CHARACTERIZATION OF
FLASH-ELICITED VOLTAGE TRANSIENTS
IN CHLOROPLAST-LIPID BILAYERS

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ABSTRACT

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BY

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Bilayer lipid membranes (BLM) are formed from extracts of
spinach chloroplast. Electrical transients are seen to originate
across the BLM in response to microsecond flash excitation under a
variety of asymmetrical conditions. Flash excitation clearly
delineates at least two voltage generative processes often of opposite
polarity that under continuous illumination give rise to only a
single steady-state voltage value. Thus a mechanistic description
accounting for the steady-state value alone may not be sufficiently
comprehensive. The "spike" feature of the continuous illumination
dependent voltage time course can be predicted from the flash
experiments. A chemical gradient of $FeCl_3$ gives rise to a rapid
microsecond voltage transient with polarity, action spectrum, and
risetime that suggest that photo-electrons from excited chlorophyll
reduce $Fe^{+3}$. However in most cases, there exists also a slow
electrical transient responding to applied electrical field or pH
gradient. The slow component peaks at typically 200 ms, depending
upon the RC time of the BLM system, and fits the following description:
$V(t) = V_o [1 - \exp(-\frac{t}{RC})] \exp(-\lambda t)$. Specific agents such as ascorbic
acid and p-benzoquinone quench completely the slow photosensitive

process (voltage or pH gradient-induced) leaving the fast ($FeCl_3$ chemical gradient-induced) photoeffect unmodified; others such as iron and bromide similarly enhance selectively the slow response. Clearly, the steady state membrane potential under continuous illumination depends upon whether such quenching or enhancing agents are present. To the extent that this system retains the machinery requisite for elements of the primary event of photosynthesis, it is useful to have such controls for purposes of identifying excited states operative in photosynthesis.

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CHAPTER I

INTRODUCTION

All biological machinery requires fuel in the form of energy-rich
chemical bonds such as the anhydride pyrophosphate bond of the nucleo-
tide adenosine triphosphate (ATP). In aerobic organisms chemical free
energy is made available in this form as reduced foodstuffs are
oxidized by highly efficient mechanisms to $CO_2$. The major energy
packaging process, oxidative phosphorylation, occurs as electrons
are passed along the respiratory electron transport chain to oxygen
within the membranous cristae of mitochondria; electron transfer
through this redox system can be monitored with sensitive differential
cytochrome adsorption spectroscopy techniques. Thus, in this case
energy is conserved and transferred in a manner relatively open to
study.

However, the primary photosynthetic electron transfer reaction,
which ultimately provides the energy required for the synthesis of
these fuel molecules while most commonly oxidizing water to oxygen in
green plants, occurs within stacked thylakoid membranes (grana) of
chloroplasts and involves membrane-dependent energy transduction. As
a consequence of the structural and functional complexity of photo-
synthetic membranes, the investigator encounters unique obstacles.
Biochemical reactions subsequent to formation of the primary reductant
and primary oxidant via chlorophyll-sensitized energy transduction

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are relatively easily delineated. According to the Z-Scheme (Arnon, 1967; Boardman, 1968) nicotineamide adenine dinucleotide phosphate ($NADP^+$) is reduced and ADP phosphorylated during non-cyclic photophosphorylation. These products are utilized to reduce $CO_2$ to hexoses and other carbohydrates via the Calvin Cycle (Bassham, 1957). However, the precise in vivo mechanism of chlorophyll-sensitized primary reductant and oxidant formation is as yet obscured by the following features:

i) Regardless of the electron donor and acceptor, in all photosynthetic organisms light energy enables electrons to flow from electron donor to electron acceptor against the normal gradient of standard reduction potentials. Light energy, therefore, causes electrons to flow in reverse toward an energy-rich state as opposed to their flow in respiration. The chloroplast's system for constraining this to occur rather than simple energy degradation is of a unique design not readily amenable to analysis.

ii) Photoexcitation of pigment molecules is very rapid, requiring less than $10^{-15}$ seconds, and singlet excited molecules possess mean lifetimes of approximately $10^{-5}$ to $10^{-9}$ seconds (Moore, 1963); consequently, rapid detection instrumentation is necessary.

iii) The grana, flattened vesicles or thylakoids stacked transversely across the chloroplast, contain essentially all the chloroplast's photosynthetic pigments and the enzymes necessary for the primary light-driven reactions (Lehninger, 1970). The fundamental importance of the thylakoid membrane is implicit. Thus an investigation of chlorophyll photochemistry in solution is not necessarily a study of the role of chlorophyll in photosynthesis. A membranous matrix for

the chlorophyll would render the system more highly representative of the in vivo arrangement.

Mueller, Rudin, Tien, and Wescott prepared the original artificial bimolecular lipid membranes (BLM) (Mueller, Rudin, Tien, and Wescott, 1962), the first successful attempt since the postulation of the lamellar bilayer structure for the plasma membrane (Gorter and Grendel, 1925). The bimolecular nature of BLM has been demonstrated (Goldup, 1970; Tien, 1971); amphipathic constituents are oriented such that aliphatic chains are immersed in the hydrophobic lipid interior of the BLM while charged and polar regions extend into the surrounding aqueous medium. Electron microscopy, x-ray diffraction and birefringence as well as circular dichroism studies corroborate that the photosynthetic apparatus consists of such a highly ordered lamellar array (Menke, 1967; Branton, 1969; Mühlethaler, 1966).

Tien recently undertook to investigate whether a BLM exhibiting energy transducing properties could be constituted from photoactive pigments to facilitate the study of photosynthetic primary processes (Tien, 1968). Indeed, chlorophyll/lipid membranes formed from spinach chloroplast extract exhibited the photovoltaic effect, a direct demonstration of in vitro energy transduction originating from a primitive reconstituted thylakoid membrane.

This work has undertaken to investigate the nature of the photo-flash stimulated voltage transient in chlorophyll bilayer lipid membranes under application of chemical and electrical gradients. By permitting voltage generative kinetics to be clearly delineated, micro-second flash illumination offers the optimum method for identifying reaction orders and lifetimes.

There are two basic photoresponse initial phase kinetics, viz.
a microsecond time duration chemically induced response and a slower
pH or voltage gradient-induced transient peaking typically at 200 ms
after illumination. In particular this work has provided identification
of the kinetics of the mechanism responsible for the slow response
(component C) of the chl-BLM to flash illumination. Since previous
kinetic models for the slow transient (Tien and Huebner, 1973) were
unable to describe precisely the slow response waveform, they were
thought to be at best marginally adequate. The following kinetic
description, however, fits the data quite satisfactorily. Specifically
this model is original in two major respects:

I. The decay process had been assumed to be first order RC; we
now understand that it is first order with rate constant $\lambda < (RC)^{-1}$ and
any process slower than $(RC)^{-1}$ across the membrane network is
kinetically independent of RC. This possibility was originally not
considered. Also the initial phase kinetics of the slow response
are now seen to be first order with $(RC)^{-1}$ as rate constant; prior
opinion was that some rate constant unrelated to dark membrane electrical
characteristics was appropriate. Thus the slow transient is described
by a unique mathematical expression that assumes a time constant
identical with dark membrane first order RC and a first order exponential
decay independent of RC, i.e.,

$$V(t) = V_o[1 - \exp(-Kt)]\exp(-\lambda t)$$

where $K = (RC)^{-1}$ and $\lambda$ is a first order rate constant. The fit to
experimental waveforms is excellent. This expression, then, yields novel
information concerning BLM photoproperties; that is, two diametrically
dissimilar mechanisms are operative: the current-generating fast effect

driven by chemical reduction potential gradients and the slow effect due to somewhat enhanced membrane conductivity.

II. Secondly, recent evidence indicates that we may begin to assign chlorophyll triplet character specifically to the slow phenomenon. Since conductivity returns slowly to equilibrium in a first order manner, responsible metastable state lifetimes are easily calculated. In addition, without modifying the microsecond transient, heavy atoms known to enhance singlet* $\longrightarrow$ triplet* intersystem crossing (McGlynn, 1969) selectively augment the slow response magnitude while other in vitro chlorophyll triplet quenchers (Livingston, 1960; Fujimori, 1957; Livingston, 1963) eliminate only the slow transient. Together with calculated lifetimes this provides compelling evidence of the molecular nature of the slow response.

CHAPTER II

LITERATURE REVIEW


In the brief space of time since the original reports of photo-
electric effects from BLM (Tien, 1968), several other investigators
recognizing invariable association of in vivo light absorbing pigments
with membranes have also initiated studies of the BLM photoeffect.
Currently, perhaps ten laboratories world-wide are examining the
photoexcitability of appropriately modified artificial bilayers.
However, the researcher who gleans BLM photoeffect data and postulated
mechanisms from the accumulated literature in this infant science
anticipating that such a study will lead to a comprehensive, thorough
understanding of bilayer photosensitivity is mistaken.  This circumstance
is due to the fact that the artificial bilayer photosystems under
investigation from laboratory to laboratory are vastly different; i.e.,
membrane and bathing media compositions vary, modifiers used to confer
excitability differ, and the photovoltage detection systems are of
assorted designs and capabilities.  Nonetheless, as we are acquiring
information concerning various aspects of the underlying phenomenon,
such variegated independent investigations will ultimately lead to a
precise explanation of the BLM light response.

Soon after the appearance of Tien's announcement of light-induced
phenomena in chlorophyll BLM in Nature, July 1968 (Tien, 1968), T.R.
Hesketh undertook to determine to what extent radiation heating

contributed to the observations although Tien had employed a heat filter. Hesketh reported (Hesketh, 1969) that the waveform of the temperature variation in the membrane aperture volume detected by thermocouple correlated with Tien's voltage transients; he continued to report detection of a "genuine" light effect using an infrared background to insure heat fluctuations of less than 0.1°C. It has since been shown that the open circuit photovoltage amplitude and bias are strictly dependent upon electron acceptor gradients and polarities; also, voltage transient characteristic times are unrelated to membrane and bulk heat capacity values (Tien and Verma, 1970; Huebner and Tien, 1972).

There followed reports of assorted photoeffects in the presence of various modifiers. Mauzerall and Finkelstein pointed out photo-conductivity in cholesterol/α-tocopherol BLM in the presence of polyiodide (Mauzerall and Finkelstein, 1969). They postulated that the polyiodide ion is a photosensitized charge carrier across the membrane. Ullrich and Kuhn using egg lecithin in octane membranes observed photoeffects employing dyes in one aqueous compartment (Ullrich and Kuhn, 1969). They reported a photovoltage action spectrum corresponding to the dye absorption spectrum and suggested that electrons are donated across the bilayer from the oxidized dye. Tien, incorporating visual pigments all-trans-retinal and β-carotene into oxidized cholesterol BLM, measured β-carotene-sensitized photoresponses and photoconductivity effects possibly induced by cis-trans conformation changes subsequent to the absorption of photons (Tien and Kobamoto, 1969). In all of the above investigations it was found that the action spectra for the photoelectric effect compared well with the

absorption spectra of the bilayer or its constituents indicating that interactions between the light and the membrane were responsible for the observed effects.

Several publications next appeared which suggested a growing interest in BLM light phenomena from a purely physical point of view as reports of organic membrane photo energy transduction to electrical and chemical form captured the imagination of physical chemists and solid state physicists alike. Kay and Chan at Philco-Ford Corporation undertook to ascertain whether bona fide charges are induced in the BLM upon irradiation (Kay and Chan, 1969). Using α-tocopherol in decane membranes and UV radiation in the presence of iodide, they concluded on the basis of current-voltage characteristics of the membrane that generated charges were essential as part of the mechanism leading to the photoelectric effect. A similar publication later followed supporting these observations (Kay and Bean, 1970).

Suspecting that since the bilayer forms a distinct phase from the film-forming solution, the chlorophyll to lipid ratio in the membrane may be quite different, Alamuti and Läuger further characterized chlorophyll bilayers in terms of pigment concentration and relative amounts of chlorophyll and lecithin, a stabilizing surface active amphipathic phospholipid (Alamuti and Läuger, 1970). Dioleoyl lecithin in n-decane membranes with various proportions of chlorophyll $\underline{a}$ exhibited a fluorescence maximum at a composition indicating a 3:1 phosphatidyl choline to chlorophyll $\underline{a}$ ratio and $\sim 10^{13}$ chlorophyll $\underline{a}$ molecules per $cm^2$. Trissl and Läuger later confirmed Tien's observations of electrical potentials elicited from chlorophyll BLM using redox agents and agreed that a redox process occurs after optical excitation

of pigment molecules in the membrane (Trissl and Läuger, 1970). The physical characterization of photoactive BLM continued as Cherry, Hsu, and Chapman developed a technique for measuring absorption spectra of pigments incorporated into lipid membranes (Cherry, Hsu, and Chapman, 1971). They employed a differential Cary 14 Spectrophotometer absorption cell containing a series of bilayer-supporting apertures to overcome the low optical density afforded by a single thin membrane. However, a more sensitive method was proposed by Van and Tien who observed that the photoelectric effect action spectrum correlated closely with the BLM absorption spectrum (Van and Tien, 1970).

Tien, meanwhile, continued his investigations into various aspects of the bilayer light phenomenon itself. The redox nature of the process was all but confirmed as photoemfs were elicited according to the redox properties of modifiers added to the BLM system (Tien and Verma, 1970). Studies into visual early receptor potentials using retinal bilayers proceeded as Tien and Kobamoto determined that the time course of photopotentials showed a biphasic mode quite similar, under appropriate conditions, to that of early receptor potentials (Kobamoto and Tien, 1971). An extension and refinement of this work appeared later (Kobamoto and Tien, 1972).

A series of papers reported results from a somewhat altered membrane/modifier arrangement. Oxidized cholesterol BLM, inert to visible light due to the lack of pigment among the bilayer constituents, exhibited photosensitivity in the presence of particular aqueous dyes and light sensitive inorganic ions (Verma, 1971; Pant and Rosenberg, 1971). Verma indicated that prior postulated redox mechanisms for pigment-containing BLM (Tien and Verma, 1970) occur also in the

non-pigmented bilayer instance although the response characteristics
reflect the altered arrangement of dye, membrane, electron donor, and
electron acceptor; and Pant and Rosenberg observed a correlation
between the classical Becquerel Effect of excited ion and metallic
electrode electron exchange and the BLM light phenomenon, the membrane
in its capacity as electronic conductor fulfilling the role of the
matallic electrode.

