absorption spectroscopy on six chlorophyll-egg lecithin bilayers in series separated by aqueous phases, Cherry and co-workers obtained orientation angles of 26° and 29.5° for the chlorophyll a and chlorophyll b blue transition moments, respectively (Cherry et al., 1972). They found angles of 36.5° for both the chlorophyll a and chlorophyll b red transition moments. From these angles, they calculated values of 48° for chlorophyll a and 51° for chlorophyll b as the angle between the porphyrin ring and the plane of the membrane.

Hoff criticized the work of Cherry et al. on the grounds that (a) their technique is inherently much less sensitive than his multilayer technique, and permits only measurement of the dichroic

ratio at one fixed angle, and (b) their values are calculated by assuming that only one dipole moment contributes to the blue absorption band. (Hoff,1974)

Biological membranes

The first experimental results of Menke, Frey-Wyssling and Steinmann, and Ruch with polarized light microscopy on unicellular algae, Mougeotia and Closterium, were interpreted by these researchers as an effect of the stacking of lamellae in the grana (i.e., textural dichroism) rather than as an orientation of pigments. (Menke, 1938; Menke, 1958; Frey-Wyssling and Steinmann, 1948; Ruch, 1957)

Goedheer made absorption measurements in polarized monochromatic light on Mougeotia and found a weak dichroism in light of 680 nm. (Goedheer, 1955) He concluded that there was a slight orientation of chlorophyll a molecules.

Later, by means of linear dichroism and polarized fluorescence measurements on the unicellular algae Mougeotia and Euglena, Olson and co-workers detected a form of chlorophyll with maximum dichroism at about 705 nm and maximum polarized emission near 716 nm. (Olson et al., 1961; Olson et al., 1962; Olson et al., 1964a; Olson et al., 1964b)

Thomas et al. found a distinct dichroism at about 680 nm in spinach chloroplasts oriented at steel-water interfaces. They interpreted this to be due to 2% of the chlorophyll a-680 being oriented in the plane of the chloroplast lamellae. (Thomas et al., 1967)

Sauer and Calvin oriented spinach chloroplast fragments by electric field (Sauer and Calvin, 1962) or by velocity gradient (Sauer, 1965) and found a dichroic ratio significantly different from unity only at 695 nm.

In the case of orientation in a hydrodynamic gradient, they showed that the long wavelength absorption oscillator lies parallel to the streamlines of the sheer gradient, and assumed this to be the direction in which the planes of the chloroplast lamellae are oriented. They interpreted this dichroism at 695 nm as resulting from 5% of the chlorophyll a which is strongly oriented.

Morita and Miyazaki oriented the rod-shaped photosynthetic bacterium Rhodopseudomonas palustris cells in a flow-gradient and lamellae in a thin film. (Morita and Miyazaki, 1971) They found small dichroism at 590 nm and large dichroism at 800 nm and 870 nm.

Geacintov et al. oriented Chlorella cells and spinach chloroplasts in aqueous suspension by means of a static magnetic field. (Geacintov et al., 1971; Geacintov et al., 1972; Van Nostrand et al., 1973) They found significant dichroism in the chlorophyll absorption band at 675 - 678 nm and polarized fluorescence at about 685 nm, the chlorophyll a fluorescence band. They concluded that the bulk of the chlorophyll in vivo is highly oriented with its red transition moment preferentially parallel to the plane of the lamellae.

Breton and fellow researchers oriented spinach chloroplasts by application of a static magnetic field or by brushing them onto an optically polished quartz plate. (Breton et al., 1973) They oriented spinach chloroplast lamellae by brushing them onto a

polished quartz plate or by air-drying a drop of a suspension of isolated lamellae on the plate. They measured the linear dichroism spectrum of the oriented chloroplasts or lamellae with a spectropolarimeter and calculated the orientations of the dichroic absorption bands' transition moments.

They found that the y-polarized transition moments of chlorophyll a-680 and longer wavelength forms of chlorophyll a lie at angles close to the lamellar plane (i.e., at angles less than 25°- 30° with the plane). Chlorophyll a-670 is less oriented or oriented at an angle slightly less than 35° with the plane. "Negative" dichroism in the Soret band of chlorophyll a implies that the directions of x-polarized transitions are at angles of about 42°. Chlorophyll b-650 exhibited a low degree of order, making an angle less than 35° with the lamellar plane.

CHAPTER III

EXPERIMENTAL

Apparatus

The equipment and parts required for the measurement of the spinach chloroplast extract chlorophyll black lipid membrane photo-emf action spectrum and the chlorophyll orientation in the membrane are listed in Table 1. The experimental set-up is illustrated in Figure 2 (Feng, 1972; Van and Tien, 1970) and Figure 3.

One requirement for the membrane chamber is that the directions of the light beam incident on the membrane and the light beam reflected from the membrane be perpendicular to the plane of the glass through which each passes. The other requirements are that the membrane chamber provide a stable support for the Teflon beaker and be of sufficient height to allow the outer aqueous medium to extend above the hole in the beaker.

The membrane chamber must be isolated from the vibrations caused by the cooling fan for the arc lamp. This was achieved by placing styrofoam pads under the lamp housing and under the stand supporting the membrane chamber. Good electrical insulation was obtained by the use of non-metallic supports and coaxial cable.

The 2 mm diameter hole for the membrane was bored in the side of Teflon beaker below the level to which the aqueous medium,

Table 1. List of the equipment and materials necessary to perform the chlorophyll black lipid membrane experiments described in the text.

Item	Model and Catalogue No.	Manufacturer
Electrometer, vibrating reed	Cary Instruments 31	Applied Physics Corp. 2724 S. Peck Road Monrovia, California
Electrodes (2)	Fiber junction calomel 39270	Beckman Instrument Co. 25511 Southfield Rd. Southfield, Michigan 48075
Light source, D.C. Xenon arc lamp	Hanovia, Type 976C (1000 W)	Engelhard Hanovia Inc. Newark, New Jersey
Lamp housing	Schoeffel LH-151N	Schoeffel Instr. Co. 24 Booker St. Westwood, New Jersey
Lamp power supply	Schoeffel LPS 255	"
Eyepiece	No. 70,266	Edmund Scientific Co. Harrington, New Jersey 08007
Magnetic bars	No. 40,418	"
Optic lenses	----------	"
Cell assembly Glass cup, Teflon beaker	10 ml	Will Scientific Co. Box 63 Ann Arbor, Michigan 48107
Micropipette	Sampler	Oxford Laboratories
Shutter	Photographic	Obtained locally
Monochromator visible grating	Bausch and Lomb, Model 5	Bausch and Lomb, Inc. Rochester, New York 14602
Synchronous motor	Hurst, Model AR-DA (1/3 RPM)	Hurst Co. Princeton, Indiana

Table 1 (cont'd).

Item	Model and Catalogue No.	Manufacturer
Coaxial cable	----------	Amphenol-Borg Electronics Co. Broadview Chicago, Illinois
Electrical connectors	No. 202 HH Bakelite insulated phone tip jack	Obtained locally
Syringe-dispensor	Model PB-600-1 (100 µl)	Hamilton Co. Whittier, Calif.
Electrical noise shield	Copper wire mesh	Obtained locally
Light polarizer	Polaroid	Obtained locally

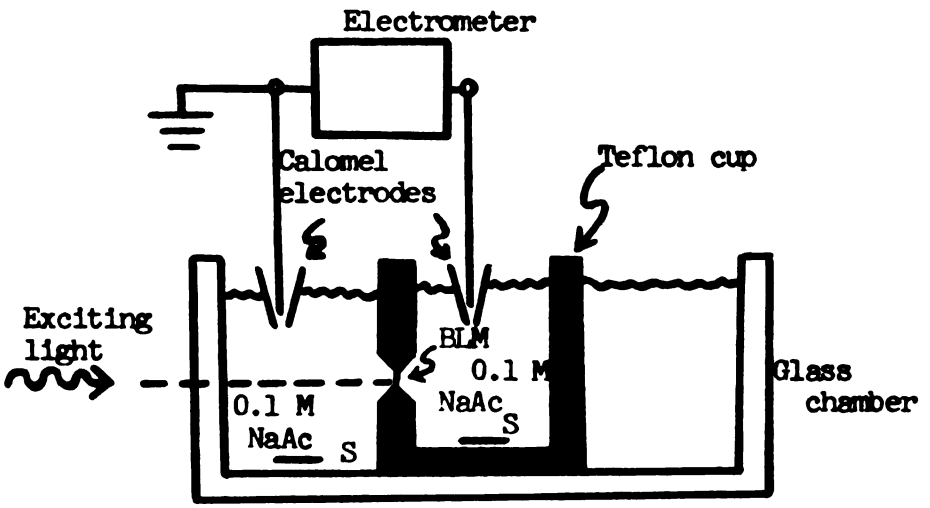


Figure 2. Schematic diagram of the membrane chamber and the electronics for measuring the photo-emf. S, magnetic stirring bar. BLM, black lipid membrane.

0.1 M potassium acetate, was added. The membrane was formed by applying a small amount of the membrane-forming solution with a micro-syringe. To facilitate application, a 2 - 4 mm piece of 0.038" polyethylene tubing was placed on the end of the micro-syringe.

The positions of the lenses were adjusted until the exciting light was focused upon the membrane. The visible wavelengths were scanned by rotating the monochromator cam with a small motor (Hurst Model AR DA) at approximately 2.5 nm/sec. The light-induced membrane emf was monitored with a calomel electrode in each aqueous phase and a Cary 31 electrometer. A convenient recorder speed was 2 in/min.

Extraction Techniques

The membrane-forming solution was prepared by isolating chloroplasts from commercial spinach and then extracting chlorophyll and lipoid materials. All glassware employed was rinsed with acetone and hot distilled water before use in order to avoid contamination by soaps. The specific steps in the extraction are given below (Tien and Howard, 1969).

1. The ribs and stalks were removed from 10 oz fresh spinach. The leaves were washed thoroughly, then dried.

2. The leaves were added to a 300 ml solution of 0.5 M sucrose and 0.05 M $KHCO_3$ buffer (pH 7.5) in a Waring blender at low speed. When all the leaves were added, high speed was used for 30 sec. The mixture was then

filtered through four layers of cheesecloth; the residue was discarded.

3. The filtrate was centrifuged in approximately 40 ml quantities at 8700 g for 5 minutes; the supernatant was discarded.

4. The chloroplasts were re-suspended in the buffered sucrose solution (approximately 15 ml per test tube) using the Vortex mixer, then centrifuged at 8700 g for 5 minutes, discarding the supernatant.

