PHOTOELECTROSPECTROMETRY OF BILAYER LIPID MEMBRANES (BLM) AND PARTIAL RECONSTITUTION OF THE PHOTOSYSTEM 1 REACTION CENTER OF PLANTS IN ARTIFICIAL MODEL MEMBRANES

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ABSTRACT

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BLMs modified with different substances were investigated for photoelectric effects when illuminated with uv and visible light. One of the systems studied consisted of membranes formed with extracts from spinach chloroplasts. Another system investigated consisted of an otherwise non-photoactive membrane to one side of which liposomes containing purple membrane (PM) fragments from H. halobium were added. Both of these BLMs exhibited photopotentials when illuminated with uv and visible light. In both cases the photovoltaic action spectrum followed the absorption spectrum of the modifier. In the system with PM, through chemical modification of the PM, the uv-elicited photoresponse was found to be due to energy transfer from the aromatic amino acids to the chromophore and subsequent proton translocation by the chromophore. One other system investigated consisted of BLMs containing chymotrypsin adsorbed to one side. This BLM also exhibited photovoltages when irradiated with uv light whose action spectrum follows the absorption spectrum of chymotrypsin. The initial charge separation in this case is attributed to ionization of the aromatic amino acids by the uv radiation. The results obtained in this set of experiments suggest the possibility of using photoelectric spectroscopy measurements to study energy transfer and interaction between membrane components in BLMs.

In another set of experiments, the partial reconstitution of the PS1 reaction center (RC) from plants in BLMs was accomplished by two different methods. The first method consisted of the association of vesicles containing thylakoid membrane fragments (TMFs) with the BLM. The second method involved the incorporation of the TMF in the BLM through fusion of vesicles with the BLM. In both cases photoelectric effects were observed which required the addition of carriers of reducing equivalents. The action spectrum of the photoelectric response followed the action spectrum of the PS1 RC. This set of experiments gave support to the idea that charge separation in the PS1 reaction centre results in the generation of a potential difference across the thylakoid membrane. The results also support the view that the RC spans the thylakoid membrane. Moreover they show the usefulness of the planar artificial BLM for reconstitution studies.

To

my parents,

my sister Nilda,

my brothers Pedro and Carlos and

especially my wife Doris

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CHAPTER 1

INTRODUCTION

Membranes comprise a large part of cell structure. From a functional point of view, membranes are known to be involved in many of the essential processes taking place in living organisms. These include: transport phenomena, energy transduction, excitability, exo- and endocytosis, DNA anchoring, protein synthesis, cell-cell recognition, synaptic transmission drug and/or hormone interaction and many others. The diversity of the processes reflects the structural differences and variations in the membrane components. In most cases, it has been possible to associate certain membrane components, e.g. lipids, proteins, lipoprotein complexes, pigments and other molecules with the different membrane functions. In the hope of understanding how biological systems work, it seems therefore necessary to obtain information on the molecular aspects of the processes, on the physico-chemical properties of the components involved, on the interaction between them and also on structure-function relationships.

In many cases, however, the direct investigation into the function of biological membranes is hampered by their structural complexity. This has driven researchers into developing membrane models which mimic certain aspects of biological membranes. This approach has the advantage that in model systems, in general, some of the properties of the physiological system are abstracted from the many others which make the study of the whole system more difficult. Moreover, model membrane systems provide a more controlled environment and help gain access to certain experimental parameters which are otherwise inaccesible in the natural system. One important membrane component which has been extensively investigated in several different model systems is the chlorophyll molecule (for a review see Seely, 1977).

One such model system which has been extensively used is the so-called bilayer (or bimolecular) lipid membrane (BLM) developed by Mueller, Rudin, Tien and Wescott in the early 1960's (Mueller, Rudin, Tien and Wescott, 1962; 1963). One of the main advantages of this artificial membrane system is that its planar configuration allows direct characterization of electrical events taking place across it.

One area of research using the BLM model system involves the reassembly of components of biological membranes into the model system and the study of its properties and characteristics (for a comprehensive review see Tien, 1974). Following the same approach, Tien and co-workers formed BLMs with a solution containing pigments and lipids extracted from chloroplast lamellae and discovered that such membranes were capable of generating photoelectric phenomena (Tien, 1968; Tien and Verma, 1970). The photoelectric effects have been attributed to electronic processes taking place inside the membrane coupled with redox reactions taking place at the interfaces (Tien, 1972; 1979). Since then many other photoactive substances have been found to generate photoelectric effects in BLMs, including dyes (Tien, 1972; Ullrich and Kuhn, 1972; Huebner, 1978), carotenoids (Kobamoto and Tien, 1971), light-sensitive inorganic ions (Pant and Rosenberg, 1971), purple membrane from H. halobium (Dancshazy and Karvaly, 1976; Herrmann and Rayfield, 1978; Higgins, Lopez and Tien, 1979; see also Appendix B), porphyrin derivatives (Hong and Mauzerall, 1972), porphyrin complexes (Joshi, Lopez, Tien, Wang and Liu, 1982; see also Appendix C), rhodopsin (Liu and Tien, 1982) and reaction centers from photosynthetic bacteria (Packham, Packham, Mueller, Tiede and Dutton, 1980).

One apsect of the study of photoelectric phenomena in photoactive BLMs is that of photoelectrospectrometry. The term photoelectrospectrometry is used here to describe the measurement of the action spectrum of the photoelectric effect, which should resemble, in general, the absorption spectrum of the photoactive species in the model system. From this follows the most immediate and common use of the action spectrum that is as an indicator of the species responsible for the initial light-absorbing step in the photoelectric process (see Appendix C). However, since the photoactive species is present in a membrane environment which is usually different from bulk solutions, the action spectrum may also exhibit some differences from the absorption spectrum which reflect the influence from that environment. It also happens that direct measurement of the absorption spectrum of the species in the membrane phase is very difficult due to the ultrathinness ($\sim$ 100 Å) of the BLM (Cherry, Hsu and Chapman, 1971). On the other hand, the action spectrum of the photoeffects, due to its nature, is in reality an excitation type of spectrum and not an absorption spectrum (as in the case, for example of fluorescence excitation spectrum). Therefore it may also reflect differences from the absorption spectrum and should reflect characteristics of the photoelectric effect itself. This is exemplified by studies on BLMs containing chlorophyll pigments (Van and Tien, 1970) and purple membrane from H. halobium (Karvaly and Dancshazy, 1977). In both of these studies, differences between the action spectrum of the photoeffects and the absorption spectrum of the photoactive species were observed. The differences were explained in terms of the properties and characteristics of the photoactive species and the nature of the photoeffects.

This research centers around two aspects of the reassembly of membrane components into BLMs, mainly photoelectrospectrometry and reconstitution. In the first case, the main objective of the research is to perform action spectrum measurements of BLMs containing several photoactive species including proteins. The action spectrum will be measured in the visible as well as in the uv region which is where most proteins absorb. It is hoped that the spectra may give information on the interaction and/or energy transfer between the membrane components.