Using a dual beam spectrophotometer and fluorescence photomultiplier
arrangement, Läuger et al. extended their previous study of lipid
bilayer composition (Steinemann, Alamuti, Brodmann, Marschall, and
Läuger, 1971). A maximum chlorophyll concentration of $3 \times 10^{13}$ per $cm^2$
was reaffirmed, at which amount the bilayer absorption spectrum was
shifted to longer wavelengths indicating an interaction among porphyrin
rings in the film. However, they continued to report that the addition
of the oxidizing agent potassium peroxodisulfate to one external
aqueous phase "destroyed exactly half of the chlorophyll in the biface",
an inference drawn from the observation that fluorescence fell by half
upon addition of oxidant. Huebner in a subsequent letter to the
editor (Huebner, 1972) pointed out that he had observed enhanced
photoeffects from chlorophyll BLM in the presence of peroxodisulfate
indicating that fluorescence is quenched by a process which enhances
electrical charge transport suggesting that pigment is not destroyed
but excitation energy in the presence of oxidant is simply lost to
fluorescence and utilized instead in charge transport. Läuger et al.
later published chlorophyll BLM dichroism data indicative of porphyrin
ring orientation in the biface (Steinemann, Stark, and Läuger, 1972)
and offered in another paper (Trissl and Läuger, 1972) a theory of

photoelectric effects in BLM based on two different models, an approach
deemed necessary by them earlier when they acknowledged: "Ullrich and
Kuhn" noted that "a transient potential spike is observed" under
continuous illumination. "In contrast to this," other systems "develop
a stationary photovoltage under continuous illumination. This
indicates that the mechanism of charge separation is quite different
in both systems" (Trissl and Läuger, 1970). Indeed, Ullrich and Kuhn
later presented further data from egg lecithin in n-octane membranes
in the presence of an aqueous cyanine dye and restated the observation
that photopotentials are not sustained under continuous illumination
but appear rather as spike potentials (Ullrich and Kuhn, 1972). They
proceeded to construct an interfacial dipole polarization model highly
analogous to the classical polarized electrode problem in electro-
chemistry in which charge separation at the electrode interface
introduces a capacitive element which effectively blocks constant
current flow. Perhaps a more useful enterprise would have been to
assemble and analyze a membrane photo system capable of sustained
charge transport facilitating chemical energy transfer relevant to
the in vivo modus operandi.

A careful study of chloroplast extract bilayers in the presence
of inorganic modifiers and exciting continuous illumination was
initiated by Ilani and Berns who asserted on the basis of an equivalent
electrical circuit approach that an effective low internal resistance
photobattery may be considered introduced upon excitation in response
to a redox potential gradient (Ilani and Berns, 1972). Meanwhile,
Hong and Mauzerall employed the voltage clamp technique to eliminate
current flow to and from the BLM capacitive element in order to isolate

trans-membrane charge flow in response to microsecond flash exposure (Hong and Mauzerall, 1972). They concluded that photoresponses to pulsed light are specific to the thin bimolecular region rather than the thick annular region.

Tien continued his investigation into properties of photoactive BLM as he reported the results of a careful characterization of chlorophyll bilayer photosensitivity and rectification produced by ferric chloride in addition to open circuit voltage, current, and resistance measurements as a function of light intensity (Loxsom and Tien, 1972); Tien also directly addressed the question of whether genuine electronic processes can occur in a bilayer structure immersed in an aqueous environment and concluded that such processes might indeed occur (Tien, 1972). Also, Huebner and Tien presented the results of an analysis of microsecond flash excitation of chlorophyll BLM using an improved low output impedance fast voltage transient detection system (Huebner and Tien, 1972) that revealed the interesting combination of an extremely rapid voltage rise concomitant with a much slower independently induced transient upon flash illumination. This arrangement provided a convenient means for measuring both the large microsecond current necessary to charge the membrane capacitance within the given risetime and simultaneously, the distinct photoconductivity transient which occurs over an extended period; this conductivity alteration merits attention since it represents a slight shorting pathway shunted across the dark conduction paths which serves to additionally diminish the true photoeffect. The entire merit of their approach was that it afforded an elegant means to concurrently measure both true photoemf and conductivity enhancement.

Tien and Huebner subsequently characterized the individual components comprising the photoeffect (Tien and Huebner, 1973) but acknowledged some difficulty in interpreting the slow response, "Component C", as a current generating process with RC decay although the fast response fit this assumption quite well; that is, a satisfactory kinetic description of the slow component was lacking. This thesis addresses itself to resolving this difficulty and further characterizing the effect in terms of recently discovered chemical quenchers and enhancers specific to the slow response in the hope that an accurate kinetic description and modifier sensitivity profile of the response might extend our understanding of its molecular details.

CHAPTER III

EXPERIMENTAL

## Apparatus

The characteristic high electrical impedance properties of
chlorophyll bimolecular lipid membranes along with their unique optical
features and delicate structural nature require that a suitable exper-
imental apparatus be employed; also since upon microsecond flash
excitation extremely rapid electrical transients originate across
chlorophyll BLM under a variety of asymmetrical conditions, it was
necessary to detect their kinetics using special instrumentation.

An excellent site for stable membrane formation was afforded by
a millimeter circular aperture in a 10 ml teflon cup. Extremely high
teflon resistivity guaranteed negligible shunting currents across the
highly resistive membrane and in addition, the teflon cup contributed
only about 3 picofarads capacitance, again negligible compared to the
typical BLM capacitance of 5000 pf. The teflon cup rested in a glass
cell designed to present surfaces normal to both the incident illumi-
nating light beam and the direction of observation, minimizing light
scattering and image distortion. Each of the chambers separated by
the membrane aperture was provided with a saturated KCl asbestos
fiber-junction salt-bridge calomel electrode to maintain electrical
continuity from solution to external circuitry. The electrodes were
connected to a high input impedance buffer amplifier mounted as close

as possible to the electrodes to minimize input cable length, a
precaution necessary because the high impedance BLM circuit is quite
sensitive to spurious external signals and, in addition, cable
capacitance increases with length, augmenting the amplifier input RC
time and thus impairing instrumental response capabilities. A Philbrick
FET operational amplifier (model 1021) was employed as an excellent
impedance buffer since it features a common mode rejection ratio of $10^{10}$
and input impedance of $10^{12}$ ohms. As a result of its stability and
low drift characteristics, this amplifier is well suited for unloading
the BLM high impedance source and providing rapid signal rise time. The
amplifier output was displayed and recorded on a Tektronix dual beam
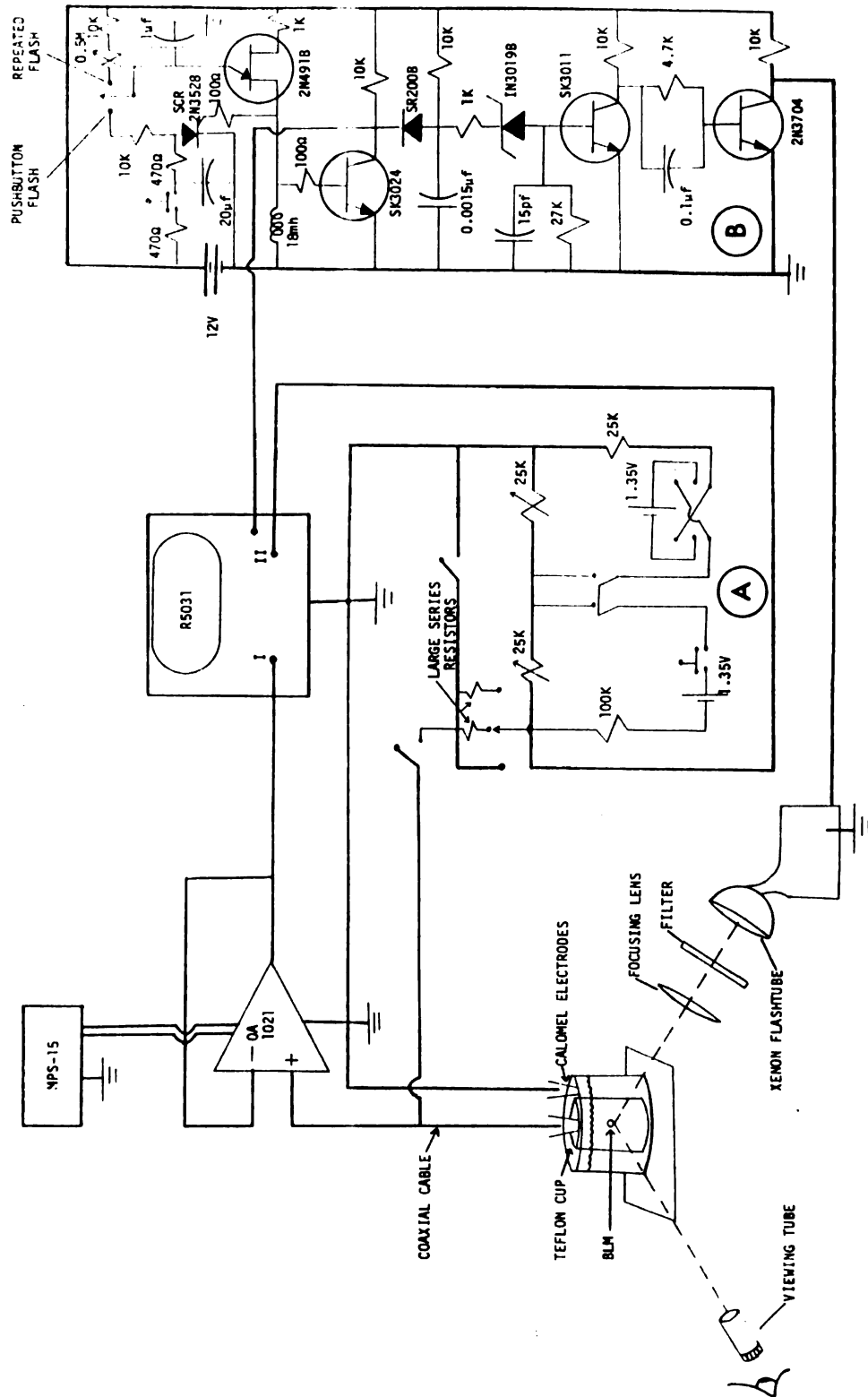storage oscilloscope screen.

The electrodes were shunted with a variable voltage source also
monitored by the oscilloscope and large series resistances for applying
trans-membrane potentials and measuring membrane resistance and
capacitance. Illumination of the membrane area was furnished by a
General Radio Stroboslave xenon flash tube assembly that produced a
peak value of $10^9$ lux at the membrane with 1/3 peak times separated
three microseconds. For resolving microsecond transients, a transistor
triggering device synchronized stroboscopic flash and oscilloscope
sweep.

To shield the BLM support cell including electrodes and buffer
amplifier from undesirable external illumination and electrical noise
a large Faraday cage was constructed of a wooden frame, fiberboard, and
copper screening grounded to the outer chamber electrode. The entire
apparatus is schematically shown in Figure 1.

Figure 1. Chl-BLM flash illumination apparatus with delay

system for resolving microsecond transients.

A. Series resistance and variable applied voltage

source.

B. Oscilloscope and strobe trigger.

REPEATED FLASH

PUSHBUTTON FLASH

0.5µf

1µf

SCR
2N3528

100Ω

2N491B

1K

10K

470Ω

470Ω

470Ω

20µf

18mh

1000

SK3024

100Ω

SR200B

1K

10K

0.0015µf

15pf

1N3019B

27K

SK3011

10K

0.1µf

4.7K

10K

2N3704

B

12V

RS031

I

II

A

25K

25K

1.35V

LARGE SERIES RESISTORS

25K

100K

1.35V

MPS-15

OA 1021

COAXIAL CABLE

CALOMEL ELECTRODES

TEFLON CUP

BLM

FOCUSING LENS

FILTER

XENON FLASHTUBE

VIEWING TUBE

## Extraction Techniques

The following procedure was followed in the preparation of chloroplast BLM-forming solutions. Fresh spinach was purchased from a local market and ribs and stalks were removed from a 10-oz. portion. The leaves were washed with distilled water, dried thoroughly, and then added slowly to a Waring blender at low speed with 300 mls of isotonic 0.5 M sucrose plus buffer at pH 7.5. When all the leaves had been added high speed was used for 30 seconds; the homogenized mixture was filtered through 4 to 6 layers of cheesecloth to further remove structural cellulose and isolate chlorophyll-rich chloroplasts. The filtrate was next centrifuged in a Sorvall centrifuge (Model SS-1, Ivan Sorvall, Inc., Norwalk, Conn.) in approximately 40 ml quantities (8 tubes) at low speed for 5 minutes, i.e., Variac (Type 116B, Superior Electric Co., Bristol, Conn.) at 40 volts. The supernatant was discarded. The remaining crude chloroplast was resuspended in buffered sucrose solution using 25 mls per 2 test tubes bringing the total stock to 4 tubes containing 25 mls each. Resuspensions were facilitated by a Vortex mixer (Model M-10715, Thermolyne Corporation, Dubuque, Iowa). These tubes were again centrifuged at 40 volts for 5 minutes, the supernatant discarded and the residue washed and mixed in hypotonic 25 ml glass-distilled water per tube and allowed to stand for 5 minutes. These tubes were centrifuged at high speed (45 volts) for at least 10 minutes and the supernatant discarded leaving a residue that was next extracted with 90 mls of 2/1 (v/v) petroleum ether/methanol solution in the blender at medium speed for one minute. Using two tubes this mixture was centrifuged (Variac at 30 volts) for 10 minutes and the resulting top layer was pipetted off into a dry

round-bottomed flask and evaporated to dryness using a flash-evaporator (Buchler Instruments, Fort Lee, N.J.). This residue was resuspended in 5 mls of 1/1 n-butanol/dodecane and placed in a clean covered storage vial.