5. The chloroplasts were broken by adding 25 ml of glass-distilled water to each test tube, mixed with the Vortex mixer, then allowed to stand for 5 minutes. The mixture was then centrifuged at 9700 g for 10 minutes; the supernatant was discarded.

6. The residue was extracted with 90 ml of 2:1 petroleum ether: methanol (by volume) in the Waring blender at medium speed for 1 minute, then centrifuged at 5100 g for 10 minutes.

7. The top layer was pipetted off into a dry flask and evaporated to dryness. It was then re-suspended in 5 ml of 1:1 n-butanol: dodecane (by volume), and stored in the dark at 0 - 4°C.

Procedure

The membrane was formed in 0.1 M acetate buffer, pH 5, across a circular aperture of 2 mm diameter in a Teflon beaker set inside a glass cup. After the membrane had reached the black stage, $FeCl_3$ was added to the inner chamber to bring the Fe^{3+} ion concentration to 1 mM. The open-circuit potential difference across the membrane was monitored by a Cary 31 electrometer via a calomel electrode in the aqueous phase on each side of the membrane (Tien and Howard, 1969).

The black lipid membrane was excited with light from a 1000 W D.C. Xenon arc lamp (Hanovia, Type 976 C) which was passed through a heat filter, a visible grating monochromator (Bausch and Lomb, Model 5), a plano-convex lens, a shutter, a converging lens, a polarizer, and a collimator (Figure 3). During formation, the membrane was observed with dim green light($\sim$525 nm).

After application of the membrane-forming solution across the aperture, the membrane thinned first to a thickness of less than 1 μm. At this stage, if the membrane is observed at an angle with the normal equal to the angle of incidence of illuminating light, interference fringes are seen (Figure 4a). In this way, the orientation of the membrane relative to the direction of propagation of the incident light was determined.

Most of the light reflected from the membrane is polarized with the direction of vibration of the electric vector parallel to the plane of the membrane, i.e., perpendicular to the plane of incidence (Figure 4b). The direction of polarization of the

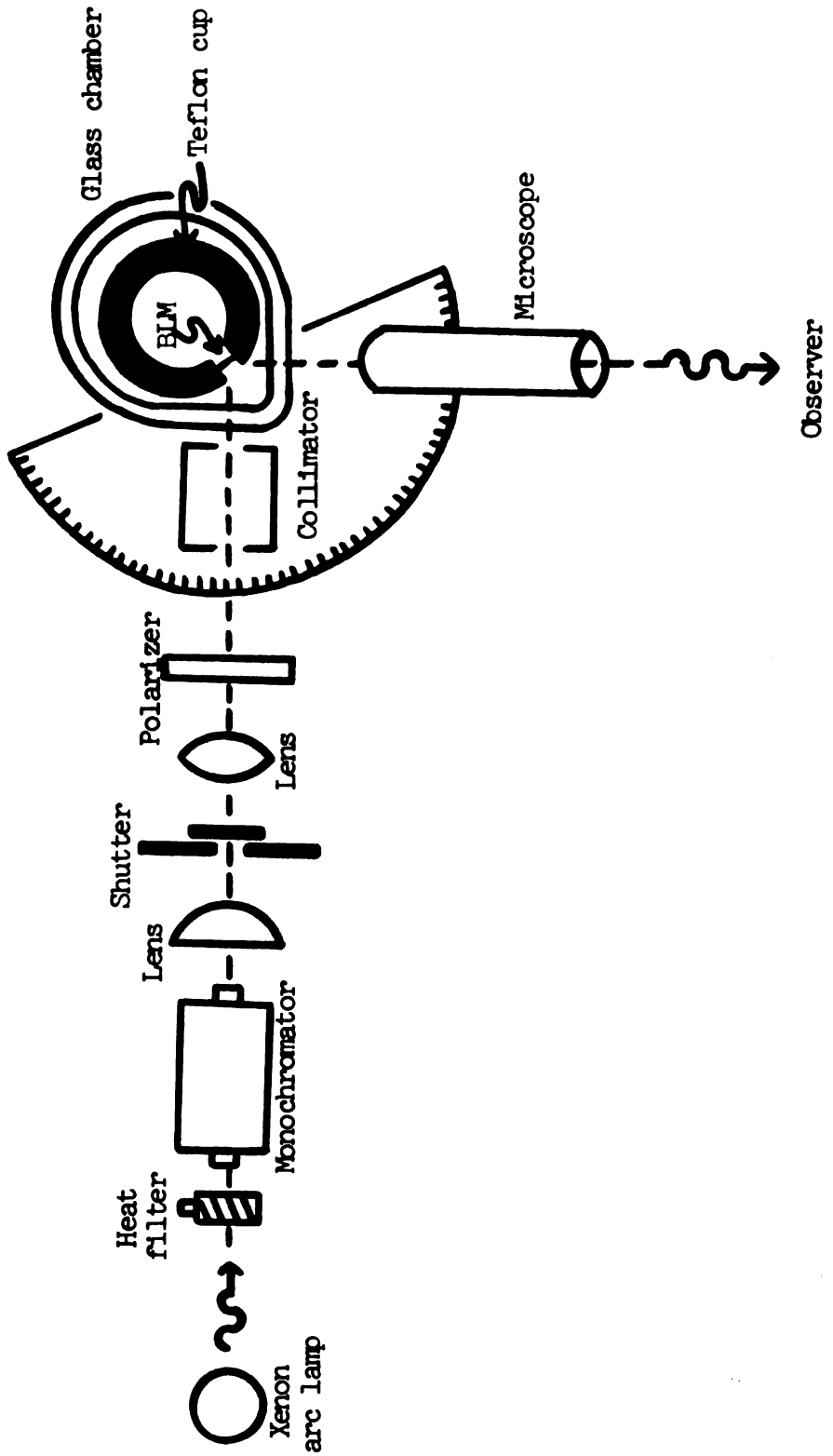
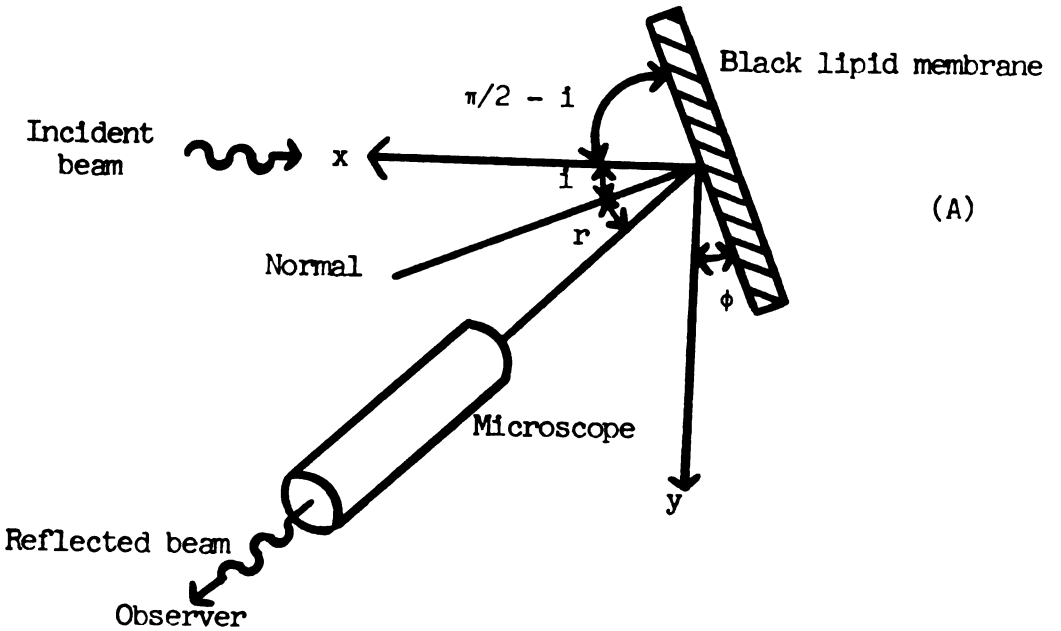


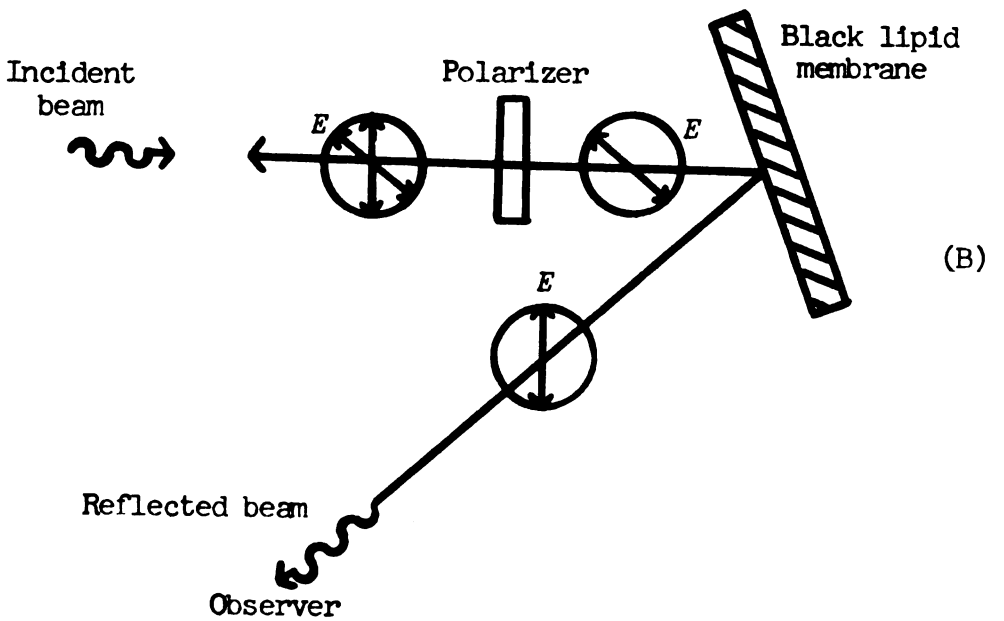
Figure 3. Schematic diagram of optic set-up (top view).

Figure 4. (A) Schematic diagram illustrating the determination of the orientation of the membrane relative to the direction of propagation of the incident light, -x (top view). i, angle of incidence; r, angle of reflection. $i = r = \phi$.

(B) Schematic diagram illustrating the establishment of the direction of polarization of the incident light with the electric vector, *E*, vibrating parallel to the plane of incidence (top view).


Black lipid membrane
π/2 - i
Incident beam
x
i
r
Normal
ϕ
Microscope
y
Reflected beam
Observer

(A)


Black lipid membrane
Incident beam
Polarizer
E
E
E
Reflected beam
Observer

(B)

illuminating light was varied, by rotating the polarizer about the direction of propagation, until the direction for which the interference fringes were observed to have a minimum intensity. This established the direction of polarization of the incident light with the electric vector vibrating parallel to the plane of incidence.