The other main objective of this research is to go one step ahead in the reassembly of components of the photosynthetic apparatus of plants and try to reconstitute, at least partially, the photosystem 1 (PS1) reaction center of plants in a BLM model system. The word reconstitution is meant to describe the reassemly of the component in such a way that at least some of its functional activity is retained. Under such circumstances it may be possible to obtain direct evidence that a potential difference develops across the thylakoid membrane of chloroplasts as a result of illumination. Reconstitution experiments of this type have been successful in the case of isolated reaction centers from photosynthetic bacteria (Barsky _et_ _al_., 1976; Packham _et_ _al_., 1980). However, when working with isolated PS1 reaction centers from plants the results have not been as successful (Barsky _et_ _al_., 1976; Lopez, unpublished observations). For this reason it was decided to try to reconstitute not the isolated reaction centers but whole membrane fragments obtained from thylakoid lamellae.

The results of the experiments on photoelectrospectrometry appear in Chapter 4 and also in Appendix A. The results of the experiments on the partial reconstitution of the thylakoid membrane fragments are presented in Chapter 5. Also, because of the wide range of subjects dealt with in this research the literature review has been divided into three parts: the BLM model system, the photosynthetic apparatus of plants, and reconstitution of proteins from biological membranes into planar model membrane systems. This literature review is presented in Chapter 2.

CHAPTER 2

LITERATURE REVIEW

THE BLM MODEL MEMBRANE

(a) General:

Biological membranes are complex and highly variable structures. The variability is reflected in both function and composition. At the same time, however, there are also some properties and characteristics of biological membranes which are common to almost all biological membranes found in living organisms. On the one hand biological membranes are usually composed of two classes of macromolecules, proteins and lipids. Also, stained electron micrographs of membranes from different sources all exhibit the same pattern, two dark electron dense regions sandwiching a not so dense light region. From electron microscope studies the thickness of most biological membranes has been found to range between 40 and 110 Å. It has been proposed that all membranes have the same basic structure and a large number of models have been proposed over the years since the original proposal of the bimolecular leaflet model of Gorter and Grendel (1925), followed later by the "unit membrane" hypothesis of Robertson (1959). Up to now, no single model can explain all of the data obtained on the structure of biological membranes, however a large number of observations can be explained in terms of the model of Singer and Nicholson (1972). The model proposes a fluid lipid bilayer interdigitated by proteins.

As mentioned previously, due to the complexity of biological membranes, several investigators decided to develop and study membrane models. Of the several model systems which have been developed, the liposome, introduced by Bangham (1963) and the BLM developed by Rudin and co-workers (Mueller et al., 1962) have received the most attention. Both of these artificial membrane systems consist of an ultrathin (∿100 Å in thickness) lipid bilayer matrix separating two aqueous phases. The popularity of both these models lies mainly in the ease of their preparation and handling, the way they mimic biological membranes under certain circumstances and their significant role in membrane reconstitution studies. Several detailed reviews on both of these model systems have been published over the years (see Shamoo and Trivol (1980), Tien (1974) and Tien (1981) for reviews on BLMs and see Bangham, Hill and Miller (1974), Szoka and Papahdjopolous (1980) and Tyrell, Heath, Colley and Ryman (1976) for reviews on liposomes).

Of the two model systems, the BLM has proven especially useful in studies involving electrical characterization, diffusion and permeability since its planar configuration allows easy access to both sides of the bilayer.

Some of the areas of study involving BLMs include: formation techniques and materials (Takagi, Azuma and Koshimoto, 1965; Tien, 1974; Vodyanoy, and Murphy, 1982; Waldbillig and Szabo, 1979) mechanical, physical and chemical properties of BLMs, e.g. thickness (Tien, 1974), stability (Chizmadzhev, Abidor, Postushev and Arakelya, 1979), bifacial tension (Tien, 1974), fluorescence spectroscopy (Alamuti and Lauger, 1970, electrical breakdown (Benz, Janko and

Lauger, 1979), diffusion and permeability (Fettiplace and Haydon; Orbach and Finkelstein, 1980) and ion transport (Andersen, 1978; Jordan, 1980); the effect of antibiotics on membrane permeability (Andreoli and Tosteson, 1971; Muller and Rudin, 1967); the interactions of toxins with BLMs (Donovan, Simon Draper and Montal, 1981); the interaction of liposomes with BLMs (Duzgunes and Ohki, 1981; Zimmerberg, Cohen and Finkelstein, 1980); reconstitution of membrane components on planar membranes (Montal, Darszon and Schindler, 1981; Shamoo and Trivol, 1980).

(b) Photoelectric effects on BLMs containing chlorophyll pigments:

Photoelectric phenomena on BLMs was first observed by Tien (1968) on artificial membranes formed with an extract obtained from chloroplast lamellae (Chl-BLM). Since then, photoelectric effects for several other modified BLM systems have also been observed, including BLMs containing; carotenoids (Kombamoto et al., 1971), purple membrane from H. halobium (Dancshazy et al., 1976; Herrman et al., 1978; Higgins et al., 1979), porphyrin derivatives (Hong et al., 1972), prophyrin complexes (Joshi et al., 1982; Wang, Tien, Lopez, Liu, Joshi and Hu, 1982), rhodopsin (Liu et al., 1981; Montal, 1979) and bacteriochlorophyll reaction centers (Packham et al., 1980; Schonfeld, Montal and Feher, 1979). Photoelectric effects have also been observed when certain modifiers (so-called extrinsic) are added to the bathing solution at either side of a BLM, including: dyes (Huebner, 1978; Tien, 1972; Ullrich et al., 1972) and light-sensitive inorganic ions (Pant and Rosenberg, 1971). Several reviews on this subject have been published and the reader is referred to these for more details, including Tien (1974) and Tien (1979). (Two of the previous systems, namely, purple membrane-BLM and bacteriochlorophyll reaction centers - BLM will be discussed in more detail in the next section).

The observation of photoelectric effects on BLMs suggest the generation of light-induced charge carriers in the membrane. Originally, the photoelectric effect from a Chl-BLM was found to be very small since the system was set up to be symmetric (Tien, 1968). Later the photoresponse was found to increase dramatically under assymetric conditions, such as: externally applied electric field, the presence of electron acceptors and/or donors on opposite sides of the BLM and also a pH gradient across the BLM (Tien, 1974). The photoelectric effect consisted of a photovoltaic and a photoconductive effect. The action spectrum of the photovoltaic effect identified the chlorophyll pigments as the photoactive species responsible for the photoeffects. The photovoltage was found to vary linearly with an externally applied potential difference. This is attributed to the large magnitude of the electric field (up to 10^5 V/cm) present across the membrane which can cause the oppositely charged species, generated either directly or indirectly by light, to move to opposite sides of the BLM depending on the direction of the electric field. Such a tremendous electric field is possible due to the small thickness ($\sim$ 100 Å) of the BLM and its ability to withstand potential differences of 100 mV or more. The magnitude of the open-circuit photovoltage was found to increase with the logarithm of the incident light intensity. Moreover, when examined under continuous illumination the photovoltage was found to consist of two components, with one much faster than the other. By illuminating the BLM with a short duration (8 μs) light flash, the rise time of the first component was found to be faster than 8 μs (Heubner and Tien, 1972).

