This is to certify that the

thesis entitled

LOCATION OF SPIN LABELS IN

OAT LEAF PROTOPLASTS

presented by

Steven Paul Briggs

has been accepted towards fulfillment
of the requirements for

Master of Science degree in Botany and Plant Pathology

Robert F. Scheffer

Major professor

Date 5 May 1980

O-7639

LOCATION OF SPIN LABELS IN OAT LEAF PROTOPLASTS

By

Steven Paul Briggs

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1980

ABSTRACT

LOCATION OF SPIN LABELS IN
OAT LEAF PROTOPLASTS

By

Steven Paul Briggs

The spin label method was applied to the study of lipid mobility in the plasmalemma of oat leaf protoplasts. The criteria which are commonly used to ascertain the location of spin labels were found to be inadequate. Fatty acid spin labels partitioned throughout the membranes of protoplasts. The electron spin resonance line shape and intensity varied with the redox state of the cells. A spin label derivative of phosphatidylcholine was incorporated into the plasmalemma and did not partition into the other membranes.

Isolated chloroplasts reduced spin labels in response to white light. This reduction was inhibited by dichlorophenyldimethylurea (DCMU) and reversed with far-red light. Chloroplasts within protoplasts oxidized spin label in both white and far-red light, independent of DCMU. Nitroxide spin labels apparently donate electrons to the photosynthetic electron transport chain after the DCMU block; electrons are recieved from photosystem I.

To Gardner and Nancy

who taught me perseverance

ACKNOWLEDGMENTS

My graduate committee members, Drs. Scheffer, Haug, and Ellingboe, have provided me with encouragement, support, and guidance for an interdisciplinary line of research which was full of surprises.

Tom Graan taught me how to isolate chloroplasts.

Mark Lesney came to my rescue and helped type this thesis so that it could be submitted on time.

My wife, Marsha, helped with the Figures and cooked.

I am deeply grateful.

PREFACE

The blight of oats caused by Helminthosporium victoriae is of interest because the fungus produces a compound known to be essential for pathogenicity. Details of this work have been reviewed by Scheffer (48). The toxic determinant, a low molecular weight compound known as HV-toxin or victorin, has been isolated and partially characterized. The molecule consists of a terpenoid and a small peptide containing aspartic acid, glutamic acid, glycine, valine, and leucine. The molecular weight is approximately 1000 d. The toxin can completely inhibit root growth of seedlings at concentrations as low as 0.2 ng/ml.

There are several lines of evidence which suggest that HV-toxin is required for pathogenicity and for disease development. All pathogenic strains of H. victoriae produce the toxin; non-pathogenic strains don't. Hosts which are susceptible to the fungus are sensitive to the toxin; resistant plants are insensitive to the toxin. Spores release toxin upon germination and the fungus produces toxin in the host tissue. All of the biochemical symptoms of disease can be reproduced with the toxin. Non-pathogenic mutants of H. victoriae, which have lost the ability to produce toxin, can colonize susceptible plants if toxin is added exogenously. The role of the toxin in pathogenesis appears to be to create conditions in the host which are conducive to colonization.

Exposure to HV-toxin disturbs various plasmalemma properties in sensitive cells: the membrane potential drops; active uptake of solutes stops; electrolytes rapidly leak out; the ability to plasmolyze is lost; the apparent free space of tissue increases; and coleoptile protoplasts burst within minutes after toxin treatment. Certain membrane-binding

compounds protect sensitive tissues from toxin.

A greater understanding of how the toxin exerts its effects and of the role it plays in pathogenesis may come from structural studies of the plasmalemma. The changes in membrane function listed above appear to be manifestations of changes in the structure of the membrane. Knowledge of such structural changes could lead to the identification of the primary effect of HV-toxin.

Toward this end, I have endeavored to develop a method for selectively monitoring changes in the structure of the plasmalemma of intact protoplasts. While this thesis is solely concerned with the development of this method, the ultimate goal is the elucidation of the mechanism of action of HV-toxin.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

INTRODUCTION

The Need for the Spin Label Method

Many phenomena have been ascribed to changes in the structure of the plasmalemma of higher plants (e.g., cold acclimation, salt tolerance, host-pathogen interactions, hormone responses, photosensing, turgor-pressure sensing, and senescence). Yet, few experiments directly test such hypotheses. Most attempts to study membrane structure have been made with fixed tissue or isolated membranes (20). While useful, such approaches cannot directly measure membrane events as they occur in living cells. Only recently have the appropriate techniques been developed for the study of plasma membranes of plant protoplasts. Borochov et al. (11) observed an increase in microviscosity of rose petal protoplast membranes as the protoplasts senesced. Borochov and Borochov (10) observed a decrease in microviscosity of rose petal protoplast membranes as a result of osmotic swelling. In both cases, microviscosity was determined by the fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). The authors asserted that the DPH was localized in the plasmalemma as evidenced by the fluorescence observed microscopically. Vigh et al. (59) claimed that they could specifically label the plasmalemma of wheat protoplasts with the fatty acid spin label I(1,14). The fluidity of the membranes increased as the protoplasts became cold-hardened. Their criteria for establishing the location of the spin label were not given. This report (59) may be a harbinger of widespread application of spin-labeling

with protoplasts; animal cell physiologists have for years employed the spin label method to probe plasma membranes of intact cells. This method provides information about the chemical polarity and microviscosity of specified regions of the membrane, from the external aqueous interface to the center of the hydrocarbon interior. The utility of spin labels has been recognized by plant scientists who work with highly pigmented materials (42). Optical interference by the sample renders fluorescence probes useless. Electron spin resonance (ESR) spin labels are detectable at concentrations which are three orders of magnitude lower than their nuclear magnetic resonance (NMR) counterparts. These are some of the advantages of spin labels. There is much flexibility in the application of the method. Various lipid classes and membrane proteins can be selectively labeled, using analogs and by direct, covalent bonding. Azethoxyl nitroxides can be incorporated into hydrocarbon chains with little or no change in the original shape (30). Paramagnetic ions and reducing agents can reversibly "turn off" the spin labels on one side of the membrane, to study only one leaflet of the bilayer. In addition, the rate of transverse and vertical motion of spin-labeled enzymes and lipids can be determined. Magnetic interactions between spin labels allows for observations of lateral diffusion of spin-labeled molecules within the plane of the membrane and lateral thermotropic and ionotropic phase separations. Spin labels are used to study conformational changes of proteins. Clearly, the spin label method holds promise for answering some longstanding questions in plant biology.

Introduction to the Theory of Spin Labeling

The term "spin labeling" generally refers to a branch of electron spin resonance (ESR) spectroscopy in which a stable free radical molecular probe (a spin label) monitors the motion and chemical environment of macromolecules and the molecular constituents of organelles. The following is a qualitative discussion of the rudimentary concepts of spin labeling theory; there are sources for a detailed treatment of the subject (8, 53, 49).

An electron can be thought of as a rapidly rotating, charged sphere. As such, it generates a magnetic field along the axis of rotation. This can be pictured as a ball with an arrow through it, pointing in the direction of the generated field. This magnetic property of an electron is called "spin" because of the classical description of its origin; actually, the magnitude of the magnetic moment of an electron is far greater than that which could be generated by rotation. When an unpaired electron is placed in an external magnetic field, it will orient so that the "arrow" is pointing either parallel (with) or antiparallel (against) to the external field. That there can be only these two "spin states" is a quantum mechanical restriction. The magnetic energy difference between the two spin states is proportional to the magnitude of the external field. In the absence of an external field, an ensemble of unpaired electrons will possess no net magnetization. When an external field is imposed, the electrons will populate the two spin states according to the Boltzmann distribution. In most spin label experiments conducted at ambient temperature, the magnetic energy difference between the two spin states is so small (ca. 1 cal/mol) that there are only 0.1% more electrons

in the lower spin state than in the higher.

The fundamental equation of ESR is:

$$h\nu = gBH$$

where h = Planck's constant; B = Bohr magneton; g = electronic g-factor; ν = frequency of the microwave radiation; and H = magnitude of the external magnetic field. The major variables in the equation are ν and H. ESR spectrometers hold ν at a constant (e.g., 9.5 GHz) and sweep the magnetic field intensity through various ranges centered around 3400 gauss. When the magnetic energy potential difference between the two spin states becomes equal to the energy of the microwave quanta, resonance occurs. The energy potential difference between the two spin states is determined by the magnitude of the magnetic field at the electron. This magnitude is the vector sum of the external and local magnetic fields. The g-factor differs from the universal constant, g_e, because of an orbital angular momentum induced by the external field. The effect of g is to shift the center of gravity of the absorbance spectrum along the abscissa. The g-values are helpful in identifying paramagnetic entities. The g-factor is a measure of externally-induced local magnetic fields.

Irradiation with microwaves (wavelength is approximately 3 cm) induces both upward and downward transitions between the two magnetic energy states with equal probability. As a result of the initially larger population in the low energy state there is a net absorbance of radiation. ESR spectrometers detect this absorbance. In the absence of radiationless relaxation mechanisms, the numbers of electrons in the two spin states would become equal. However, the electrons can exchange thermal energy with the environment. This provides a means to maintain an asymmetric population distribution in the presence of resonant frequency radiation.

The radiationless release of magnetic energy to the environment is known as spin-lattice relaxation and is characterized by a rate constant, T_1, known as the spin-lattice relaxation time (approximately 10^{-8} sec for spin labels; 37). High-intensity radiation can overcome the system's ability to relax resulting in equal numbers of electrons in the two spin states. This is known as saturation.

The experimentally observed ESR linewidth is normally broader than T_1 (if calculated in frequency units). Fluctuating local magnetic fields spread out the region of magnetic field intensity over which the population of electrons will resonate, resulting in an "envelope" of discreet spectra which blend into a single, broad absorbance. These fluctuating, local fields are caused by the spin of paramagnetic nuclei or electrons in the vicinity of the spin label. The observed linewidth is characterized by a term, T_2, where

$$dH = \hbar(gBT_2)^{-1}$$

and dH = half-width of the absorbance curve at half-maximum amplitude (53). The difference between the observed linewidth and the inherent linewidth (due to T_1) is known as the spin-spin relaxation time, T_2':

$$\frac{1}{T_2'} = \frac{1}{T_2} - \frac{1}{2T_1} \qquad (53).$$

In addition to fluctuating and induced local magnetic fields, there are also permanent local magnetic fields. These are of the utmost importance in spin labeling. Permanent local magnetic fields originate in the nuclei of atoms which possess spin. The naturally abundant isotopes of some magnetic nuclei which occur in organic molecules and their spin quantum numbers are: ^{1}H, 1/2; ^{14}N, 1; and ^{31}P, 1/2. The common isotope,

^{16}O, is not magnetic. The spin quantum number, I, determines the number of possible spin states of the nucleus, J, where J = (2I + 1).

For our purposes, all spin labels are nitroxides (Figure 1). The unpaired electron is localized in the $2p\pi$ orbital of the nitrogen atom. The conventional coordinate system sets the x-axis parallel to the N-O bond, the z-axis parallel to the $2p\pi$ orbital, and the y-axis perpendicular to the x-z plane. There are three possible spin states for ^{14}N. From the point of view of the unpaired electron, the magnetic field vector of the nitrogen atom is either parallel, antiparallel, or perpendicular to the $2p\pi$ orbital. As a result, this permanent local magnetic field either increases, decreases, or does not affect the magnetic field at the electron. This implies that resonance can occur at three discreet values of the external magnetic field, resulting in a 3-line ESR spectrum. The splitting of ESR lines into multiplets by paramagnetic nuclei is called nuclear hyperfine splitting.

Imagine a nitroxide held rigid in a crystal. If the crystal is placed in an ESR cavity and rotated so that the z-axis ($2p\pi$ orbital) is parallel to the magnetic field, then the hyperfine splitting which will result is of the largest possible magnitude. This is because the entire vector component of the nuclear spin either adds to or subtracts from the external magnetic field. At all other orientations, only part of the nuclear spin vector is oriented along the lines of force of the external field. As the nitroxide is rotated so that the z-axis is perpendicular to the external field, the hyperfine splitting decreases to a minimum. The minimum splitting is determined by the magnitude of the nuclear spin along the

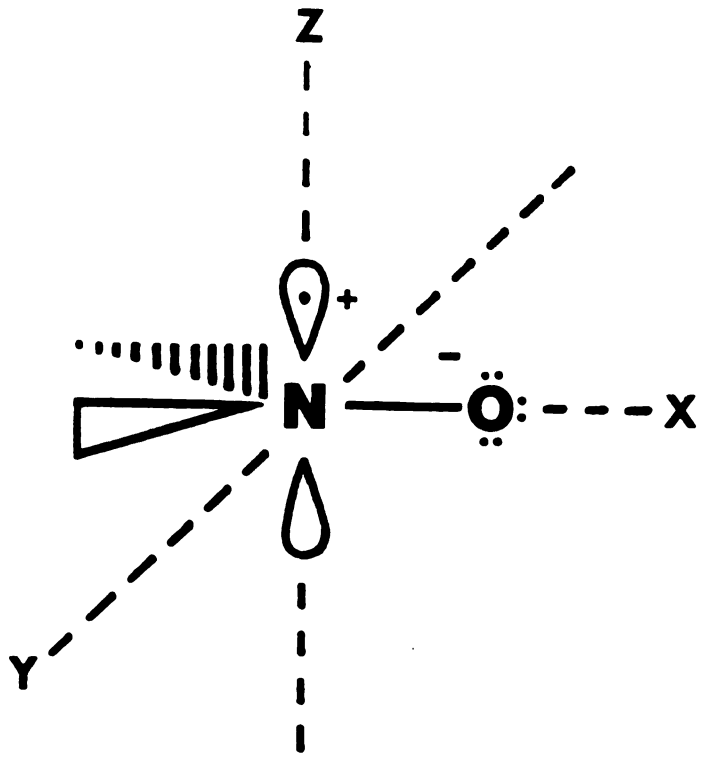

Figure 1. Relationship between the principal axes and the atomic structure of the nitroxide radical.

x- and y-axes. Because the 2pπ orbital is axially symmetric about z, the components of the nuclear spin along the x- and y-axes are equal. These crystal values of the nuclear hyperfine splitting are actually second order tensor rather than vector quantities and are denoted T_{xx}, T_{yy}, and T_{zz} (some authors prefer A instead of T). Obviously, T_{zz} is greater than T_{xx} and T_{yy} which are equal to each other.

Now imagine a crystal which was made by freezing a solution of nitroxide-containing molecules. All of the nitroxides are completely immobile and randomly oriented. Rotating this crystal in the ESR cavity will not affect the spectrum. The so-called "powder spectrum" which one obtains from a randomly oriented population of immobile spin labels is the sum of the spectra from each nitroxide. For any given nitroxide, the nuclear spin "vector" through the 2pπ orbital can be resolved into two components: the part which is oriented along the external lines of force (parallel and antiparallel) and the part which is perpendicular to the lines of force. The magnitude of these components determines the values of the external magnetic field at which resonance occurs. The average splitting produced by the component of the nuclear spin along the lines of force is denoted $T_{||}$ ($2T_{||}$ is the distance between the low-field and high-field resonance lines which result from a parallel and antiparallel orientation, respectively). In a powder spectrum $T_{||}$ is approximately equal to T_{zz} and an upper limit for $T_{yy} = T_{xx}$ can be obtained as 1/2 the distance between the midfield peaks, $T_{\perp}$ (Figure 3).

The nitroxides can be divided into two equal populations: those whose average orientation of the 2pπ orbital lies along the external magnetic lines of force and those whose average orientation is

perpendicular to the external field. However, because the first component is split into parallel and antiparallel populations, the magnitude of the central ESR line will be approximately twice that of the low- and high-field lines.

As stated earlier, spin labels are useful because they provide information about the motion and chemical environment of a molecule. Motional information is obtained as a result of the time-averaging of the nitroxide orientation (i.e., the g and T tensors) with respect to the external magnetic field. We have already examined the case of complete immobilization (the powder spectrum). In that case, each nitroxide had a unique, fixed value of the external magnetic field at which it would resonate. This value was determined by the orientation of the $2p\pi$ orbital with respect to the external field. Now let us consider a population of nitroxides undergoing rapid, isotropic motion. For each nitroxide, the orientation with respect to the external magnetic field is now an average of all of the orientations experienced during the lifetime of the excited state. Every nitroxide will experience "all" orientations under these conditions and, hence, they will be magnetically identical to each other. The splitting of the hyperfine lines narrows to a value known as the isotropic hyperfine coupling (or splitting) constant, a_N, where $a_N = 1/3\ (T_{zz} + T_{xx} + T_{yy})$. This parameter is approximated experimentally as $a'_N = 1/3\ (T_{||} + 2T_{\perp})$. Over a range of rotational correllation times of 10^{-11} to 10^{-8} sec, the spectrum of a nitroxide will shift from that of rapid, isotropic motion to the powder spectrum. This is the effective time scale of events which can be studied by conventional ESR spectroscopy.

The isotropic hyperfine coupling constant, a'_N, is used as an index

of solvent polarity. The solvent polarity affects the equilibrium distribution of the nitroxide between the two states:

$$\geq \overset{\cdot}{\underset{+}{N}}\text{-O:} \rightleftharpoons \geq \ddot{N}\text{-O}\cdot$$

with non-polar solvents shifting the equilibrium to the right. A shift to the right localizes the unpaired electron density on the oxygen atom which tends to "uncouple" the electron spin from the nitrogen nuclear spin. In other words, the local magnetic field which the unpaired electron "feels" is less, so a_N' decreases (because T_{zz}, T_{xx}, and T_{yy} decrease).

Anisotropic motion can be understood as a combination of slow and fast isotropic motion. For example, a fatty acid spin label in a membrane rotates rapidly about the long axis of the hydrocarbon chain (which is parallel to the $2p\pi$ orbital of the nitroxide) but undergoes little or no rotation about any axis which is parallel to the plane of the membrane. The nitroxide label, therefore, experiences fast rotation about the z-axis but very slow rotation about the x- and y-axes. The result is a "powder" spectrum for the T_{zz} component (the outer lines) and an "isotropic" spectrum for the $T_{xx}$and T_{yy} components (the inner lines). If all rotation is minimized by lowering the temperature, the $T_{||}$ value increases further but, more dramatically, the sharp, narrow "isotropic" lines of $T_{\perp}$ broaden to give a net spectral approximation to the powder spectrum. If only $T_{||}$ can be determined then no conclusions about the anisotropic qualities of the motion can be made. However, $T_{||}$ by itself is still a measure of microviscosity.

When both $T_{||}$ and $T_{\perp}$ can be determined experimentally, the motion of the spin label population can be described by an order parameter, S,

where:

$$S = \frac{(T_{11} - T_{\perp})\ (a_N)}{(T_{zz} - T_{xx})\ (a'_N)} \qquad (46).$$

The a_N and a'_N terms correct for the difference between the polarity of the solvent and the polarity of the crystal in which T_{zz} and T_{xx} were determined. As the motion of the molecule becomes restricted, the observed hyperfine splitting approaches the crystal values and S approaches one. For rapid, isotropic motion, S approaches zero.

When T_{11} and $T_{\perp}$ cannot be determined due to rapid, isotropic tumbling, motion is described by the rotational correllation time, τ_c,

$$\tau_c = (6.5 \times 10^{-10})\ (w_0)\ ((h_0/h_{-1})^{\frac{1}{2}} - 1)$$

where w_0 = peak-to-peak width of the mid-field line, h_0 = peak-to-peak height of the mid-field line, and h_{-1} = peak-to-peak height of the high-field line (26). The rotational correllation time is the time required for rotation through 1 radian (Figure 10) and is a valid measure of isotropic motion in the 5×10^{-11} to 10^{-9} sec range.

Changes in the microviscosity of membranes can be determined by the relative solubility of certain spin labels in the membrane. This method requires rapid, isotropic tumbling of the molecule. 2-doxyl-n-octane (2N8) will partition into a membrane such that part of the population is in the aqueous phase and part is in the membrane. Each population has a characteristic ESR signal but the two spectra are only resolved in the high-field line. This line is split into components from each population. The magnitudes of the hydrophobic (h) and polar (p) components will vary

with the membrane fluidity since the amount of 2N8 in the membrane is proportional to the fluidity of the membrane. The fluidity parameter, f, is calculated by $f = h/(h + p)$ (50) (Figure 12).

Chemistry and Structure of Spin Labels

Nitroxides are remarkably stable relative to most free radicals. However, they can be reversibly reduced to the corresponding hydroxylamine by compounds such as ascorbic acid, sodium dithionite, reduced glutathione, cysteine, and mercaptoethanol. Many living cells reduce nitroxides *in situ*. Further reduction to the corresponding secondary amine is usually not reversible in biological systems. Oxidation of the hydroxylamine back to the nitroxide is spontaneous with O_2 and ferricyanide. Maruyama and Ohnishi (33) have estimated the standard reduction potential of a nitroxide, $E_0' = -0.31$ V.

The structures of some commonly used spin labels are shown in Figure 2. The notation for fatty acid and phosphatidylcholine spin labels is I(m,n) and PC(m,n), respectively, where m and n refer to the number of methylene groups subsequent to and preceding the nitroxide (with respect to the polar end). Me-I(m,n) refers to the methyl ester of the fatty acid. The notation for 2-doxyl-n-octane is 2N8. Maleimide, iodoacetamide, and steroid spin labels are also shown. Note that the orbital of the unpaired electron is parallel to the hydrocarbon chain of phospholipids, fatty acids, and fatty esters.