When the thickness of the membrane has fallen much below 1000 Å, destructive interference gives rise to the optically "black" appearance (Tien and Howard, 1969). When the "black" membrane is illuminated with exciting light, an electromotive force (open-circuit voltage) is generated across it, with the side in contact with Fe^{3+} ion becoming more negative than the other side. The magnitude of this "photo-emf" is dependent upon the wavelength of the exciting light, and a "photo-emf action spectrum" can be obtained by scanning the visible wavelengths (Van and Tien, 1970).

CHAPTER IV

THEORETICAL CONSIDERATIONS

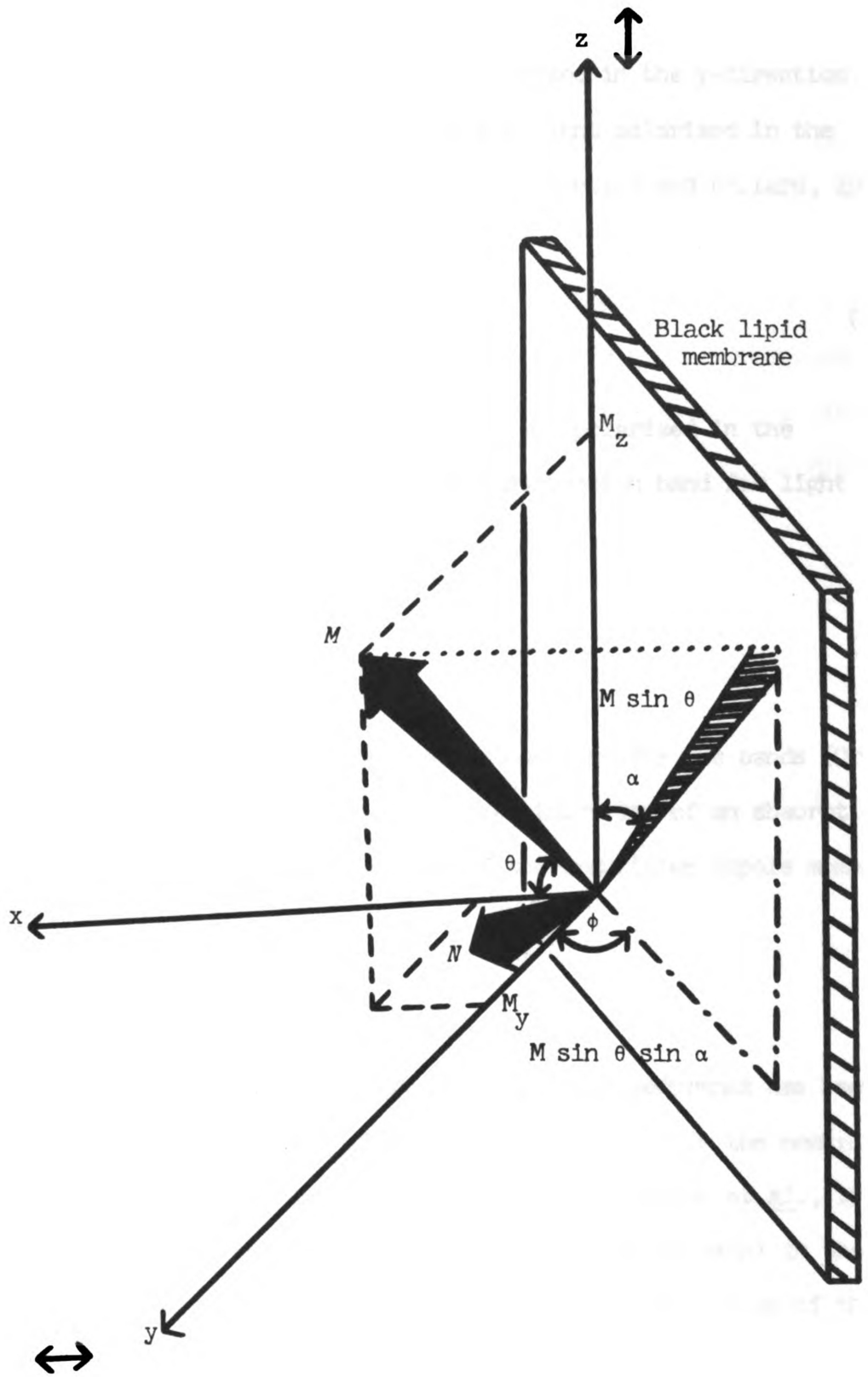
Before presenting the results and discussing their significance, a consideration of some aspects of theoretical background upon which the present interpretation is based is in order. First, it is assumed that the chlorophyll black lipid membrane is a lyotropic liquid crystalline system with smectic structure. In a smectic structure the molecules are arranged in layers, with their long axes parallel to each other and approximately normal to the plane of the layers. The molecules can move in two directions in the plane and can rotate about one axis (Brown, 1967). In the chlorophyll black lipid membrane, each transition moment is probably restricted to any direction which lies on a conical surface making an angle of θ with the normal, N, as depicted in Figure 5.

The orientation of each transition moment, M, is given by the angle between M and the normal, N, to the membrane. The components of M along the two directions, y and z, of polarization of the incident light are M_y and M_z, where

$$M_y = M \sin \theta \sin \alpha \cos \phi + M \cos \theta \sin \phi \qquad (1)$$

$$M_z = M \sin \theta \cos \alpha \qquad (2)$$

Figure 5. Schematic diagram of the transition dipole moment, ***M***, of either the red or the principal blue chlorophyll absorption band (see text). -x, the direction of propagation of the exciting light.


z
Black lipid membrane
M_z
M
M sin θ
α
θ
x
ϕ
N
M_y
M sin θ sin α
y

The dichroic ratio, D, which is defined as the ratio of the absorbance for monochromatic light polarized in the y-direction (i.e. horizontally polarized) to that for the light polarized in the z-direction (i.e., vertically polarized) (Setlow and Pollard, 1962), is given by

$$D = \frac{A_y}{A_z} \tag{3}$$

If the absorption band for light polarized in the y-direction has the same shape as the absorption band for light polarized in the z-direction, then

$$\frac{A_y}{A_z} = \frac{I_y}{I_z} \tag{4}$$

where I_y and I_z are the integrated intensities for the bands (Orchin and Jaffé, 1971). Since the integrated intensity of an absorption band is proportional to the square of the transition dipole moment,

$$\frac{A_y}{A_z} = \frac{I_y}{I_z} = \frac{M_y^2}{M_z^2} \tag{5}$$

Dichroism of chlorophyll black lipid membranes has been attributed to orientation of chlorophyll molecules in the membrane (Cherry et al., 1971; Cherry et al., 1972; Steinemann et al., 1972). The dependence of the magnitude of the blue and red peaks in the photo-emf action spectrum on the direction of polarization of the exciting light is apparently a direct consequence of the dichroism of the membrane. If so, then ratios of emf magnitudes of each peak

for the stated two directions of polarization of incident light should allow calculation of the orientations in the membrane of the transition dipole moments of the blue and red absorption bands. For each peak the ratio of photo-emfs, $(E_{h\nu})_y/(E_{h\nu})_z$ varied less than 8% with a fourfold increase in light intensity.

If the photo-emf, $E_{h\nu}$, is proportional to the amount of light energy absorbed by the membrane, then the ratio of the emf magnitudes for horizontally polarized light to vertically polarized light for the red peak or the principal blue peak gives the ratio of the absorbances for these directions of polarization (see Appendix A), and

$$\frac{(E_{h\nu})_y}{(E_{h\nu})_z} = \frac{M_y^2}{M_z^2} \tag{6}$$

Since each transition moment is probably restricted to any direction which lies on a conical surface making an angle of θ with the normal, M_y and M_z must be integrated over all α,

$$\frac{(E_{h\nu})_y}{(E_{h\nu})_z} = \frac{(2\pi)^{-1} \int_0^{2\pi} M_y^2 \, d\alpha}{(2\pi)^{-1} \int_0^{2\pi} M_z^2 \, d\alpha} \tag{7}$$

$$\frac{(E_{h\nu})_y}{(E_{h\nu})_z} = \cos^2 \phi + 2 \operatorname{ctn}^2 \theta \sin^2 \phi \tag{8}$$

Since the orientation, ϕ, of the membrane is known, the last equation can be used to calculate the direction, θ, of each

transition moment from the magnitudes of the polarized light induced photo-emf peaks. Since there are both chlorophyll a and chlorophyll b in the spinach chloroplast (Park and Biggins, 1964), the value for θ thus obtained is actually an average over both types of chlorophyll present in the membrane.

Polarized absorption and fluorescence measurements with chlorophyll have shown that the two transition moments responsible for the red and principal blue absorption bands are perpendicular to each other and lie in the plane of the porphyrin ring (Rabinowitch, 1956; Goedheer, 1966). The orientations, θ_R and θ_B, of the red and principal blue dipole moments with respect to the normal, N, to the membrane then supply enough information that the orientation of the chlorophyll porphyrin ring can be calculated. With the assumptions that the red and principal blue dipole moments are mutually perpendicular and lie in the plane of the porphyrin ring, it may be shown (see Appendix B) that the angle, β, between the plane of the porphyrin ring and the normal to the membrane is given by the expression