As mentioned previously, it was found that addition of certain oxidizing agents (electron acceptors) to one side of the BLM, enhanced dramatically the photoresponse (Tien and Verma, 1970). At the same time, addition of electron donors (reducing agents) to the opposite side of the membrane further enhanced the photovoltage and/or photocurrent (Tien, 1974). Both of these facts taken together have been interpreted to indicate that electron transfer reactions are taking place at each interface (Ilani and Berns, 1972; Tien, 1968; Trissl and Lauger, 1972). The question is then 'What is the nature of the charge carriers inside the bilayer?' Two mechanisms have been proposed to answer this question. In one case the migrating species are said to be radicals (Trissl and Lauger, 1972). In the other mechanism it is proposed that electrons and holes are the charge carriers, suggesting that the membranes are capable of electronic conduction (Ilani _et al._, 1972; Tien, 1968). It has been suggested that carotenoids, which are present in the BLM, provide a pathway of low enough resistance to electron movement across the "bulk" membrane (Mangel, Berns and Ilani, 1975). For more details on the mechanism for electronic conduction by electrons and holes across the Chl-BLM the reader is referred to a recent review by Tien (1979) on this subject.

REVIEW ON RECONSTITUTION IN PLANAR MEMBRANES

(a) _Introduction_:

Recently, an increased number of reports on reconstitution of certain membrane components on planar bilayer (model) membranes have appeared in the literature (for a review see Montal, et al., 1981). This increase in the number of reports follows some new methods and techniques used to incorporate membrane components, including proteins and lipoprotein complexes, into planar bilayer membranes (see Montal et al., 1981). This is tied in with some recent progress in membrane research which makes it possible to associate complex biological processes with membrane components, including protein lipids, pigments and others, and with technical advances in the biochemical characterization and purification of those components.

The main features of the reconstitution approach are that it "allows, in principle, the dissection of a complex phenomenon into its components with the subsequent gain of experimental control over parameters that are otherwise inaccesible or highly varible in the natural membrane". Also "it establishes general principles for the reassembly of biologically active membranes providing insight into the interplay between the membrane environment and the functional unit".

Model membranes have proved very valuable in membrane reconstitution studies in terms of providing a more controlled environment. The most widely used model membranes are the liposome or vesicle of spherical configuration and the planar bilayer lipid membrane, first introduced by Mueller et al. in 1962. Liposomes have been used widely especially in spectroscopic studies and have the advantage of being easy to handle, relatively stable and that proteins are relatively easy to be incorporated in them. Planar membranes, on the other hand, although more unstable have the advantage that they allow easy access to both of its sides and therefore provide a direct way for the characterization of electrical events taking place membranes. Unfortunately, the functional reassembly of proteins in planar membranes is difficult and has therefore advanced more slowly. As mentioned previously however, recent basic conceptual and methodological advances in protein reconstitution in planar membranes are providing new hope and perspectives in this approach.

In this review section mostly reconstitution studies involving planar membranes will be discussed although in some cases and when relevant, work done with model membranes of special configurations will also be mentioned.

(b) Methods and techniques for the reassembly of proteins and membrane fragments in planar bilayers:

At present time there are two main approaches used to incorporate proteins into planar membranes. One approach involves the formation of planar bilayers in such a way that the proteins become incorporated at the moment of formation. In the other approach the proteins are added into the aqueous phase of an already preformed planar membrane with the protein adsorbing into the membrane and eventually becoming incorporated into it. In both cases the planar bilayers can be formed either as a bimolecular (black) lipid membrane (Mueller *et al.*, 1962) or assembled from monolayers (Takagi *et al.*, 1965).

In the simultaneous approach the material to be incorporated has to be either suspended in a lipid organic solvent phase (Montal, 1974) or incorporated in a vesicle suspension (Schindler and Quast, 1980). The rationale for the transfer of proteins in active form into organic solvents is based on the fact that membrane proteins usually have hydrophobic and hydrophilic regions. It can be assumed that the hydrophobic parts will be at least partly soluble in the apolar solvents while the hydrophilic regions will not. A procedure had been established by Das and Crane in 1964 in order to extract complexes of cytochrome c and acidic and neutral lipids in isooctane. They found that the partition of the complexes into the apolar solvents was enhanced by the charge neutralization between the positive charges of cytochrome c and the acidic phospholipids. Then Gitler and Montal (1972) found that the partition of these complexes in apolar solvents could be enhanced by adding cations like Ca^{2+} and Mg^{2+} and by lowering the pH. Following these results several groups have succeeded in transfering certain protein and proteolipid complexes into alkanes. These include cytochrome oxidase lipid complex in hexane (Montal, 1974) and octane (Chien and Mueller, 1976), rhodopsin in hexane (Darszon, Philipp, Zarco and Montal, 1978) and octane Liu *et al.*, 1982) and reaction centers from photosynthetic bacteria in hexane (Schonfeld, *et al.*, 1979, Kendall-Tobias and Crofts, 1979) and octane (Packham *et al.*, 1980).

Two methods are at present generally used to transfer the proteins into the apolar solvent. In one case the proteins are first solubilized by detergent, purified and delipidated if needed. Then the protein can be added directly to solvent containing lipids or recombined with lipids to form vesicles which are then extracted with the organic solvents. This allows for some control over the protein and its reconstituted lipid environment. In the other methods, the protein is transfered directly into the non-aqueous phase from the biological membrane. This is accomplished by mixing the biological membrane with the organic solvent and sonicating followed by addition of cations and separation of the two phases by centrifugation.

One other interesting technique, which follows the simultaneous approach, was recently developed by Schindler (1980) and it is based on the results of an experiment by Verger and Pattus (1976) (see also Pattus, Desmuelle and Verger, 1978; Pattus, Piorant, Lardunski, Desmuelle and Verger, 1978 and Schindler, 1979) who found that monolayers can form spontaneously at air water interfaces containing liposomes, or vesicle suspensions derived from biological membranes. Using this result and following the method of Takagi *et al.*, (1965), Schindler (1980) was able to form planar bilayer membranes. The membranes were found to be stable if the liposomes or vesicles used for spreading the monolayers were > 50 nm in diameter. It was found that if vesicles obtained from biological membranes containing proteins were used to spread the monolayers, the planar bilayers obtained from them would also contain the proteins (Schindler *et al.*, 1980). Using this technique a functional acetyl choline receptor from *Torpedo marmorata* was reconstituted in planar membranes (Schindler *et al.*, 1980). The technique also worked when the acetyl choline receptor was isolated, purified, and incorporated into liposomes which were spread to form monolayers and eventually planar bilayers (Nelson, Anholt, Lindstrom and Montal, 1980). The results obtained in this study were similar to the ones obtained with native bio-membrane vesicles by Schindler *et al.*, (1980).