## Procedure

The bulk BLM bathing medium was 0.1 M acetate titrated with potassium hydroxide to pH 5.1 in laboratory distilled water re-distilled in an all-glass still. Preliminary to membrane formation undesirable input currents from the buffer amplifier were offset using the trim adjustment to correct the output signal to zero when the electrodes were shorted. Also, electrode asymmetry potentials typically did not exceed a millivolt. The bimolecular lipid membrane (BLM) was formed by sweeping across the aperture with the tip of a microsyringe after a two microliter extrusion using the repeating dispenser attachment (Tien and Howard, 1972).

Membranes formed in 0.1 M acetate buffer solution thinned to the black condition in approximately ten minutes. This thinning was observed directly using a green filter and dim flash light triggered repeatedly by the timing circuit or more carefully monitored by comparing membrane RC transient times as thinning progressed, as capacitance is inversely related to thickness. Since BLM resistance was measured independently using the voltage divider technique, $R_m = R_s \dfrac{E_m}{E - E_m}$, $C_m$ was calculated using $R_m$ and $R_s$ in parallel as the R of RC for the circuit given in Figure 1. The final bilayer lipid membrane (BLM) exhibited uniform stable resistive and capacitive properties which were measured and recorded since photovoltage features

were determined largely by these characteristic electrical properties. Typical values of $R_m$ and $C_m$ were $10^8$ $\Omega$ and 5000 pF ($10^6$ $\Omega$-cm$^2$ and 0.5 $\mu$F/cm$^2$), respectively. Subsequent to membrane stabilization, chemical or pH differences across the chl-BLM were established by the addition of a small volume of concentrated HCl, KOH, FeCl$_3$, ascorbate, etc., to the inner chamber with the aid of an Oxford Sampler followed by careful magnetic stirring. A listing of the equipment used follows in Table 1.

TABLE 1

List of Equipment

| Item | Model | Manufacturer |
|------|-------|--------------|
| Cell assembly<br>Glass cell<br>Teflon cup | Design suggested<br>in Figure 1 | Will Scientific Co.<br>Ann Arbor, Mich. 48107 |
| Viewing tube | No. 70266 | Edmund Scientific Co.<br>Barrington, N.J. 08007 |
| Microsyringe<br>(100 μl) | Model PB-600 | Hamilton Co., Inc.<br>Whittier, Calif. 90605 |
| Micropipette | Sampler | Oxford Laboratories<br>San Mateo, Calif. 94401 |
| Xenon flashtube<br>assembly | Stroboslave<br>1539-A | General Radio Co.<br>West Concord, Mass. 01781 |
| Electrodes | Fiber junction<br>calomel, #39270 | Beckman Instr., Inc.<br>Fullerton, Calif. 92634 |
| Operational<br>amplifier | Model 1021 | Philbrick Nexus<br>Dedham, Mass. 02026 |
| O.A. power<br>supply | MPS-15 | Transidyne General Corp.<br>Ann Arbor, Mich. 48103 |
| Dual beam storage<br>oscilloscope | R5031 | Tektronix, Inc.<br>Beaverton, Oregon |
| $10^8$ to $10^{11}$ ohm<br>series resistors | Hi-meg resistors | Victoreen Instr. Co.<br>Cleveland, Ohio 44104 |

CHAPTER IV

RESULTS

## Introduction

The microsecond photo-flash illumination of chlorophyll bilayer

lipid membranes technique has resolved single-flash voltage responses

into clearly defined distinct components representative of kinetically

independent membrane photo-processes indistinguishable under continuous

illumination circumstances.  A variety of combinations of applied

voltage, chemical gradients, and pH differences were imposed across

the membrane to induce observable effects.  Such asymmetries were

necessary since, alternatively, excitation would have been randomly

partitioned equally to both interfaces, yielding no photoeffect.  The

voltage transients elicited were in general composed of four processes:

i)   a fast, microsecond risetime component induced by electron

acceptors such as $Fe^{+3}$ (the Fast Component);

ii)   a slower 100 to 300 ms initial phase component in the

presence of pH gradient or voltage applied across the membrane (the

Slow Component); and two distinct voltage decay kinetics, viz.

iii)   a simple 100 to 500 ms RC decay corresponding exactly to

the membrane RC transient in the dark; and

iv)   a slower decay transient, "lambda", a feature of pH gradient

or applied voltage-induced effects only.

These four basic components essentially characterize all the

portions of waveforms elicited from chl-BLM (chlorophyll BLM) under

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flash excitation regardless of the nature of the trans-bilayer asymmetry. Also, the large difference in component rates has permitted clear delineation of both the fast and slow processes in response to a single flash, facilitating simultaneous evaluation of several components.

## The Fast Component

The Fast Component was detected only in response to certain chemical gradients. In the case of $FeCl_3$, a maximum photoresponse was elicited at a concentration of $2 \times 10^{-3}$ M $FeCl_3$ in the inner ungrounded chamber. The $Fe^{+3}$ chamber potential was driven negative typically 3 to 5 mV upon flash illumination within 10 microseconds, maximum instrumental response rate, and decayed according to the RC exponential transient characteristic of the BLM in the dark (Figure 2). These results were common to all chl-BLM tested. Other electron acceptors, ceric ion and methyl viologen, induced responses of somewhat smaller magnitudes but with similar kinetics; however, since ferric ion was the most effective sensitizer, it was employed in the majority of experiments designed to investigate features of the Fast Component. As previously mentioned, the bathing medium was buffered with 0.1 M acetate and titrated to pH 5.1.

The Fast Component and accompanying RC decay appeared alone only in the total absence of applied voltage or pH gradient; otherwise the Slow Component appeared to some extent. This presented moderate experimental difficulties, viz. $FeCl_3$ in aqueous solution is an electron pair acceptor and hence a Lewis acid. Although the inner chamber was buffered with 0.1 M acetate, $FeCl_3$ decreased the inner
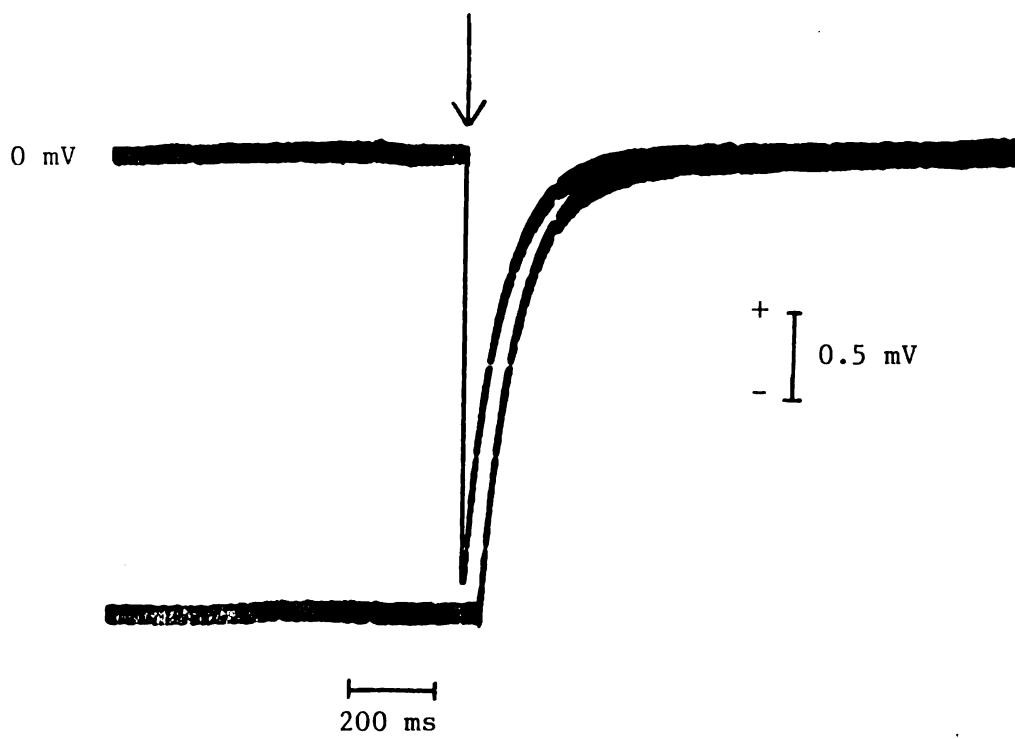
Figure 2. Flash response in the presence of $10^{-2}$ M $FeCl_3$ in the inner chamber compared to the dark chl-BLM RC transient. Arrow denotes flash.

0 mV

0.5 mV

200 ms

chamber pH typically 0.2 units, imposing a small pH gradient across the chl-BLM and inducing therefore slight Slow Component character in the response waveform. The Slow Component contribution, usually essentially insignificant, could be additionally minimized in most cases via the external application of a slight reverse biasing potential.

A distinctive feature of the Fast Component was the fact that maximum possible applied voltages of ±70 mV, corresponding to ±70,000 Volts/cm field strength within the chl-BLM did not alter the polarity of the fast response; it continued to exhibit a polarity indicative of negative charge flow to the ferric/chl-BLM interface. The magnitude of the response was altered ±15% under application of these intense fields, augmented when the field was of a polarity to assist negative charge flow to the ferric/chl-BLM interface and diminished when the field was oriented to retard current flow to this interface.

## The Slow Component

Upon flash excitation the Slow Component appeared in the presence of applied voltages or transmembrane pH differences. Its risetime was on the order of 0.5 seconds and its polarity in the case of applied voltage was such that the absolute magnitude of potential across the BLM in the dark prior to flash was reduced. Also, the flash response amplitude was directly proportional to the potential applied. An example of the addition of a positive Slow Component to a negative iron-induced Fast Component when a negative potential is applied is given in Figure 3.

The magnitude of the Slow Component light response was increased by the addition of bromide or iron in equal concentrations to both sides of the membrane. This concentration symmetry minimized fast effect contributions to the photoresponse, facilitating study of the Slow Component alone. It was found that $FeCl_3$, in addition to inducing the large fast photoresponse described earlier also enhanced the kinetically independent slow response approximately twentyfold at $10^{-4}$ M concentrations. Bromide also enhanced the Slow Component photovoltage magnitude somewhat (Figure 4). The pH gradient-induced photoresponse exhibited kinetics similar to those of the applied voltage response but its polarity was always negative with respect to the high pH side of the membrane.

## Lambda Decay

Whereas the Fast Component was discharged at a rate given by the chl-BLM RC time, the Slow Component invariably returned to the dark voltage at a much slower rate. Figure 5 compares an RC transient with a voltage gradient-induced rapid repeated flash photoeffect decay process for the same membrane. This multi-flash technique yielded a better-resolved decay process than attainable using single flash excitation. Clearly, the rates are not the same. The rate constant for this decay, lambda ($\lambda$), was typically 2 $sec^{-1}$ while the RC rate constant was 5 $sec^{-1}$. (This corresponds to a 200 ms "RC time".) Though $\lambda$ varied from one chl-BLM to another within values of 0.5 to 2.5 $sec^{-1}$, for any single membrane $\lambda$ remained constant and was not a function of time, applied voltage, total membrane illumination time or membrane resistance values.
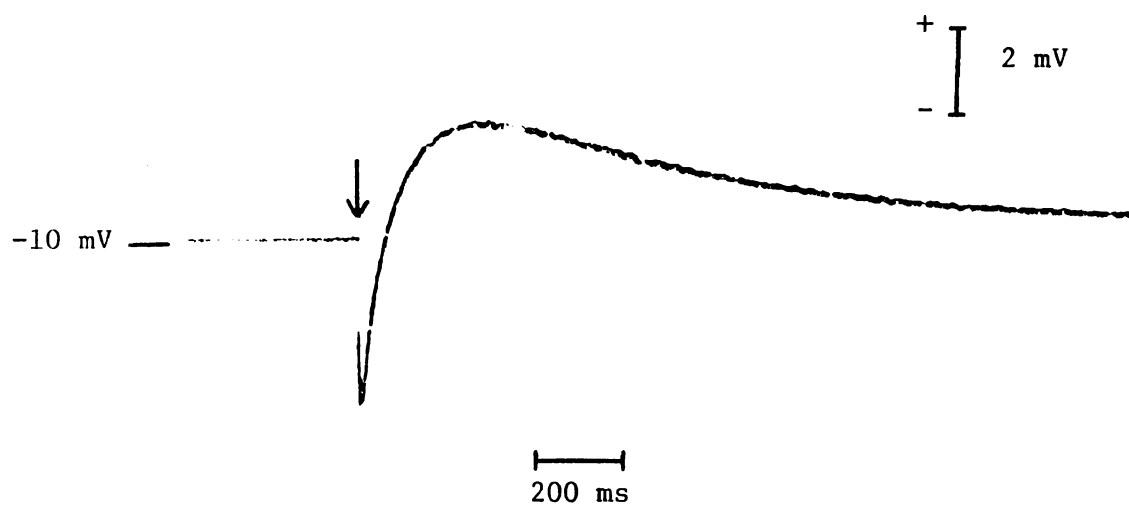
Figure 3.  Biphasic response in the presence of $FeCl_3$ and
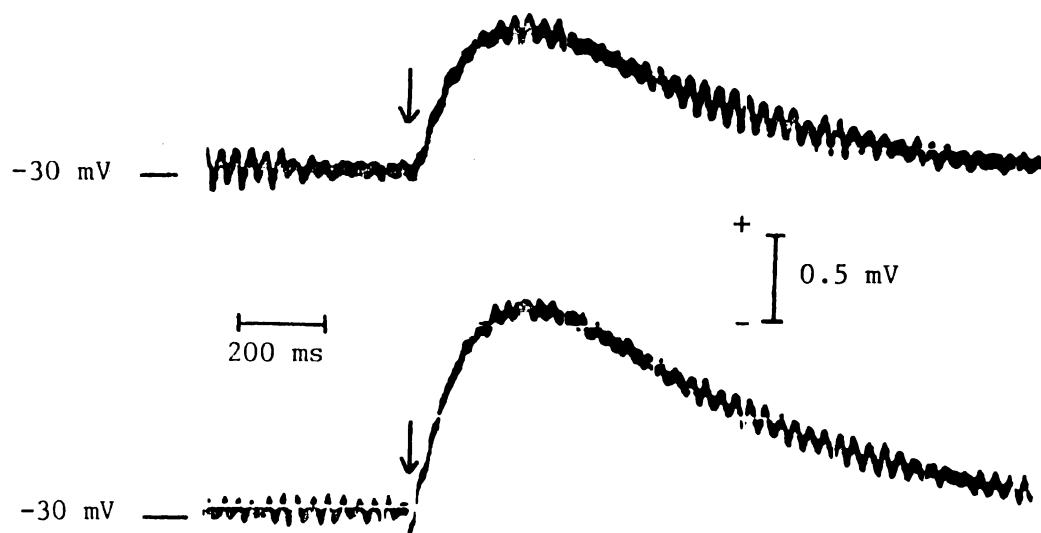
negative applied voltage.