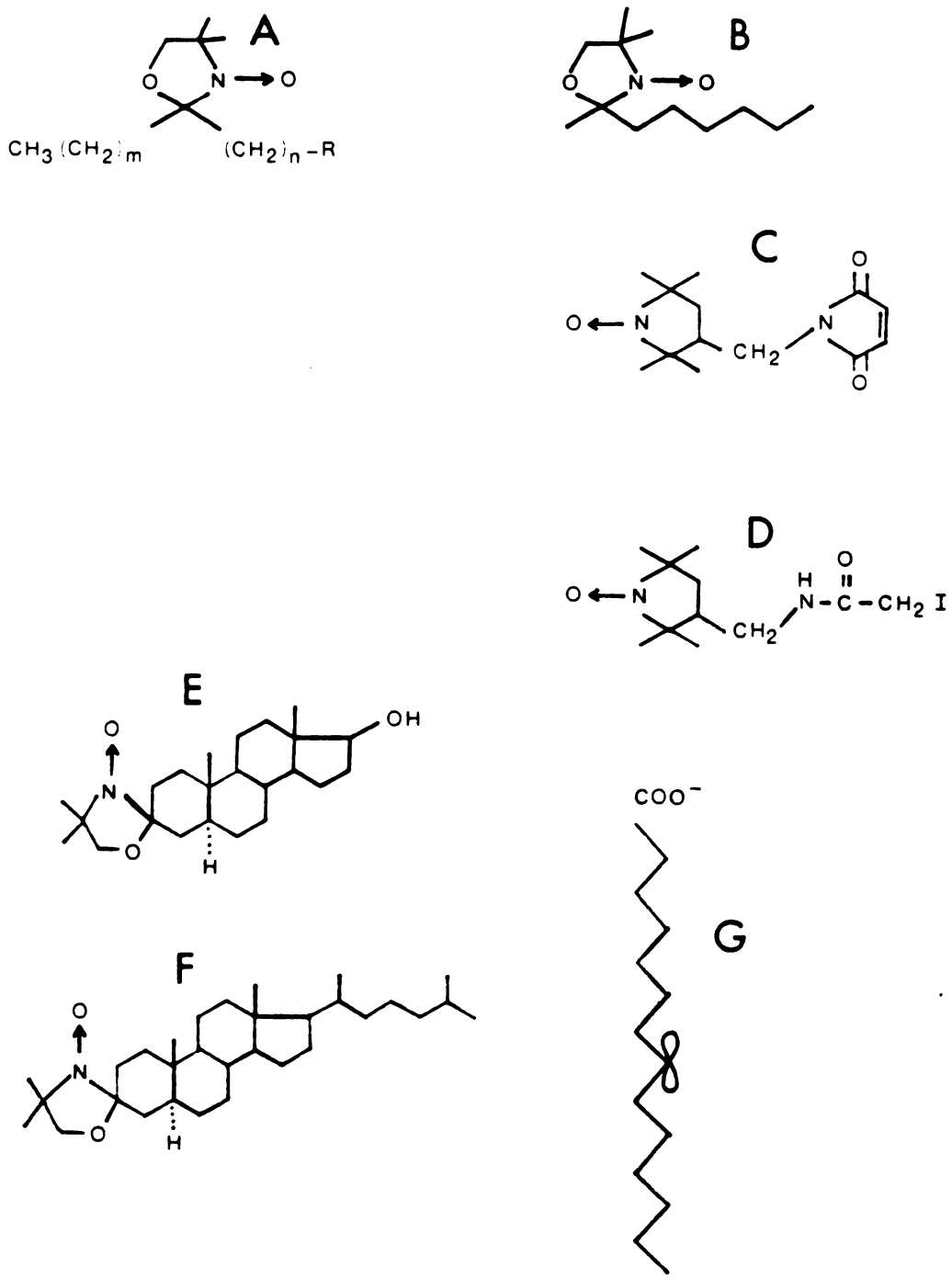

Figure 2. Structures of spin labels used: (A) general structure for fatty acid (I(m,n)), phosphatidylcholine (PC(m,n)), and fatty acid methyl ester spin labels; (B) 2N8; 2-doxyl-N-octane; (C) maleimide spin label; (D) iodoacetamide spin label; (E) androstane spin label; (F) cholestane spin label; (G) I(5,6) showing orientation of the 2pπ orbital with respect to the acyl chain.

Spin Label Studies of Cells

In order to selectively probe the plasma membrane of an intact cell, the probe must only be reporting from the plasma membrane. This condition can be met if the probe is: (a) only present in the plasma membrane; and/or (b) spectroscopically inactive everywhere except in the plasma membrane. The problem is trivial if erythrocytes or mycoplasmas are used since there is only one membrane in which the spin label can be located. However, determining the location of "active" spin label in nucleated, eukaryotic cells is difficult but necessary for a correct interpretation of the data.

Kaplan et al. (23) first reported the use of spin labels as selective probes of the plasma membranes of intact, multi-membranous cells. They observed that the fatty acid spin labels I(12,3) and I(5,10) were gradually reduced by mouse L-cells and human lymphocytes. Addition of $K_3Fe(CN)_6$ re-activated the signal although the degree of recovery was not indicated. The authors claimed that $K_3Fe(CN)_6$ could not enter the cells and, therefore, only spin label in the outer leaflet of the bilayer was oxidized. Under these conditions, they believed that all of the ESR signal was from probe in the plasma membrane (condition (b) listed above). However, if the spin label molecules were rapidly exchanging between different membranes then the oxidized spin label could have moved into the cell and reported from an intracellular membrane. When the various membrane fractions were analyzed, 87% of the spin label was found in the low-speed pellet of lysed cells rather than in the high-speed plasma membrane pellet, suggesting that rapid exchange may have occurred. The order parameters of spin label in each of the membrane fractions were identical, therefore, exchange

between membranes would not have altered the order parameter.

Gaffney(16) also used fatty acid spin labels to study the plasma membrane of intact mammalian cells but $K_3Fe(CN)_6$ was not applied. Gaffney postulated that the probe must be in the plasma membrane because the ESR spectra were recorded 30 to 40 minutes after labeling, and because the order parameter remained constant during the rapid cellular reduction of the spin label (condition (a) listed above). The distribution of spin label throughout the membranes of the cell and the order parameters of the various membranes were not determined. Without such data, Gaffney's criteria for spin label location must be taken on faith.

Nevertheless, the claim is seen in several papers that spin-labeled fatty acids selectively labeled the plasma membrane of nucleated, eukaryotic cells (2, 3, 4, 21, 59, 60). In some cases $K_3Fe(CN)_6$ was included in the aqueous solution but in most cases it was not. Most authors relied upon Gaffney's postulates to support their claims of selective labeling. Other papers have been published in which fatty acid spin labels were used to study problems directly associated with the plasma membrane of intact cells but no claims were made regarding the location of the probe (5, 47). The report by Vigh et al. (59), that the fatty acid spin label I(1,14) is selective for the plasmalemma, lacks a description of the criteria which they used to establish spin label location. This is unfortunate in light of my results which do not support the conclusion that fatty acid spin labels show membrane selectivity. An evaluation of their method must await publication of their background work.

Hammerstedt et al. (19) disputed the belief that fatty acid spin labels are selectively retained in the plasma membrane of mammalian cells. They provide direct evidence that I(13,2) partitions into all of the

cellular membranes of sperm cells. A statement was made in an earlier paper from the same lab (31) that "most spin labels designed to probe membranes penetrate throughout the membrane system resulting in an average signal of all membranes present." Whether or not these conclusions are applicable to the cell types for which claims of selective labeling have been made (human lymphocytes, mouse L-cells, 3T3 mouse fibroblasts, mouse ascites tumor cells, Dictyostelium discoideum, wheat protoplasts, and XC sarcoma cells) remains to be determined.

Objectives

My objectives in thesis are to: (a) evaluate whether or not fatty acid spin labels are selective for the plasmalemma of plant protoplasts; (b) develop adequate criteria for ascertaining the selectivity of a spin label for the plasmalemma; (c) test the various classes of spin labels and identify those which have the highest selectivity for the plasmalemma; and (d) determine whether spin label incorporation into other membranes would be likely to lead to artifacts.

MATERIALS AND METHODS

Avena sativa L. cultivars Garry and Park were grown in the laboratory at 22 C in vermiculite irrigated with White's nutrient solution (12). Illumination with fluorescent and incandescent bulbs provided a 16 hr. photoperiod. Primary leaves were harvested from 1-2 week-old seedlings.

Protoplasts were isolated by peeling away the lower epidermis of the leaves with forceps and floating the peeled surface in a solution composed of 0.5% Cellulysin (Calbiochem) and 0.6M sorbitol, adjusted to pH 5.7 with KOH. The preparation was incubated at 28 C for 3 hours in the light, then was swirled gently to release protoplasts. The protoplasts were filtered through a layer of Miracloth and the suspension was centrifuged at 40xg for 10 minutes. The supernatant was removed and the pellet was washed in a suspension medium (pH 5.7) containing 0.6M sorbitol and 10mM $CaCl_2$, unless indicated otherwise. Chloroplasts were prepared from protoplasts by passing the protoplasts through a #26 syringe at room temperature into a test tube embedded in ice. The lysate was centrifuged at 2500xg for 2 minutes. The supernatant was decanted and saved and the chloroplasts were resuspended and washed in a solution (pH 6.8) containing 0.6M sorbitol, 10mM $CaCl_2$, 10mM PIPES, unless indicated otherwise. The supernatant which had been saved was centrifuged at 75,000xg for 90 minutes. A white pellet was obtained, which will be referred to as non-green membranes.

The structures of the spin labels used in this study are shown in Figure 2. The spin labels I(1,14),I(12,3),Me-I(5,10), iodoacetamide, maleimide, androstane, and cholestane were purchased from Syva Associates, Palo Alto, CA., and were used without further purification. Distearoylphosphatidylcholine, spin-labeled at carbon 12 in the B chain (PC(5,10)), was obtained from Serdary Research Laboratories, Ontario, Canada; 2-doxyl-n-octane was a gift of Dr. J. Raison, Macquarie University, North Ryde, Australia.

Protoplasts in thick slurry suspensions were labeled by adding them to a test tube containing a dry film of the selected spin-probe and incubating for 5 min at 23 C. Incorporation of probe PC(5,10) was difficult. First, dispersions were prepared by adding 0.5 ml suspension medium to a tube containing a dry film of the probe. The suspension was vortexed and then sonicated with either a bath or tip-type sonicator. The dispersed spin label was mixed with 0.5 ml of protoplasts and incubated for 30 minutes at 23 C; free label was then washed away. Next, the samples were pipetted into Varian low temperature quartz cuvettes which were placed in the electron spin resonance cavity and scanned. A 0.1 ml sample was required for these cuvettes. Chloroplasts were labeled in the same manner as were protoplasts.

Samples were irradiated in the electron spin resonance (ESR) cavity with a 100 W Xe-Hg arc lamp. Light was filtered with either 5 cm H_2O or 5 cm H_2O plus a 739 nm interference filter giving light intensities at the cavity of 2.7×10^5 and 2.7×10^3 ergs cm^{-2} sec^{-1}, respectively. A shutter was used to turn the light on and off. ESR spectra were recorded with a Varian model E-112 X-band spectrometer. A power setting of 15mW and a modulation amplitude of 2.5 guass were used unless stated otherwise.

Lineshape of the spectrum of I(12,3) was not affected by power even at 40 mW. The sample temperature was controlled by heating or cooling a rapidly moving column of N_2 gas in which the cuvette was placed. A Varian variable temperature controller was used to regulate the gas temperature. The sample temperature was monitored by an Omega model 250 thermocouple positioned within the cuvette.

Relative ESR signal intensities were determined by dividing the peak-to-peak height of the mid-field line by the amplifier gain. This method is valid as long as the peak width remains constant.

RESULTS

Experiments With Protoplasts

A typical ESR spectrum of oat leaf protoplasts spin-labeled with I(12,3) is shown in Figure 3. Since $T_{\parallel}$ and $T_{\perp}$ in the hyperfine splitting pattern can both be distinguished, the motion of the fatty acid is a rapid rotation about the long molecular axis. The value of the order parameter (S=0.65) confirms this. The hyperfine coupling constant (a_N') was determined to be 15.5 gauss. Such a low value for a_N' indicates that the nitroxide is in a non-polar environment (36). There data suggest that the fatty acid spin label probably is intercalated between the lipids of the membrane with the long axis of the label parallel to the acyl chain. At low cell density,free label "liquid lines" appear in the spectra as a result of spin label partitioning into the aqueous phase, where it undergoes rapid, isotropic motion (data not shown).

Protoplasts in the ESR cavity (at 23 C) caused a gradual reduction of signal intensity (Figure 4). This phenomenon has been observed in other cell types; the reduced intensity was attributed to chemical reduction of the nitroxide to the corresponding hydroxylamine by respiratory metabolites (43). Concomitant with the observed reduction in signal intensity was an increase in the maximum hyperfine splitting parameter, $2T_{\parallel}$, and a broadening of the $2T_{\perp}$ peaks. This apparent increase in membrane viscosity from a $2T_{\parallel}$ value of 56 gauss to

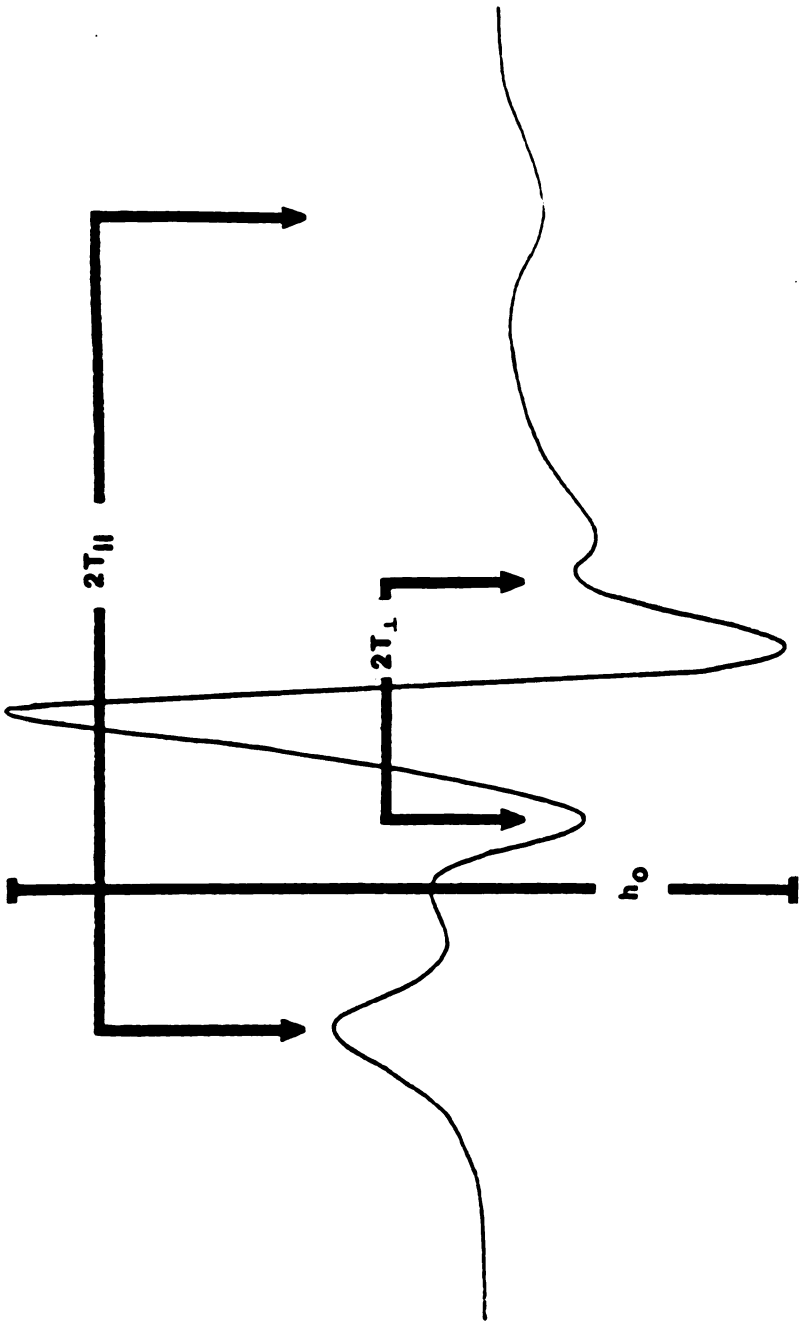

Figure 3. ESR spectrum of I(12,3)-labeled protoplasts. Freshly-labeled protoplasts were scanned at 23 C in the dark.

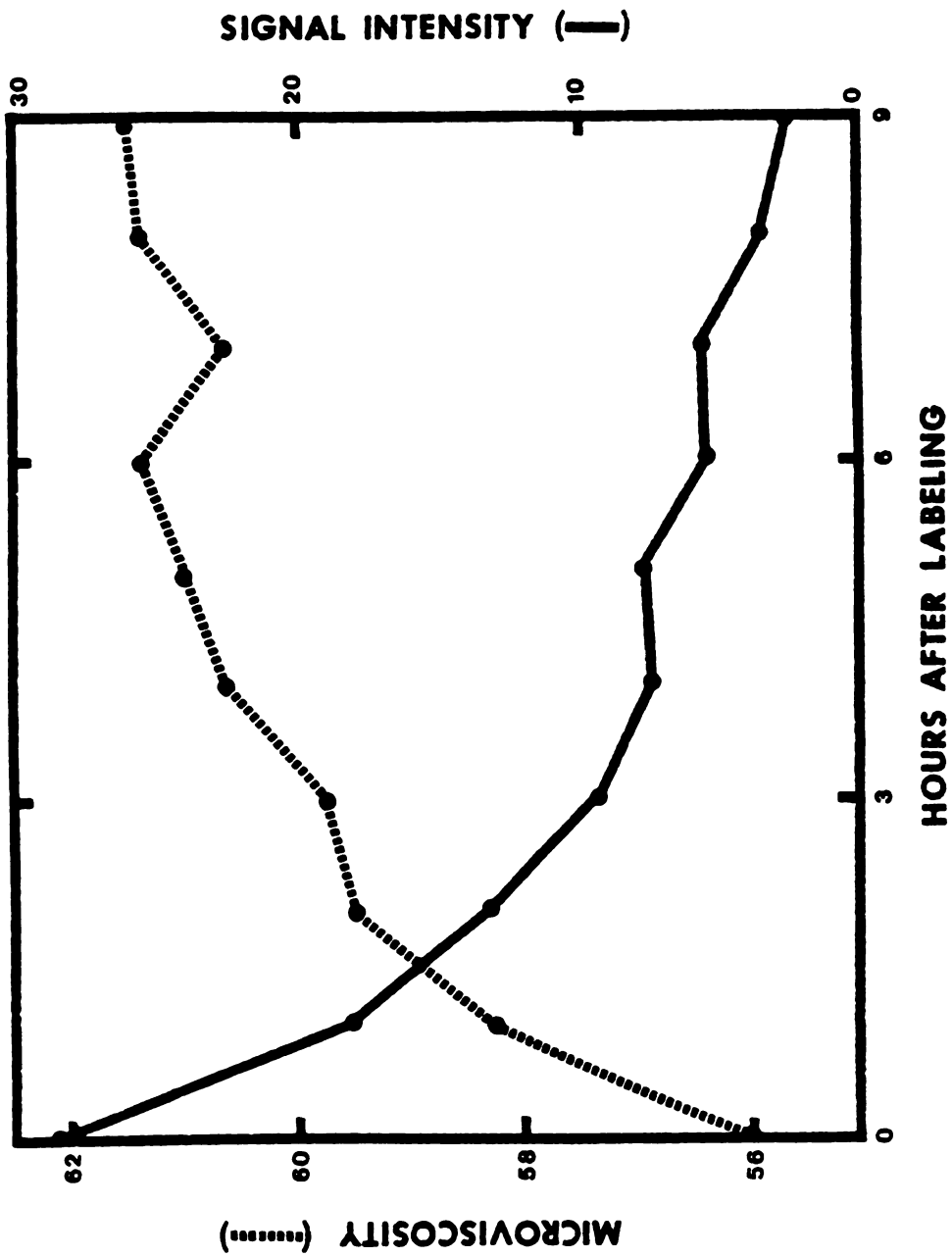

Figure 4. Kinetics of signal intensity and microviscosity changes of I(12,3)-labeled protoplasts in the dark. The protoplasts were held at 23 C in the ESR cavity. Microviscosity was measured as $2T_{II}$.

62 gauss at constant temperature corresponded to the increase observed upon lowering the temperature from 23 C to 3 C (data not shown). $2T_{\perp}$ became too broad to detect suggesting that rotation about the long molecular axis was severely restricted. The rates of change of microviscosity and signal intensity were the same. However, peak height reduction due to line broadening could not account for the observed decreases. The cellular reduction of spin label was anticipated, but the change in membrane viscosity was a surprise since, to my knowledge,the literature contains no reports of similar phenomena.