$$\cos^2 \beta = \cos^2 \theta_R + \cos^2 \theta_B \qquad (9)$$

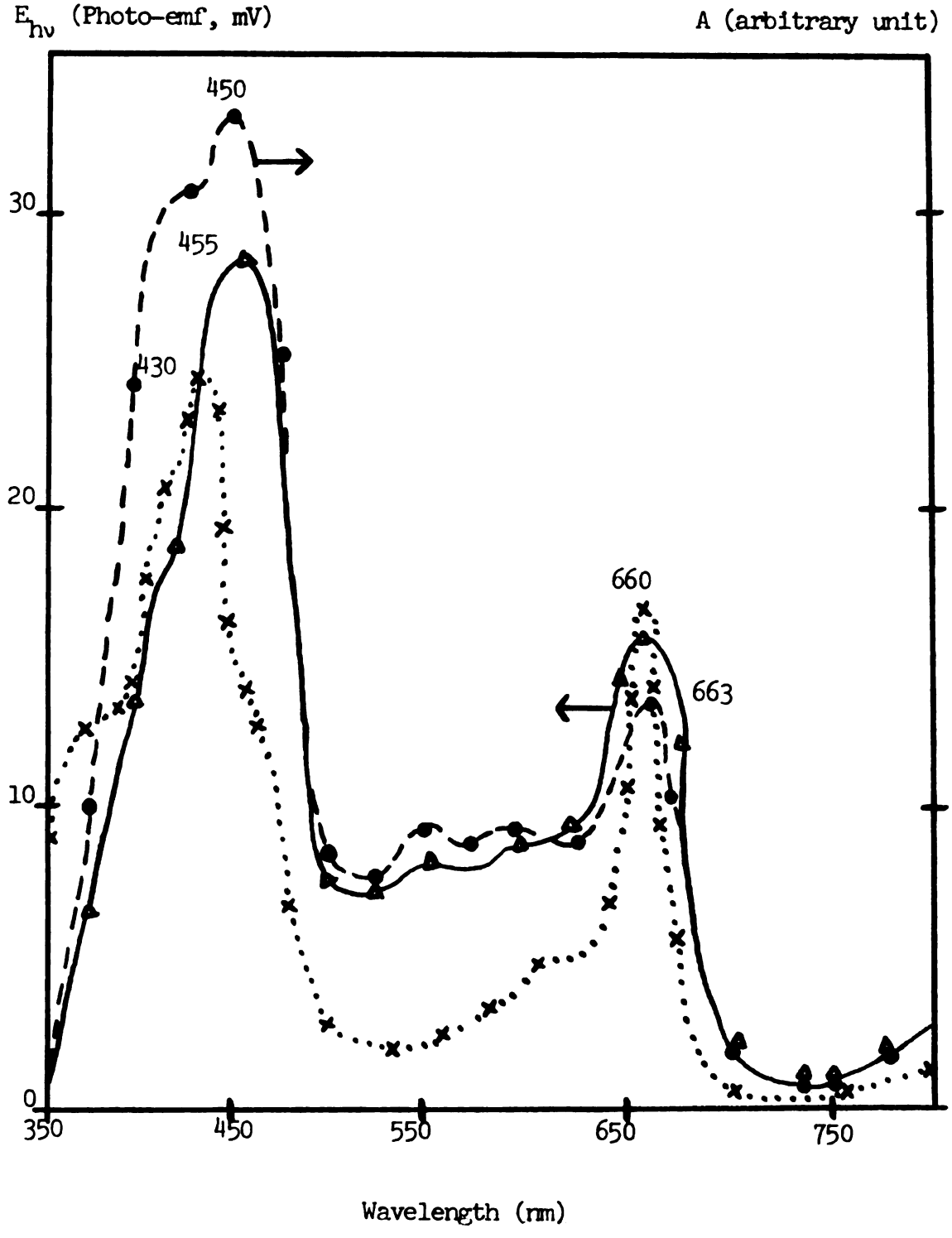
CHAPTER V

RESULTS AND DISCUSSION

A typical photo-emf action spectrum of a chloroplast extract black lipid membrane, obtained by the method outlined in the experimental section, is shown in Figure 6.. The photo-emf action spectrum shows a slight dependence on the direction of wavelength scan, with peaks shifting about 5 nm and each peak magnitude changing by a factoer of about 1.2. The peaks in the action spectrum are shifted to the red from the peaks for the bulk solution absorption spectrum. Otherwise, the photo-emf spectrum bears a strong resemblance to the absorption spectrum for chlorophyll a, with peaks at 430 and 660 nm. Other researchers have observed a red shift in the red and blue peaks from the chlorophyll absorption spectrum in bulk to the absorption spectrum in chlorophyll black lipid membranes (Steinemann et al., 1971; Cherry et al., 1971). It is likely that this is responsible for the red shift in the photo-emf action spectrum.

The photo-emf blue peak is of greater magnitude for the incident light polarized perpendicular to the plane of incidence than for it polarized parallel to it. The situation is opposite for the red peak. From the photo-emf peak values for horizontally and vertically polarized light, the orientation θ of each transition

Figure 6. Absorption spectrum and photo-emf action spectra of the spinach chloroplast extract black lipid membrane. .... , absorption spectrum of the spinach chloroplast extract membrane-forming solution in bulk. ----, photo-emf action spectrum of spinach chloroplast extract black lipid membrane, scan from 350 to 800 nm. ____, photo-emf action spectrum of spinach chloroplast extract black lipid membrane, scan from 800 to 350 nm. The action spectra have been corrected to show emf per unit light intensity.


$E_{h\nu}$ (Photo-emf, mV)
A (arbitrary unit)
450
455
430
660
663
30
20
10
0
350
450
550
650
750
Wavelength (nm)

moment can be calculated with the aid of the equations developed above. The results are summarized in Table 2. The principal blue transition moment was calculated to make an angle of 21 ± 2° with the plane of the membrane; the red transition moment, an angle of 38 ± 2°. From these angles, an angle (averaged over the chlorophyll a and chlorophyll b in the membrane) of 45 ± 5° is calculated for that between the plane of the porphyrin ring and the plane of the membrane (Weller and Tien, 1973).

These results can be compared with values for chlorophyll porphyrin ring orientation obtained from polarized absorption spectroscopy on artificial chlorophyll membranes by other researchers. For chlorophyll-egg lecithin black lipid membranes, Cherry et al. found angles of 48° for chlorophyll a and 51° for chlorophyll b (Cherry et al., 1972). Steinemann and co-workers found angles of 44 ± 3°, 46 ± 3°, and 49 ± 5° for chlorophyll a-phosphatidyl ethanolamine, chlorophyll a-dioleoyl-phosphatidyl choline, and chlorophyll a-phosphatidyl serine membranes, respectively. They found 42 ± 4° for chlorophyll b-dioleoyl-phosphatidyl choline membranes.

There exists the possibility that the acidic bathing solution employed in these experiments converts some or all of the chlorophyll a and chlorophyll b in the artificial membrane to pheophytin a and pheophytin b, respectively, by the removal of the Mg atom from the center of each porphyrin ring. The central Mg atom of chlorophylls is readily displaced by strong and weak acids (Willstatter and Hocheder, 1907).

Table 2. Orientations in the spinach chloroplast extract chlorophyll black lipid membrane of the transition dipole moments responsible for the chlorophyll red and principal blue absorption bands, and orientation of the chlorophyll porphyrin ring.

These values are averaged over the chlorophyll a and chlorophyll b present in the membrane.

Absorption peak	$\frac{(E_{h\nu})_y}{(E_{h\nu})_z}$	θ (°)	"Average" angle between transition moment and plane of the membrane ($\pi/2 - \theta$) (°)	"Average" angle between plane of porphyrin ring and plane of membrane ($\pi/2 - \beta$) (°)
Blue peak (455 nm)	0.69	69 ± 2	21 ± 2	45 ± 5
Red peak (660 nm)	1.08	52 ± 2	38 ± 2	

In aqueous acetone, the rate of pheophytinization is first order in acid concentration (Joslyn and Mackinney, 1938) and in chlorophyll concentration (Mackinney and Joslyn, 1940). The rate constant for Mg displacement in 20% aqueous acetone is 5 - 6 times larger for chlorophyll a than for chlorophyll b (Schanderl et al., 1962). Activation energy for chlorophyll a was about 11 kcal.

The pheophytinization of chlorophyll in the lipid bilayer would depend on the degree of exposure of the membrane chlorophyll porphyrin rings to the acid. Loss of Mg from chlorophyll has been found to be 13 times as fast in a chlorophyll monomolecular layer at an air-water interface (pH 4) as in acetone (Rosoff and Aron, 1965). The rate in the monolayer is sensitive to pressure and the presence of O_2, Ca^{++}, and Mg^{++}.

The rate constant for the monolayer pheophytinization decreased with increasing pressure. For example, the rate constant was $1.36(10^3)$ min^{-1} M^{-1} at an initial pressure of about 6 dynes/cm (i.e., a molecular area of 120 A^{o2} per chlorophyll molecule) and $1.43(10^2)$ $min^{-1}M^{-1}$ at 16 dynes/cm. These results suggested that the change in orientation of the chlorophyll molecules in the monolayer is responsible for the availability of the porphyrin ring Mg for reaction.

However, the chlorophyll embedded in a lipid bilayer matrix may not be as exposed to the acidic bathing solution as chlorophyll in solution or in a monolayer. The porphyrin ring orientation angle determination in the experiments described herein does not supply enough information to ascertain the availability of

the chelated chlorophyll Mg atom to the acid.

Comparison of the photo-emf action spectrum and the absorption spectra of chlorophyll in solution and in a lipid bilayer with the absorption spectra of pheophytin in solution seems to indicate that a very small amount of chlorophyll in the chloroplast extract chlorophyll-lipid bilayer is pheophytinized.

The absorption maxima for chlorophyll a and chlorophyll b in ether have been reported as 430 nm, 662 nm and 453 nm, 642 nm, respectively. The maxima for pheophytin a and pheophytin b in ether are 408 nm, 667 nm and 434 nm, 655 nm, respectively (Goedheer, 1966). Pheophytinization shifts the principal blue peak about 20 nm to a lower wavelength and the red peak 5 - 13 nm to a higher wavelength.

However, Cherry et al. found maxima at 439 nm, 672 nm and 466 nm, 653 nm for chlorophyll a and chlorophyll b, respectively, in the chlorophyll-egg lecithin bilayer (Cherry et al., 1972). These are red shifts from the bulk spectrum of 9 - 13 nm for the principal blue peaks and about 11 nm for the red peaks. Steinemann and co-workers found maxima at 437 nm and 672 nm for chlorophyll a in the chlorophyll-dioleoyllecithin bilayer (Steinemann et al., 1971). These are red shifts of about 5 nm for the principal blue peak and 10 nm for the red peak from the bulk spectrum.

The photo-emf actiwn spectrum peaks (Figure 6) for the chloroplast extract chlorophyll black lipid membrane are red-shifted 20 - 25 nm from the bulk absorption spectrum for the principal blue peak and about 3 nm for the red peak.

Pheophytinization of chlorophyll, or even subsequent

complexing of a Fe atom in the center of the porphyrin ring, would change only slightly the directions of the red and principal blue transition moments relative to the symmetry axes of the porphyrin ring. For example, the direction of the chlorophyll red transition moment vector is shifted about 4° by replacement of the chelated Mg atom by a Fe atom (Platt, 1956).

The calculation of the angle of tilt, β, of the porphyrin ring is not unduly sensitive to the assumption of a right angle between the red and principal blue transition moments. For example, if this angle is perturbed up to 10° from the value of 90°, the resultant uncertainty in β is still only $\pm 5^{\circ}$ (Cherry et al., 1972).