The second approach followed in planar membrane reconstitution studies known as the sequential approach is the one which has been most widely used. In this approach the material to be incorporated can be added to the aqueous phase in purified form, as a detergent or as a vesicle suspension. This latter technique has been developed into what is referred to as fusion of vesicles with planar bilayers. The disadvantage of using detergent extracts is that detergents usually have a deleterious effect on the planar membrane.

The technique of membrane fusion for reconstitution studies was originated by Skulachev and co-workers in studies with cytochrome oxidase, H^+-ATPase and bacteriorhodopsin (Drachev, Jasaitis, Koulen, Kondrashin, Liberman, Hayrecek, Ostroumov, Semenov and Skulachev, 1974). It was found, however that total fusion of the vesicles was not taking place since the measurements were reproduced on thick planar membranes. The results were attributed to adsorption or partial fusion where the vesicle closed structure is maintained after attachment of the two structures (Drachev, Frolov, Kaulen, Kondrashin, Samuilov, Semenov and Skulachev, 1976; Herrmann _et al_., 1978).

More successful reconstitution using fusion of vesicles with planar membranes was accomplished by Miller and Racker (1976) in order to study the permeability properties of the sarcoplasmic reticulum (SR). As a result of these studies, an experimental protocol was devised which consisted of making SR vesicles interact with planar bilayers in the presence of negatively-charged lipids, calcium and an osmotic gradient across the vesicular membrane leading to the swelling of the vesicles (Miller, Arvan, Telford and Racker, 1976). The criterium for fusion was the observation of an increase in the bilayer conductance in discrete steps which were associated with fusion events. The conductance increases were composed of a sequence of spike-like events of varying size. It was suggested that each spike represents the fusion of a single SR vesicle with the planar bilayers Miller _et al_., 1976; Miller, 1978) resulting in the insertion of package of potassium channels. Incorporation of proteins for reconstitution studies using fusion of liposomes or vesicles with planar bilayer membranes has also been reported by other groups, including Repke, Berczi and Matties (1980), Hanke, Eihl and Beheim (1981) and Latorre, Vergara and Hidalgo (1982). The results of some of these experiments although all of them positive, sometimes contradict each other as well as previous results. In the case of Tepke _et al._, (1980) no requirement for osmotic gradient nor enhancement of fusion events using Ca^{2+} were observed. The planar membranes used in this case were of the black lipid membrane type. Fusion was said to depend on the type of lipids present in both membrane structures. Hanke _et al._, (1981) on the other hand, using planar membranes formed according to the method of Takagi _et al._, (1965) found that while the presence of Ca^{2+} ions is not a prerequisite for fusion, application of an osmotic gradient appears to be necessary for high fusion rates. It was also found that maximum fusion rates occur at the calorimetric phase transition temperature and that the addition of cholesterol to the membrane lipid stops fusion. Latorre _et al._, (1982) followed the protocol of Miller _et al._, (1976) in their studies. In all previous cases the criteria for successful membrane fusion was the observation of sharp increases in the membrane conduction indicative that the channel forming proteins had been incorporated in the membrane.

Fusion of liposomes or vesicles with planar membranes has also been observed by other groups interested in studying the process of exocytosis (Zimmerberg, Cohen and Finkelstein, 1980; Duzgunes and Ohki, 1981). In both of these studies the divalent ion Ca^{2+} was found to be a requirement for fusion to cocur. Duzgunes _et al._, (1981) used gramicidin A present in the liposomes as the assay for fusion with appearance of conductance channels in the planar membrane as the criterium that fusion had taken place. Zimmerberg _et_ _al_., (1981) and Cohen _et_ _al_., (1981) found that an osmotic gradient across the planar membrane was a requirement for fusion to take place. In these studies the authors used multiamellar vesicles containing a voltage-dependent anion channel (VDAC) from mitochondria incorporated in n number lamellae and the fluorescent dye carboxy-fluorescein incorporated in the aqueous space between the lamellae. The two criteria for fusion included: 1) the incorporation of the VDAC protein in the planar membrane as assayed by monitoring changes in conductance and 2) the transfer of an n-1 lamellar liposome across the planar membrane as assayed by detecting the fluorescent liposomal particle in the opposite side to the one in which it was originally located. Both of these results were observed in these experiments and they were explained in terms of the n lamellar liposome (containing the VDAC) fusing its outer membrane with the planar membrane and the n-1 lamellar particle moving to the other side of the planar membrane. The discharge of vesicular contents across the planar membrane represents the most convincing evidence of vesicle-membrane fusion hitherto obtained.

It should be mentioned that in the studies mentioned above true fusion between the two membranes has not been unequivocally shown except perhaps in the studies by Zimmerberg _et_ _al_., (1980). The reason for this being that, as mentioned earlier, in most cases the assay for fusion involves the incorporation into the planar membrane of a membrane associated marker from the vesicular membranes. Some authors have not ruled out (Cohen and Moronne, 1976) that the incorporation of such markers can occur via its transfer by a process other than fusion. However, for reconstitution studies the important thing is that the material intended for reconstitution becomes incorporated in a functional way independent of the process used. It is also important to indicate that all the data obtained from biological membrane systems reconstituted using different techniques and different preparations will provide information that help in trying to resolve the question of how the particular system works.

(c) Biological membrane components reconstituted in planar model membranes:

One biological membrane component which has been reconstituted in planar membranes by various research groups is the protein bacteriorhodopsin which is present in the purple membrane patches of *H. halobium*. The first report was published by Drachev *et al*., in 1974. In this study purple membrane containing bacteriorhodopsin was mixed with phospholipids and incorporated first into liposomes which were then added to one side of a thick (not bilayer) planar phospholipid membrane in the presence of Ca^{2+} ions. After waiting for some time the system developed photopotentials when illuminated. The action spectrum of the photopotentials followed the absorption spectrum of bacteriohodopsin which indicated that the protein was responsible for the generation of the photovoltage. The photopotential was attributed to the proton pump activity which is associated with the bacteriorhodopsin protein (for review see Stoeckenius, Lozier and Bogomolni, 1978). In these studies, however, it appears that the bacteriorhodopsin protein did not become incorporated into the thick planar membrane (Drachev *et al*., 1976a). Following the same approach, Shieh and Packer (1976) obtained similar results using planar bilayer lipid membranes stabilized with polysterene. This same approach was also used by Herrmann and Rayfield (1976) and Higgins, Lopez and Tien (1979) (see Appendix 2) using BLM and also by Blok and Van Dam (1978) who substituted the planar membrane with a lipid-impregnated millipore filter. Another approach which was first used by Dancshazy et al., (1976) and then adopted by others (Bamberg, Apell, Dencher, Sperling, Stieve and Lauger, 1979) consisted of adding the purple membrane patches to one side of a positively charged BLM. After some time the system developed photopotentials in response to light due to the adsorption of the purple membrane patches to the planar membranes. As in all other studies, the action spectrum of the photoresponses followed the absorption spectrum of bacteriorbodopsin. However, as in previous studies the fact that the steady-state photocurrent increases when the lipid bilayer is supplemented with proton conductors such as CCCP and gramicidin A (Bamberg et al., 1979; Herrmann et al., 1978) suggests that the protein does not become actually incorporated into the BLM but only becomes associated with the membrane in a preferential orientation. Up to the present, there is no report in the literature of a genuine incorporation of bacteriorhodopsin into a planar bilayer where bacteriohodopsin regains transmembrane orientation analogous to that existent in the bacterial membrane.