Different results were obtained when a new, unscanned sample from the same preparation was examined (Figure 5). Each sample, once placed in the cavity, behaved the same as shown in Figure 2; i.e., there was an increase in $2T_{\parallel}$. There did not appear to be a change occurring in the sample while it sat on the bench. When a sample was removed from the cavity the original signal was gradually restored (data not shown). This suggested that the environment in the cavity caused a change in the membrane viscosity. In every case, as the hyperfine splitting ($2T_{\parallel}$) increased, the signal intensity decreased.

The two properties of the ESR cavity most likely to affect oat leaf protoplast physiology are: (a) the relatively anaerobic conditions of the N_2 atmosphere; and (b) the low light intensity within the cavity. Changing the bathing gas from N_2 to atmospheric did not prevent the ESR signal change. Next, two samples were placed in airtight cuvettes,with one incubated in a foil covered flask and the other in a clear flask. Only the sample in the covered flask gave changes in signal intensity and membrane microviscosity. These results suggested that low light intensity within the cavity was responsible for the ESR signal changes. This hypothesis

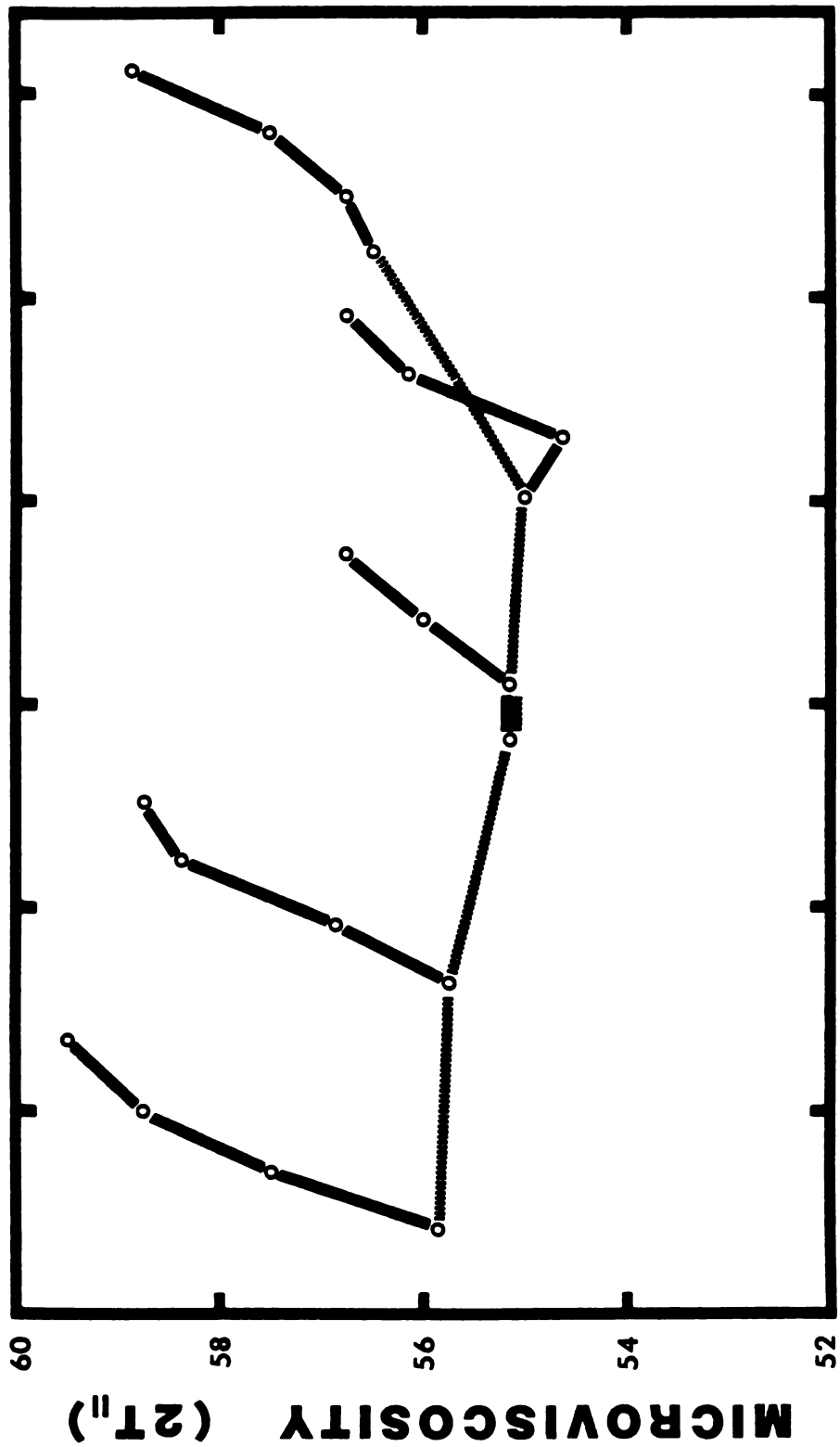

Figure 5. Microviscosity changes in the ESR cavity (—) and on the bench (▪▪▪). Sample temperature was 23 C.

was tested directly by irradiating the sample in the cavity with white light. Turning the light on caused a rapid increase; when the light was turned off, there was a decrease in the signal intensity (Figure 6). The chemical reduction of the nitroxide in the dark appeared to be reversed when the protoplasts were irradiated. However, there was a gradual reduction in the light; obviously, this was not reversed by white light. The light effect was temperature-dependent, with inhibition below 12 C. There was a break in the plot of the temperature dependence of spin label motion at 12 C (data not shown). The increase in microviscosity in the dark was also reversed by light (Table 1).

These results raise three questions: (a) what is the pigment(s) responsible for the light-mediated oxidation of the nitroxide; (b) what is the pigment(s) responsible for the change in membrane microviscosity; and (c) where are the pigment systems located (i.e., where is the spin label)? Localization of the photoreceptor was attempted by use of spin labels known to permeate all of the cellular membranes and which can be used to selectively probe certain regions of a bilayer (e.g., headgroup region or hydrocarbon interior).

The most likely photoreceptor candidates were: (i) the photosynthetic pigments (with the assumption that the spin labels permeate to the chloroplast membranes); (ii) flavin oxidases; and (iii) phytochrome via the high-irradiance response. Light is known to cause extensive changes in the structure of chloroplast membranes (61). The large number of redox processes associated with the light reactions of photosynthesis are also well known. The location and behavior of flavin oxidases and phytochrome are controversial (38,40). The action spectra of flavins and photosynthesis indicate that little or no use can

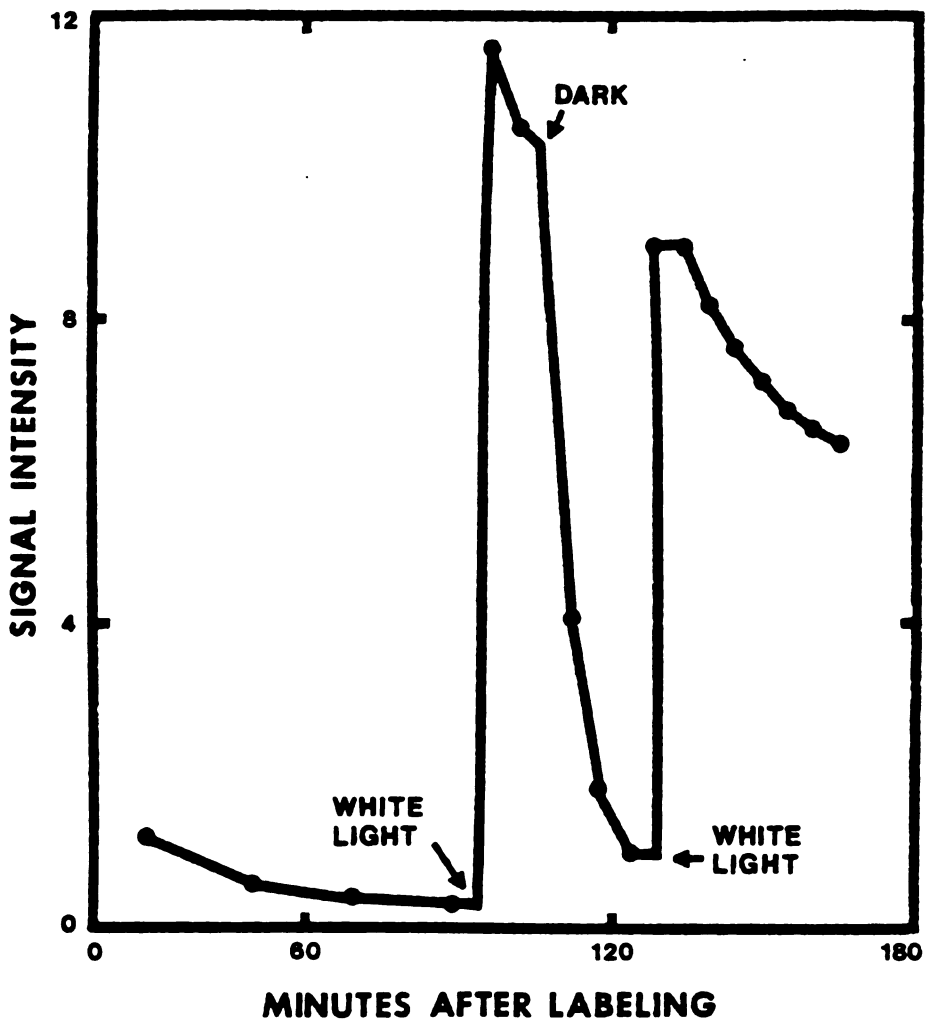

Figure 6. Effect of white light on I(12,3)-labeled protoplasts. Protoplasts were held in the dark at 23 C until irradiation.

Table 1. Effects of white light on the I(12,3) ESR signal from protoplasts.

t[a]	$2T_{II}$	H[b]	Conditions
20	56.0	39.0	Dark @ t = 20
345	59.5	4.6	Dark
390	56.1	120.0	White light @ t = 360

[a] t = minutes after labeling sample

[b] H = signal amplitude

be made of the far-red region of the spectrum. Therefore, spin-labeled protoplasts were placed in the ESR cavity, allowed to undergo the dark-induced changes, and then irradiated with far-red (729nm) light. Far-red light reversed both the increase in microviscosity and the decrease in signal intensity (Figure 7). The rate of response to far-red light was less than the rate of response to white light, due perhaps to the greater intensity of the white light. The response to far-red light was not reversed with red light and required continuous irradiation to be sustained.

The response of protoplasts to light in the presence of the photosynthetic electron transport inhibitor 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) was determined as a further test of the possible involvement of photosynthesis. A comparison of the maximum rates of photoreduction of the spin label in response to white light showed that DCMU did not inhibit the light reaction (Figure 8). Irradiation of labeled protoplasts with far-red light in the presence of DCMU gave a response similar to that observed without DCMU (Figure 9). Both the signal intensity and the membrane microviscosity decay in the dark were restored by the light. These data are evidence that photosynthesis is not responsible for the light effect. However, photosystem I of the light reaction complex could still function with far-red irradiation and exposure to DCMU.

The results are consistent with the hypothesis that the light effect is either a high-irradiance response of the phytochrome system or due to spin label interaction with photosystem I. High-irradiance responses require sustained irradiation (hence a failure of the law of reciprocity), are strongly dose-dependent, lack photoreversibility, and have an action spectrum with a sharp peak at 720 nm with some broader peaks below 500 nm.

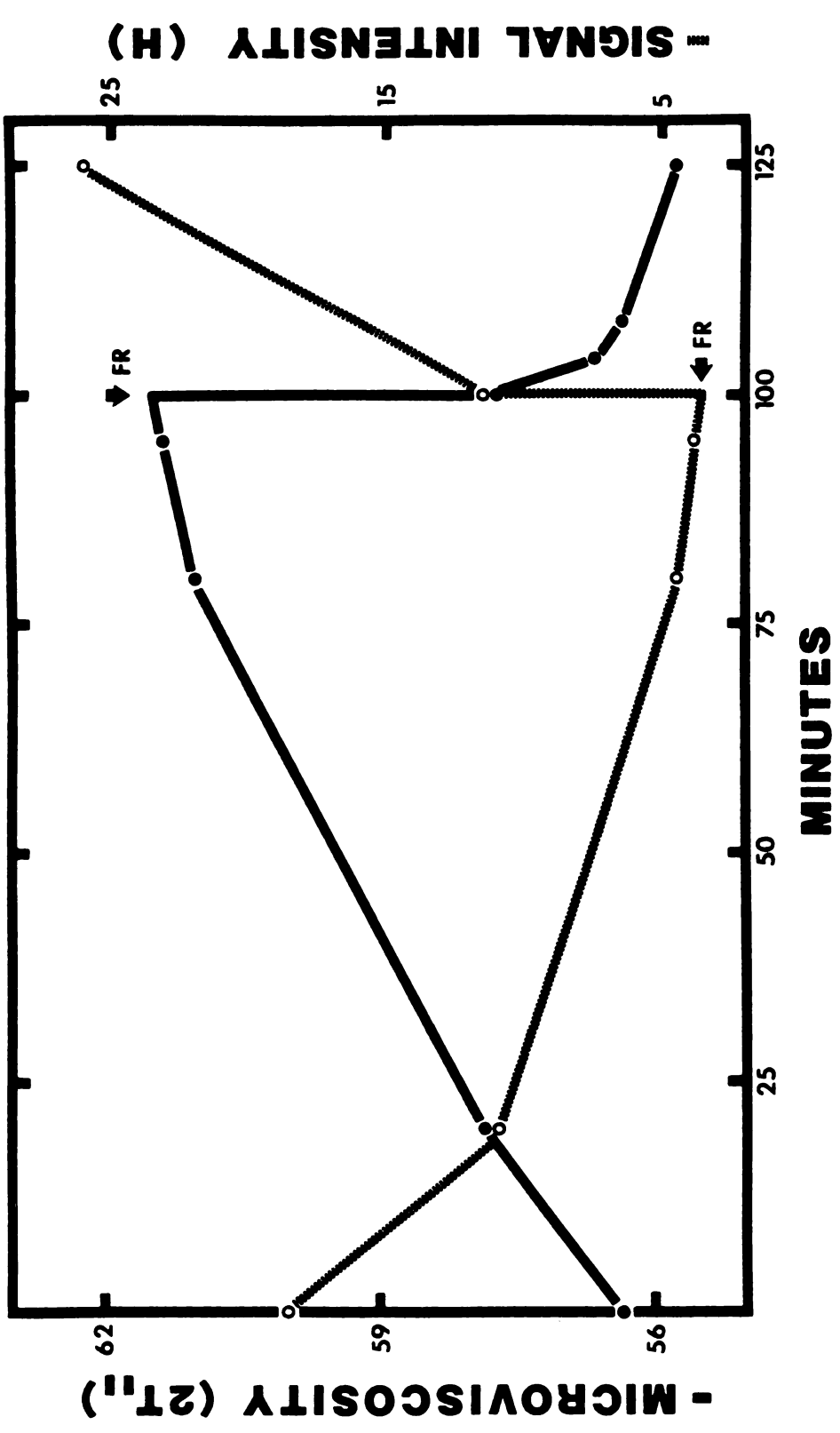

Figure 7. Effect of far-red light on I(12,3)-labeled protoplasts. Protoplasts were held in the dark at 23 C until irradiation with far-red (FR) light.

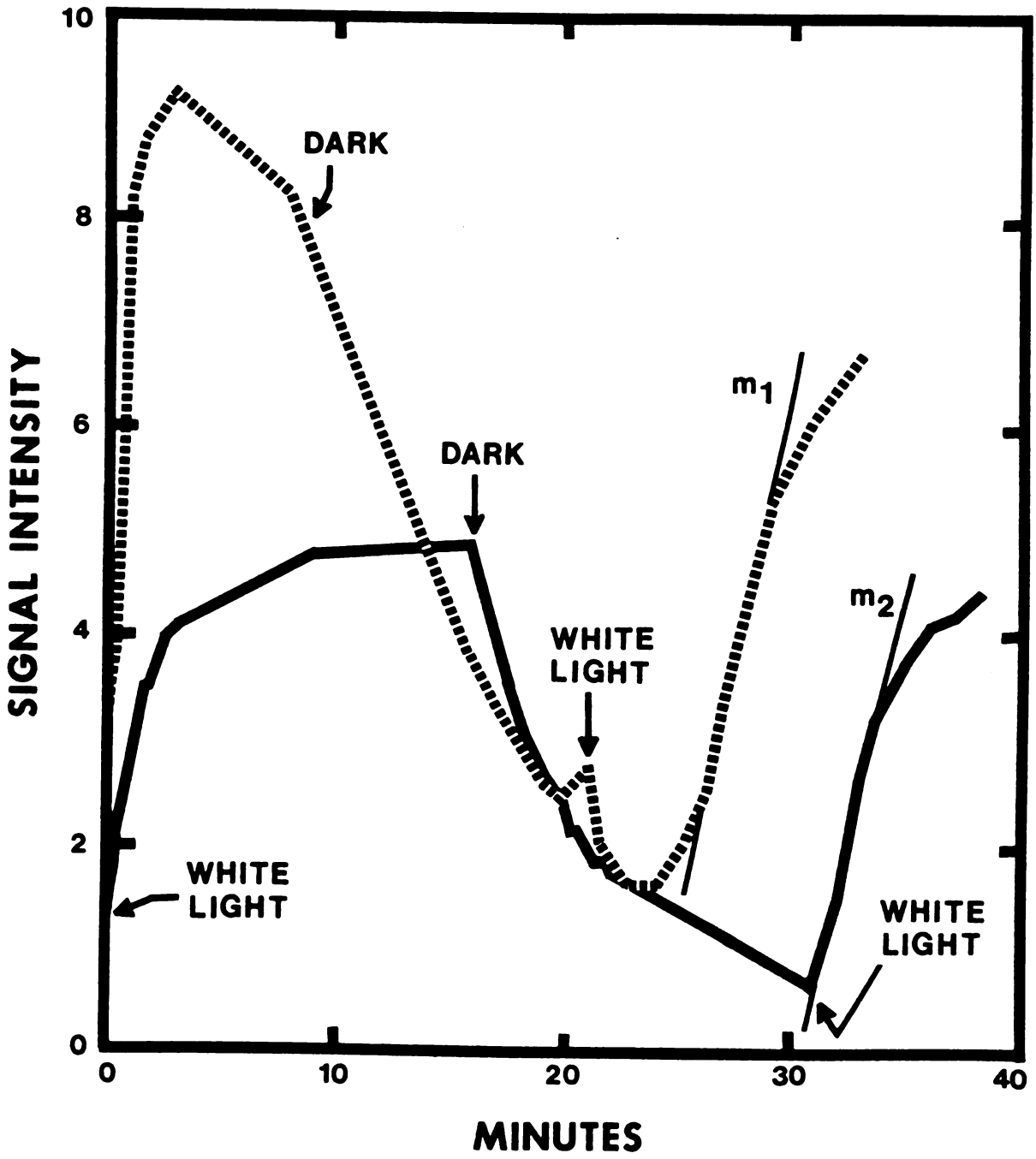

Figure 8. Effect of white light on I(12,3)-labeled protoplasts in the presence of DCMU. Protoplasts were irradiated in wash medium (▪▪▪▪) or wash medium plus 10 μM DCMU (▬). The maximum rates of photooxidation were: control (m_1) = 0.96 min^{-1} and DCMU (m_2) = 1.08 min^{-1}.

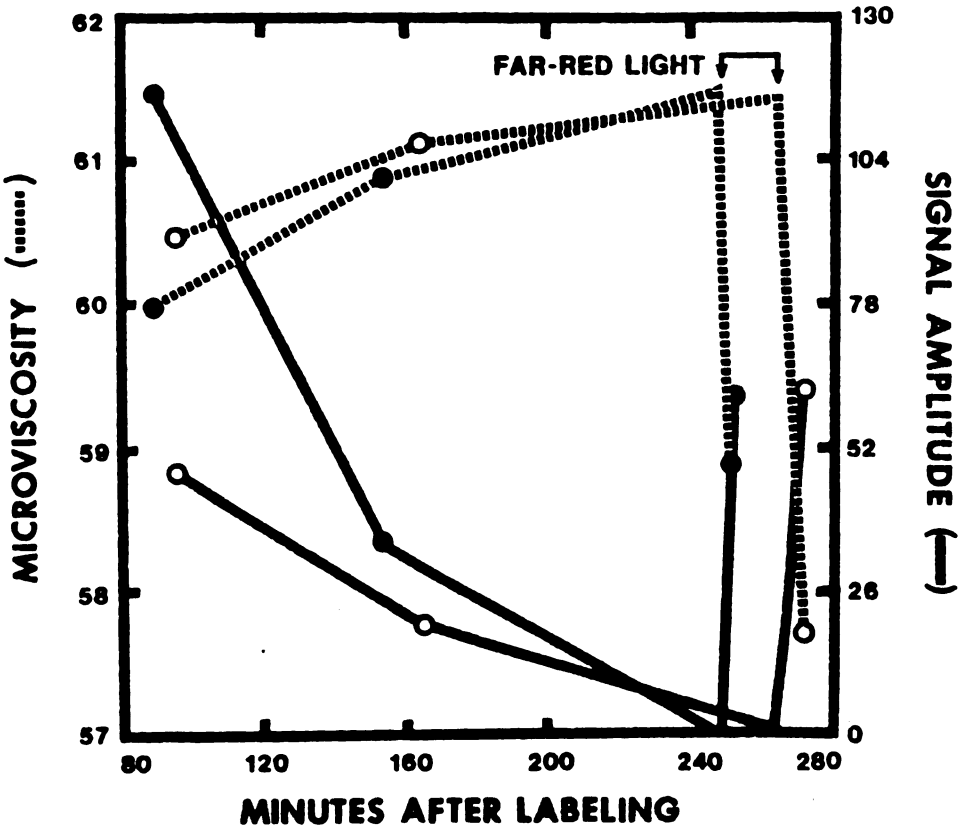

Figure 9. Effect of far-red light on I(12,3)-labeled protoplasts in the presence of DCMU. Protoplasts were suspended in wash medium (○) or wash medium plus 10 μM DCMU (●) and held at 23 C. Microviscosity was measured as $2T_{II}$.