−10 mV

2 mV

200 ms

Figure 4.  Top trace:  Slow Component in the presence of applied

voltage only.

Bottom trace:  enhanced photovoltage transient

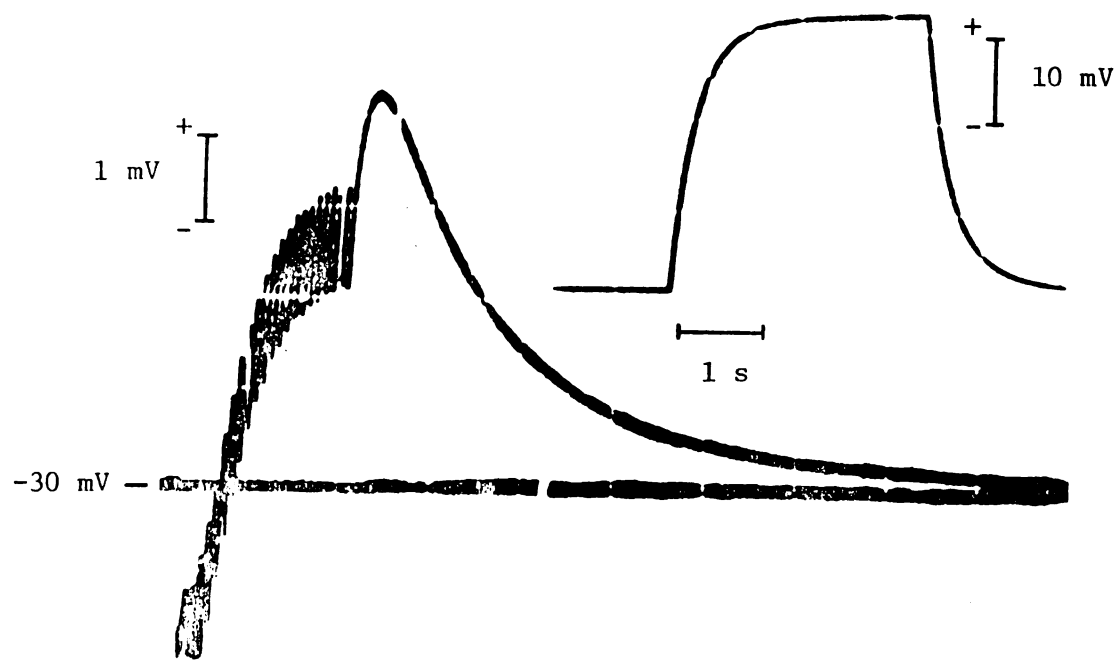subsequent to the addition of 0.023 M NaBr.

-30 mV ——

0.5 mV

200 ms

-30 mV ——

Figure 5. Chl-BLM Slow Component decay kinetic (left) compared

to membrane RC transient (right).

1 mV

−30 mV

+
10 mV
−

1 s

## The Quenching Phenomenon

Certain compounds were found to eliminate completely or "quench" the pH or voltage-induced slow response without modifying the Fast Component. In order of quenching effectiveness, these include: ascorbic acid, tannic acid (dicatechin), p-benzoquinone, and sodium azide. The quenchers exhibited identical quenching properties in either single chamber or both chambers. Ascorbate at $10^{-3}$ M eliminated the Slow Component immediately upon addition prior to stirring, as seen in Figure 6, while at $10^{-4}$ M 20 seconds of stirring was necessary in order for a similar degree of quenching to be observed. Figure 7 compares the relative quenching efficiencies of ascorbic acid and sodium azide. The quenching phenomenon was essentially irreversible for any single BLM; i.e., once quenched the Slow Component did not reappear.

Figure 8 demonstrates quencher elimination of a bromide-enhanced slow response while Figures 9 and 10 illustrate the ability of tannic acid and ascorbate, respectively, to quench slow responses of both polarities. These results were independent of which chamber contained the quenching agent.

Further, the light and dark current-voltage characteristics of chl-BLM in the presence and absence of Slow Component quencher were measured and are shown in Figure 11. As indicated, after the addition of quencher, the I-V characteristics of the membrane were identical in both the dark and upon illumination.

Figure 6. Top trace: immediate elimination of the Slow

Component upon addition of $10^{-3}$ M ascorbate (indicated

by noise);

Bottom trace: gradual disappearance of the slow

transient in the presence of $10^{-4}$ M ascorbate.

Figure 7. Top traces 1 and 3: biphasic responses in the absence

of quencher;

Bottom traces 2 and 4: reduced Slow Component in the

presence of $2 \times 10^{-3}$ M $NaN_3$ (trace 2) and $2 \times 10^{-3}$ M

ascorbate (trace 4).

Figure 8.  Quencher elimination of a bromide-enhanced slow
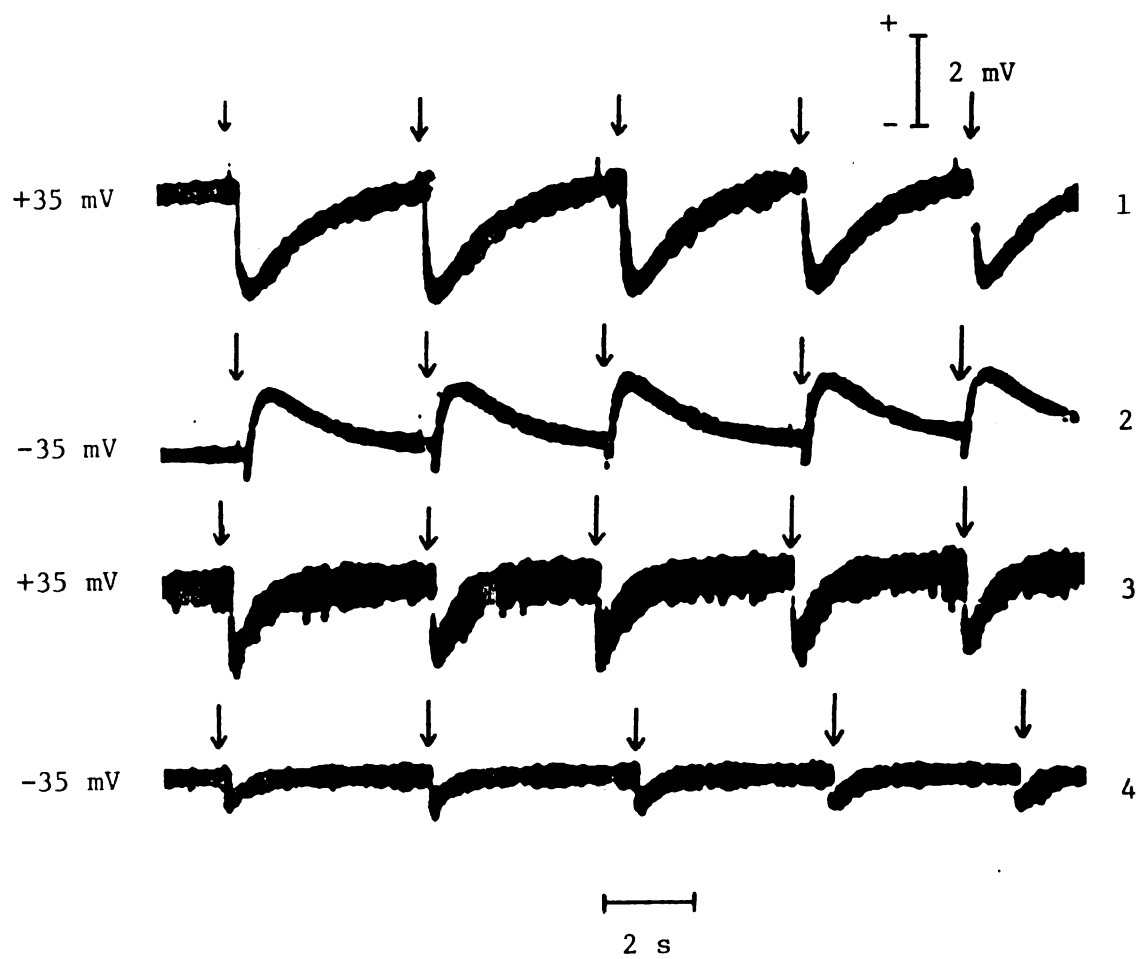
response.

Trace 1:  applied voltage only.

Trace 2:  enhancement in the presence of bromide.

Trace 3:  no response after $10^{-3}$ M ascorbate

addition.

Figure 9.  Slow transient quenching by $10^{-3}$ M tannic acid

while $10^{-3}$ M $FeCl_3$-induced Fast Component remains

unmodified.

Traces 1 and 2:  prior to tannic acid addition.

Traces 3 and 4:  subsequent to tannic acid

   addition.

Figure 10. Quenching by $10^{-3}$ M ascorbate in the presence of repeated flash illumination ($\sim$50 Hz). Equal applied voltages of opposite polarity provide the only asymmetry across the membrane. The noise spike indicates addition of quencher; subsequently the chl-BLM is rendered relatively light-insensitive.
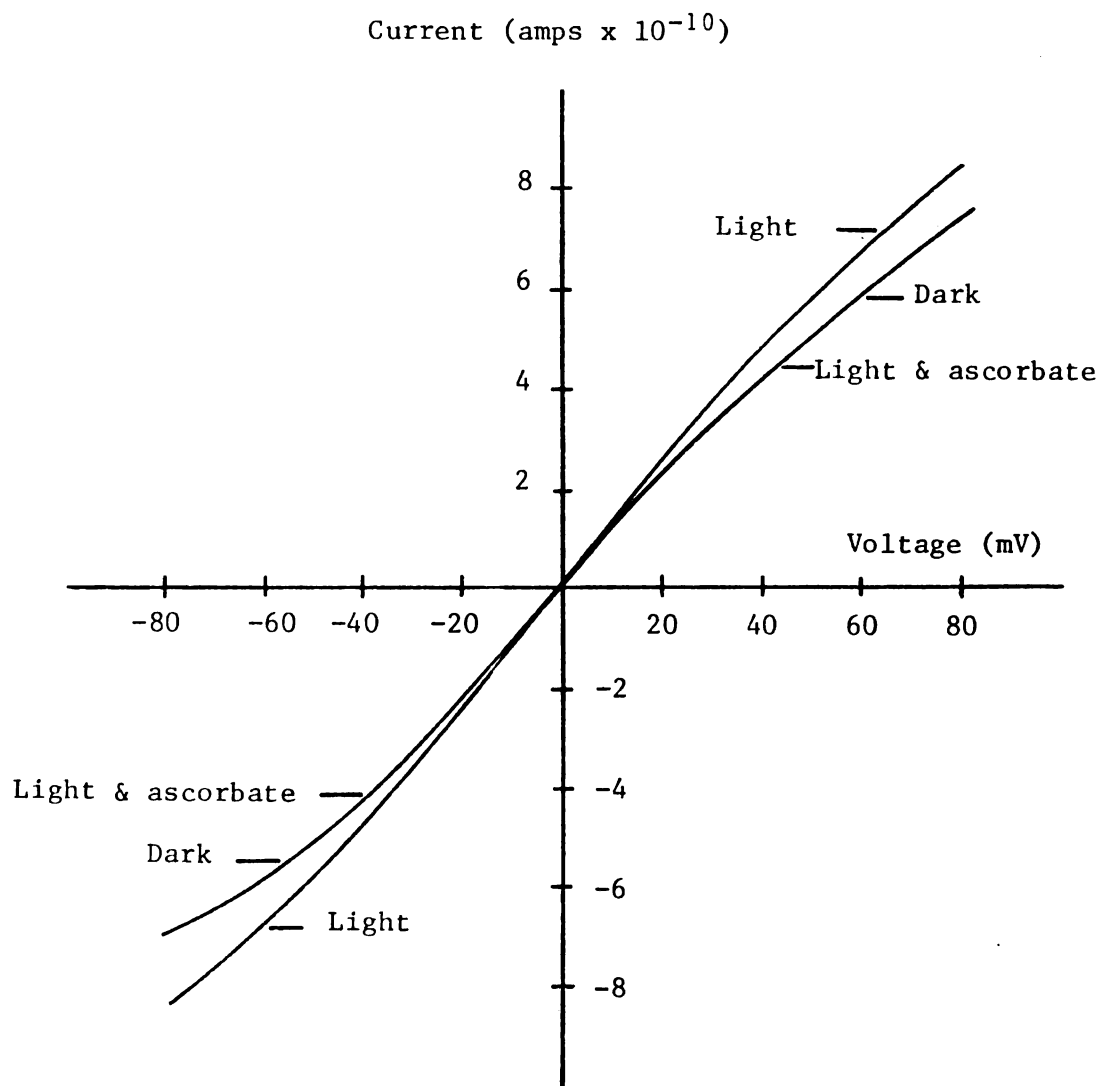
Figure 11. Light and dark current-voltage relationship of chl-BLM prior and subsequent to the addition of $10^{-3}$ M ascorbate.