Another membrane component which has been reconstituted in planar membrane systems is rhodopsin, the visual pigment involved in the primary event in visual excitation. It is believed that light-induced conformational changes in rhodopsin eventually lead to cellular excitation by modulating the ionic conductance of the photoreceptor cell membrane (Haggins, 1972). However, the coupling mechanism of the rhodopsin photochemical changes to the cell electrical response is not known (Hubbell and Bownds, 1979). By incorporating rhodopsin in planar membranes insight into this problem may be obtained.

Different rhodopsin preparations including rhodopsin-containing vesicles, detergent solubilized rhodopsin and purified rhodopsin have been used for reconstitution studies in planar membranes.

In studies by Montal and his collaborators planar bilayers containing rhodopsin were formed by apposition of two preformed monolayers (Montal, Darszon and Trissl, 1977). The monolayers were formed by spreading at an air-water interface a mixture made up of an organic solvent (hexane or diethyl ether), rhodopsin and phospholipids. They found that the membrane conductance increased with illumination. The increase was to be irreversible and to have a latency period from one up to several hundred seconds. This increase in conductance was attributed to the light induced formation of channels in the rhodopsin-containing bilayers. The same group apposed a monolayer containing rhodopsin one side of a thin 16 μm Teflon film separating two aqueous compartments (Trissl, Darszon and Montal, 1976). The Teflon septum acts as an electrical insulator and couples the compartments only capacitatively. Fast photoelectric signals were recorded when the membranes were illuminated with light flashes of short duration. The photosignals were attributed to capacitative charge displacements in rhodopsin as it changes conformations. These photoresponses are comparable to the early receptor potential (ERP) of photoreceptor cells which is also believed to originate from capacitative charge displacements in rhodopsin (Cone, 1967; Hagins and

McGaughy, 1968).

In another model system consisting of squid outer segment vesicles adsorbed onto a thick ($\sim$1000 Å) planar membrane formed with monoolein and decane, Takagi and Kishimoto (1977) have also recorded photosignals similar to the ERP. More recently Liu and Tien (1982) have also observed photosignals similar to the ERP in a model system consisting of rod outer segment (ROS) vesicles adsorbed to one side of a BLM. The kinetics of the rise and decay of the photosignals followed the kinetics of the transitions from lumirhodopsin to metarhodopsin I (Meta I) and from Meta I to metarhodopsin II (Meta II) respectively (Liu, Tien, Lopez and McConnell, submitted for publication).

A similar model system, BLM formed in the presence of sonicated rod outer segments, had been previously used by Fesenko and Lyubarskiy (1977). In their studies an increased in the conductance of the BLM in response to a single light flash was observed. The conductance increased to its maximum value within 60 ns at 17°C and decayed to a somewhat higher steady-state conductance than the original dark level in about 1 s. No definite conclusion could be made as to the mechanism of the photoeffect since it was not known if the rhodopsin had been incorporated into the BLM or if ROS vesicles had just sorbed to one side of the membrane.

One other report of reconstitution of rhodopsin in planar membranes involves the sonication of ROS with phospholipids followed by lyophylization and resuspension in decane (Antanavage, Chien, Ching, Dunlap and Mueller, 1977). The BLM formed with this suspension exhibit a transient light-induced conductance increase, with a rise time of 5 ns and selective for H^+. The response was optimum in the presence of

2% ethanol. This last condition raises the possibility that the results might have been due to released retinal since rhodopsin can be denatured by the ethanol. BLM containing retinal are known to exhibit light-induced H^+ specific conductance changes (Kobamoto et al., 1971).

Up to the present, however, there is no report of a planar bilayer model system containing rhodopsin similar to the way it is present in the natural membrane. The planar membranes containing rhodopsin actually incorporated into them which have been prepared are all symmetric.

Cytochrome c oxidase, the enzyme which catalyses the reduction of molecular oxygen to water during respiration, has also been incorporated in planar model membranes (for a detailed review see Montal et al., 1981). In studies by Drachev et al., (1974) vesicles containing cytochrome oxidase were added to one side of a thick planar membrane. The vesicles adsorbed to the thick film and upon addition of cytochrome c and ascorbate a cyanide-sensitive transmembrane potential was generated. The results were said to be in agreement with Mitchel's idea of transmembrane electron flow in the cytochrome oxidase segment of the respiratory chain (Drachev et al., 1974). In studies by Montal (1974) planar bilayer membranes were formed by apposing two lipid monolayers containing cytochrome oxidase. The membranes developed a potential when oxidized cytochrome c was added to one compartment which was reversed or prevented by cyanide. The origin of the potential difference was attributed to a transmembrane electron transfer reaction (Montal, 1976). Similar results were obtained by Chien and Mueller (1976), who extracted cytochrome oxidase with phospholipids into octane or decane and formed BLMs with the mixture. Tredgold and Elgamal (1978) added cytochrome oxidase and lipids to hexane and formed bilayer membranes by two techniques, with the usual brushing technique and by putting together two opposing monolayers containing the enzyme, subsequently on top of a mylar sheet. In both cases addition of cytochrome c and ascorbate generated membrane potentials which were sensitive to cyanide. The question of the nature of the photopotentials was not directly addressed by the authors (Tredgold _et al_., 1978). Recently there has been a suggestion that cytochrome oxidase may work as a redox-linked proton pump (Wikstrom and Krab, 1979). This view is contrary to the electron-translocating model of the enzyme as proposed by Mitchel (1968). The use of planar bilayer membranes may help resolve this question.

The first reports on reconstitution of photosynthetic reaction centers from plants and bacteria in planar membranes were published by Skulachev and collaborators in 1975 (Drachev, Kondrashin, Samuilov, 1975; Barsky, _et al_., 1976; Drachev _et al_., 1976). In their studies isolated reaction centers from both plants and bacteria were incorporated into liposomes which were then added to one side of a thick (not bilayer) planar phospholipid membrane in the presence of divalent cations. The proteoliposomes containing bacteriochlorophyll RC complexes from _Rhodospirillum rubrum_ associated with the planar membranes and photopotentials were observed in response to illumination. The polarity of the light-induced potentials was consistent with the proteoliposomes charging negative inside. The photoelectric effect was shown to increase on addition of tetramethyl-p-phenylenediamine (TMPD), coenzyme Q_6 (CoQ_6), and vitamin K_3 and to decrease on addition of fericyanide, o-phenanthroline, and tricholorocarbonylcyanide phenylhydrazon (CCCP). All the agents were added to the same side as the proteoliposomes. This group also did studies with plant chlorophyl complexes of PS1, however, no direct measurement of light-induced electric generation were obtained (Barsky et al., 1976). The generation of an electric field across the proteoliposomal membrane was demonstrated in this case by means of the anion probe phenyldicarbaundecaborane (PCB^-). The effect required addition of phenazinemethosulfate (PMS) and its polarity was consistent with the liposomes charging positive inside. Photopotentials were also obtained in studies where R. rubrum chromatophores were added to one side of a thick planar membrane. The photoresponse required TMPD (or PMS) and could be enhanced by naphtaquinone and ascorbate. In all of these studies the action spectrum of the photoresponse followed the absorption spectrum of the reaction centers. It was suggested that perhaps proteoliposomes with RCs from plants could not associate with the planar membrane or that electron transport in associated proteoliposomes could be inactivated by decane which was present in the planar membrane.