The polarized light-induced emf in black chlorophyll-lipid membranes is apparently a sensitive technique for investigating the orientation of chlorophyll in a bilayer lipid membrane. It requires only one membrane and a simple electronic set-up. Furthermore, the existence of the phenomenon of light-induced emf in artificial chlorophyll membranes suggests that a similar phenomenon may occur in vivo in the chloroplast thylakoid membrane during the process of transduction of light energy into chemical energy.

CHAPTER VI

MISCELLANEOUS

Photoelectric Action Spectra with Various Additional Components in the Bathing Solutions

The dependence of the photo-emf action spectrum of the spinach chloroplast extract chlorophyll black lipid membrane upon the direction of polarization of the exciting light was measured for the following four sets of bathing solution components (Figures 7 - 10). The exciting light was incident initially upon the solution shown at the left side of the membrane.

1.	Fe^{3+} (1mM) HAc (pH 5)	Black Lipid Membrane	"Water-soluble" chlorophyll HAc (pH 5)
2.	Fe^{3+} (1mM) HAc (pH 5)	Black Lipid Membrane	Thionine (10^{-4} M) HAc (pH 5)
3.	Fe^{3+} (1mM) HAc (pH 5)	Black Lipid Membrane	p-Benzoquinone (3)(10^{-4} M) HAc (pH 5)

Figure 7. Photo-emf action spectra of the spinach chloroplast extract black lipid membrane with 1 mM $FeCl_3$ in the outer chamber and "water-soluble chlorophyll" in the inner chamber. 0.1 ml of "water-soluble chlorophyll" from K&K Laboratories, Inc., Plainview, New York (no concentration value was supplied by them) was added. ____, action spectrum with unpolarized exciting light. ----, action spectrum with the exciting light polarized perpendicular to the plane of incidence. .... , action spectrum with the exciting light polarized parallel to the plane of incidence.

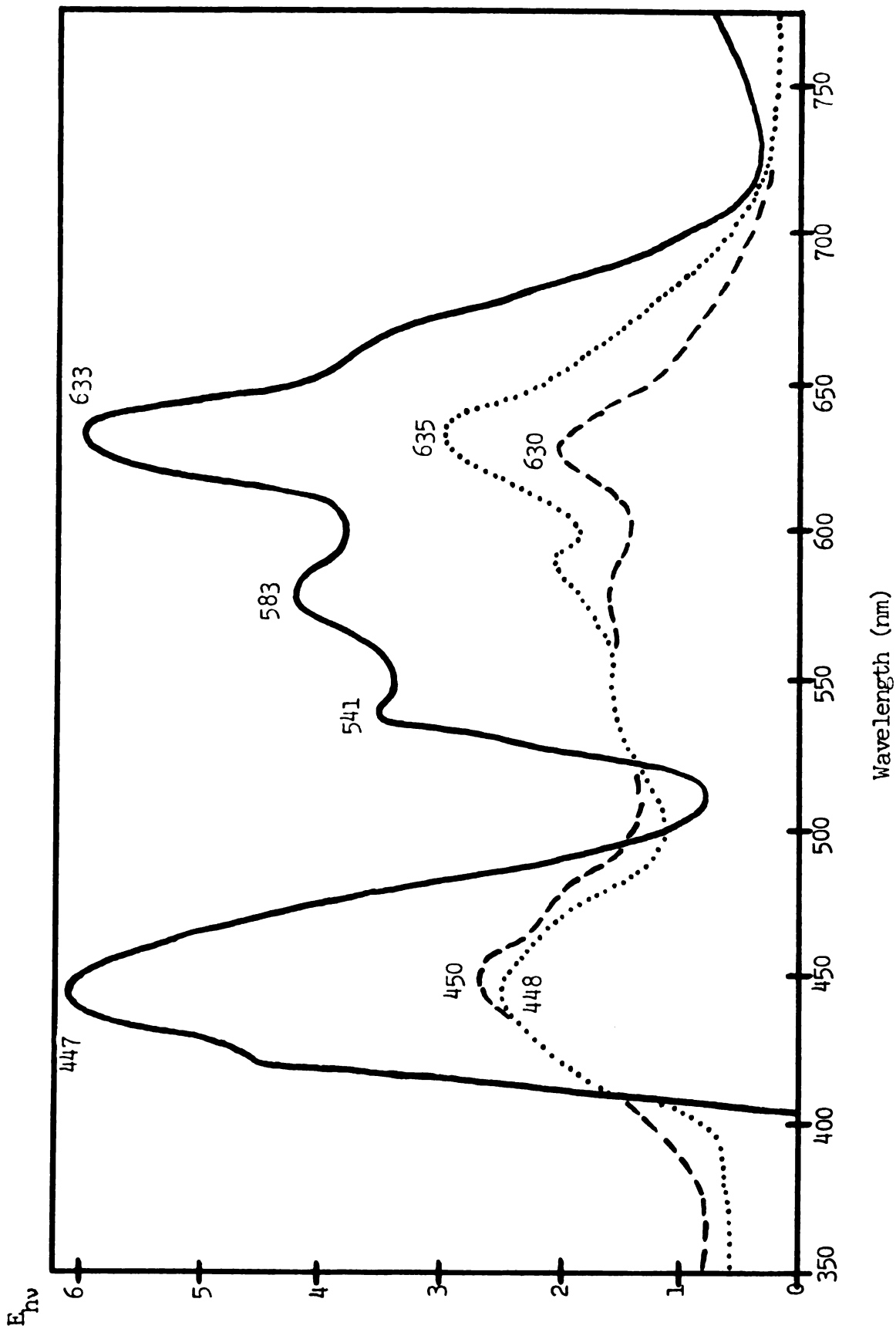

$E_{h\nu}$
0
1
2
3
4
5
6
350
400
450
500
550
600
650
700
750
Wavelength (nm)
447
448
450
541
583
633
630
635

Figure 8. Photo-emf action spectra of the spinach chloroplast extract black lipid membrane with 1 mM $FeCl_3$ in the outer chamber and 0.1 mM thionine in the inner chamber. ____, action spectrum with unpolarized exciting light. ----, action spectrum with the exciting light polarized perpendicular to the plane of incidence. .... , action spectrum with the exciting light polarized parallel to the plane of incidence.

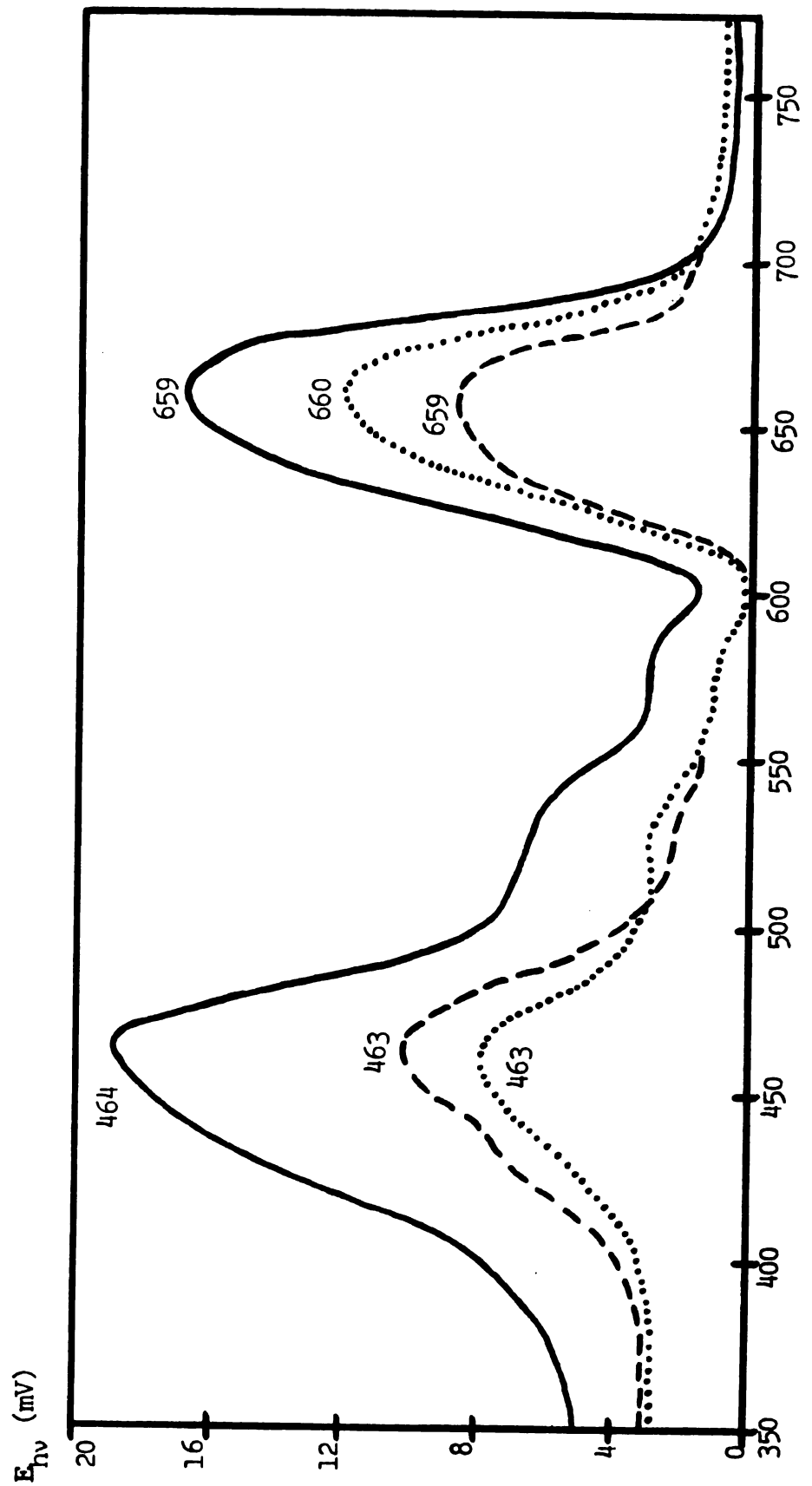

$E_{h\nu}$ (mV)
20
16
12
8
4
0
350
400
450
500
550
600
650
700
750
Wavelength (nm)
464
463
463
659
660
659

Figure 9. Photo-emf action spectra of the spinach chloroplast extract black lipid membrane with 1 mM $FeCl_3$ in the outer chamber and 0.3 mM p-benzoquinone in the inner chamber. ____, action spectrum with unpolarized exciting light. ----, action spectrum with the exciting light polarized perpendicular to the plane of incidence. .... , action spectrum with the exciting light polarized parallel to the plane of incidence.