Current (amps x $10^{-10}$)

Voltage (mV)

Light

Dark

Light & ascorbate

Light & ascorbate

Dark

Light

# CHAPTER V

## DISCUSSION

As an ultrathin ($\sim 10^{-6}$ cm), relatively large surface area, low dielectric-constant partition between highly conducting aqueous phases, the chl-BLM possesses an appreciable electrical capacitance, usually $\sim 5 \times 10^{-9}$ F or 5000 pF ($C_m$). Additionally, the 1 mm$^2$ membranes employed in these investigations exhibited electrical resistances of $1 \times 10^7$ $\Omega$ to $5 \times 10^8$ $\Omega$ ($R_m$). The BLM, then, is electronically equivalent to a capacitor shunted with a resistor; it is, in effect, a "leaky" capacitor. As any voltage is applied to this system, either through an external voltage source or via the action of light, the voltage transients appearing across the effective membrane $R_m$, $C_m$ combination obey the following relationship: as the membrane capacitance $C_m$ charges and discharges, the BLM exhibits voltage transients given by $V(t) = V_0 (1 - e^{-t/RC_m})$ and $V(t) = V_0 (e^{-t/RC_m})$, respectively, where $V_0$ is the maximum voltage appearing across the BLM in either case, $V = Q/C_m$, and most importantly, R is the Thevenin equivalent resistance with respect to $C_m$ (Korneff, 1968). For the chl-BLM this value is given by $R_m$ in parallel with the internal or series resistance of the EMF shunted across the membrane; i.e., $R = R_s R_m / (R_s + R_m)$. In the simple case of a voltage discharge when the charging element has been removed, $R_s$ is nearly infinite and thus $R = R_m$ and the discharge rate constant is simply $1/R_m C_m$, typically 5 sec$^{-1}$. For a

potential applied through a resistance $R_s$ of $10^8$ $\Omega$, R becomes

$5 \times 10^7$ $\Omega$ ($R_m = 10^8$ $\Omega$) and the charging rate constant is equal to

10 sec$^{-1}$.

The chl-BLM flash-elicited voltage waveform in the instance of an

imposed transmembrane chemical gradient of $FeCl_3$ indicates rapid

negative charge flow to the chl-BLM/$FeCl_3$ interface that rapidly (<1 ms)

charges the BLM capacitance followed by the total disappearance of

charging current flow resulting in discharge of the membrane capacitance

in precisely the same kinetic manner as $C_m$ is discharged in the dark

according to membrane $R_m C_m$ characteristics. The rapid initial phase

voltage transient represents charge separation across the membrane by

a current-delivering element not hindered by high internal resistance.

The condition in which charge separation can be established across the

chl-BLM capacitive element within only 1 ms is that the input Thevenin

equivalent resistance be reduced to less than $10^5$ $\Omega$, yielding an RC

charging time of less than 0.5 ms. The experimental decay of the Fast

Component is seen to be kinetically equivalent to the $R_m C_m$ transient

exhibited by the chl-BLM in the dark when the charging element is

suddenly removed (cf. Figure 2); i.e., the Thevenin equivalent resistance

is again simply $R_m$. Figure 12 illustrates an electronic circuit voltage

waveform response to a brief introduction of a current-generating

element.

This analysis, then, indicates that a transmembrane current of

typically $\sim 10^{-8}$ amps flows to the membrane capacitance upon flash

excitation in the case of $FeCl_3$-induced fast responses. This value is

corroborated by actual short circuit current measurements (cf. p. 83).

These data support the view of Tien and Huebner (Tien and Huebner, 1973)

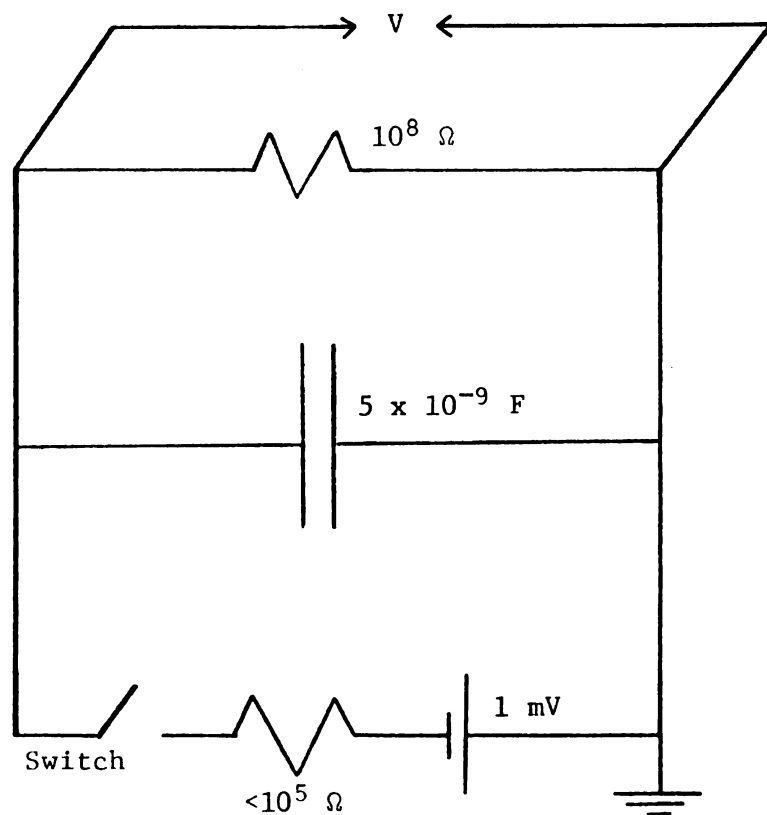Figure 12.   RC system analogous to ch1-BLM and voltage waveform

for switch closed 1 ms (denoted by arrow).  The

charging rate constant is greater than 2000 sec$^{-1}$,

corresponding to an RC time of less than 0.5 ms;

and the discharge rate constant is 2 sec$^{-1}$, corre-

sponding to an RC time of 500 ms (cf. Figure 2).

V

$10^8$ Ω

$5 \times 10^{-9}$ F

Switch

$<10^5$ Ω

1 mV

Voltage

0 mV

-1 mV

500 ms

Time
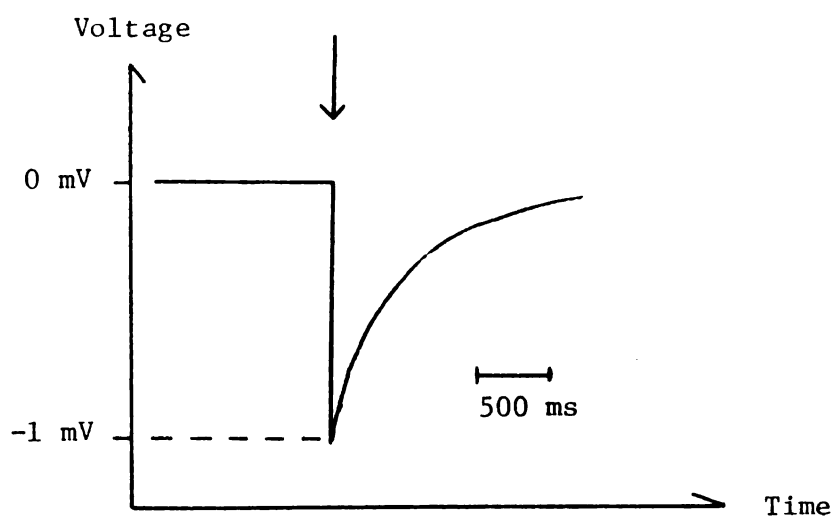
that the chlorophyll molecule excited by light to a reduction

potential more negative than that of the $Fe^{+3}/Fe^{+2}$ reduction-oxidation

couple may donate an electron to the ferric ($Fe^{+3}$) ion, the oxidized

chlorophyll subsequently oxidizing and being reduced by substrate,

most likely $H_2O$, at the opposing interface. These coupled oxidation-

reduction reactions driven by the photoenergy harvested by the chl-BLM

imply a net electron flow to the $FeCl_3$/chl-BLM interface. After

illumination and the charging of $C_m$ due to this current flow, the

decay process follows $R_m C_m$ exponential decay kinetics, indicating

that light-stimulated redox processes have ceased; indeed, the

capacitance dissipates this accumulated charge precisely as it does

any charge added to it in the dark.

In addition, these decay kinetics illustrate that the membrane

capacitance becomes charged and thus the initial photo-stimulated

charge flow must be a transmembrane one, not merely an electrical

double-layer charging current localized at a single interface.

As demonstrated above, the shape of the voltage transient

responding to flash excitation in the case of $FeCl_3$-induced effects

provides specific information concerning the current-generating nature

of the fast photoresponse. Consequently a study of features of the

Slow Component response elicited in the presence of voltage gradients

follows. Transient responses in the case of pH gradients displayed

waveforms kinetically identical to applied voltage responses; therefore

applied voltages were employed in the majority of experiments

investigating the slow response, eliminating several limitations and

precautions necessary in the pH gradient instances, viz.: applied

voltages were easily varied or removed and reapplied during experiments

while pH gradients could not so easily be varied or eliminated; chl-BLM functions as a hydrogen electrode, exhibiting the hydrogen ion Nernst potential in the dark and this "dark potential" invariably introduced a voltage bias requiring difficult normalization of data; and also pH differentials were likely to result in membrane surface asymmetries.

Initially the slow response decay process was examined. As pointed out earlier, the rapidly repeating flash illumination approach was optimal for this investigation as it provided a large amplitude photoresponse concomitant with long-lived easily-resolved decay. It will be shown later that the decay waveform is not altered by the number of flashes incident upon the membrane prior to decay. The results are indicated in Figure 13. Significantly, the decay process was found to be a simple first order exponential one. That is, for the beginning of decay at $t = 0$,

$$V(t) = V(0)e^{-\lambda t}$$

where $\lambda$ (lambda) is the rate constant. Although for different chl-BLM $\lambda$ varies from $0.5 \text{ sec}^{-1}$ to $2.5 \text{ sec}^{-1}$, corresponding to $1/e$ times (duration of time in which V falls to $V/e$ or $0.368 V$) of 2000 ms and 400 ms, respectively, $\lambda$ remains essentially constant for any single BLM.

A major point is that $\lambda$ is not equal to or a function of $R_m C_m$; $\lambda$ is always greater than $(R_m C_m)^{-1}$. In other words, $\lambda$ decay is always slower than $R_m C_m$ decay, as exemplified in Figures 5, 13, 15, and 16. An earlier model for this Slow Component assumed $R_m C_m$ decay resulting in the problem that "the theoretical voltage. . . falls too rapidly after the peak value" (p. 71, Tien and Huebner, 1973).

Figure 13. Experimental Slow Component decay ($\lambda$) and plot

of log V values versus time. The linearity of

this plot demonstrates the first order exponential

character of the decay. ($R_m C_m$ decay is again

included for purposes of comparison.)

λ

+
5 mV
−

$R_mC_m$

−100 mV

1 s

Voltage (mV)

40
30
20
10
9
8
7
6
5
4

1    2    3    4    Seconds
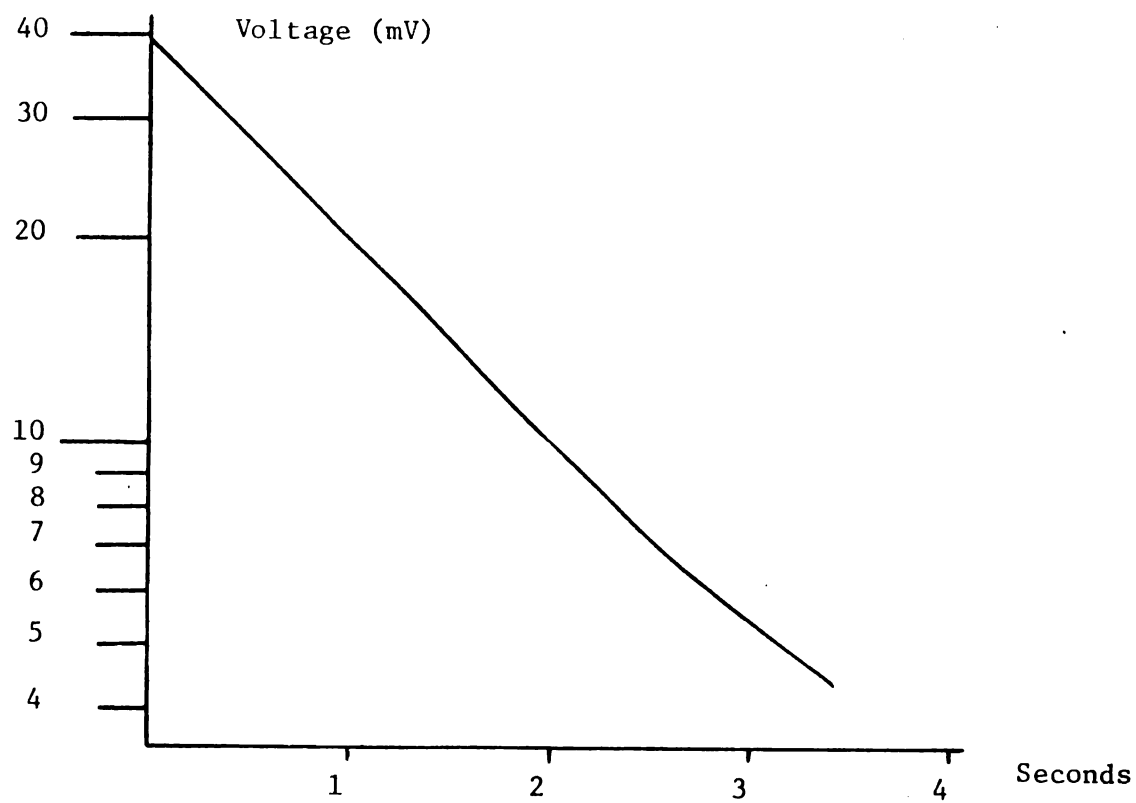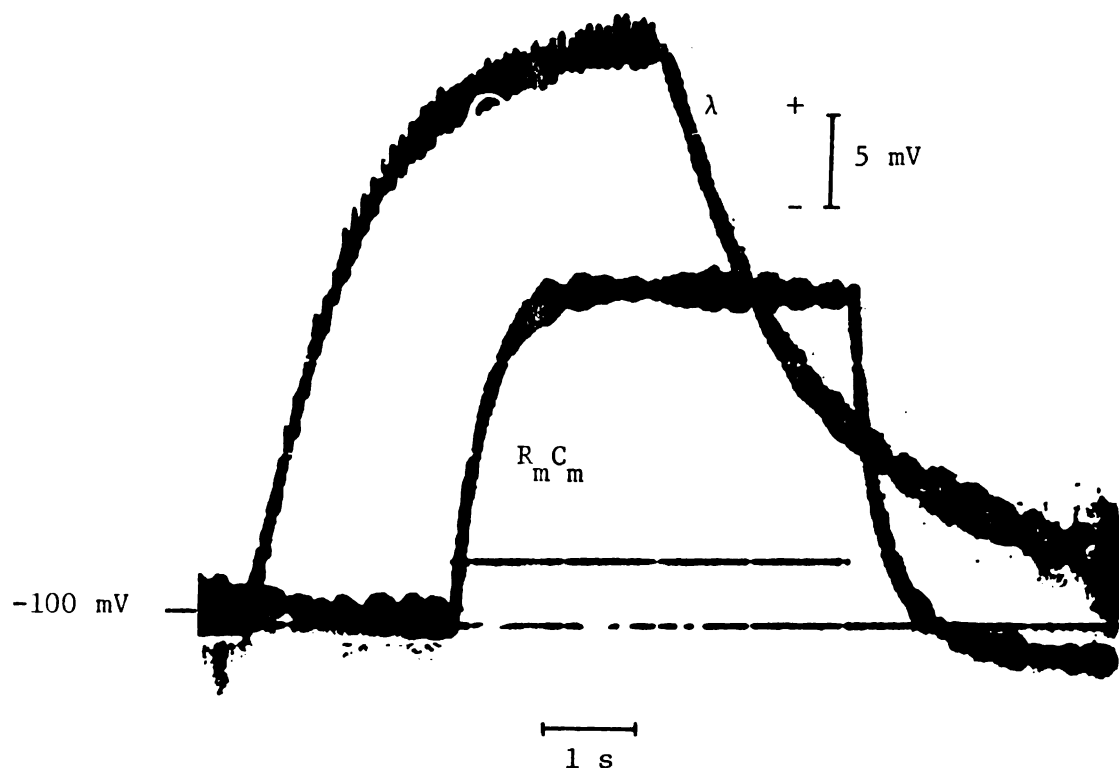
The next step in the effort to describe mathematically the entire experimental waveform was to assume the most likely, simplest case for the initial phase of the response: that it is also a first order exponential process. In the idealized absence of decay and for strobe flash at t = 0,