RC from Rhodopseudomonas Sphaeroides extracted in hexane were mixed with phospholipids and used to formed monolayers which were then assembled into bilayers. The bilayers generated photovoltages and photocurrents when supplemented with secondary donors, such as cytochrome c, and acceptors as ubiquinone-o, on opposite sides of the membrane. The photocurrent was biphasic, followed the absorption spectrum of the RCs and could be abolished by o-phenantroline.

Similar results were obtained by Packham _et al_., (1980) using bacterial RCs extracted into octane. In this case the RC-octane mixture was used to form BLMs by the brushing technique. Two kinds of membranes were obtained "black" (bilayer) and thick (> 500 Å). No consistent difference was observed in the photoresponses exhibited by the two kinds of planar membranes (Packham _et al_., 1980). The RCs in the phospholipid octane solution were shown to retain photochemical activity. In both cases the generated photoelectric signals were attributed to the transfer of electrons from a secondary donor to a secondary acceptor located on opposite sides of the membrane (Packham _et al_., 1980; Schonfeld _et al_., 1979).

THE PHOTOSYNTHETIC APPARATUS OF PLANTS

(a) The chloroplasts:

In higher plants the process of photosynthesis takes place within specialized cell organelles called chloroplasts. These chloroplasts exhibit a characteristic lens shape with a long axis of 5 to 10 μm when looked under the microscope. However, when sectioned in a plane perpendicular to the lens shape, the chloroplasts appear discoid. They are surrounded by two non-pigmented membranes (inner and outer) which enclose the highly organized internal lamellar structure and the amorphous stroma. The lamellar inner structures are often referred to as thylakoids. Usually a major part of the thylakoids is oriented in an appressed form to constitute granum stacks or grana lamellae. Single thylakoids usually connecting granum stacks are called stroma lamellae. It has been proposed (Heslop-Harrison, 1966) that the complex internal system of lamellae divides the volume inside the chloroplasts into two compartments, each forming a separate continium. It is suggested that the two compartments are formed by the folds and connections of a single continuous sheet of membrane (lamellae). One compartment is the stroma and the other represents the inner thylakoid volume. This view is supported by the realization that the intergranal lamellae were perforated, forming an interconnecting fretwork system (Heslop-Harrison, 1963; Weier and Thomson, 1962) with layers connected to lamellae at several levels within the same granum (Weier, Stocking, Thomson and Drever, 1963). Some good reviews and monograms on the structure and function of chloroplasts have been published and the reader is referred to those for more detailed information. These include descriptions of the structure of mature chloroplasts (Thomson, 1974), relationship between structure and photosynthetic function (Arntzen and Briantais, 1975; Park and Sane, 1971) and aspects of chloroplast development (Kirk, 1971). Also a comprehensive review has been published by Gunning and Steer (1975). The more specialized function of chloroplasts in providing and organizing the machinery for converting light energy into useful biological energy is associated with the internal lamellar systems of the chloroplasts (Park and Sane, 1971). On the other hand, the dark biochemical reactions of CO_2 are dependent on the soluble proteins of the stroma.

(b) The thylakoid membrane:

The internal lamellar system of the chloroplasts is the one that contains the pigments, proteins and other molecules responsible for the photochemical conversion of light energy into chemical energy. When examined with the electron microscope at a high level of resolution the appearance of the thylakoid membranes depends to a large extent on the fixation procedure and the electron-opaque materials used in sample preparation. In general, the various images obtained are similar to those recorded for most membranes, ranging from the tri-partite (unit) membrane structure consisting of two electron-dense regions about 2 nm thick with a central translucent region of comparable width to a membrane composed of globular subunits (Hohl and Hepton, 1965; Murakami, 1964; Weier, Engelbrecht, Harrison and Risley, 1965). X-ray structure studies on the thylakoid membrane are consistent with the tri-partite model (Kreutz, 1969). Using electron microscopy and the techniques of freeze-etch and freeze-fracture, it has been possible to obtain information on the surface contours of thylakoids, and the contours of fractured planes between the inner and outer membrane surfaces. Four regions of the thylakoid membrane can thus be exposed: inner and outer surfaces, and two complementary faces of an interface generated by fracture. With these techniques particles of about 5 nm in diameter can be observed attached to the outer surfaces (A' faces) of the lamellar system (unstacked region). These particles however, can be removed by washing the thylakoids with EDTA and buffers of low ionic strength prior to freeze-etching (Arntzen, Dilley and Crane, 1969; Park and Pheichofer, 1968). They have been associated with the enzyme ribulose diphosphate carboxylase (RuDP carboxylase) which is responsible for CO_2 fixation and the CF_1, which is the main part of the enzyme responsible for synthesis of ATP from ADP and phosphoric acid in photosynthesis (for a review see McCarty, 1979). The particles are scarce or absent in the stacked regions outer surfaces (A). The membranes are assymetric and when fractured exhibit different types and number of particles on each face. In the unstacked region one finds many bumps on the B' face (fracture face outer lamellae), roughly of two sizes, about 8 and 11 nm in diameter. The fracture face inner thylakoid side (C' face) has as expected the complementary appearance of many pits and a few 11 nm bumps. In stacked regions the B ace exhibits large bumps of about 16 nm in diameter, plus some 11 nm bumps. The C face has large pits (complementary to the large bumps of the B face), and also some small 8 nm bumps. The inner lamellar surface of both stacked and unstacked thylakoids (D' and D faces) exhibit medium and large bulges which may be reflections of bodies seen on the fractured faces as well as other components of the photosynthetic apparatus. In general, the results indicate that the large bodies (16 nm) are found in the stacked regions and the small (8 and 11 nm) bodies exist in both stacked (granal) and unstacked (stromal) lamellae.

Since the discovery that photosynthesis in plants involves the cooperation of two photosystems, many investigators began trying to isolate specific components or fractions identifiable with one or other photosystem. Using disruptive techniques like sonication, detergents, french press and others plus differential centrifugation it has been possible to obtain two fractions, a light buoyant fraction (Fraction I) enriched in photosystem 1 (PS1) activity and a dense heavy fraction (Fraction II) having both activities but enriched for photosystem 2 (PS2) activity (Boadman and Anderson, 1964; Michel and Michel-Wowertz, 1969; Ke and Vernon, 1967; Anderson, 1975; Becker, Shefner and Gross, 1965). When the fractions were examined with the electron microscope it was observed the Fraction I consisted of small vesicles with surfaces resembling those of stroma lamellae, while Fraction II looked more like pieces of membrane in the stacked region, the grana lamellae.