Phytochrome is thought to be a photocoupler (rather than a photosensor) in the high-irradiance response. Phytochrome might also be a proton pump (41) which might help to explain photooxidation of spin label. In the well-known Mougeotia system, the red absorbing form of phytochrome (the form obtained in the dark) is believed to be embedded in the plasma membrane with the long axis of the phytochrome molecule perpendicular to the plane of the membrane. Red light causes a reorientation of the phytochrome, probably on the membrane surface, so that the long axis of the molecule is parallel to the plane of the membrane (65). Even though this is not a high-irradiance response, it suggests an intriguing explanation for the effect of light on the mobility of the spin label. Specifically, if the high-irradiance response involves phytochrome embedded in the membrane in the dark and surface - localized in the light, the I(12,3) mobility should be more restricted in the dark (due to protein-lipid interactions) than in the light. This was observed with I(12,3) in protoplasts (as previously described), which probes the membrane just below the phospholipid headgroups; therefore, I decided to probe other regions of the membrane.

The spin labels I(1,14) and Me-I(5,10) probe the central region of the hydrocarbon interior. The motion of these probes was much greater than I(12,3) indicating that the fluidity of the bilayer is greatest in the hydrocarbon interior. This is consistent with studies of model bilayer membranes (22) and with microbial and animal membranes (22) . The effect of incubation in the dark followed by irradiation with white or far-red light on the motion and redox states of these probes was similar to the results obtained with I(12,3) (data not shown). Therefore, the effector of the light response must penetrate

deep into the membrane bilayer, possibly traversing the membrane.

Spin label derivatives of iodoacetamide and maleimide are used to covalently label proteins by reacting with amino and sulfhydryl groups, respectively. When either one of these labels was mixed with protoplasts, a spectrum characteristic of rapid, isotropic motion was observed (Figure 10). This indicated that the spin labels probably were not binding to membrane proteins. However, their motion was restricted since the rotational correlation time was longer in a slurry of protoplasts (22×10^{-11} sec) than in the suspension medium alone (5×10^{-11}sec). The isotropic hyperfine coupling constant ($a_N' = 16.25$ gauss) indicated that the labels were in a polar environment (not in the membrane). The spin label probably is distributed throughout the aqueous compartment of the cell and in the external solution. The ESR spectrum obtained from such a sample would yield an average rotational correllation time from the spin labels in every environment. The rotational correlation time obtained for dark-incubated protoplasts (22×10^{-11}sec) is about half that of a similar spin label (TEMPAMINE; 2,2,6,6-tetramethyl piperidine-N-oxyl-4-amine) located in the lumen of thylakoids (40×10^{-11}sec) (6). In the latter case, the ESR signal from the spin label in the aqueous phase was eliminated by 80 mM $K_3Fe(CN)_6$ which acts as a broadening agent at high concentrations. When protoplasts labeled with either the iodoacetamide or the maleimide spin label were incubated in the dark, the rotational correlation times increased (Figure 11) and the signal intensity decreased. Irradiation with far-red light reversed this trend. Apparently the effector of the light response can interact with the aqueous phase bounding the membranes.

The spin label 2-doxyl-n-octane (2N8) partitions between the aqueous

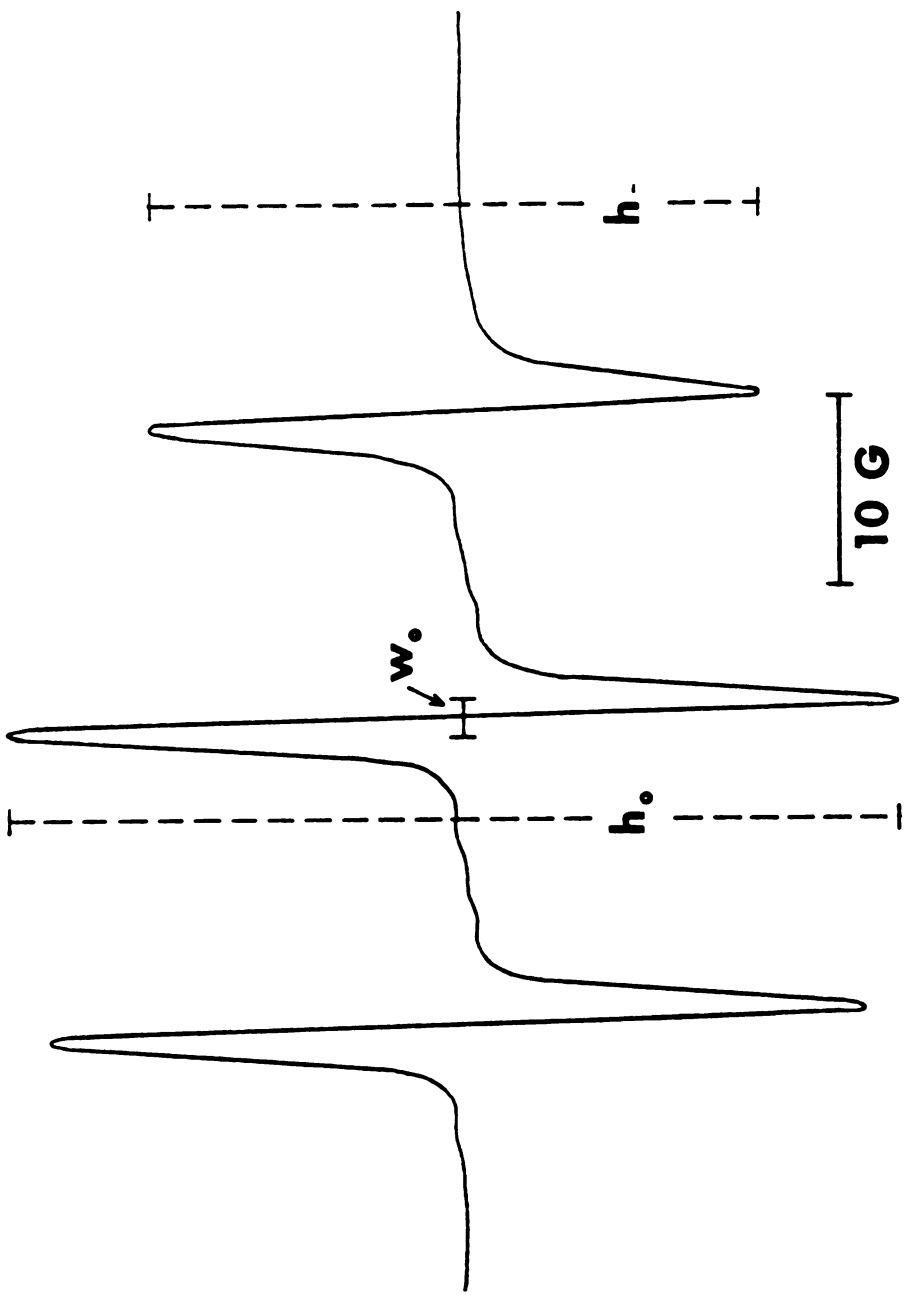

Figure 10. ESR spectrum of maleimide spin-labeled protoplasts. The sample was scanned at 23 C in the dark with a modulation amplitude of 0.032.

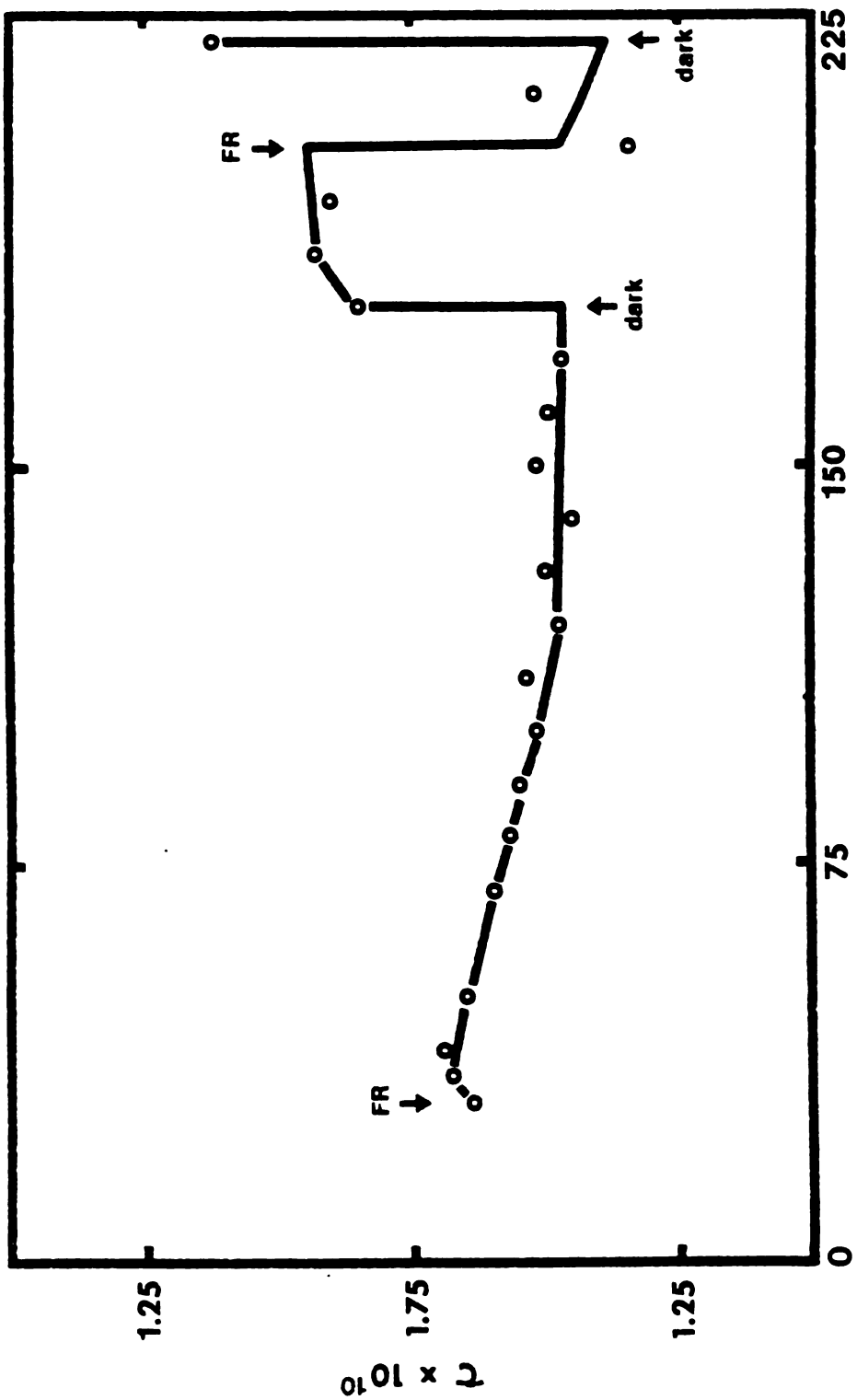

Figure 11. Effect of far-red light on maleimide spin-labeled protoplasts. The sample was alternately exposed to far-red light and darkness at 23 C.

phase bounding the membrane and the hydrocarbon phase of the membrane. The ESR signals differ for each of these two populations. When the membrane becomes more fluid, the partition coefficient of the spin label changes such that the fraction of 2N8 in the membrane increases. This is reflected by a change in the relative peak heights of the two ESR signals.

A spectrum of 2N8 labelled protoplasts (Figure 12) shows that the high field line is clearly resolved into the components from the hydrocarbon (h) and polar (p) phases. When the labeled protoplasts were incubated in the dark, the fluidity index, f, ($f = h/(h + p)$) increased (Figure 13). When the sample was irradiated with far-red light, f rapidly decreased. This effect is exactly the opposite of that observed with the other spin labels, where microviscosity increased in the dark and decreased in the light. However, the signal intensity followed the same pattern as the other spin labels by decreasing in the dark and increasing in the light (data not shown). Partitioning spin labels are known to permeate plasma membranes and partition into all of the membranes of the cell (19). The changes in membrane fluidity observed are probably due to changes occurring in the majority of the cellular membranes. In oat leaf protoplasts, most of the cellular membranes are thylakoids.

Attempts to incorporate PC(5,10) into oat leaf protoplasts were successful (Figure 14). The ESR spectrum shows that the label is rapidly rotating about the long molecular axis of the hydrocarbon chain. The label is not in a micelle or spin label vesicle since this would bring the nitroxides into such close proximity with each other that the resultant spin-spin interactions would obscure the hyperfine structure seen in the ESR spectrum. The intensity of the signal gradually decayed with

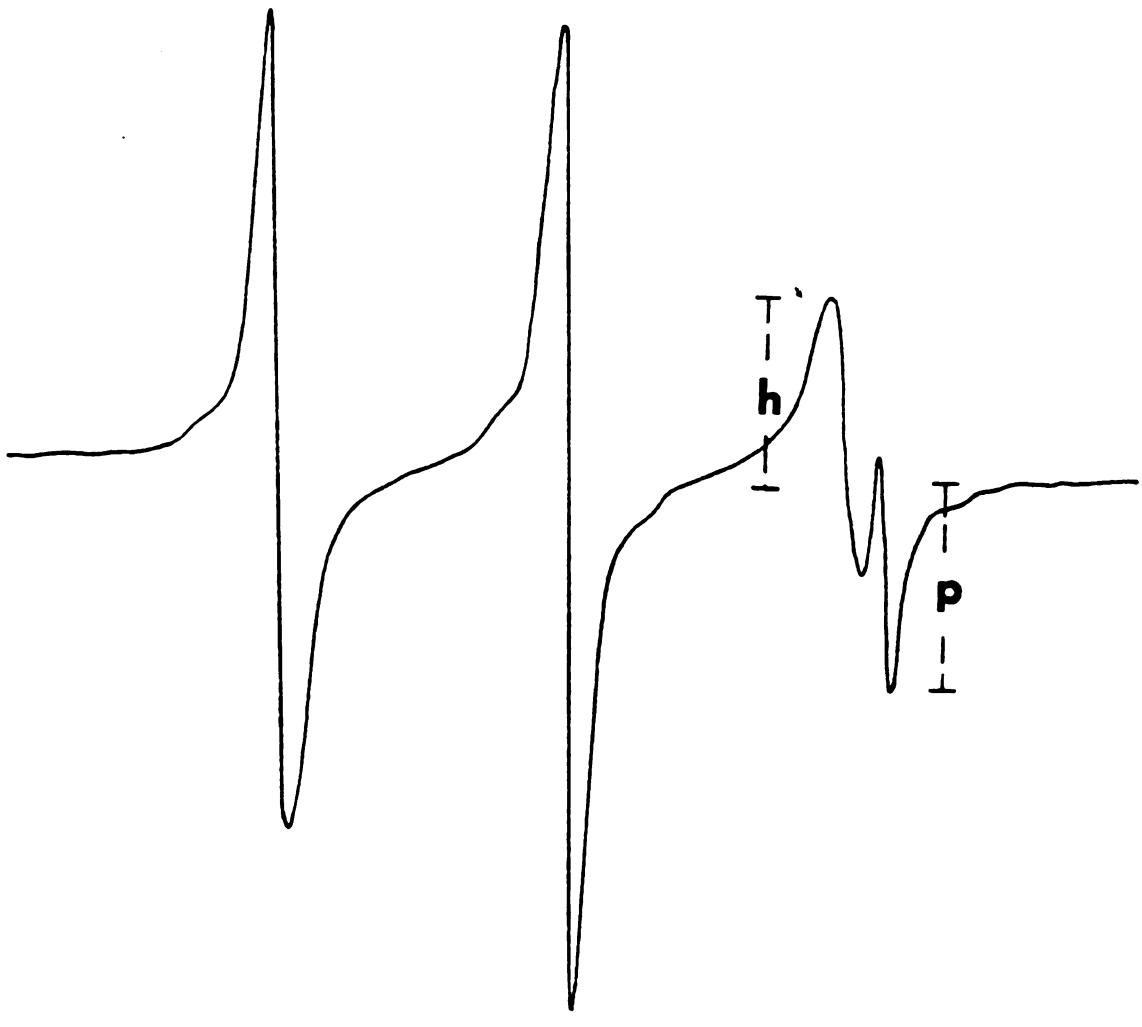

Figure 12. ESR spectrum of 2N8-labeled protoplasts. The sample was scanned at 21.8 C in the dark with a modulation amplitude of 0.25.

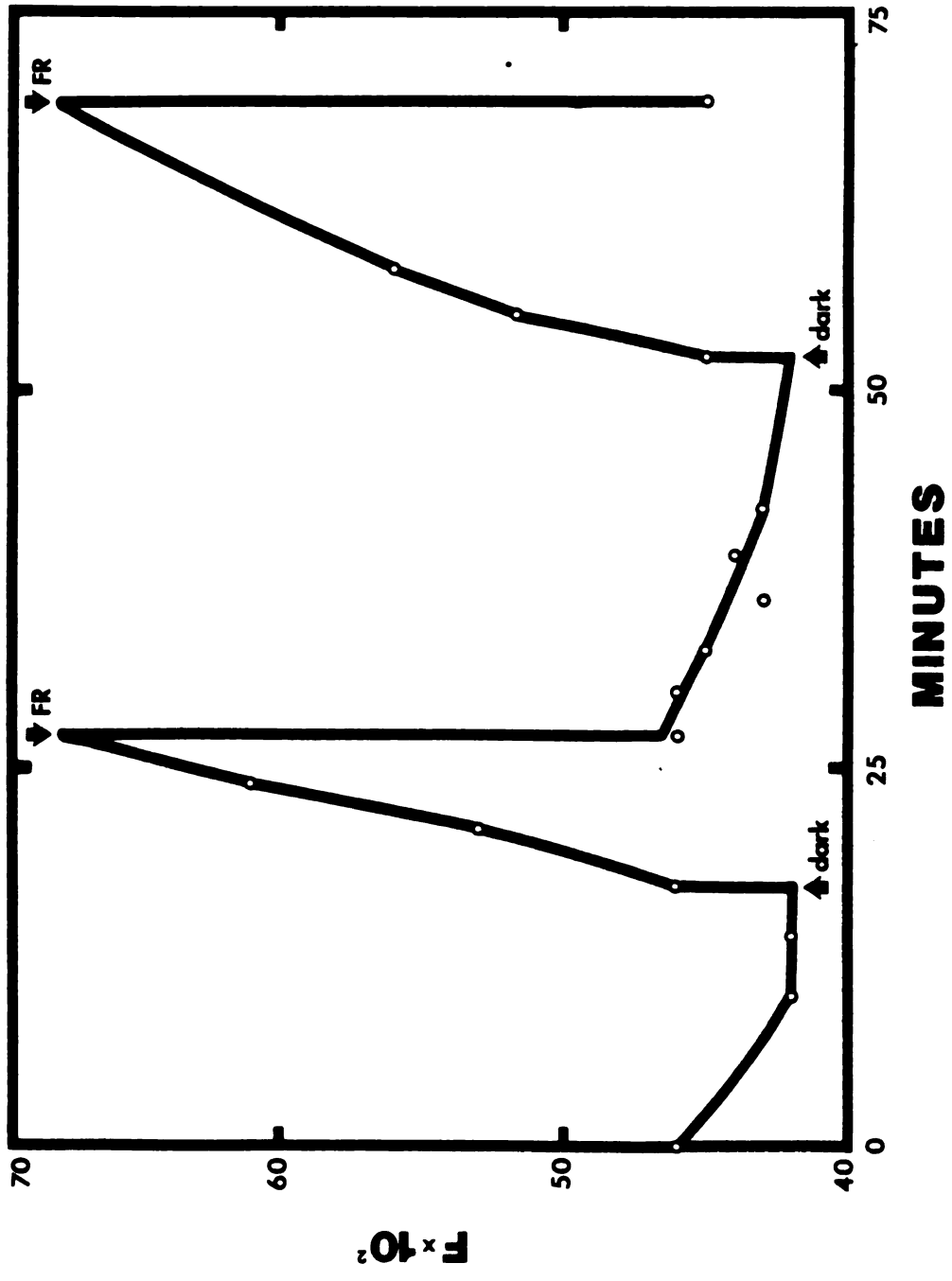

Figure 13. Effect of far-red light on 2N8-labeled protoplasts. Fluidity was measured as F = h/(h + p) where h and p are the portions of the spin label population in the membrane and in the aqueous phase, respectively. The sample was held at 23 C during the dark and far-red (FR) irradiation periods.