$$V(t) = V_o(1 - e^{-Kt})$$

where $V_o$ is the voltage value at t = ∞.

Whether this assumption holds for experimental waveforms was determined in the following manner: if the initial phase is first order exponential,

$$V(t) = V_o(1 - e^{-Kt})e^{-\lambda t}$$

will accurately describe the entire waveform. The constant $\lambda$ was measured experimentally and K was determined numerically for experimental waveform sets of values of V and t. This computation was facilitated by taking the first derivative of V(t) with respect to time and measuring the experimental time interval from t = 0 to the moment of peak voltage, $t_{Max}$. Thus

$$K = \lambda(e^{Kt_{Max}} - 1).$$

Now for any t,

$$V(t) = V_o(1 - e^{-Kt})e^{-\lambda t}$$

yields a voltage value that may be compared with the experimental voltage at that instant. The fit achieved is quite good, as shown in Figure 14. The fact that lambda values derived from rapid repeating flash illumination provide accurate fits to single flash waveforms is clear indication that the decay form is not altered by the number of flashes incident upon the membrane prior to decay.

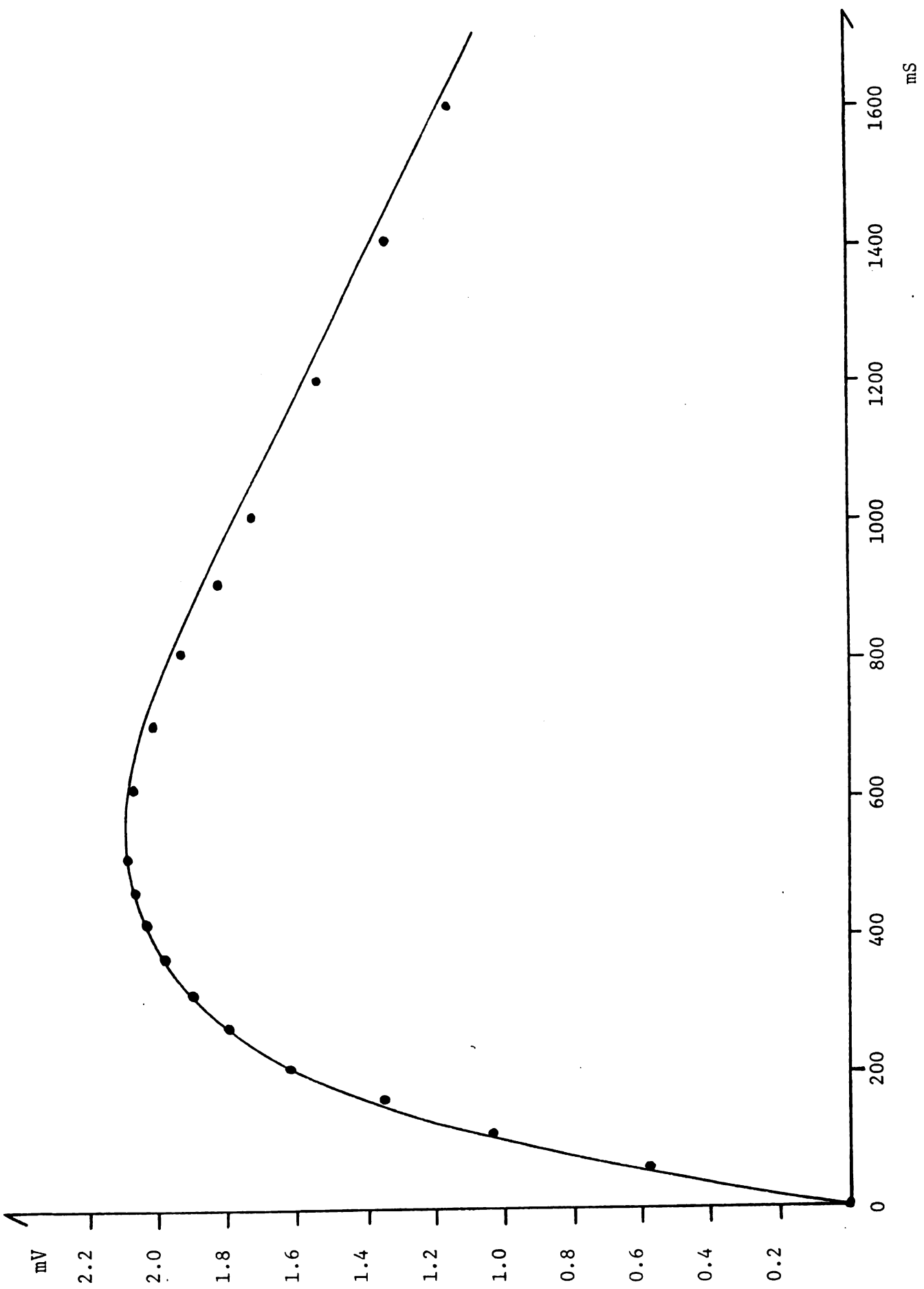Figure 14. Experimental ch1-BLM slow response waveform

and points predicted from $V(t) = V_o(1 - e^{-Kt})e^{-\lambda t}$

approximation.

For this membrane:

$K = 3.68 \text{ sec}^{-1}$

$\lambda = 0.69 \text{ sec}^{-1}$

$V_o = 3.53 \text{ mV}$.

In the course of verifying this Slow Component fit for several chl-BLM, it was noticed that the constant K for a particular membrane was invariably equal to $(R_m C_m)^{-1}$ for that BLM. This observation introduced distinct advantages: the numerical method for solving for the value of K could be abandoned and chl-BLM Slow Component waveform shapes could be predicted exactly, prior to any flash illumination with $\lambda$ having been measured from continuous illumination response decay and $K = (R_m C_m)^{-1}$ having been determined in the dark, as shown in Figures 15 and 16.

Further, from

$$V(t) = V_o (1 - e^{-Kt}) e^{-\lambda t}$$

setting $\dfrac{dV(t)}{dt} = 0$ when $t = t_{Max}$, yields

$$t_{Max} = \frac{\ln(\frac{K + \lambda}{\lambda})}{K} \text{ sec.}$$

This expresses the voltage waveform peak time in terms of $\lambda$ and $(R_m C_m)^{-1}$. As a test of this prediction, external resistances were shunted across the chl-BLM, effectively changing $R_m$ and consequently also $t_{Max}$. Figure 17 indicates experimental peak times altered precisely as expected. The successful prediction of experimental waveform shapes justifies the original assumptions and provides a basis for identification of the photo-stimulated membrane events responsible for the chl-BLM Slow Component response to flash illumination. The current-voltage relationships of the membrane in the dark and upon illumination (Figure 11) together with the fact that the initial growth phase of the Slow Component exhibits a $(R_m C_m)^{-1}$ rate constant constitute compelling evidence that the Slow Component is a

Figure 15. Slow Component waveforms and points calculated using $R_m C_m$ and $\lambda$ data included in photographs.
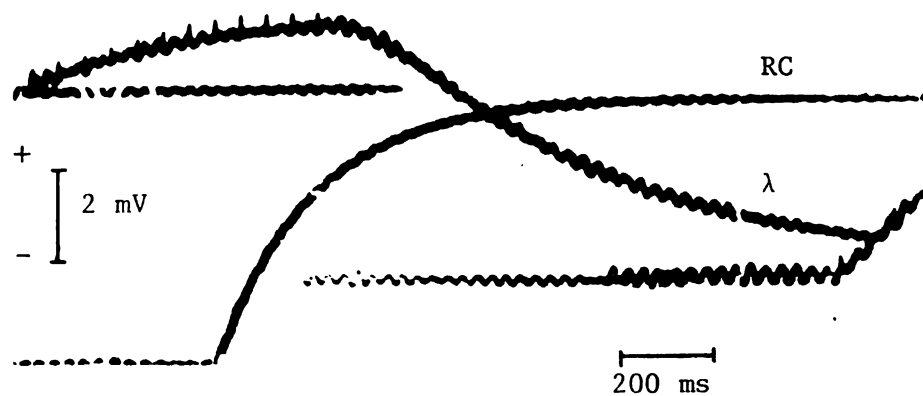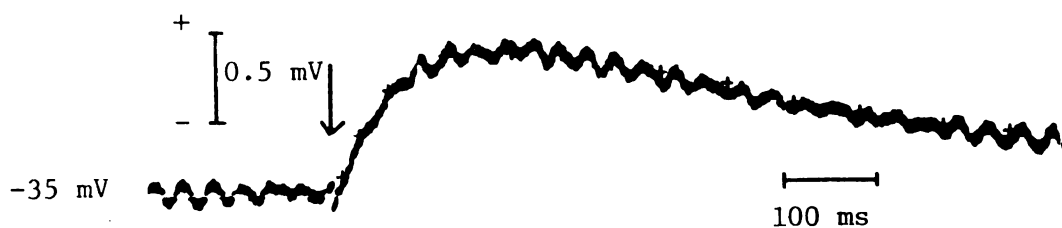
RC

1 mV

200 ms

+

0.5 mV

−35 mV

100 ms

RC

λ

+

2 mV

−

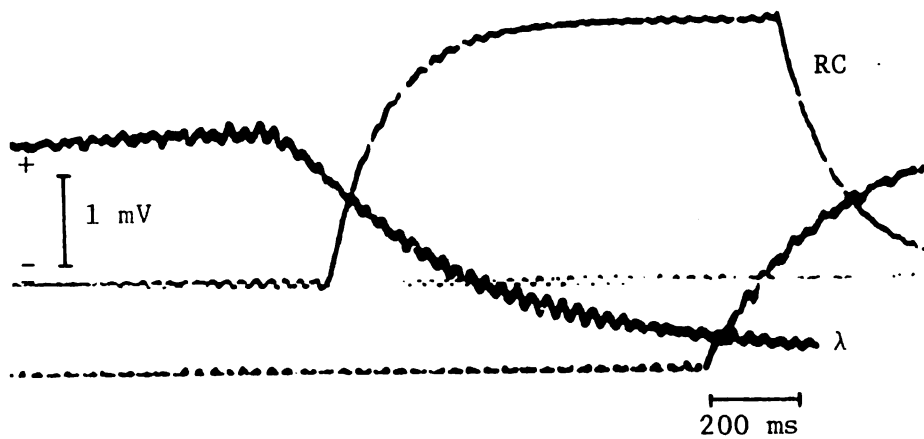200 ms

+

0.5 mV

−

−30 mV

200 ms

Figure 16.   Additional Slow Component waveforms and points

calculated from $R_m C_m$ and $\lambda$ data included in

photographs.