In general, Fraction I contains 40 chlorophyll a (long wave form) molecules per P700 (photosystem 1 pigment) and no chlorophyll b. Fraction II contains some P700, long and shorter wave forms of Chl a and a ratio of Chl a to Chl b of less than 3. By subjecting the fractions to further analytical treatments Thornber and his collaborators (1976) discovered the light harvesting complex LHa/b, a major antenna pigment-protein complex which contains equimolar amounts of chlorophylls a and b and as mentioned before, has no photochemical activity. In summary, the studies on Fractions I and II suggest that thylakoid membranes contain three principal components: a major antenna complex (LHa/b) and a component for each photosystem, consisting of reaction center and a "subantenna" of Chl a. Several polypeptides have been associated with the PS1 activity of chloroplasts having stacked regions (grana), PS2 activity is confined mainly to fractions derived from these regions; however, as yet, no morphological entity in the membrane has been identified decisively with the reaction centers PS1 and PS2.

(c) Charge separation and transport of electrons and protons in plant photosynthesis:

The most widely accepted model of photosynthesis in higher plants and algae is the so-called Z-scheme of Hill and Bendall (1960). It proposed two light-reactions in series activating the transport of electrons from H_2O to nicotinamide adenine dinucleotide phosphate (NADP) via changes in the redox potentials of components of an interconnecting electron transport chain. In all, except the two light-driven steps, the flow of electrons is exergonic, in the direction of redox equilibrium and constrained by the relative position of the electron transport intermediates in the membrane. Several detailed reviews on this model have been published by Vernon and Avron (1965). Hind and Olson (1968), Bishop (1971) and Bishop (1974).

In essence, the process is as follows. Light energy is absorbed by the light harvesting system of PS2 and trapped at a reaction centre thought to contain a specialized form of chlorophyll, P680 (Doring, Renger, Vater and Witt, 1969). Through charge separation the excited P680 (P680*) donates an electron to a primary electron acceptor Q(E'_0=0.0 V) reducing it and producing a strong oxidant, Z, (E_0'=+ 0.8 V) capable of oxidizing H_2O to O_2. The identities of the primary electron acceptors and donors of PS1 (Q and Z respectively) have not been clearly established yet. There is some evidence which suggest that Q may be a quinone (Van Gorkom, 1974; Bensasson and Land, 1973). There is however, some evidence (Van Best and Duysens, 1977) that still another earlier acceptor (referred to as I in the literature) may be present between P680* and Q, and it has been suggested that pheophytin (Pheo a) plays this role (Klimov, Klevanik, Shuvalov and Krashnovsky, 1977). Very little is known about the entities that carry electrons from H_2O and act as donors to oxidized P680 but the process is not a direct reaction between $P680^+$ and H_2O (Amesz and Duysens, 1977). It is a controlled sequence in which the H^+ and O_2 liberated from H_2O are deposited inside the thylakoids and the O_2 diffuses out. One thing that is known however, is that manganese (Mn^{2+}) is essential for photosynthetic oxygen evolution (Cheniae and Martin, 1972; Den Haan, Gorter de Vries and Duysens, 1976).

As in the case of PS2, light absorbed in the light-harvesting system of PS1 is trapped at a reaction centre, in this case believed to

contain a photooxidisable form of chlorophyll, P700 (Kok, 1956; Ke, 1973). Charge separation at this reaction center leads to the oxidation of P700 (E_0'=+ 0.4 V) and the reduction of a primary electron acceptor referred to as A_1 (E_0' - 0.6 V) in the literature. Another acceptor usually referred to as X in the literature was believed to be the primary acceptor, however, some recent evidence suggests there is still one earlier acceptor A_1 (Shuvalov, Dolan and Ke, 1979). The reduced form of Q or I and the oxidized form of P700 are re-oxidized and re-reduced respectively by electron transport via the electron transport chain connecting the two photosystems. The net results of the simultaneous operation of the two photosystems is then the transfer of electrons from H_2O to A_1. Reduced A_1 can, in turn, be re-oxidized by the transfer of electrons to X (referred to also as A_2) then to A and B (collectively referred to as P430) and from here to ferredoxin, ferredoxin-NADP reductase and finally NADP (noncyclic mode). The electrons can also be transferred from FD to cytochrome b_6 (Cyt b_6), also known as cytochrome 563 (Cyt 563), to the plastoquinone (PQ) pool and back to oxidized P700 ($P700^+$) in what is known as cyclic electron transfer. The ferredoxins are Fe-S-proteins that appear in many pathways of electron transport, especially in anaerobic (not necessarily photosynthetic) bacteria.

Electron transport in the pathway interconnecting the two photosystems is thought to take place in the following way. Electrons from the primary electron acceptor of PS2, Q or I is transferred to the plastoquinone (PQ) pool and then to cytochrome f (Cyt f). Plastoquinone is the name given to a mixture of closely related electron transport intermediates whose principal component is