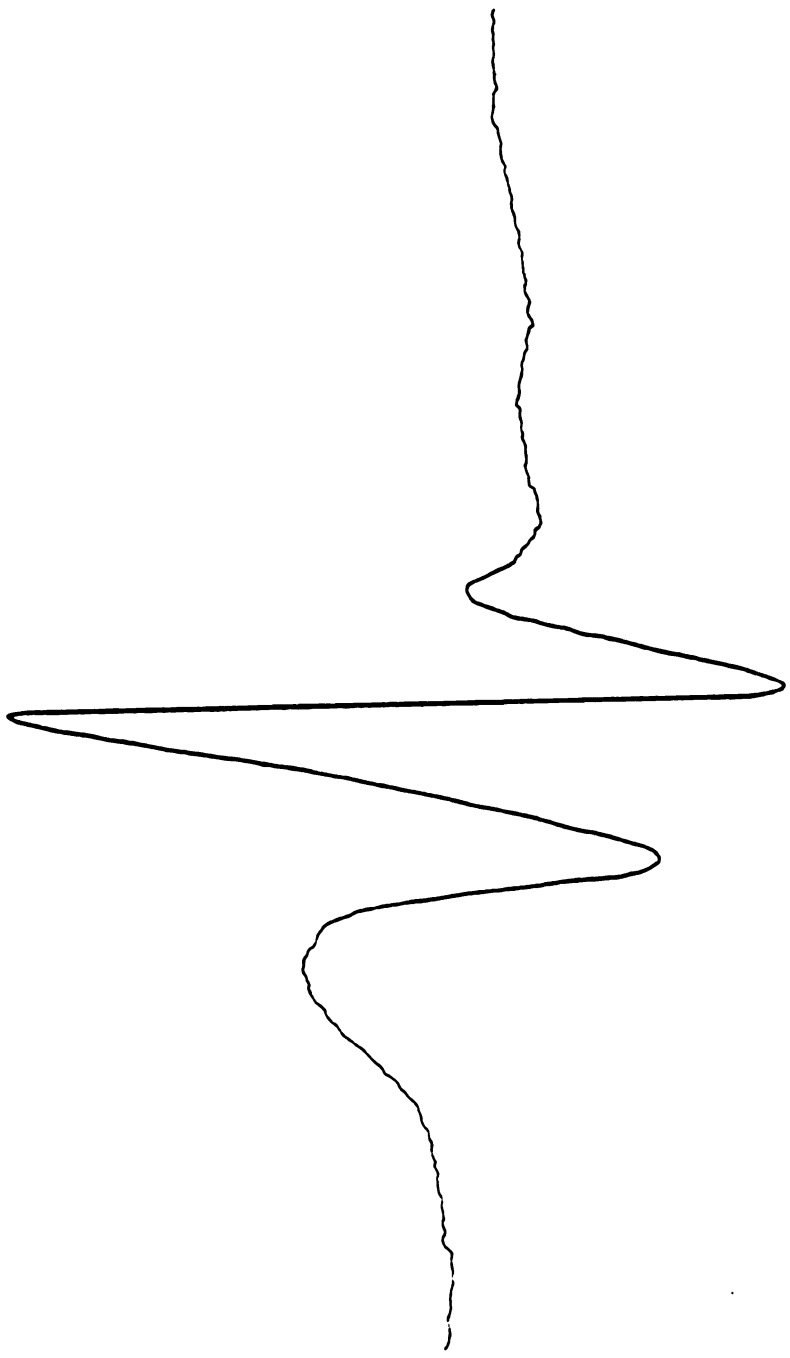

Figure 14. ESR spectrum of PC(5,10)-labeled protoplasts. The sample was scanned in the dark at 23 C.

time (Figure 15). When the sample was irradiated with white light a transient P-700 signal appeared (to be discussed) which caused the mid-field line to increase. However, the signal intensity due to the nitroxide continued to decrease. The rotational correlation time prior to irradiation (5.03×10^{-9}sec) was not significantly different after irradiation (4.47×10^{-9}sec). These results indicate that the membrane effector of the light response cannot affect the location probed by PC(5,10).

A spin labeled analog of androstane was incorporated into protoplasts. Irradiation with white light caused an immediate increase in the signal intensity (data not shown) indicating that sterols are able to penetrate to the site of photooxidation. Attempts to incorporate the cholestane spin label into protoplasts were unsuccessful.

When oat leaf protoplasts were spin labeled with I(12,3), exposed to ascorbate, and incubated, all at 0 C, the reduction kinetics of the signal intensity were distinctly biphasic (Figure 16). If the temperature of the sample was allowed to increase after exposure to ascorbate, the break in the reduction kinetics was not observed. This indicates that there are two populations of spin label being sequentially reduced: the population in the outer leaflet of the plasmalemma, followed by the population in the inner leaflet and the intracellular membranes. When the temperature of the sample was increased to 23 C after complete reduction of the spin label, irradiation of the protoplasts rapidly regenerated the ESR signal (data not shown). The time elapsed from initial exposure of the cells to ascorbate to the initiation of the second phase of reduction (21 minutes) was identical to the half-life for penetration of ascorbate into electric eel membrane vesicles at 0 C (35). These results indicate

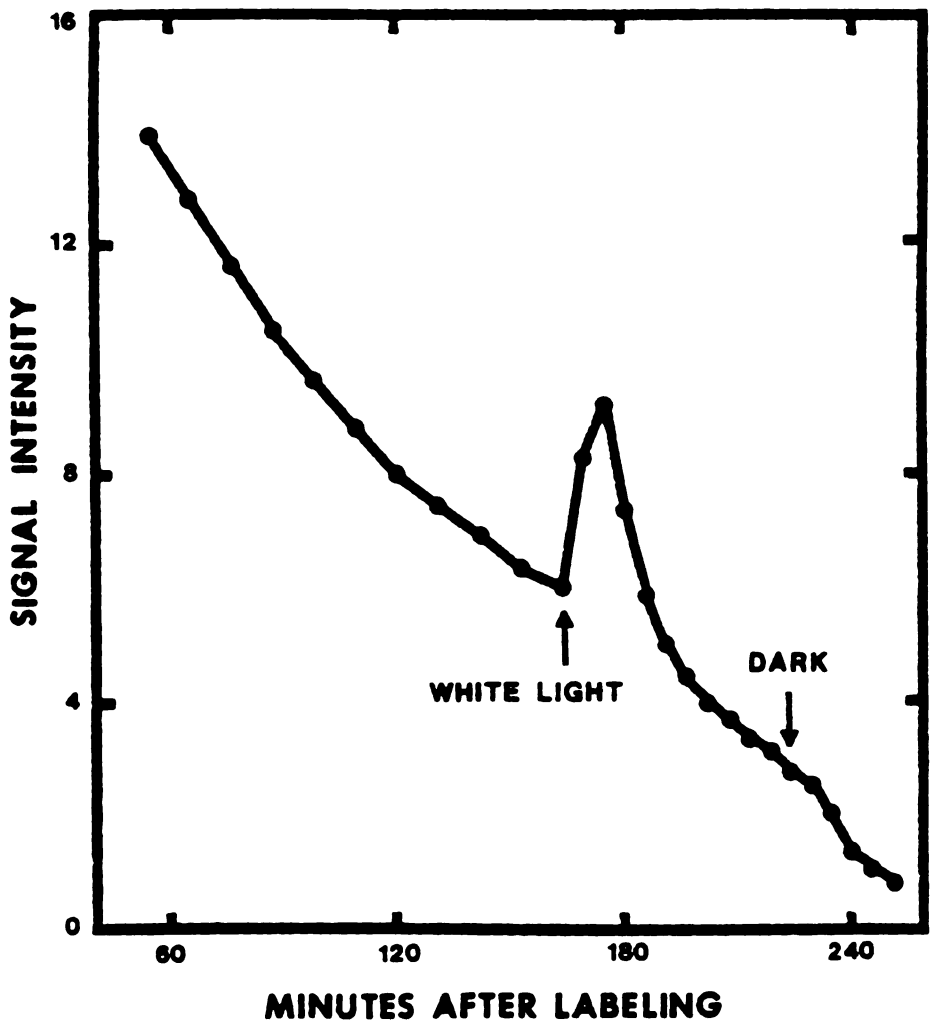

Figure 15. Effect of white light on PC(5,10)-labeled protoplasts. The sample was held at 23 C in the dark until irradiation.

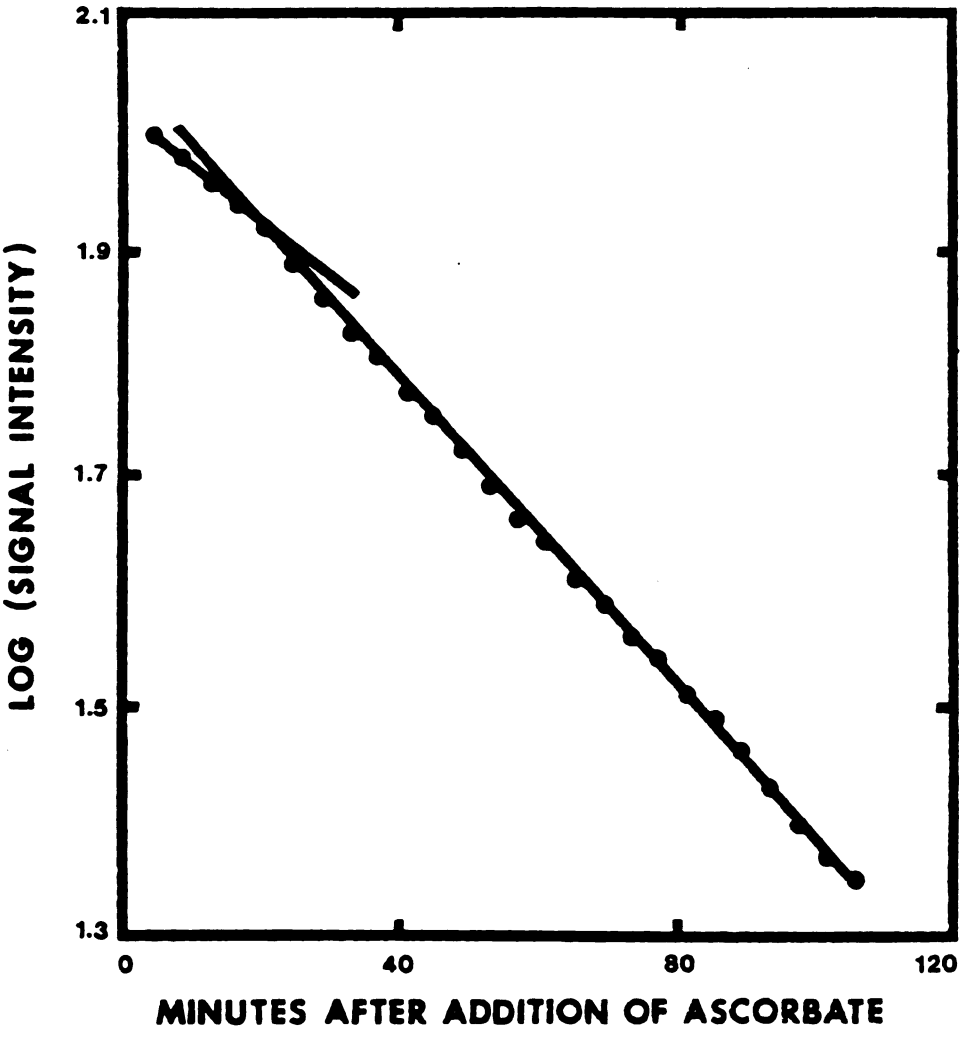

Figure 16. Effect of ascorbate on I(12,3)-labeled protoplasts. After labeling, the sample was cooled to 0 C, exposed to 10 mM ascorbate, and repeatedly scanned in the dark. A break in the line is seen at 21 minutes after addition of ascorbate.

that fatty acid spin labels are not selectively retained in the plasmalemma.

Protoplasts which were spin labeled with PC(5,10) gave an undiminished ESR signal when incubated with ascorbate at 0 C for 1 hour (Figure 17). The failure of ascorbate to reduce PC(5,10) is not surprising. Rousselet et al.(44) found that it took 1 hour to completely reduce PC(10,3) in the outer leaflet of erythrocyte membranes but 4 hours to reduce PC(7,6). PC(m,n) spin labels are probably more resistant to ascorbate reduction than I(m,n) spin labels as a result of their greater stability in the membrane. I(m,n) spin labels may be able to orient in bilayers with their nitroxyl and carboxyl groups at the membrane interface (14). This would greatly facilitate interaction with ascorbate.

Oat leaf protoplasts were spin labeled with I(12,3) and divided into two portions. One portion was washed with solutions containg 100 mM $NiCl_2$, an ESR signal broadening agent; the other portion was washed with solutions that lacked $NiCl_2$. The relative intensity of the Ni^{++} -treated sample varied widely (25-65% of control values) from experiment to experiment. Such a high concentration of Ni^{++} could be toxic to the protoplasts. This, in turn, could lead to an increased permeability to Ni^{++} or to a greater rate of chemical reduction of the probe. These possibilities make the results ambiguous, even though they are comparable to the results of others (19). Line broadening agents are of questionable value as tools to locate spin labels unless there is a stable background signal intensity and a proven lack of permeability.

Oxidizing agents such as $K_3Fe(CN)_6$ are often used to stabilize or regenerate signals which would otherwise be reduced by cellular metabolism, (see "Introduction"). There was little signal decay in the dark when

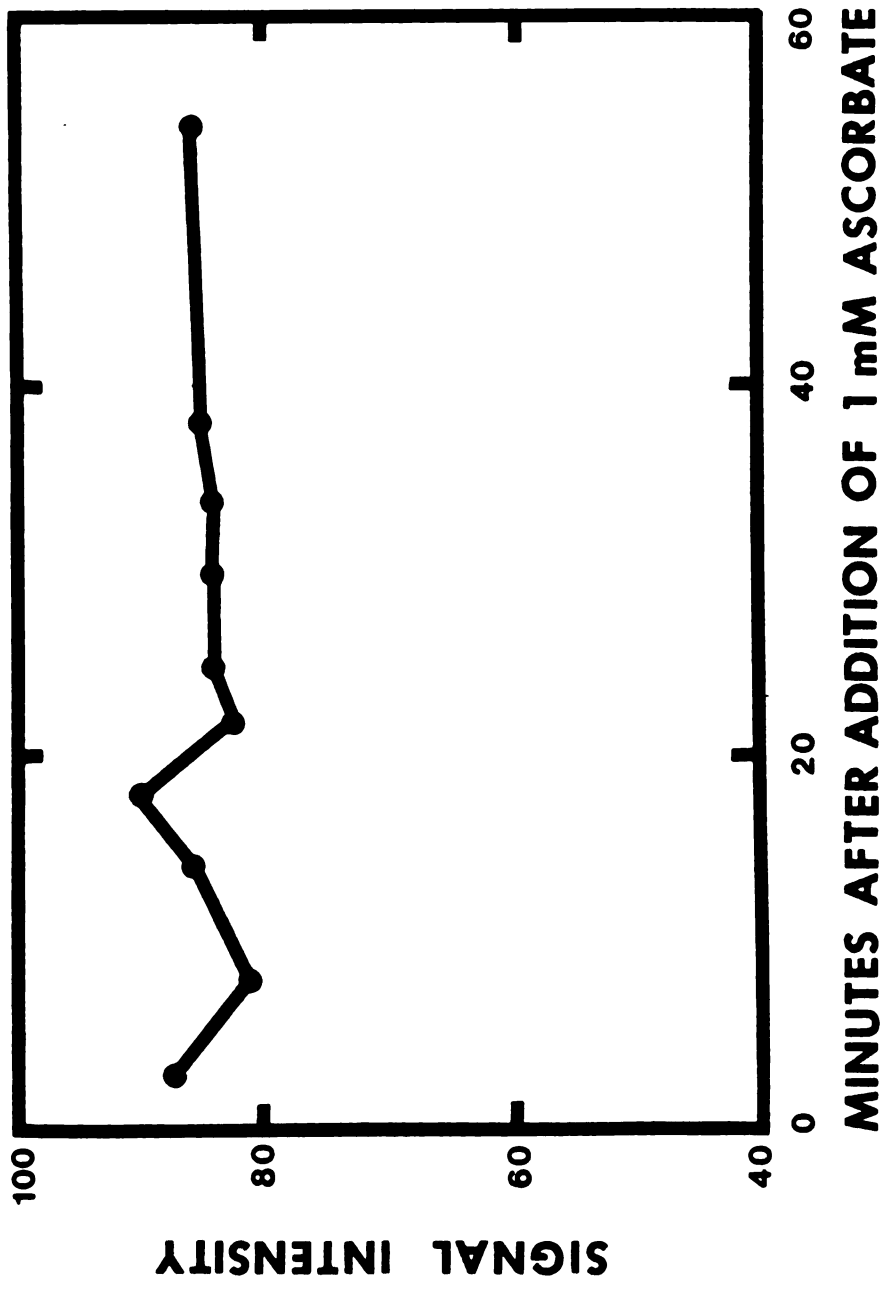

Figure 17. Effect of ascorbate on PC(5,10)-labeled protoplasts. All procedures were conducted at 0 C in the dark.

when 1 mM $K_3Fe(CN)_6$ was included in the wash medium of oat leaf protoplasts spin labeled with I(12,3). Irradiation with white or far-red light did not change the signal intensity or the motion parameter. Unless all of the probe was in the outer leaflet of the plasmalemma (which is inconsistent with the ascorbate reduction data), these results suggest that either $K_3Fe(CN)_6$ penetrated into the cell interior or else the spin label inside the cell was rapidly exchanging with the spin label in the outer leaflet of the plasmalemma.

Experiments with Chloroplasts

Chloroplasts were isolated and spin labeled with I(12,3) in an attempt to resolve the questions of spin label localization and the origin of the light effect. The ESR spectrum was the same as that observed from freshly-labeled or irradiated protoplasts ($2T_{\parallel}$ = 56 gauss). The chloroplasts caused a much slower reduction of the signal intensity in the dark than did protoplasts. When irradiated with white light, the chloroplasts rapidly reduced the spin label (Figure 18). This is the opposite of the response observed in protoplasts. Far-red light had the reverse effect, causing a slow oxidation of the spin label. These results are incompatible with the idea that the light effect may be a high-irradiance response of phytochrome. The results can be reconciled, however, with a photosystem I interaction with spin label. The generation of excess reducing equivalents by white light could produce free radicals or reducing agents which would react with the nitroxide. This could account for the signal loss due to white light. Far-red light, which only drives photosystem I, does not cause electrons to accumulate. On the contrary,

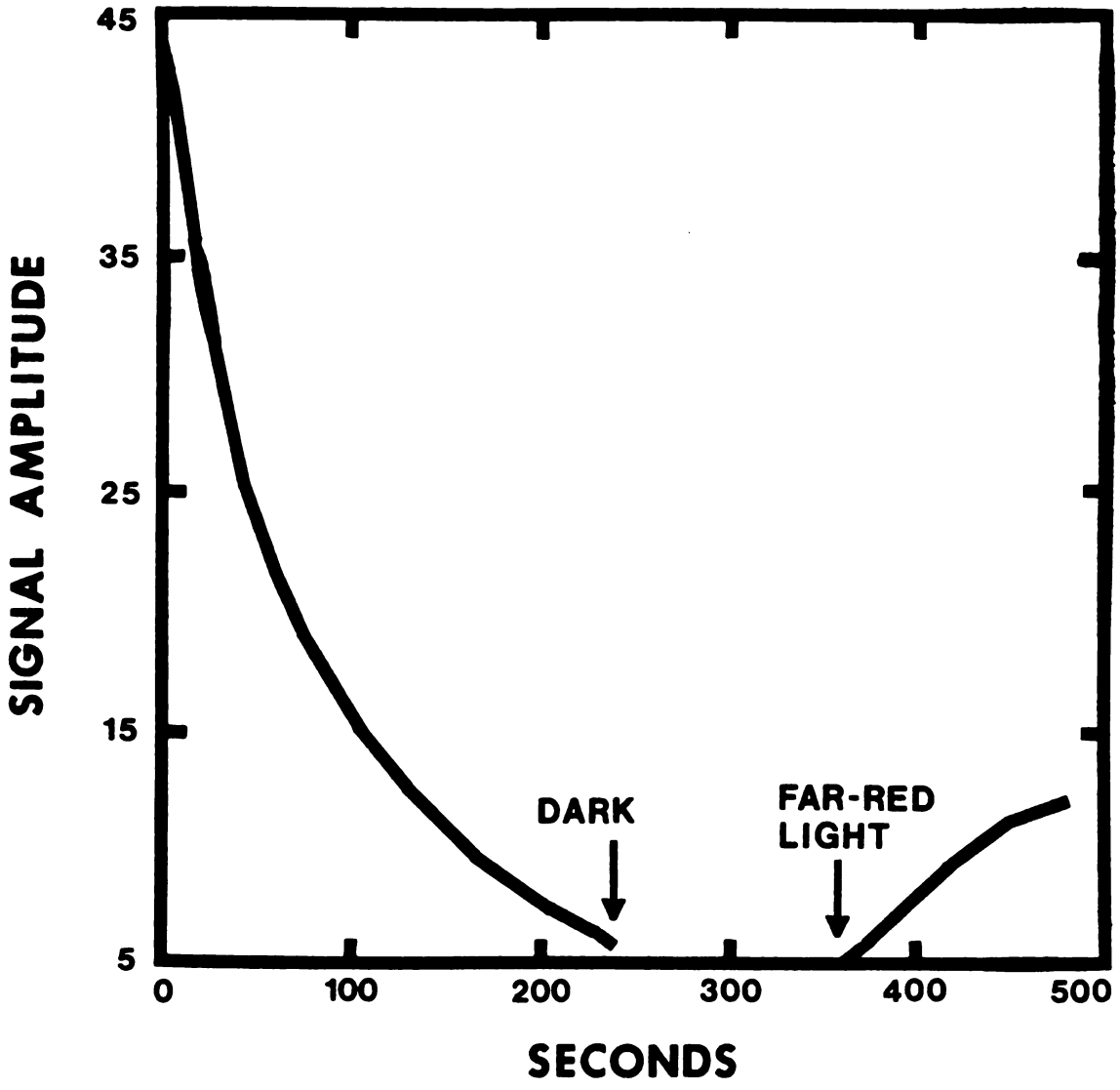

Figure 18. Effect of white and far-red light on I(12,3)-labeled chloroplasts. The sample was irradiated with white light at T = 0.

oxidation of the photosystem I reaction center by far-red light might create "holes" in the electron transport chain which could accept electrons from reduced nitroxides. This would account for the signal gain due to far-red light.