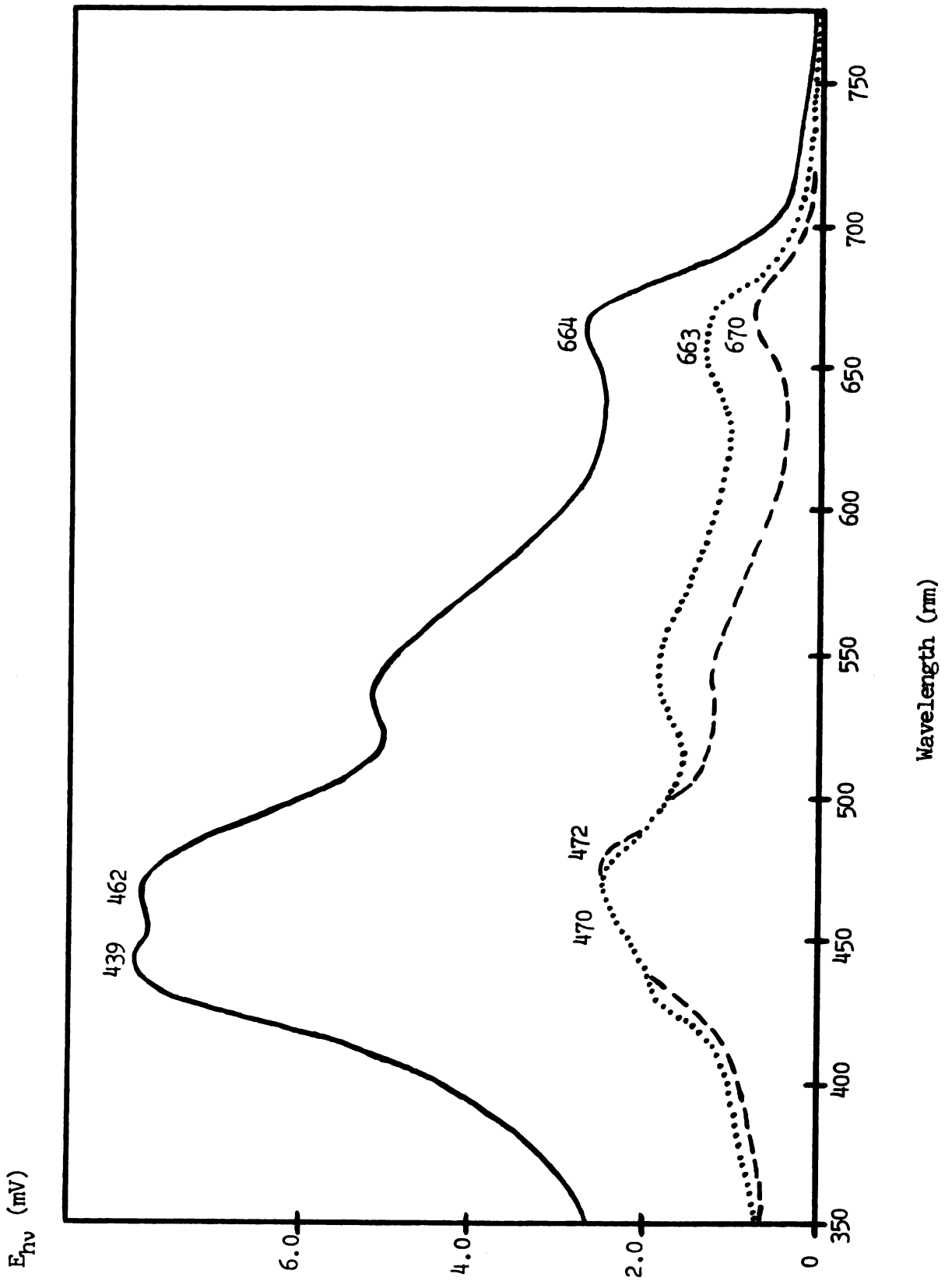
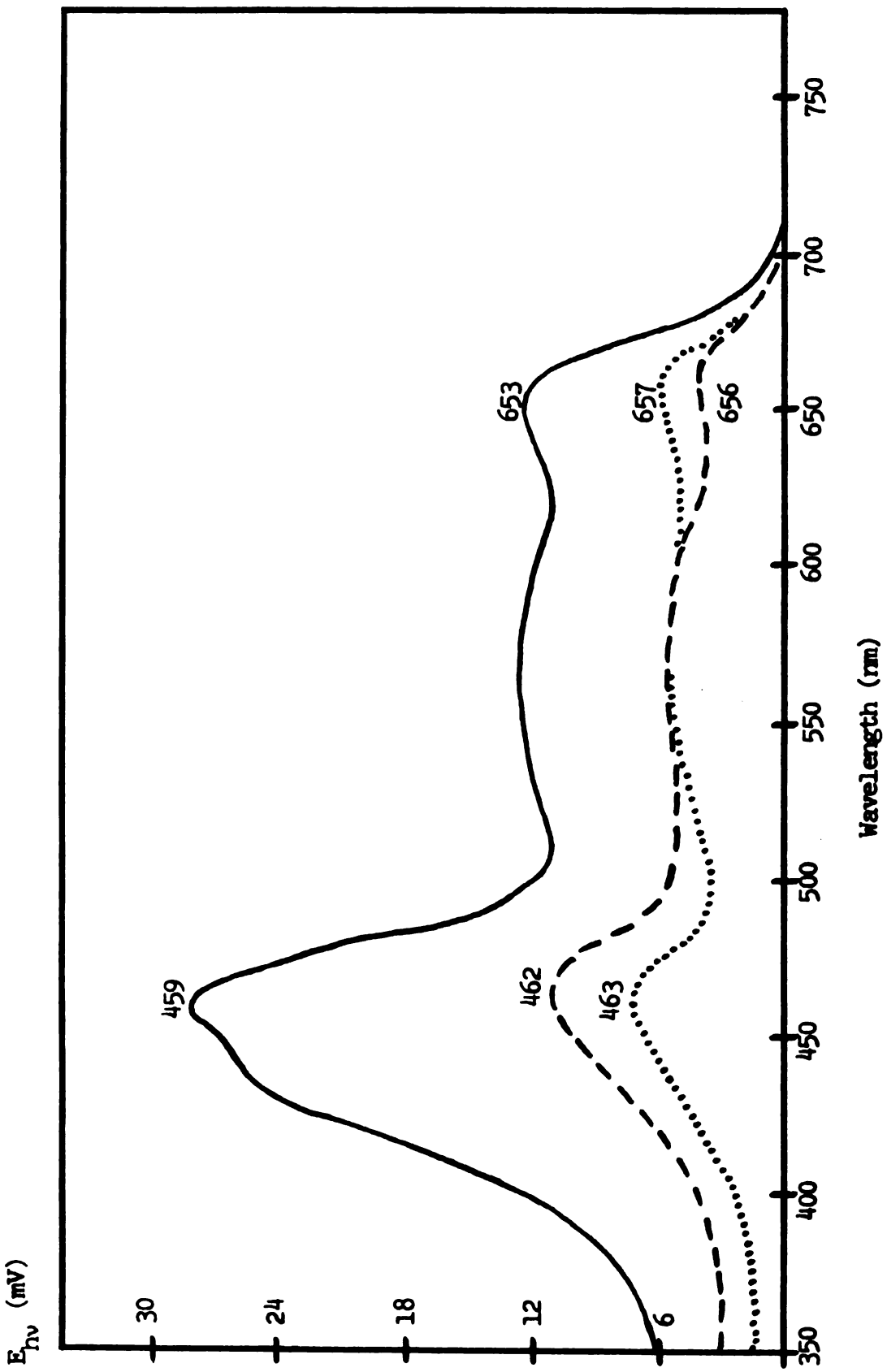

$E_{h\nu}$ (mV)
6.0
4.0
2.0
0
350
400
450
500
550
600
650
700
750
Wavelength (nm)
439
462
664
663
670
472
470

Figure 10. Photo-emf action spectra of the spinach chloroplast extract black lipid membrane with 1 mM $FeCl_3$ in the outer chamber and 4 mM L-ascorbic acid in the inner chamber. ____, action spectrum with unpolarized exciting light. ----, action spectrum with the exciting light polarized perpendicular to the plane of incidence. ...., action spectrum with the exciting light polarized parallel to the plane of incidence.


$E_{h\nu}$ (mV)
30
24
18
12
6
350
400
450
500
550
600
650
700
750
Wavelength (nm)
459
462
463
653
657
656

4.	Fe^{3+} (1mM) HAc (pH 5)	Black Lipid Membrane	L-Ascorbic acid (4) (10^{-3}) M HAc (pH 5)

All of these spectra exhibit a dependence of the red and principal blue peaks upon the direction of polarization of the exciting light which is similar to that exhibited by the membrane with only Fe^{3+} in one bathing solution. However, the addition of another species of chromophore ("water-soluble" chlorophyll or thionine) and/or the alteration of the photochemistry probably render incorrect an interpretation of changes in the ratio $(E_{h\nu})_y/(E_{h\nu})_z$ as reflecting only changes in the orientation of the membrane chlorophyll rings.

Preferential Polarized Light "Photo-Bleaching" of Chlorophyll Molecules in the Membrane and the Attempt to Employ it for the Determination of a Rotational Diffusion Relaxation Time for the Membrane Chlorophyll Molecules

Under continuous illumination of the spinach chloroplast extract chlorophyll black lipid membrane with light of either the red or the principal blue photo-emf peak wavelength, the photo-emf first (a) increases linearly (Figure 11), then (b) the rate of increase of $E_{h\nu}$ decreases until (c) $E_{h\nu}$ remains constant or decreases. The existence of this saturation of the capacity of the membrane to transduce light energy into energy stored in an electric field suggests a possible method for the determination of the diffusional