+

1 mV

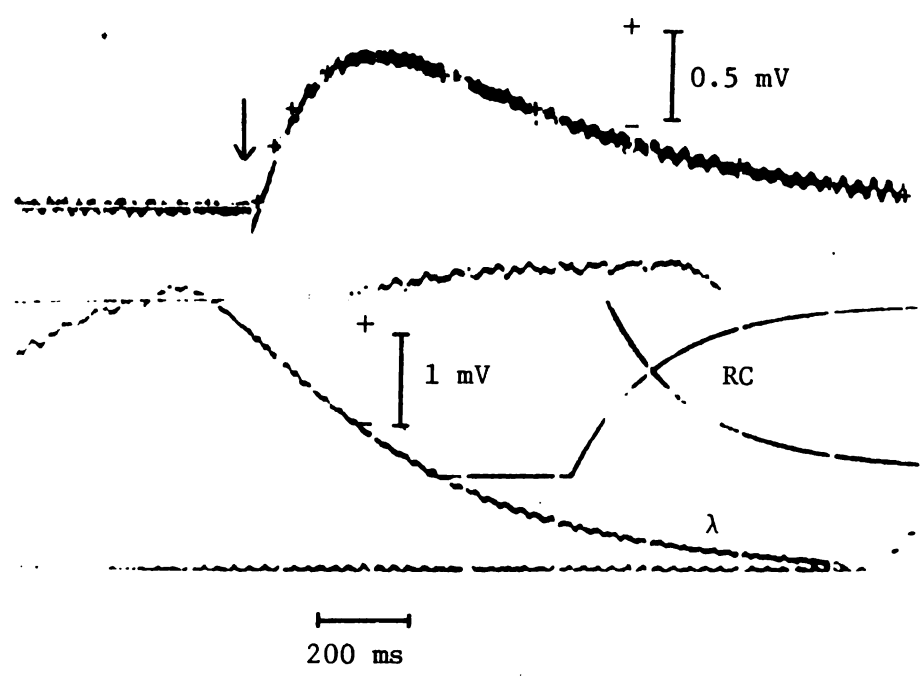RC

λ

+

0.5 mV

−50 mV

200 ms

+

0.5 mV

+

1 mV

RC

λ

200 ms
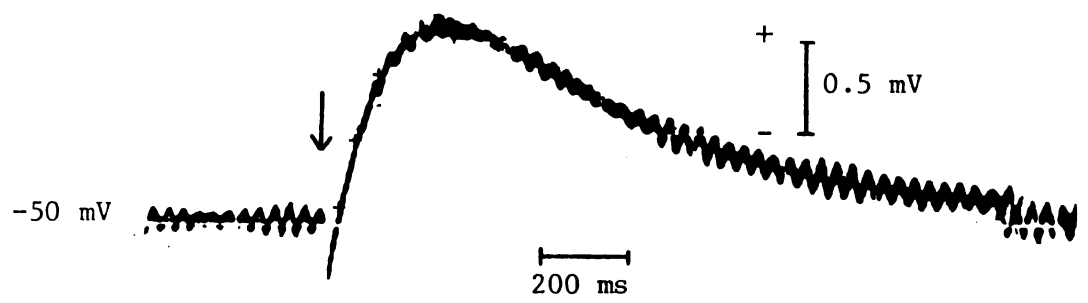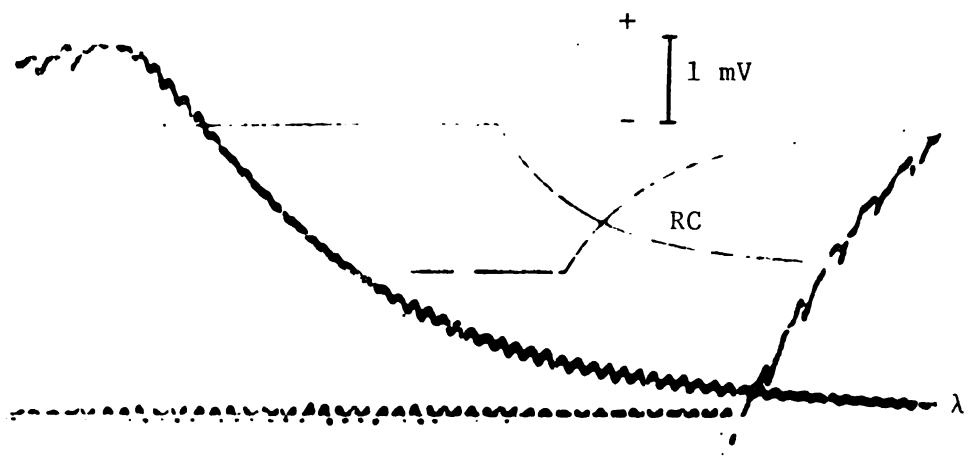
Figure 17. Slow Component peak time, $t_{Max}$, dependence upon effective $R_m$. In both cases

$$t_{Max} = \frac{\ln(\frac{K + \lambda}{\lambda})}{K} \text{ sec,}$$

$C_m = 3 \times 10^{-9}$ F and $\lambda = 1.9$ sec$^{-1}$.

Upper trace: $R_m = 3.21 \times 10^7 \ \Omega$ ($R_s = 9 \times 10^7 \ \Omega$);

  calculated $t_{Max} = 180$ ms.

Lower trace: $R_m = 4.76 \times 10^7 \ \Omega$ ($R_s = 1 \times 10^9 \ \Omega$);

  calculated $t_{Max} = 221$ ms.

-30 mV ⎯

180 ms

+
⎤
⎥ 0.5 mV
⎦
−

-30 mV ⎯

220 ms

⊢——⊣
100 ms
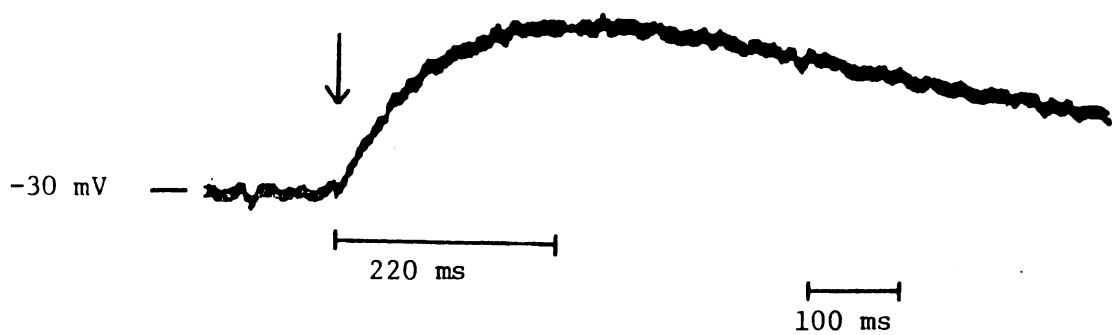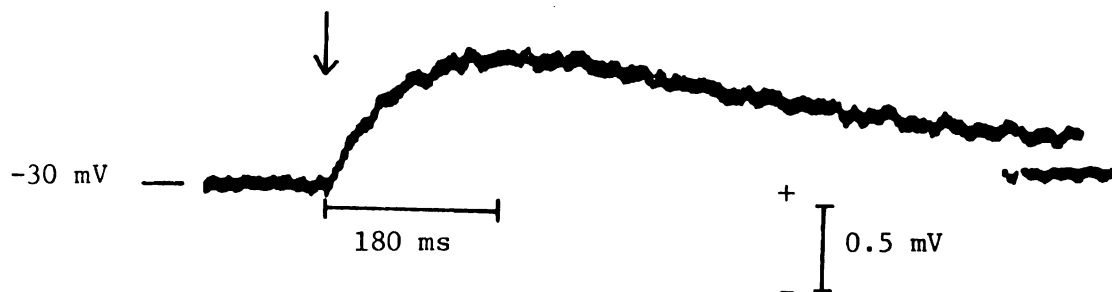
result of chl-BLM conductivity enhancement upon illumination. All

of the results are in accord with this interpretation.

That this conductivity enhancement or resistance decrease is 3%

at most accounts for the fact that $R_m C_m$ values measured in the dark

were sufficiently close to the slightly (<3%) smaller $R_m C_m$ values

actually characteristic of the membrane upon illumination that excellent

fits were obtained regardless. Note in Figure 14 that the experimental

voltage increase is very slightly more rapid than the rise predicted

according to the dark $R_m C_m$ values. The sudden introduction of slight

additional conductivity across the membrane simply decreases the

voltage dropped across the BLM, whose resistance in relation to the

fixed shunt resistance determines the amount of transmembrane potential.

This transmembrane potential decrease would be expected to display

$R_m C_m$ kinetics. The $\lambda$ decay of the Slow Component, the first order

exponential return to the dark resistance value, is slower than the

$R_m C_m$ transient and hence is not at all modified by the membrane $R_m C_m$

components.

The quenching phenomenon, then, represents an elimination of

light-induced conductivity enhancement. An ascorbate concentration

versus photoconductivity quenching ability profile is given in Figure

18. Since prior to quencher addition the enhanced conductivity decays

with rate constant $\lambda$ of $\sim$2 sec$^{-1}$, a metastable state of lifetime $\sim$500 ms

is responsible. Recognizing that the conductivity enhancement action

spectrum correlates with the chlorophyll absorption spectrum, that the

chlorophyll triplet excited state may possess lifetimes of this duration

(Vernon and Seely, 1966), that Slow Component enhancers iron and

bromide enhance also excited singlet to triplet intersystem crossing

Figure 18. Transient BLM photoconductivity dependence upon

quencher concentration. Dark conductivity =

$\sim 1.5 \times 10^{-6}$ ohm$^{-1}$ cm$^{-1}$.

Log [Ascorbate]

ΔConductivity x $10^{-8}$ ohm$^{-1}$ cm$^{-2}$
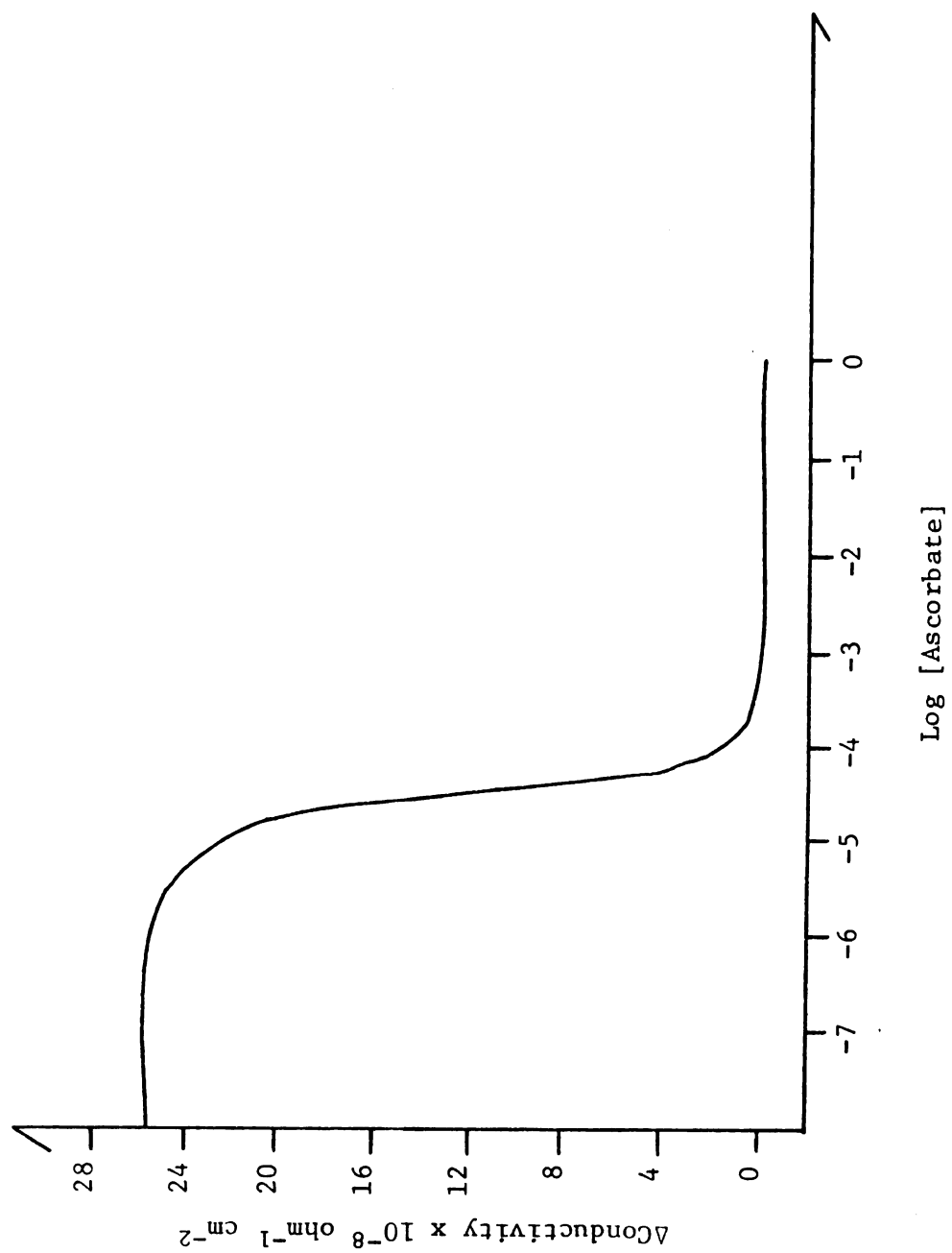
(McGlynn, 1969), that the slow transient quenchers ascorbate and p-benzoquinone are reported to be excellent in vitro chlorophyll triplet quenchers (Livingston, 1960; Fujimori, 1957; Livingston, 1963), and that at concentrations of chlorophyll likely in the chl-BLM (Alamuti and Läuger, 1970) chlorophyll triplet states would be expected to decay in a first order exponential manner (Vernon and Seely, 1966), it is reasonable to suppose that photo-populated chlorophyll triplet states are responsible for the conductivity enhancement in chl-BLM manifested as the Slow Component. In addition, the fact that there exists a pH gradient-induced Slow Component suggests that the chlorophyll triplet conductivity channels are relatively specific for protons.

The Fast Component represents a current delivering process at a polarity fixed only by the arrangement of redox agents; the Slow Component represents a conductivity increase with kinetic features containing information concerning chlorophyll metastable states in chl-BLM.

CHAPTER VI

ADDITIONAL RESULTS

## Dual Component Voltage Decay

In the course of the measurement of dark $R_m C_m$ values for chl-BLM

it was observed that the $R_m C_m$ transients in most cases followed

exactly the logrithmic decay pattern expected from the membrane as

detailed on page 47 of this thesis. However, decay transients from

potentials applied longer than 5 seconds exhibited a slower decay

contribution whose magnitude was directly dependent upon the duration

of voltage application. Figure 19 illustrates the appearance of this

secondary decay. The linearity of the secondary portion indicates

the exponential character of this transient. Also, voltages applied

for periods longer than $\sim 20$ seconds induced secondary contributions

no greater than that characteristic of the 20 second applied potential

case and as indicated this maximum secondary decay does not appear

until V has fallen to $\sim V_o/10$, accounting for the accuracy of the $R_m C_m$

approximation of this decay waveform.