Further evidence that the photosynthetic pigments are involved with the light response was obtained by treating chloroplasts with 3(3,4 - dichlorophenyl) -1,1-dimethyl urea(DCMU) (Figure 19). Irradiation with white light failed to cause the rapid signal reduction in DCMU-treated chloroplasts which is seen in untreated chloroplasts. This supports the idea that reduction is caused by the accumulation of reducing equivalents in photosystem I. Possible reasons that white light induces the oxidation of the spin label in protoplasts is that (i) there is ample supply of endogenous terminal electron acceptors to protect the spin label; and (ii) white light can generate a continuous supply of "holes" in the electron transport chain. Regardless of the light status of the chloroplasts, the hyperfine splitting was always the same (Table 2). Therefore, the light-associated decrease in membrane microviscosity which was observed in protoplasts is not caused by structural transitions within the thylakoid membranes.

Chloroplasts were also spin-labeled with PC(5,10). In the dark, there was a very slow reduction of the signal intensity. Irradiation with white light greatly accelerated the reduction of the spinlabel (Figure 20). This contrasts with the absence of a light effect on the ESR signal of PC(5,10)-labeled protoplasts. Since it is clear that PC(5,10) can go into isolated chloroplast membranes and react with the photosynthetic pigment complex, the results obtained with protoplasts suggest that PC(5,10) is retained within the plasmalemma. If PC(5,10) were partitioning between

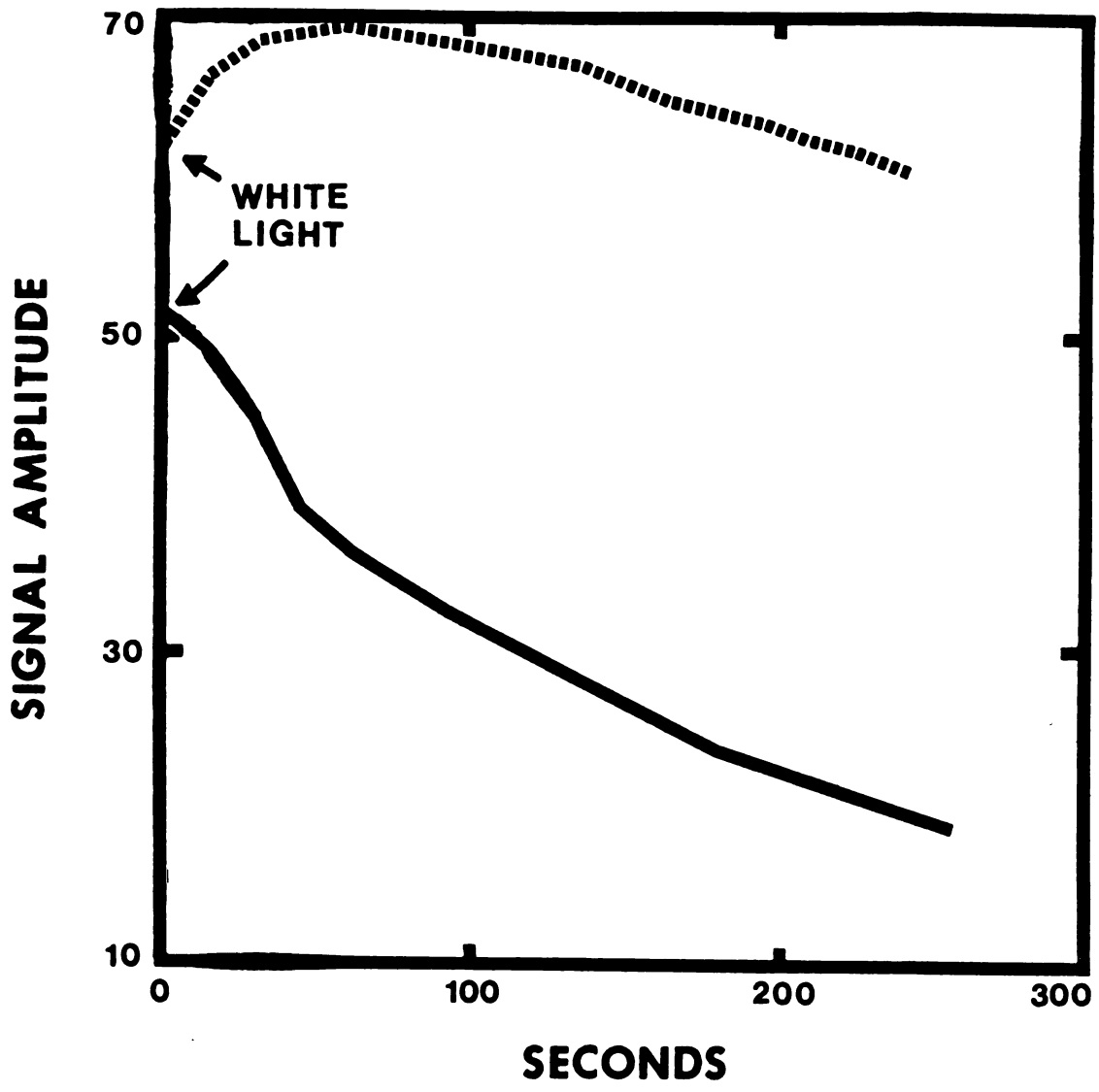

Figure 19. Effect of white light on I(12,3)-labeled chloroplasts in the presence of DCMU. Chloroplasts were irradiated in wash medium (▬) or wash medium plus 5 μM DCMU (▪▪▪▪).

Table 2. Effects of white light on the I(12,3) ESR signal from chloroplasts.

t[a]	$2T_{II}$	H[b]	Conditions
36	56.8	28.0	Dark @ t = 20
66	57.0	6.5	White light @ t = 60

[a] t = minutes after labeling sample

[b] H = signal amplitude

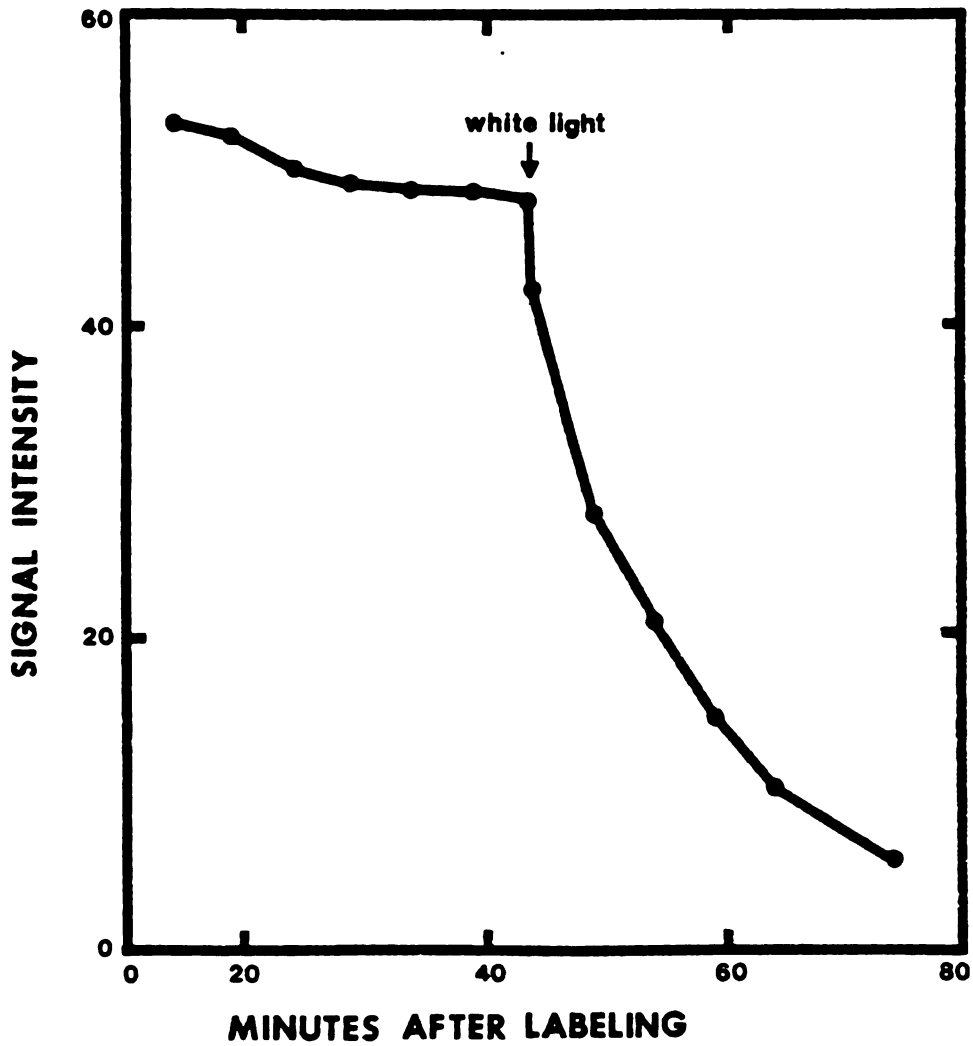

Figure 20. Effect of white light on PC(5,10)-labeled chloroplasts. Chloroplasts were held in the dark at 23 C until irradiated.

all of the cellular membranes, as do the other spin labels, some would have been incorporated into thylakoid membranes and been oxidized by light.

Experiments with Protoplast Lysates

Protoplasts which had been precooled to 0 C were very resistant to lysis. Therefore, protoplasts were spin labeled with I(12,3) at 23 C and then were simultaneously lysed and cooled to 0 C. The lysate was divided into chloroplast and non-green membrane fractions (see Materials and Methods) and the relative proportion of spin label was determined for each fraction. $K_3Fe(CN)_6$ was included in the suspension medium to oxidize spin label which had been reduced. The weight of the two fractions was not determined. However, the volume of the chloroplast pellet was typically 0.1 ml, whereas the non-green membrane pellet was > 0.01 ml before being resuspended. More than 95% of the I(12,3) spin label was associated with the chloroplast pellet. Similar experiments with PC(5,10)-labeled protoplasts indicated that approximately 50% of the spin label was associated with the chloroplast pellet. No attempt was made to biochemically characterize the membranes of either fraction. Although the chloroplasts were excluded from the non-green membranes,(based on visual observation), most other types of membranes (plasmalemma, tonoplast, endoplasmic reticulum, mitochondria, etc.) were probably present in both fractions. The results are consistent with the hypothesis that I(12,3) was present in all membrane fractions, and PC(5,10) was preferentially associated with the non-green membranes, presumably the plasmalemma.

The microviscosity of the non-green membranes was high, greater than that observed in dark-incubated protoplasts ($2T_{//}$ = 63 gauss with I(12,3)) (Table 3). Intact, freshly labeled protoplasts had to be cooled to 0 C to obtain a similar spectrum. The signal was stable in the dark and neither white nor far-red light had any effect. The same results were obtained whether the membranes were labeled before or after isolation. Membrane viscosities of protoplasts, chloroplasts, and non-green membranes were compared (Table 3). In the light, protoplast membranes are similar to chloroplasts, but in the dark, they are similar to non-green membranes.

Spectral Anomalies

The line shapes of the spin label signals often became distorted when dark-incubated protoplasts were scanned at high gains. Typically, a broad "dip" (line A) appeared at the low-field end of the spectrum. When the protoplasts were irradiated with either white or far-red light, not only did the nitroxide signal become larger, but a "dip" (line B) appeared just above the midfield line. In addition, the midfield line became broader. These "spectral anomalies" are shown in Figure 21. As the nitroxide signal intensity increased, lower gains were required to fit the spectrum onto the recorder. In contrast, the intensity of the anomalous signals remained constant. At sufficiently low gains, only the nitroxide signal was observed.

The g-values of nitroxides are similar to most radicals (approximately 2); therefore, the appearance of ESR signals in this region does not help to identify the radicals. To determine whether or not the

Table 3. Comparison of I(12,3) motion in protoplast, chloroplast, and non-green membranes.

Sample	$2T_{II}$	
	Light	Dark
Protoplasts	56.4	62.0
Chloroplasts	56.8	57.5
Non-green Membranes	63.5	63.5

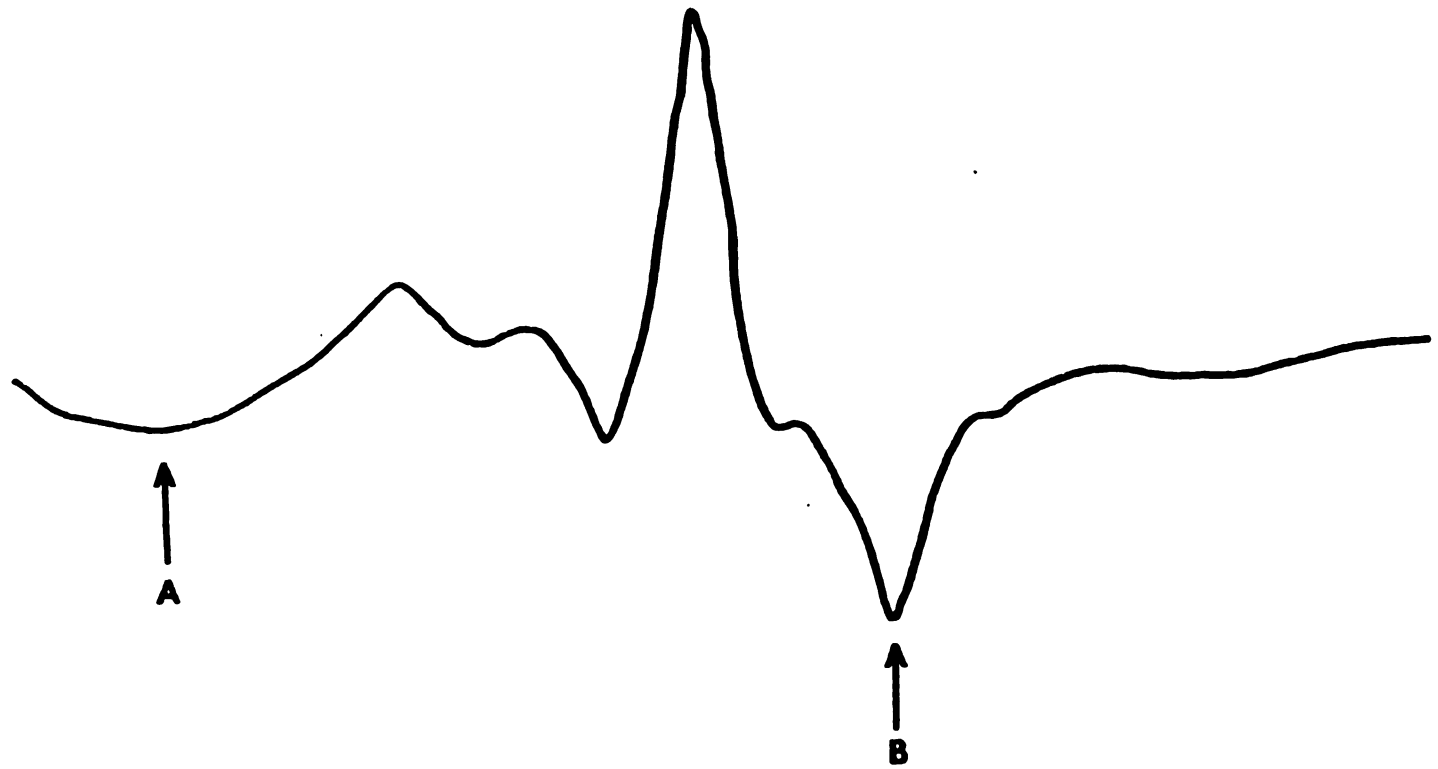

Figure 21. ESR spectrum of I(12,3)-labeled protoplasts showing endogenous signals. At low amplitudes, signal A becomes apparent in the dark and signal B appears upon irradiation.

signals are endogenous (rather than being associated with the nitroxide), protoplasts without a spin label were scanned. In the dark a shallow wave was observed (line A). This spectrum was not affected by the addition of $K_3Fe(CN)_6$. Upon irradiation, an intense line appeared at the same position as line B (Figure 22); this line disappeared in the dark. Neither 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) nor ascorbate inhibited the generation of line B. When line B was generated by far-red light and red light was suddenly added, the intensity of line B diminished quickly and then rapidly recovered (data not shown). Similar results were obtained with isolated chloroplasts, except that $K_3Fe(CN)_6$ caused the appearance of line B in the dark. The results suggest that line B is due to the oxidized photosystem I reaction center, P-700$^+$ (1).

Line A was identified when the scan range was expanded to 1000 gauss (Figure 23). Tanner et al. (54) observed a similar spectrum in Chlorella cells after one hour of CO_2-deprivation. This signal is due to free Mn^{++}, which has a nuclear spin quantum number of I = 5/2 and can, therefore, be in six spin states. These correspond to the six lines of the ESR spectrum. Protoplasts and chloroplasts are deprived of CO_2 when they are held at high cell density in the ESR cuvettes.

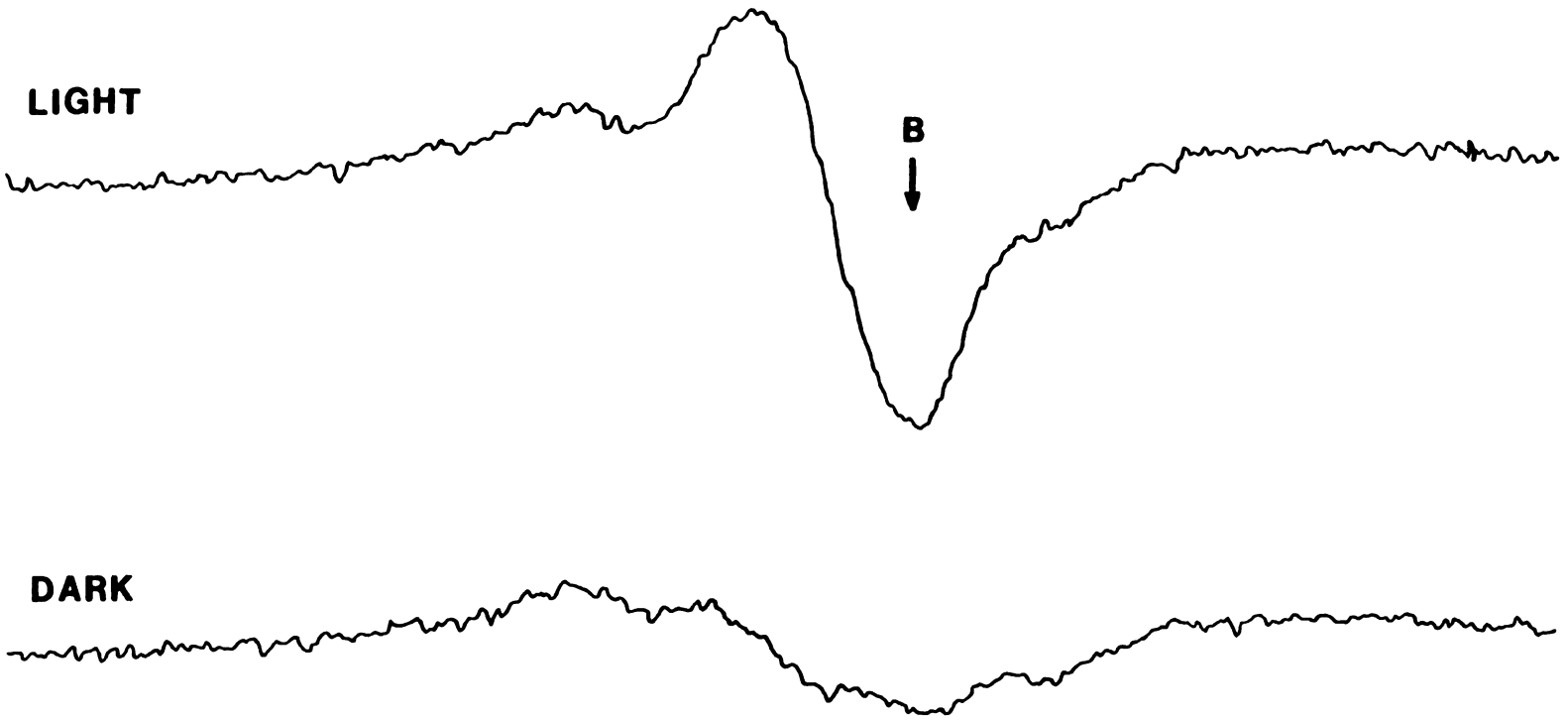

Figure 22. Endogenous ESR signals of non-labeled protoplasts in the light and dark. The sample was scanned at 22.8 C with a normal (100 gauss) scan range.