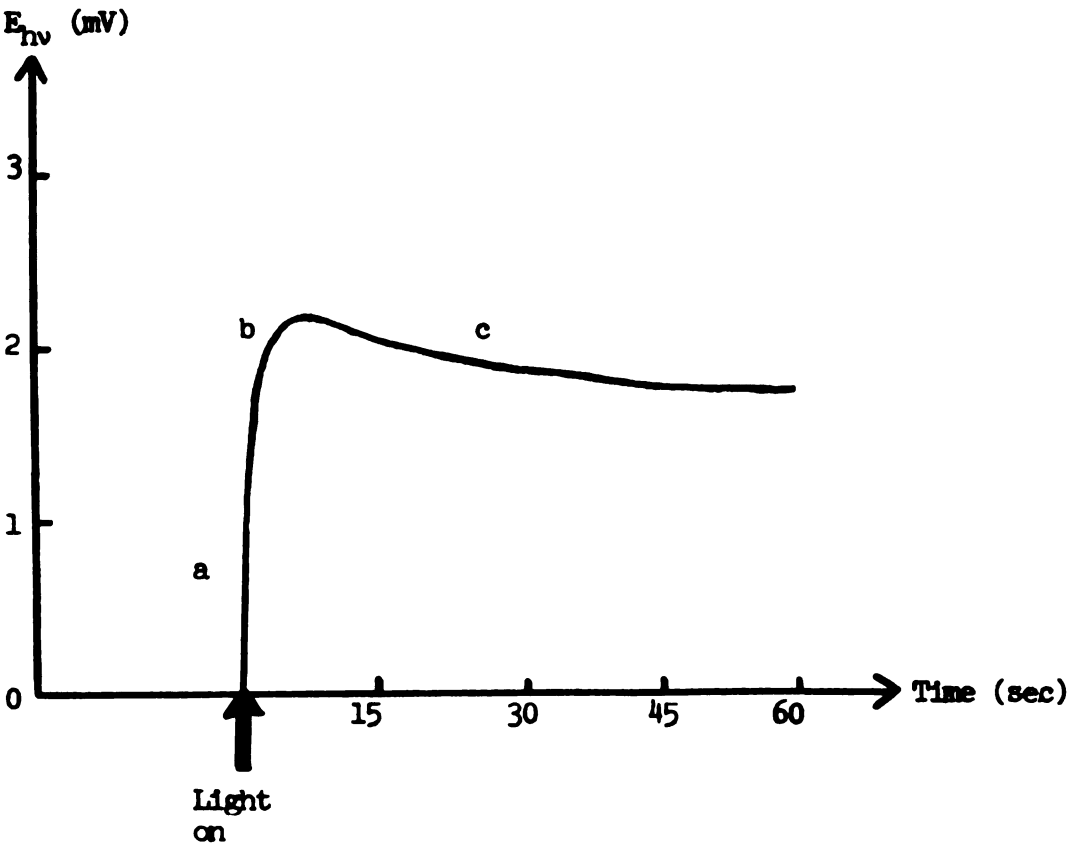


Figure 11. Shape of a typical plot of the photo-emf versus time. Excitation of the spinach chloroplast extract chlorophyll black lipid membrane is at either the principal blue peak or the red peak wavelength. The bathing solutions are 0.1 M acetic acid (pH 5) with 10^{-3} M $FeCl_3$ in the inner chamber. See text for further details.

rotation frequency of the chlorophyll molecules in the membrane.

If the membrane chlorophyll molecules with their red (or principal blue) transition moments more aligned in a certain direction --- e.g., more vertically oriented than horizontally --- could be preferentially "photo-bleached" with plane-polarized light, then there would be a subsequent amount of time before rotational diffusion would again randomize the azimuthal orientations of the "bleached" chlorophyll molecules.

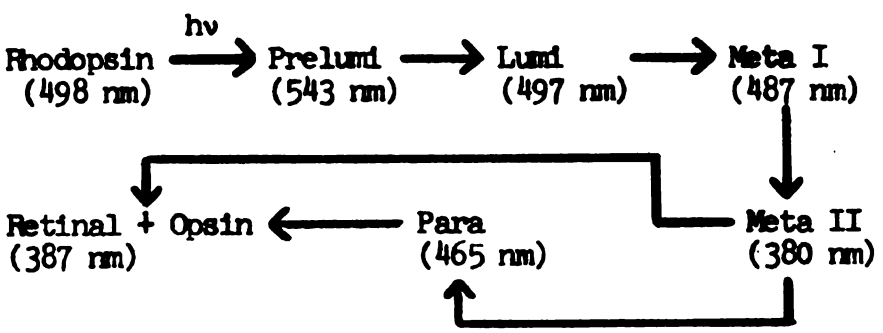
During this randomizing interval, exciting light plane-polarized normal to the polarization direction of the previously employed "bleaching" light would give rise to a photo-emf of nearly "unbleached" magnitude, whereas light polarized in the "bleaching" plane would result in a photo-emf of smaller magnitude than that when "unbleached." I.e., the ratio $(E_{h\nu})_y/(E_{h\nu})_z$ will have a value different than when the membrane is "unbleached." The time required to return to the "unbleached" value of $(E_{h\nu})_y/(E_{h\nu})_z$ is then related to the rotational diffusion relaxation time for the membrane chlorophyll molecules.

In order for an experimental determination of the rotational diffusion frequency to be performed using preferential "bleaching" of membrane chlorophyll molecules with plane-polarized light, it would be desirable first to show that the preferential "bleaching" can occur. I.e., it must be shown that the membrane chlorophyll molecules can be "fixed" (e.g., with glutaraldehyde, $KMnO_4$, or OsO_4) so that preferential "photobleaching" can be observed over a period of time of the order of a few seconds or more.

This technique for the determination of the rotational diffusion relaxation time for the chlorophyll molecules in the membrane is in some aspects similar to the techniques of Brown and Cone who established first that (a) dichroism of frog retinal rod outer segment viewed end-on can be photo-induced by partially bleaching rhodopsin with plane-polarized light following fixation with glutaraldehyde (Brown, 1972), and then that (b) the rapid decay of the dichroism induced by a flash of plane-polarized light provides a direct measure of the relaxation time — 20 μsec — of rhodopsin in the receptor membrane (Cone, 1972).

Isolated rhodopsin is highly dichroic, absorbing light most strongly when the electric vector is parallel to the long conjugated chain of 11-cis retinal (Clayton, 1971). Rhodopsin consists of the chromophore 11-cis retinal attached by a Schiff's base linkage to the lipoprotein opsin. Several chemical and structural properties of rhodopsin suggest that rotations of the chromophore accurately reflect rotations of the entire molecule.

Photoisomerization of retinal from 11-cis to all-trans initiates a bleaching process which consists of a series of configuration transitions in opsin, and leads eventually to the release of retinal:



The absorption maxima are shown below the configurations. Retinal in rhodopsin and lumirhodopsin has an absorption maximum at about 500 nm, whereas in prelumirhodopsin the maximum shifts to 543 nm. At the meta II stage, the spectrum shifts far to the blue, becoming similar to that of free retinal.

Attempts were made to "fix" the chlorophyll molecules in the spinach chloroplast chlorophyll black lipid membrane with glutaraldehyde, and subsequently to induce a change in the ratio $(E_{h\nu})_y/(E_{h\nu})_z$ with plane-polarized light at the photo-emf red peak (Table 3). It was found that the presence of glutaraldehyde has no effect, or perhaps only a small effect, on the membrane photo-emf action spectrum. However, no change in the ratio $(E_{h\nu})_y/(E_{h\nu})_z$ upon excitation with plane-polarized light was observed. Similar concentrations of $KMnO_4$ also proved ineffective.

Some comments may be in order on possible reasons for the ineffectiveness of glutaraldehyde in "fixing" the membrane chlorophyll molecules sufficiently to enable photo-induction of dichroism. Glutaraldehyde has been used by researchers principally to stabilize proteins by cross-linking them. It may only react with lipids containing free amino groups (e.g., phosphatidyl ethanolamine) (Johnston and Roots, 1972). The reaction of an aldehyde with a primary amine is expected on the basis of classical organic reactions (i.e., Schiff base formation).

Although Brown observed very strong photoinduced dichroism with a 2.5% glutaraldehyde concentration fixation of rhodopsin in the disk membranes (Brown, 1972), there are important differences between

Table 3. Four attempts at fixation of the membrane chlorophyll molecules with glutaraldehyde.

Following exposure to glutaraldehyde, the spinach chloroplast extract chlorophyll black lipid membrane was "bleached" with vertically polarized 660 nm light (the photo-emf action spectrum red peak wavelength).

Amount of 25% glutaraldehyde added		Time stirred	Time bleached	$(E_{h\nu})_y/(E_{h\nu})_z$			Photo-induced change in emf ratio
(ml)		(min)	(min)				
Inner chamber	Outer chamber			Initial	Immed. after bleaching	15 sec later	
0.1	----	10	2	2.0	----	2.0	None
0.1	0.1	11	3	2.0	1.8	2.0	None
0.5	0.5	10	5	1.8	1.8	1.6	None
1.0	1.0	20	7	2.0	1.8	1.8	None

his system and an artificial membrane. The retinal rod outer segment is a tissue and rhodopsin is largely protein. The black lipid membrane contains only chlorophyll and lipids.

It must be noted that Vásquez and co-workers (Vásquez et al., 1971) employed a 2% glutaraldehyde concentration in fixation of black lipid membranes for electron microscopy. The black lipid membrane types which they fixed were (a) lipidic --- total phospholipids of the cerebral cortex and cholesterol, (b) lipidic-proteolipidic --- with small amounts of proteolipid from Electrophorus electroplax, and (c) proteolipidic --- from Electrophorus electroplax.

Effect of Voltage Applied to Spinach Chloroplast Extract Black Lipid Membrane on the Dependence of the Photo-emf Action Spectrum upon the Direction of Polarization of the Exciting Light

Externally applied voltages across the membrane up to 35 mV had no observable effect on the dependence of the photo-emf action spectrum upon the direction of polarization of the exciting light.

APPENDICES

APPENDIX A

DIRECT PROPORTIONALITY OF THE PHOTO-EMF, $E_{h\nu}$, TO THE AMOUNT OF LIGHT ENERGY ABSORBED BY THE MEMBRANE LEADS TO DIRECT PROPORTIONALITY OF $(E_{h\nu})_y/(E_{h\nu})_z$ TO THE DICHROIC RATIO

The fraction of the light incident on the membrane which is absorbed is

$$\rho = \frac{I_o - I_T}{I_o} \tag{1A}$$

where I_o and I_T are the intensities of the incident and the transmitted light, respectively. The fraction, ρ, is related to the absorbance in the following manner

$$A = -\log(1 - \rho) = -(1/2.303)\ \ln(1 - \rho) \tag{2A}$$

The red or principal blue peak absorbance of a chlorophyll-lipid bilayer is at best of the order of 0.010 (Cherry et al., 1971; Cherry et al., 1972; Steinemann et al., 1971), which implies a value of about 0.023 for ρ. Expansion by Taylor series of the expression for the absorbance in terms of the fraction, ρ, absorbed yields

$$A = +(1/2.303)\ (\rho + \rho^2/2 + \rho^3/3 + \rho^4/4 + \cdots) \tag{3A}$$

for ($-1 < \rho < +1$). (Clayton, 1970)

For the absorbance of the order of 0.010 (i.e., for a value of ρ of about 0.023) the following approximation holds, with an error of less than ±2%,

$$A = -\rho/2.303 \tag{4A}$$

Then, the ratio of the absorbances for monochromatic light polarized horizontally to that for the light polarized vertically is given by

$$\frac{A_y}{A_z} = \frac{\rho_y}{\rho_z} = \frac{\{(I_o - I_{T,y})/(I_o)\}}{\{(I_o - I_{T,z})(I_o)} \tag{5A}$$

where $I_{T,y}$ and $I_{T,z}$ are the transmitted intensities for the incident light polarized horizontally and vertically, respectively. Since the incident light intensity, I_o, is the same regardless of its polarization,

$$\frac{A_y}{A_z} = \frac{(I_o - I_{T,y})}{(I_o - I_{T,z})} \tag{6A}$$

If the assumption is made that the photo-emf, $E_{h\nu}$, is directly proportional to the amount of light energy absorbed by the membrane

$$E_{h\nu} \propto (I_o - I_T)tS \tag{7A}$$

where t is the time of exposure of the membrane to the light and S is

the membrane area.