When an impressed potential induces charge flow across the membrane,

the most permeable charge carriers may accumulate in the region of

the interface to which they are driven. If so, upon removal of the

applied field these permeable charges may simply diffuse across the

chl-BLM according to their concentration gradient, resulting in an

exponentially decaying voltage of a polarity tending to sustain the

69

Figure 19. Logarithmic plots of chl-BLM voltage decays for

potentials applied.

A. 0 seconds (pulse). Simple $R_m C_m$ exponential as

expected.

B. 6 seconds. Another slower first order decay

becomes apparent.

C. 20 seconds. Maximum secondary decay contribution.
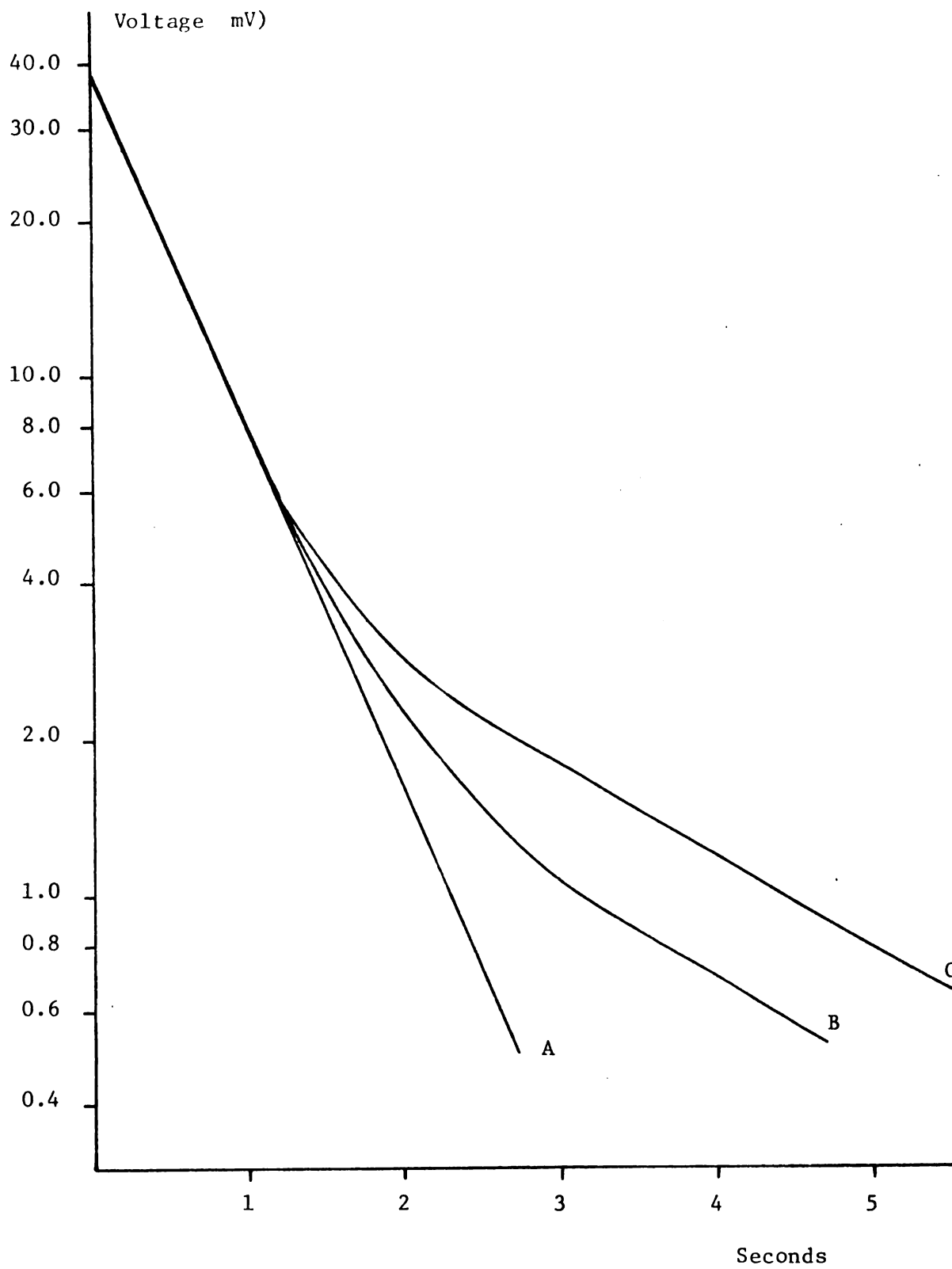
transmembrane potential drop that existed during external voltage application.

## Chl-BLM Mechano-Electric Oscillations

When the chl-BLM support cell was vibrated at uniform frequencies, the membrane responded with mechanical oscillations, from the planar to alternately nearly hemispherical configurations in each chamber, at a frequency identical with the driving vibration. Interestingly, this mechanical oscillation was accompanied by a membrane $\pm 1$ mV electrical oscillation of the same period.

The stroboscope trigger rapid repeated dim flash mode was employed to determine membrane mechano-electric oscillation frequencies and, further, to establish the correlation between polarity of voltage and direction of membrane distortion with respect to the electrically grounded chamber; only when the flash frequency was tuned to equal the chl-BLM vibrational frequency, flash-denoting spikes were distributed on the BLM voltage output display at one spike per electrical oscillation, and the membrane mechanical vibration was visually totally arrested. Simply, the chl-BLM was being illuminated at only one particular moment in each of its vibrational cycles, giving the visual appearance of no movement. The synchronous strobe flash frequency, typically $\sim 30$ Hz, was then altered slightly, introducing a phase difference resulting in very slow apparent membrane vibration. A mark was easily made at the moment the chl-BLM appeared maximally distended into the ungrounded electrode chamber (Figure 20), and the strobe spike, then, indicated the polarity of membrane potential at this configuration by its position on the voltage waveform.

The chl-BLM potential was established in this manner to be negative when the membrane is distorted toward the ungrounded electrode compartment.

As a possible explanation: for the hemispherical configuration of a 100 $\overset{\circ}{A}$ ($10^{-6}$ cm) thick bilayer of planar diameter 1 mm (0.1 cm), the inner radius, $R_i$, is 0.05 cm or $5 \times 10^6$ $\overset{\circ}{A}$ while the outer radius, $R_o$, is 0.050001 cm or $5.0001 \times 10^6$ $\overset{\circ}{A}$. So the area difference is given by

$$\Delta A = 2\pi (R_o^2 - R_i^2) = 2\pi \times 10^9 \ \overset{\circ}{A}^2.$$

Assuming a negative chl-BLM membrane surface charge of 1 esu/200 $\overset{\circ}{A}^2$, the outer surface of the membrane holds $\sim 3 \times 10^7$ excess negative unit charges. For a membrane capacitance of $5 \times 10^{-9}$ F, according to the relation $V_m = Q/C_m$ (1 esu = $1.6 \times 10^{-19}$ coulombs) $V_m = \sim -1$ mV.

The polarity and magnitude of this result compare favorably with the data given in Figure 20.

## Photo-Emf Internal Resistance

Measurements of the equivalent internal resistance of the $FeCl_3$ induced photo-emf under flash illumination and continuous illumination are useful since this indicates whether the chl-BLM photovoltage device can be loaded, i.e., deliver appreciable currents. As shown in Figures 2 and 12, the initial rapid voltage change requires that a current of at least $10^{-8}$ amps be delivered in response to microsecond illumination.

A more precise determination of the value of the photobattery internal resistance was made by establishing the value of external

Figure 20.  Open circuit BLM potential oscillation synchronous

with mechanical vibrations.  Superimposed spikes

indicate strobe light flashes; arrow denotes moment

at which membrane appears maximally distended

into the ungrounded electrode chamber.

+

⊥ 5 mV

−

100 ms
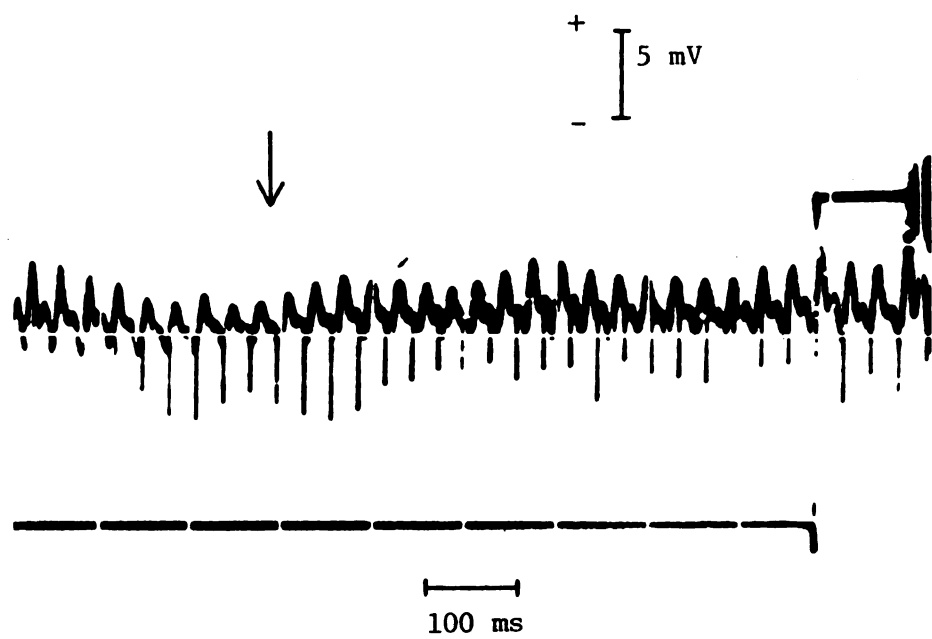
shunt resistance $R_s$ capable of reducing the absolute magnitude of the Fast Component by half. According to the data recorded in Figure 21, this value of $R_s$ was found to be typically on the order of $5 \times 10^4$ ohms. (The electrodes, in series with $R_s$, presented resistances of approximately this value; therefore this represents a lower limit on possible membrane shunt resistance values.) Notice the extremely short $R_m C_m$ times as expected for Thevenin equivalent resistances reduced to this extent.

Under rapidly repeating flash illumination, an approximation of continuous illumination, a constant photovoltage becomes established across the chl-BLM. As illustrated in Figure 22, the closed circuit current available from the membrane may be determined easily in this case by applying sufficient voltage through shunt resistor $R_s$ to bias the photovoltage to zero. It may be shown that when $V = 0$,

$$E_L/R_L = V_{app}/R_s .$$

In other words, the short-circuited current available from the membrane is simply $V_{app}/R_s$. This method has been tested experimentally using an ammeter to actually measure these short-circuited currents after expected values of current were calculated as outlined above. The agreement obtained is indicated in Figure 23. These current values correspond to a photobattery internal resistance of $\sim 10^7$ ohms, much greater than the $\sim 5 \times 10^4$ ohms measured as being characteristic of the single flash response photobattery internal resistance.

Concomitantly, membrane $FeCl_3$-induced photocurrents fall from a $\sim 10^{-8}$ amp current pulse under brief single flash illumination to approximately $10^{-11}$ amp under 50 Hz repeated flash illumination.

Figure 21.  Effect of low external shunt resistance on the

$FeCl_3$-induced Fast Component.  $R_s$ equals:

1.  $2.5 \times 10^5 \ \Omega$

2.  $1.5 \times 10^5 \ \Omega$

3.  $5.0 \times 10^4 \ \Omega$

4.  $1.0 \times 10^8 \ \Omega$

5.  $5.4 \times 10^5 \ \Omega$

6.  $4.0 \times 10^4 \ \Omega$

7.  $3.0 \times 10^4 \ \Omega$

8.  $2.0 \times 10^4 \ \Omega$

Figure 22. Arrangement used to measure photocurrent output

of chl-BLM. At V = 0, set by varying $V_{app}$,

$$E_L/R_L = V_{app}/R_s$$

Figure 23.  Membrane photovoltages (upper trace) returned to

zero by application of external voltage biases

(lower traces).

Above:  $V_{app}/R_s$ = 8.8 x $10^{-11}$ amps; ammeter current

measurement, 8.9 x $10^{-11}$ amps ± 5%.

Below:  $V_{app}/R_s$ = 8.9 x $10^{-11}$ amps; ammeter current

measurement, 8.5 x $10^{-11}$ amps ± 5%.

$(R_s$ = 9.1 x $10^7$ Ω)

# CHAPTER VII

## SUMMARY

Chlorophyll Bimolecular Lipid Membranes are most faithful
laboratory reproductions of the essential components of the natural
photosynthetic energy transduction device. They successfully meet
the cardinal requirements for chlorophyll-dependent photosensitivity
and membranous insulating properties to maintain charge separation
and insure subsequent chemical reaction rather than energetically
wasteful charge recombination. These artificial membranes exhibit
photovoltaic responses of two basic types: one represents a reduction
potential gradient dependent current that flows in a direction
established by the arrangement of redox agents, and the other represents
a conductivity increase with kinetic features containing information
concerning chlorophyll metastable states in chl-BLM. A study of the
latter process offers at least two immediate, direct benefits:

i)  Several investigators make extensive use of continuous
illumination photoeffects as a tool, but unless the specific molecular
excited states responsible for the photoeffect are more fully charac-
terized, interpretations of data can easily be only speculative.

ii)  Also, much effort is currently being expended in the study
of chlorophyll excited states in vitro (Dutton, Leigh, and Seibert,
1972; Netzel, Rentzepis, and Leigh, 1973) and, provided that $\lambda$ decay
indeed represents chlorophyll triplet decay we have presented a

significant new tool to investigators of chlorophyll photosynthetic excited states as we have incorporated chlorophyll into a membranous matrix akin to the thylakoid membrane of the chloroplast and more highly representative of the in vivo arrangement.

Offering a convenient method for monitoring membranous chlorophyll excited state properties, chlorophyll Bimolecular Lipid Membranes are useful probes into the pathways of photosynthetic energy transduction.

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