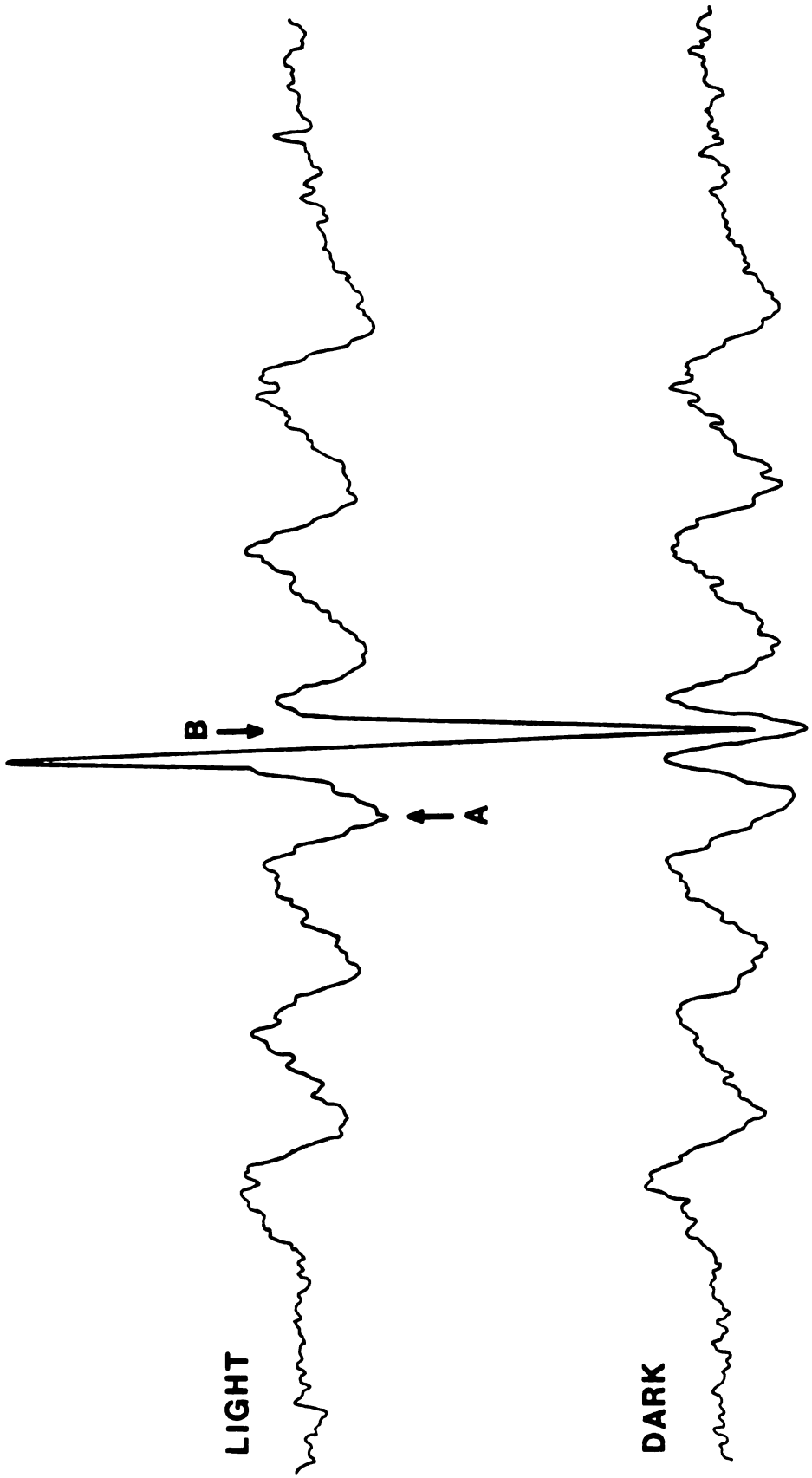

Figure 23. Wide-field scan of endogenous ESR signals. Unlabeled protoplasts were scanned at 23 C in the light or dark with a scan range of 1000 gauss.

DISCUSSION

Changes in Signal Intensity

I. Review of the Literature

The mechanism of reduction of spin label in the dark is understood in some cases. Dark reduction by rat liver mitochondria has been attributed to a component of the electron transport chain located in the phospholipid headgroup region of the membrane; the component probably is a semiquinone of ubiquinone (43). Dark reduction of spin label in chromatophore membranes of the photosynthetic bacterium, Rhodospirillum rubrum, is associated with a cytoplasmic reductase system (33).

The mechanism of photoreduction and photooxidation of spin label is also understood in some cases. Washed chromatophore membranes of R. rubrum do not reduce spin label in the dark (33). When irradiated, the chromatophores rapidly reduced 2,2,6,6-tetramethylpiperidine-n-oxyl (TEMPOL) and I(5,10). The electron transport inhibitor, Antimycin A, blocked the photoreduction of TEMPOL and reversed the light effect on I(5,10), resulting instead in photooxidation. If I(5,10) was first reduced in the dark, subsequent irradiation caused 20 to 30 % of the spin label population to be reoxidized. If reduced TEMPOL was used instead, light had no effect. The authors suggest that photoreduction of spin label is due to the generation of soluble reducing agents by the photosystem. This is blocked by Antimycin A. Photooxidation is due to a direct coupling with the electron transport chain (i.e., I(5,10) donates electrons to

the system) somewhere after the Antimycin A block. TEMPOL, being excluded from the hydrocarbon interior of the membrane, does not have access to the oxidizing site.

Corker et al. (15) were the first to study spin label interactions with isolated chloroplasts. They used the spin label di-tertiarybutyl-nitroxide (DTBN) and observed that freshly isolated chloroplasts caused a rapid, irreversible destruction of spin label in the dark. Aged chloroplasts or chloroplast fragments caused little or no reduction in the dark but when irradiated caused a rapid reduction of DTBN. The response was abolished by 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). In contrast, the reduced form of DTBN was rapidly oxidized to the nitroxide and then slowly destroyed when aged chloroplasts or fragments were irradiated. The authors believe that the nitroxide is photochemically destroyed by adduct formation with a free radical which is generated by the light reactions.

Torres-Pereira et al. (56) observed that chloroplasts failed to reduce nitroxides in the dark but rapidly reduced them in the light. Subsequent incubation in the dark caused a slow reoxidation of the probe. I(13,2) was reduced 30 % faster than I(5,10), suggesting that the sight of reduction is near the membrane interface. The reducing equivalents generated by light could not be transferred to BSA-adsorbed I(5,10) in the solution unless the electron carrier phenazine methosulfate (PMS) was present. Pre-reduced TEMPOL was oxidized by chloroplasts in the light.

The photoreduction of water-soluble nitroxides (Monoradical A and Biradical X) by isolated chloroplasts can be prevented by the addition

of ferredoxin and $NADP^+$ to the incubation mixture (52). The ability to protect other types of spin labels was not tested. These authors suggest that the site of reduction in the light reaction scheme is close to where $NADP^+$ is reduced. DCMU inhibited photoreduction of the spin labels.

The slow photoreduction of another water soluble spin label, 2,2,6,6-tetramethylpiperidine-N-oxyl-4-amine (TEMPAMINE) has been observed in the presence of isolated thylakoids (6).

When chlorophyll a is incorporated into phospholipid membrane vesicles the motion of I(12,3) and I(5,10) is restricted but the motion of I(1,14) is not (39). Irradiation of the vesicles causes I(12,3) to be rapidly reduced but I(5,10) and I(1,14) are only slowly reduced. These results indicate that chlorophyll a is capable of photoreducing spin labels and that the active site is associated with the macrocyclic ring near the headgroup region of the membrane. It has been suggested that the electron donor in such systems is H_2O or OH^- (28).

The response of protoplasts to light which I observed are similar to those reported for Chlamydomonas which was spin-labeled with the water-soluble probe 2,2,5,5-tetramethyl-3-carbamidpyrroline-1-oxyl (TEMPAMIDE) (62). Chlamydomonas cells were grown in the presence of TEMPAMIDE, washed, and then spin-labeled with fresh TEMPAMIDE. Irradiation of these samples with white light caused a rapid, reversible increase of the ESR signal intensity. Labeled cells grown without TEMPAMIDE in the medium rapidly decreased the ESR signal during irradiation. This reduction was not inhibited by DCMU. Cells grown in the light in the presence of TEMPAMIDE developed a large Mn^{++} signal over a few hours whereas cells grown in the

dark or without TEMPAMIDE did not develop the Mn^{++} signal. This indicated that bound Mn^{++} is released. Mutants which could not evolve oxygen but still had photosystem I activity did not reduce the spin label in the light. The authors (62) suggest that a semiquinone of plastiquinone is responsible for the light-mediated reduction and that O_2 evolution caused the light-mediated oxidation. When the spin label was photooxidized, there was a slight broadening of the spectral lines, indicative of restricted motion.

II. Discussion of the Data

The results presented in this thesis confirm much of the earlier work. Spin-labeled oat leaf chloroplasts slowly reduce nitroxides in the dark and rapidly reduce them in white light. The light-catalyzed reduction is inhibited by DCMU and partially reversed by irradiation with far-red light. The far-red light effect is not inhibited by DCMU. Prereduced TEMPO is transiently oxidized by white light. Spin-labeled protoplasts reduce nitroxides in the dark but oxidize them in both white and far-red light. This effect is not inhibited by DCMU. The oxidized spin label is slowly destroyed in the light.

This body of evidence strongly suggests that spin labels in chloroplasts are photoreduced by photosystem I, perhaps through an intermediate redox carrier(s), in the absence of terminal electron acceptors. The reduced forms of the spin labels are oxidized back to the nitroxide by an element(s) of the electron transport chain somewhere between the DCMU block and the photosystem I reaction center. These suggestions are consistent with the estimated standard reduction potential of a nitroxide,

E_o' = -0.31 V (33). In addition, an irreversible reduction or destruction of nitroxides appears to be occurring in the light. This destruction is probably the result of the formation of an adduct between the nitroxide and a free radical product of the light reactions as first suggested by Corker et al. (15). A diagram of this reaction scheme is shown in Figure 24. The reactions which would be occurring in both protoplasts and isolated chloroplasts are:

$$\text{N-O-A} \xleftarrow{k_2} \text{N-O}\cdot \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{N-O-H}$$

where $k_1 > k_{-1}$, k_2 in isolated chloroplasts and $k_{-1} > k_1 > k_2$ in protoplasts (and chloroplasts which contain ferredoxin + $NADP^+$).

Changes in Microviscosity

I. Review of the Literature

Protoplast membrane changes which are independent of light/dark include an increase of microviscosity due to senescence (11) and a decrease due to osmotic swelling (10). There are only two reports of light-induced changes in chloroplast membrane microviscosity detected by spin labels. Tzapin et al. (57) reported that the membranes of chloroplasts which had swelled in response to light were more viscous than the dark controls. The membranes of chloroplasts which had shrunk in response to light were less viscous than dark controls. Torres-Pereira et al. (56) questioned these results on the basis that the spin label used was probably hydrolyzed *in vitro* resulting in spectral artifacts. Torres-Periera

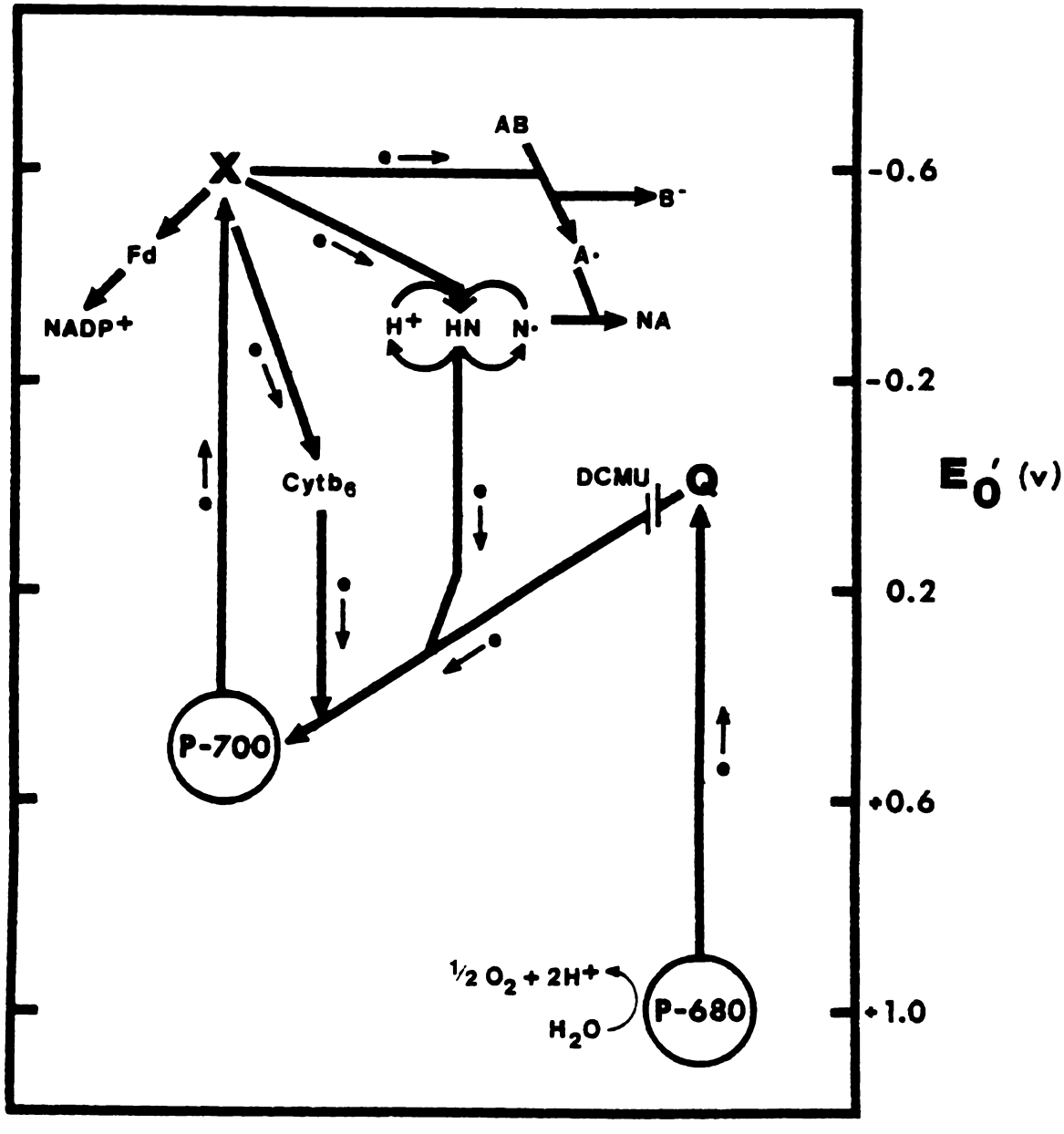

Figure 24. Proposed scheme of electron exchange between nitroxide free radicals and chloroplasts. A· is a hypothetical radical intermediate, generated by the light reactions, which forms an adduct with the nitroxide radical, N·. The reduced form of the nitroxide is HN. The estimated stansard reduction potential of the nitroxide is -0.31 V (33).

et al. (56) claim that the membranes of swollen chloroplasts are more viscous than those of shrunken chloroplasts. Both of these groups fixed their chloroplasts with gluteraldehyde after swelling or shrinking them and prior to being spin labelled. When unfixed, spin-labeled chloroplasts were irradiated there was a small decrease in the microviscosity of the membranes (56).

The apparent light-induced changes in protoplast membranes are similar to those observed in rod outer segment membranes (58). In rod outer segments, light causes a decrease or increase in membrane microviscosity depending upon the presence of Na^{+} or Ca^{++}, respectively. Photoreduction of the spinlabel is also observed.

II. Discussion of the Data

The membrane microviscosity of spin-labeled chloroplasts from oat leaves generally did not change when the chloroplasts were irradiated. Occassionally, when the chloroplasts were irradiated with far-red light there appeared to be a slight decrease in microviscosity, but this was attributed to contamination with non-green membranes (to be discussed).

The microviscosity of protoplast membranes appeared to increase in the dark and decrease in the light. The following arguement suggests, however, that the membranes of protoplasts did not undergo measurable changes in microviscosity, despite the large changes in the spin label motion and partitioning parameters which were observed.

The apparent changes of membrane microviscosity were probably a direct result of the redox reactions previously described. The majority of the membranes in an oat leaf protoplast are thylakoid. With the

exception of PC(5,10), which will be discussed later, none of the spin labels showed any membrane selectivity. Therefore, with the hydrophobic spin labels, the bulk of the population should partition into the thylakoids and the remainder should be in the non-green membranes. The population of probe in the thylakoids was rapidly reduced in the dark and oxidized in the light. The spin label residing in the non-green membranes was probably reduced and oxidized at a much slower rate. Table 3 shows that: (i) the non-green membranes were rigid and unaffected by light; (ii) the chloroplast membranes were fluid and unaffected by light; and (iii) the protoplast membranes varied in fluidity between the values of the chloroplast and the values of the non-green membranes, depending on light conditions. These findings suggest that the net ESR signal, which is the sum of the spectra from all of the spin label populations, was dominated during irradiation by the population of spin label located in the thylakoids (because that population was fully oxidized). In the dark, the thylakoid population was chemically reduced and the signal was dominated by the population which was in the non-green membranes. The apparent increases and decreases of microviscosity were the result of the changes in the ratio of these two spin populations with respect to one another.

These results should serve as a warning to investigators who are using spin labels to study light-induced structural changes in chloroplast membranes. The presence of non-green membrane contaminants could result in apparent changes in microviscosity, especially if the signal intensity is changing. These comments apply to experiments on other organelles as well.

Data with the partitioning spin label, 2N8, indicated that the

membrane microviscosity decreased in the dark and increased in the light in direct contradiction to the results obtained with other spin labels. Keep in mind that the fraction of 2N8 which was in the membrane (h) would be oxidized more quickly than the fraction which was in the aqueous phase (p) causing an increase in the fluidity index, f , ($f = h/(h + p)$). This is consistent with the reports that the sites of spin label oxidation and reduction are in the phospholipid headgroup region of the membrane (33,39,43,56). In the dark the opposite occurs. It is significant that both h and p increased in the light and decreased in the dark. If there was a change in partitioning, p might have decreased in favor of h and vice versa.

Location of Spin Labels

The fact that a spin label is oxidized by protoplasts in white light strongly suggests that the label is partitioning throughout the cell. This effect could not be caused by chloroplasts released into the medium from ruptured protoplasts because chloroplasts would reduce the spin label, not oxidize it. The failure of the protoplasts to oxidize a spin label suggests that either the probe is: (i) retained in the plasmalemma; (ii) excluded from the thylakoids; or (iii) incorporated into the thylakoids, but unable to donate electrons to the electron transport chain. Isolated chloroplasts can be used to determine which of these three possibilities is the case. Failure to incorporate into the plasma membrane would result in either no signal, or strong spin-spin interaction. If incorporation occurred but the chloroplasts could not reduce spin label in the light, then possibility (iii) would be correct. If the

spin label could incorporate into the chloroplast membranes and was rapidly reduced in the light, then it probably is selectively retained in the plasmalemma. Most likely, it results from a very high energy of activation for transverse motion through the bilayer (i.e., a low flip-flop rate) and a low solubility in polar solvents.

Spin-labelled phospholipids have been studied extensively in model bilayers (34). Their rate of transverse motion (flip-flop) varies widely depending upon the cell type used. In Electrophorus membrane vesicles the half-time for flip-flop was 3.8-7 minutes at 15 C (35); this is an order of magnitude faster than the rate observed in phospholipid vesicles (27). The fastest rate described is for Acholeplasma laidawii (15 sec)(17). The rate in erythrocytes is considered to be zero (44). With such heterogeneity, it is imposssible to predict the behavior of these types of spin labels in an untested membrane.

Ascorbate reduction at 0 C has been used to determine the location of spin labels. Ascorbate is unable to penetrate the membranes of phosphatidylcholine vesicles (37) and erythrocytes(44). Only the spin label located in the outer leaflet of the bilayer is reduced. This method has been used to study the rotation of the ATPase in sarcoplasmic reticulum vesicles (55). However, it is unrealistic to assume that ascorbate will not penetrate the membranes of nucleated cells. Ascorbate is known to rapidly penetrate Electrophorus membrane vesicles, (35).

Biphasic reduction kinetics in the presence of ascorbate at 0 C were observed. This indicates a heterogeneous distribution of fatty acid spin label throughout the cell, confirming other workers (19). This method must be used with caution because unintentional warming of the sample for a matter of seconds can eliminate the "break" in the reduction kinetics.

The use of oxidizing and broadening agents has also been suggested to determine whether a spin label is inside or outside a cell, or is in the outer leaflet or the inner leaflet of a membrane. However, this method is of questionable value unless the agent is known to be excluded by the membraneduring the experiment. The permeability of a membrane to oxidizing and broadening agents is difficult to determine if endogenous reduction, oxidation, or destruction of the spin label is occuring. A further problem with oxidizing agents is that they "activate" all of the spin label in the solution, including that which is incorporated into cell fragments and debris.