Since the membrane area, S, remains constant and is exposed to either polarization of the incident light for the same time interval, t,

$$\frac{(E_{h\nu})_y}{(E_{h\nu})_z} = \frac{(I_o - I_{T,y})}{(I_o - I_{T,z})} = \frac{A_y}{A_z} \tag{8A}$$

$$\frac{(E_{h\nu})_y}{(E_{h\nu})_z} = \frac{M_y^2}{M_z^2} \tag{9A}$$

APPENDIX B

DERIVATION OF THE ANGLE, β, BETWEEN THE PLANE OF THE PORPHYRIN RING AND THE NORMAL, *N*, TO THE MEMBRANE

Two vectors of any magnitudes, one along the direction of the red transition moment, M_B, and the other along the direction of the principal blue transition moment, M_R, determine the plane of the porphyrin ring. Thus, for ease of calculation, select both vectors, *BC* and *BD*, with magnitude m (Figure 12).

M_B and M_R are mutually perpendicular and make angles of θ_B and θ_R, respectively, with the normal, *N*.

$$\text{Angle CBD} = \pi/2 \tag{1B}$$

N is drawn long enough that its projection, n cos β, upon the porphyrin plane has an endpoint, E, on the diagonal $\overline{DC}$. Then,

$$\overline{AE} = n \sin \beta \tag{2B}$$

$$\text{Angle AEB} = \text{Angle AED} = \text{Angle AEC} = \pi/2 \tag{3B}$$

$$\overline{CE} = \overline{DE} = m\sqrt{2} \tag{4B}$$

Since triangle BCE is an isosceles right triangle,

$$\text{Angle BCE} = \text{Angle BDE} = \pi/4 \tag{5B}$$

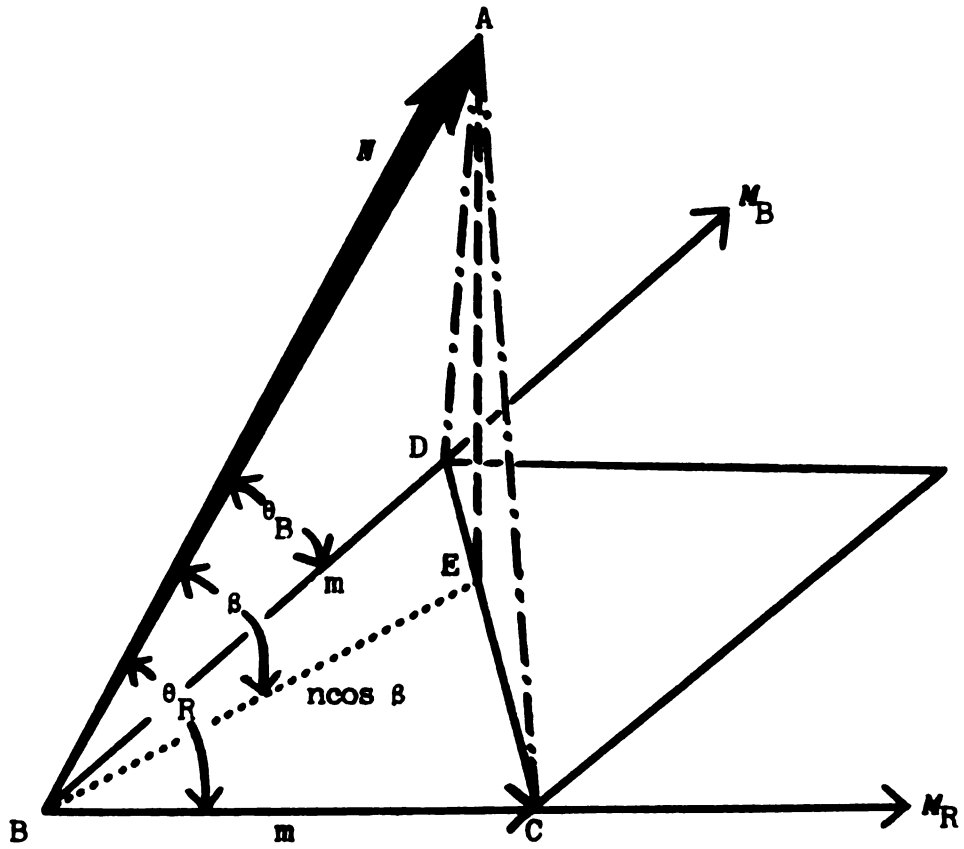


Figure 12. Schematic diagram of the relation between the red transition moment vector, M_R, the principal blue transition moment vector, M_B, and the normal to the membrane, N. See text for further details.

$$\overline{CD} = m\sqrt{2} \tag{6B}$$

Applying the Pythagorean Theorem to triangle AED,

$$\overline{AD}^2 = n^2 \sin^2 \beta + \overline{DE}^2 \tag{7B}$$

and to triangle AEC,

$$\overline{AC}^2 = n^2 \sin^2 \beta + \overline{CE}^2 \tag{8B}$$

Subtracting equation 8B from equation 7B,

$$\overline{AD}^2 - \overline{AC}^2 = \overline{DE}^2 - \overline{CE}^2 \tag{9B}$$

Applying the Law of Cosines to triangle ABD,

$$\overline{AD}^2 = n^2 + m^2 - 2mn \cos \theta_B \tag{10B}$$

and to triangle ACB

$$\overline{AC}^2 = n^2 + m^2 - 2mn \cos \theta_R \tag{11B}$$

Subtracting equation 11B from equation 10B,

$$\overline{AD}^2 - \overline{AC}^2 = 2mn (\cos \theta_R - \cos \theta_B) \tag{12B}$$

Equating $(\overline{AD}^2 - \overline{AC}^2)$ in equation 9B and equation 12B,

$$\overline{DE}^2 - \overline{CE}^2 = 2mn (\cos \theta_R - \cos \theta_B) \tag{13B}$$

Substituting for $\overline{CE}$ from equation 4B into equation 13B,

$$\overline{DE}^2 - (m\sqrt{2} - \overline{DE}^2) = 2mn (\cos \theta_R - \cos \theta_B) \tag{14B}$$

$$\overline{DE}\sqrt{2} = n (\cos \theta_R - \cos \theta_B) + m \tag{15B}$$

Squaring both sides of equation 15B,

$$2\,\overline{DE}^2 = n^2\,(\cos\theta_R - \cos\theta_B)^2 + 2mn\,(\cos\theta_R - \cos\theta_B) + m^2 \qquad (16B)$$

Substituting for $\overline{DE}^2$ from equation 16B into equation 7B,

$$\overline{AD}^2 = n^2 \sin^2\beta + n^2/2\,(\cos\theta_R - \cos\theta_B)^2 + mn\,(\cos\theta_R - \cos\theta_B) + m^2/2 \qquad (17B)$$

$$\overline{AD}^2 - n^2 = m^2/2 - n^2\cos^2\beta + n^2/2\,(\cos\theta_R - \cos\theta_B)^2 + mn\,(\cos\theta_R - \cos\theta_B) \qquad (18B)$$

Substituting for $(\overline{AD}^2 - n^2)$ from equation 10B into equation 18B,

$$m^2 - 2mn\cos\theta_B = m^2/2 - n^2\cos^2\beta + n^2/2\,(\cos\theta_R - \cos\theta_B)^2 + mn\,(\cos\theta_R - \cos\theta_B) \qquad (19B)$$

$$m^2/2 - mn\,(\cos\theta_B + \cos\theta_R) = n^2/2\,(\cos\theta_R - \cos\theta_B)^2 - n^2\cos^2\beta \qquad (20B)$$

Substituting the trigonometric identity ($\sin^2\beta = 1 - \cos^2\beta$) in equation 7B,

$$\overline{AD}^2 = n^2(1 - \cos^2\beta) + \overline{DE}^2 = n^2 - n^2\cos^2\beta + \overline{DE}^2 \qquad (21B)$$

$$n^2\cos^2\beta - \overline{DE}^2 = n^2 - \overline{AD}^2 \qquad (22B)$$

Applying the Law of Cosines to triangle BDE,

$$n^2\cos^2\beta = m^2 + \overline{DE}^2 - 2m\,(\overline{DE})\cos 45^\circ \qquad (23B)$$

$$n^2\cos^2\beta - \overline{DE}^2 = m^2 - m(\overline{DE})\sqrt{2} \qquad (24B)$$

Equating $(n^2\cos^2\beta - \overline{DE}^2)$ in equations 22B and 24B,

$$n^2 - \overline{AD}^2 = m^2 - m(\overline{DE})\sqrt{2} \qquad (25B)$$

Rearranging equation 5B,

$$n^2 - \overline{AD}^2 = 2mn\cos\theta_B - m^2 \qquad (26B)$$

Equating $(n^2 - \overline{AD}^2)$ in equations 25B and 26B,

$$m^2 - m(\overline{DE})\sqrt{2} = 2mn\cos\theta_B - m^2 \qquad (27B)$$

$$2m - \overline{DE}\sqrt{2} = 2n\cos\theta_B \qquad (28B)$$

Substituting for $\overline{DE}\sqrt{2}$ from equation 15B into equation 28B,

$$2m - \{n(\cos\theta_R - \cos\theta_B) + m\} = 2n\cos\theta_B \qquad (29B)$$

$$m = n(\cos\theta_B + \cos\theta_R) \qquad (30B)$$

Substituting for m from equation 30B into equation 20B,

$$n^2/2(\cos\theta_B + \cos\theta_R)^2 = n^2(\cos\theta_B + \cos\theta_R)^2 + n^2/2(\cos\theta_R - \cos\theta_B)^2 - n^2\cos^2\beta \qquad (31B)$$

Simplifying,

$$\cos^2\beta = \cos^2\theta_R + \cos^2\theta_B \qquad (32B)$$

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