The oxidizing agent $K_3Fe(CN)_6$ prevented the ESR signal changes in the dark. The use of "signal stabilizers" such as $K_3Fe(CN)_6$ (which keeps the nitroxide oxidized) may lead to artifacts when the location of a spin label in the cell is not known. This is especially true if the permeability to $K_3Fe(Cn)_6$ is also unknown. Some membranes may be permeable to $K_3Fe(CN)_6$ (31) but others are definitely permeable (6). A discontinuity in the plot of spin label motion vs. temperature could be caused by an increased permeability to $K_3Fe(CN)_6$ rather than to a phase transition. If the $K_3Fe(CN)_6$ entered the cell and oxidized spin label in membranes whose viscosity differed from the plasma membrane, a discontinuity in the plot would be expected. This should be associated with a sudden increase in signal intensity at the"phase transition" temperature. Of course, at actual phase transitions membrane permeability may be enhanced(9,29,32), resulting in the events described above. If so, then $K_3Fe(CN)_6$ diffusion into the cell could be an even more sensitive marker of the phase transition than the motion parameter of the probe in the outer membrane. Prevention of endogenous reduction with $Fe(CN)_6^3$ could be due to either

penetration of $K_3Fe(CN)_6$ into the cell interior or to a rapid exchange of spin label between the internal and external membranes.

With broadening agents, only spin labels which are within approximately 10 A are affected. If the broadening agent cannot enter the cell (a condition which is generally assumed) then only the ESR signal from spin label which is in the outer leaflet of the plasma membrane can be broadened. This method can be used in a manner similar to that described for ascorbate (19). It can also be used to measure the viscosity of intracellular cytoplasm (25) or organellar lumens(7). One drawback of this method is that high concentrations of the ions are required (100 - 400 mM) (31). Recently, moderate concentrations of chromium oxalate (40 mM) have been reported to broaden the ESR signal from water soluble spin labels as effectively as high concentrations of Ni^{++} or $Fe(CN)_6^{-3}$ (7). The use of broadening agents to localize spin labels is valid only if the broadening agents are known to be excluded from the cell interior. This can be established by using water solubel spin labels to which a broadening agent is added; thereafter, the observed ESR signal originates from the cell interior. The rate of decay of that signal is a measure of the rate of entry into the cell of the broadening agent. This method is difficult to use if endogenous chemical reduction of the spin label is also occurring.

I observed broadening of the ESR signal from I(12,3)-labelled protoplasts with $NiCl_2$ (25-65% of the signal was lost) but, because the signal intensity was modulated simultaneously by light/dark, the effects of Ni^{++} must be considered ambiguous.

Several classes of spin label were used in these experiments: fatty acid and ester (I(12,3), I(14,1), Me-I(5,10)); hydrophilic (iodoacetamide,

maleimide); partitioning (2N8); steroid (androstane, cholestane); and phospholipid (PC(5,10)). Only the phospholipid appeared to be selectively retained in the plasmalemma. This confirms the report by Hammerstedt et al (19) that the fatty acid spin labels become spread throughout all of the membranes of the cell.

The apparent distribution of the fatty acid and phospholipid spin labels is consistent with the reported lipid composition of oat membranes (24). The total lipid and mitochondrial lipid fractions each contain 3x more fatty acid and phosphatidic acid than the plasma membarne. Lipid hydrolysis reveals that 16:0 is the major fatty acid in the plasma membrane whereas 18:2 predominates in the total and mitochondrial lipids. These latter two fractions are 2x more unsaturated than the lipids of the plasma membrane. Phosphatidycholine comprises 18% of the phospholipid in the plasma membrane but only 15% and 10% in the total and mitochondrial fractions respectively. The predominant lipids of chloroplasts are highly unsaturated (18:3) galactosyl acyl glycerols (51). Phospholipids are only minor components.

Butler et al. (13) reported that stearic acid (18:0) spin labels preferentially partition into the more fluid membranes of a heterogeneous vesicle population. The distribution of the spin label is a function of the lipid-water partition coefficient which increases with lipid fluidity. The chloroplast membranes were shown to be much more fluid than the non-green membranes in this study, as would be predicted from the lipid composition. Therefore, the rapidlabelling of chloroplasts inside protoplasts and the stability of PC(5,10) in the plasmalemma is consistent with the known biochemistry of the membranes and spin labels.

Fatty acid spin labels can still be used to selectively probe the

plasmalemma if it can be shown that the spin label within the cell is completely reduced while the label in the plasmalemma is not. The success of the method will, hopefully, lead to a greater commercial availability of phospholipid spin labels.

The orientation and position of spin labels within the bilayer of a given membrane should also be considered. In lecithin bilayers, the carboxylate group of fatty acids associates with the choline portion of the lecithin, but the protonated form associates with the phosphate region (45). The latter association displaces the nitroxide moiety at the polar interface 4.5 A toward the interior of the bilayer. Alcohols and esters are displaced 6 and 8 A, respectively, relative to the carboxylate ion. The displacement causes a decrease in the ordering parameter due to greater chain flexibility within the hydrocarbon interior. Modification of pH and charge in the head group region may determine the depth at which I(m,n) spin labels probe.

Spin Labeling Plant Cells

A few unique problems are associated with green plant cells. The presence of the photosynthetic apparatus requires that the lighting of the sample be given careful consideration, especially if the location of the spin label is unknown. Probably the safest condition for such experiments is complete darkness, which would decrease the fluctuations of the redox status of the cell. However, a steady rate of reduction of the ESR signal intensity in the dark does not mean necessarily that each of the many spin label populations in the cell is being reduced at the same rate. A heterogeneous rate of reduction between the spin label

populations could lead to apparent changes in microviscosity as reported in this thesis.

Endogenous ESR signals associated with photosynthesis have been studied with isolated chloroplasts and algae (63,64). I am not aware of ESR studies of higher plant protoplasts. A ubiquitous, light-induced signal, called Signal I, has been described; signal I appears to be identical to line B (Figure 22). Baker and Weaver (1) have shown that Signal I is generated by the oxidized form of the photosystem I reaction center, $P\text{-}700^{+}$. This is consistent with my observations. DCMU does not interfere with the light-induced oxidation of P-700. On the contrary, it might be expected to enhance the light effect. Irradiation with far-red light would oxidize P-700 but photosystem II, which does not use far-red light, would provide very few electrons to reduce $P\text{-}700^{+}$. Adding red light would activate photosystem II, causing a rush of electrons and reduction of $P\text{-}700^{+}$. P-700 in isolated chloroplasts is known to be oxidized by $K_3Fe(CN)_6$ (1).

Conducting experiments in the dark has the added advantage of minimizing endogenous ESR signals. These endogenous signals become important if the ESR scans are being done at high gains. The large signal from $P\text{-}700^{+}$ can broaden the midfield line of the nitroxide spectrum leading to errors in the determination of $2T_{\perp}$.

Another signal which is observed at high gains is that of free Mn^{++}. This signal is reported to be enhanced by low CO_2 concentrations (such as in a cuvette) and darkness (54).

CONCLUSIONS

1. Fatty acid, fatty ester, hydrophilic, partitioning, and steroid spin labels become rapidly distributed throughout the membranes of oat leaf protoplasts. A phosphatidylcholine spin label is selectively retained in the plasmalemma.

2. Nitroxide spin labels in protoplasts are reduced to the corresponding hydroxylamines in the dark by factors associated with respiration and photosynthesis. Spin labels in isolated chloroplasts are reduced by the photosynthetic light reactions, probably from photosystem I. Light catalyzes the destruction of nitroxides, possibly through the formation of an adduct with a photosynthetically-derived free radical. Reduced nitroxides are oxidized by the photosynthetic electron transport chain somewhere between the 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) block and the photosystem I reaction center. The relative rates of these reactions are determined by the wavelengths of light and the presence or absence of terminal electron acceptors.

3. A heterogeneous distribution of spin label throughout the cell is indicated when: (i) the spin label is oxidized in white light by protoplasts but is reduced by chloroplasts; and (ii) the kinetics of spin label reduction by ascorbate are biphasic. Selective retention of spin label in the plasmalemma is indicated when: (a) the spin label is not oxidized in white light by protoplasts but is reduced by chloro-

plasts; and (b) the kinetics of spin label reduction by ascorbate are monophasic.

LITERATURE CITED

1. Baker, R. A. and E. C. Weaver. 1973. A correlation of EPR spins with P_{700} in spinach subchloroplast particles. Photochem. Photobiol. 18:237-241.

2. Bales, B. L. and V. Leon. 1977. Morphology and fluidity in monolayers of cells: a spin label study of XC sarcoma cells. Acta Cient. Venezolana 28:142-144.

3. Bales, B. L. and V. Leon. 1978. Magnetic resonance studies of eukaryotic cells. III. Spin labeled fatty acids in the plasma membrane. Biochim. Biophys. Acta 509:90-99.

4. Bales, B. L., E. S. Lesin, and S.B. Oppenheimer. 1977. On cell membrane lipid fluidity and plant lectin agglutinability. A spin label study of mouse ascites tumor cells. Biochim. Biophys. Acta 465:400-407.

5. Barnett, R. E., L. T. Furcht, and R. E. Scott. 1974. Differences in membrane fluidity and structure in contact-inhibited and transformed cells. Proc. Nat. Acad. Sci.(U.S.) 71:1992-1994.

6. Berg, S. P., D. M. Luscazzkoski, and P. D. Morse II. 1979. Spin label motion in the internal aqueous compartment of spinach thylakoids. Arch. Biochem. Biophys. 194:138-148.

7. Berg, S. P. and D. M. Nesbitt. 1979. Chromium oxalate: a new spin label broadening agent for use with thylakoids. Biochim. Biophys. Acta 548:608-615.

8. Berliner, L. J., ed. 1976. Spin labeling: theory and applications. Academic Press, New York.592pp.

9. Blok, M. C., L. L. M. van Deenen, J. de Gier, J. A. F. Op den Kamp, and A. J. Verkleij. 1977. Some aspects of lipid-phase transition on membrane permeability and lipid-protein association. Pages 38-46 in G. Semenza and E. Carafoli, eds. Biochemistry of membrane transport. Springer-Verlag, Berlin. 669pp.

10. Borochov, A. and H. Borochov. 1979. Increase in membrane fluidity in liposomes and plant protoplasts upon osmotic swelling. Biochimica et Biophysica Acta 550:546-549.

11. Borochov,A., A.H. Halevy, H. Borochov, and M. Shintzky. 1978. Microviscosity of plasmalemmas in rose petals as affected by age and environmental factors. Plant Physiol. 61:812-815.

12. Bronson, C. R. and R. P. Scheffer. 1977. Heat-and aging-induced tolerance of sorghum and oat tissues to host-selective toxins. Phytopathology 67:1232-1238.

13. Butler, K. W., N. H. Tattrie, and I. C. P. Smith. 1974. The location of spin probes in two phase mixed lipid systems. Biochim. Biophys. Acta 363:351-360.

14. Cadenhead, D. A. and F. Muller-Landau. 1973. Pure and mixed monomolecular films of 12-nitroxide stearate. Biochim. Biophys. Acta 307:279-286.

15. Corker, G. A., M. P. Klein, and M. Calvin. 1966. Chemical trapping of a primary quantum conversion product in photosynthesis. Proc. Nat. Acad. Sci. (U.S.) 56:1365-1369.

16. Gaffney, B. J. 1975. Fatty acid chain flexibility in the membranes of normal and transformed fibroblasts. Proc. Nat. Acad. Sci.(U.S.) 72:664-668.

17. Grant, C. W. M. and H. M. McConnell. 1973. Fusion of phospholipid vesicles with viable, Acholeplasma laudlawii. Proc. Nat. Acad. Sci. (USA) 70:1238-1240.

18. Griffith, O. H. and P. Jost. 1976. Lipid spin labels in biological membranes. Pages 454-524. in L. J. Berliner, ed. Spin labeling: theory and applications. Academic Press, New York. 592pp.

19. Hammerstedt, R. H., A. O. Keith, R.C. Boltz, Jr. and P. T. Todd. 1979. Use of amphiphilic spin labels and whole cell isoelectric focusing to assay charge characteristics of sperm surfaces. Arch. Biochem. Biophys. 194:565-580.

20. Helgerson, S. L., W. A. Cramer, and D. J. Morré. 1976. Evidence for an increase in microviscosity of plasma membranes from soybean hypocotyls induced by the plant hormone, indole-3-acetic acid. Plant Physiol. 58:548-551.

21. Herring, F. G. and G. Weeks. 1979. Analysis of Dictyostelium discoideum plasma membrane fluidity by electron spin resonance. Biochim. Biophys. Acta 552:66-77.

22. Jost, P., L. J. Libertini, V. C. Hebert, and O. H. Griffith. 1971. Lipid spin labels in lecithin multilayers. A study of motion along fatty acid chains. J. Mol. Biol. 59:77-98.

23. Kaplan, J., P. G. Canonico, and W. J. Caspary. 1973. Electron spin resonance studies of spin-labeled mammalian cells by detection of surface membrane signals. Proc. Nat. Acad. Sci. (USA) 70:66-70.

24. Keenan, T. W., R. T. Leonard, and T. K. Hodges. 1973. Lipid composition of plasma membranes from Avena sativa roots. Cytobios 7:103-112.

25. Keith, A. D. and W. Snipes. 1974. Viscosity of cellular protoplasm. Science 183:666-668.

26. Keith, A. D., W. Snipes, R. J. Mehlhorn, and T. Gunter. 1977. Factors restricting diffusion of water-soluble spin labels. Biophys. J. 19:205-218.

27. Kornberg, R. D. and H. M. McConnell. 1971. Inside-outside transition of phospholipids in vesicle membranes. Biochemistry 10:1111-1120.

28. Kurihara, K.,Y. Toyoshima, and M. Sukigara. 1979. Photoinduced charge separation in liposomes containing chlorphyll a. II. The effect of ion transport across membrane on the photoreduction of $Fe(CN)_6^{3-}$. Biochem. Biophys. Res. Com. 88:320-326.

29. Lee, A. G. 1977. Lipid phase transitions and phase diagrams. II. Mixtures involving lipids. Biochim. Biophys. Acta 472:285-344.

30. Lee, T. D., G. B. Birrell, P.J. Bjorkman, and J.F.W. Keana. 1979. Azethoxyl nitroxide spin labels. ESR studies involving thiourea crystals, model membrane systems and chromatophores, and chemical reduction with ascorbate and dithiothreitol. Biochim. Biophys. Acta 550:369-383.

31. Lepock, J.R., P. D. Morse II, R. J. Mehlhorn, R. H. Hammerstedt, W. Snipes, and A. D. Keith. 1975. Spin labels for cell surfaces. FEBS Lett. 60:185-189.

32. Marcelja, S. and J. Wolfe. 1979. Properties of bilayer membranes in the phase transition or phase separation region. Biochim. Biophys. Acta. 557:24-31.

33. Maruyama, K. and S. Ohnishi. 1974. A spin-label study of the photosynthetic bacterium, Rhodospirillum rubrum. J. Biochem. 75:1153-1164.

34. McConnell, H. M. 1976. Molecular motion in biological membranes. Pages 525-561 in L. J. Berliner, ed. Spin labeling: theory and applications. Academic Press, New York. 592pp.

35. McNamee, M. G. and H. M. McConnell. 1973. Transmembrane potentials and phospholipid flip-flop in excitable membrane vesicles. Biochemistry 12:2951-2958.

36. Morrisett, J. D. 1976. The use of spin labels for studying the structure and function of enzymes. Pages 274-338 in L. J. Berliner, ef. Spin labeling: theory and applications. Academic Press, New York. 592pp.

37. Morse, P. D. II, M. Ruhlig, W. Snipes, and A. D. Keith. 1975. A spin-label study of the viscosity profile of sarcoplasmic reticular vesicles. Arch. Biochem. Biophys. 168:40-56.

38. Munoz, V. and W. L. Butler. 1975. Photoreceptor pigment for blue Light in Neurospora crassa. Plant Physiol. 55:421-426.

39. Oettmeier,W., J. R. Norris, and J. J. Katz. 1976. Evidence for the localization of chlorophyll in lipid vesicles: a spin label study. Biochem. Biophys. Res. Com. 71:445-451.

40. Pratt, L. H. 1978. Molecular properties of phytochrome. Photochem. Photobiol. 27:81-105.

41. Quail, P. H. 1976. Phytochrome. Pages 683-770 in J. Bonner and J. E. Varner, eds. Plant Biochemistry, 3rd edition. Academic Press, New York. 925pp.

42. Quinn, P. J. and W. P. Williams. 1978. Plant lipids and their role in membrane function. Prog. Biophys. molec. Biol. 34:109-173.

43. Quintanilha,A. T. and L. Packer. 1977. Surface localization of sites of reduction of nitroxide spin-labeled molecules in mitochondria. Proc. Nat. Acad. Sci. (USA) 74:570-574.

44. Rousselet, A., C. Guthmann, J. Matricon, A. Bienvenue, and P. F. Devaux. 1976. Study of the transverse diffusion of spin-labeled phospholipids in biological membranes. I. Human red blood cells. Biochim. Biophys. Acta 426:357-371.

45. Sanson, A., M. Ptack, J. L. Rigaud, and C. M. Gary-Bobo. 1976. An ESR study of the anchoring of spin-labeled stearic acid in lecithin multibilayers. Chem. Phys. Lipids 17:435-444.

46. Sauerheber, R. D., L. M. Gordon, R.D. Goslard, and M. D. Kuwahara. 1977. Spin-label studies on rat liver and heart plasma membranes: do probe-probe interactions interfere with the measurement of membrane properties? J. Membrane Biol. 31:131-169.

47. Schara, M., M. Sentjurc, L. Cotic, S. Pecar, B. Palcic, and C. Monti-Bragadin. 1977. Spin label study of normal and Ki-MSV transformed rat kidney cell membranes. Studia Biophysica 62:141-150.

48. Scheffer, R. P. 1976. Host specific toxins in relation to pathogenesis and disease resistance. Pages 247-269 in R. Heitefuss and P. H. Williams, eds. Physiological Plant Pathology. Springer-Verlag, Berlin. 890pp.

49. Schreier, S., C.F. Polaszek, and I. C. P. Smith. 1978. Spin labels in membranes: problems in practice. Biochim. Biophys. Acta 515:375-436.

50. Shimshick, E. J. and H. M. McConnell. 1973. Lateral phase separations in phospholipid membranes. Biochemistry 12:2351-2360.

51. Stumpf, P. K. 1976. Lipid metabolism. Pages 428-462 in J. Bonner and J. E. Varner, eds. Plant biochemistry, 3rd edition. Academic Press, New York. 925pp.

52. Sun, A. S. and M. Caluin. 1975. Stabilization of electron spin resonance probes for photosynthesis studies. Proc. Nat. Acad. Sci. (USA) 72:3107-3110.

53. Swartz, H. M., J. R. Bolton, and D. C. Borg, eds.1972. Biological applications of electron spin resonance. Wiley-Interscience, New York. 569pp.

54. Tanner, H. A., T. E. Brown, C. Eyster, and R. W. Treharne. 1960. A manganese dependent photosynthetic process. Biochem. Biophys. Res. Com. 3:205-214.

55. Tonumura, Y. and M. F. Morales. 1974. Change in state of spin labels bound to sarcoplasmic reticulum with change in enzymic state, as deduced from ascorbate-quenching studies. Proc. Nat. Acad. Sci. (USA) 71:3687-3691.

56. Torres-Pereira, J., R. Mehlhorn, A.D. Keith, and L. Packer. 1974. Changes in membrane lipid structure of illuminated chloroplasts - studies with spin-labeled and freeze-fractured membranes. Arch. Biochem. Biophys. 160:90-99.

57. Tzapin, A. I., Y. G. Molotkovsky, M.G. Goldfield, and V. S. Dzjubenko. 1971. Light-induced structural transitions of chloroplasts studied by the spin-probe method. Eur. J. Biochem. 20:218-224.

58. Verma, S. P., L. J. Berliner, and I. C. P. Smith. 1973. Cation-dependent light-induced structural changes in visual receptor membranes. Biochem. Biophys. Res. Com. 55:704-709.

59. Vigh, L., I. Horvath, L. I. Horvath, D. Dudits, and T. Farkas. 1979. Protoplast plasmalemma fluidity of hardened wheats correlates with frost resistance. FEBS Lett. 107:291-294.

60. Von Dreele, P. H. and K. L. Williams. 1977. Electron spin resonance studies of the cellular slime mold Dictyostelium discoideum. Biocim. Biophys. Acta 464:378-388.

61. Wang, A.Y. I. and L. Packer. 1973. Mobility of membrane particles in chloroplasts. Biochim. Biophys. Acta 305:488-492.

62. Weaver, E. C. and H. P. Chon. 1966. Spin label studies in Chlamydomonas. Science 153:301-303.

63. Weaver, E. C. and G. A. Corker. 1977. Electron paramagnetic resonance spectroscopy. Pages 168-178 in A. Trebst and M. Avron, eds. Photosynthesis I., Springer-Verlag, Berlin. 730pp.

64. Weaver, E. C. and H. E. Weaver. 1972. Electron resonance studies of photosynthetic systems. Pages 1-32 in A. C. Giese, ed. Photophysiology, vol. VII., Academic Press, New York.

65. Weisenseel, M. and W. Haupt. 1974. The photomorphogenic pigment phytochrome: a membrane effector? Pages 427-434 in U. Zimmermann and J. Dainty, eds. Membrane transport in plants. Springer-Verlag, Berlin. 473pp.