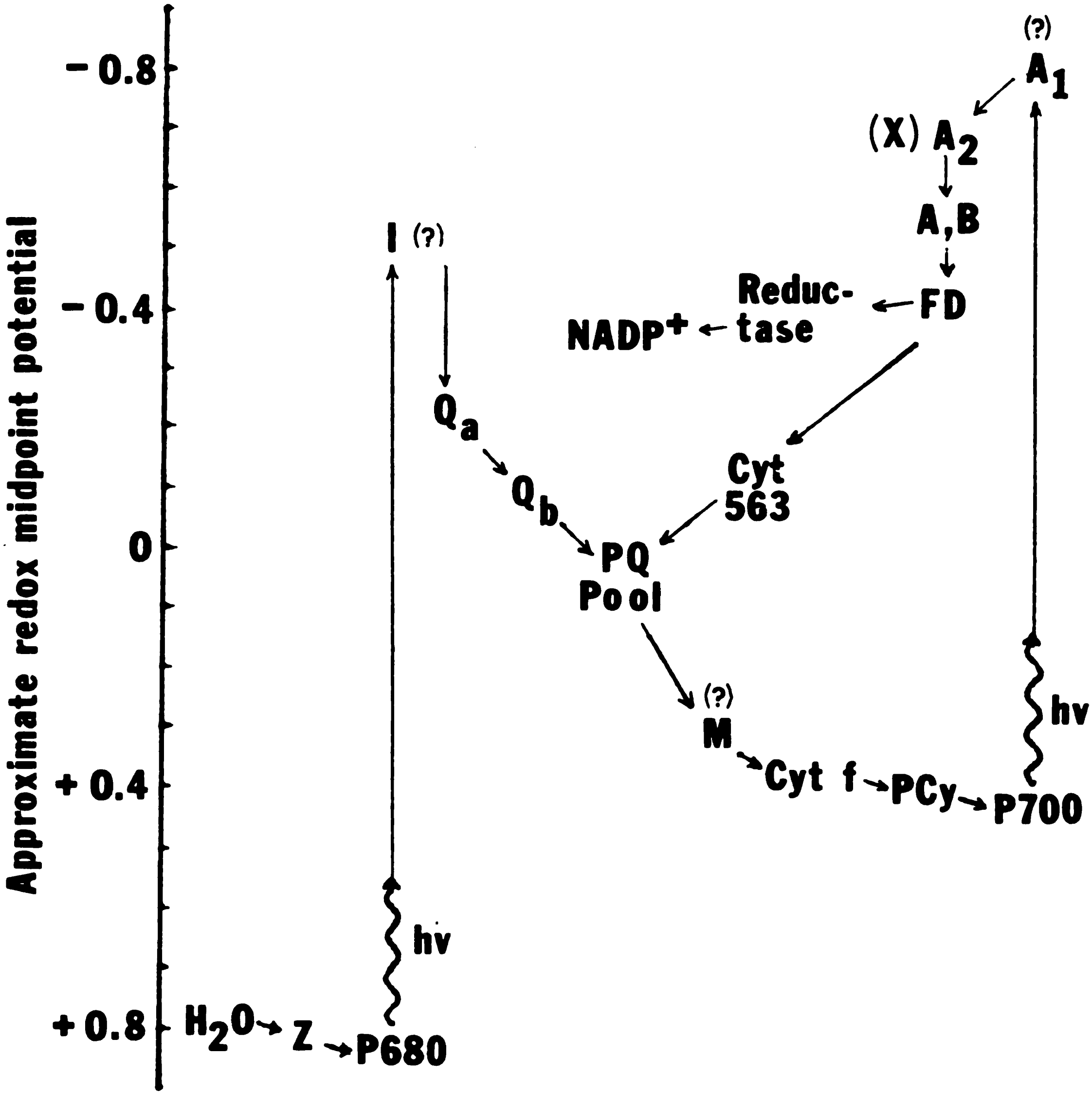


Figure 2.1 - Z-scheme of plant photosynthesis.

Current view of the so-called Z-scheme for green plant photosynthesis showing the paths of electron transfer in the RCs. The ordinate shows the approximate redox midpoint potential of each component. The question marks indicate the tentative nature of the assignments.

plastoquinone A. The concentration of this type of compound in chloroplasts is higher than that of any other electron transport intermediate (Amesz and Duysens, 1977). The concentration is normally about 5-10% of the total chlorophyll or approximately 25-50 molecules per PS1 or PS2 unit. There is some evidence that another electron transport intermediate, M, may be located between the PQ pool and cyt f (Levine, 1969). In studies with duckweed chloroplasts, Malkin and Aparicio (1975) have observed a light dependent ESR signal that could be due to the hypothetical M. The spectrum of the signal suggests that it is an Fe-S center. From cyto f electrons are then transferred to plastocyanin (PCy), a copper-protein compound, and then to oxidized P700.

Linked to this flow of electrons through the electron transport chain there is also a transport of protons whose possible role in photosynthesis will be discussed later (for review see Crofts and Dood, 1978). This transport takes place as protons are bound from outside the thylakoid when PQ is reduced, and released inside when PQ is reoxidized. This results in a net transfer of H^+ from outside the thylakoids to inside. The evolution of O_2 is also accompanied by a net release of H^+ inside the thylakoids (Saphon and Crofts, 1977).

Most of the information given above on the involvement of photochemistry and proton and electron transport in photosynthesis has come mainly from information of light induced and chemically induced changes in absorption and emission of light and ESR (see for example, Babcok and Sauer, 1975; Evans, 1977; Malkin, 1977; Mathis, 1977; Pulles, Van Gorkom and Willensen, 1976; de Grooth, van Grondelle, Romijn and Pulles, 1978). These changes usually signal the oxidation or reduction of molecules. The studies also involve manipulations of the material under investigation, as for example: (1) the use of intense short flashes of actinic light, (2) selection of the illumination wavelength so as to drive either PS2 preferentially, (3) fractionation of chloroplasts into particles enriched in PS1 or PS2 activity, (4) adjustment of the ambient redox potential, pH and temperature, (5) deletion of selected components by the use of specific inhibitors, genetic mutation and extraction, (6) replacement of deleted components or substitution with analogous, or addition of artificial electron donors and acceptors.

(d) Electric potential across the thylakoid membrane and ATP synthesis:

The most important problem in ATP synthesis involves conversion of the redox energy of electron transport into the anhydride bond of ATP. Three of the hypothesis which have been proposed involve a high energy intermediate to connect electron transport with ATP formation and are therefore known as coupling hypothesis. The difference between them lies in the nature of the high energy intermediate.

The chemical coupling hypothesis (Chance and Williams, 1956) proposes that a molecule or molecular complex plays the role of the high energy intermediate. The complex has the property that the affinity for H_2O is changed by a change in the redox state of the electron carrying part and the redox potential of the latter depends on the state of hydration. This hypothesis was successful in accounting for the formation of ATP linked to the fermentation of sugars and other organic compounds through soluble components.

In the conformational coupling hypothesis (Boyer, 1974) the energy intermediate are stresses induced by the transport of electrons which causes changes in the conformation of macromolecules. Due to these changes, in turn, the equilibrium between hydrolysis and dehydration is shifted to favor ATP formation.

This last hypothesis to be mentioned is the chemiosmotic hypothesis of Mitchel (1961), which proposes that the high energy coupling intermediate is a thermodynamic state equivalent to a proton concentration cell. A protonmotive force (pmf) established across the thylakoid membrane (in the case of plant photosynthesis) by the light-driven translocation of H^+ from outside to inside drives the movement of protons through an ATP-forming enzyme (ATPase) and ATP is synthesized.

This hypothesis to explain photosynthetic phosphorylation is the only one which offers an explanation to the observation that both oxidative as well as photosynthetic phosphorylation required an intact vesicular membraneous structure separating two aqueous regions. It also offers a very plausible explanation for the degree of chemical diversity of those substances which interfere with phosphorylation and are known as uncouplers. In terms of the chemiosmotic hypothesis uncouplers act by allowing the passive movement of H^+ and other ions dissipating the pH differential and/or the membrane potential.

Evidence has been obtained that there is a relationship between ATP synthesis and H^+ and electrical gradients. First comes the classical and dramatic experiment of Jagendorf and Uribe (1966) which showed that ATP could be synthesized, without the aid of light, with an artificially imposed pH gradient across the thylakoid membrane. In this experiment chloroplasts were added to a medium containing succinic acid (permeates thylakoid membrane) at pH 4 in order to lower the pH inside the thylakoids to 4. The external pH was then suddenly raised to 8 resulting in ATP formation. Then comes the experiment by Miles and Jagendorf (1969) who showed that ATP synthesis and light-induced proton uptake by chloroplasts have the same kinetics. More recently Graber, Schlodder and Witt (1977) showed that the membrane potential, the other component of the pmf, can also yield significant ATP formation when imposed artificially. In this experiment chloroplasts were suspended between electrodes 1 mm apart with a potential of 200 V applied across the electrodes. The artificial electric gradient which was estimated to produce a gradient of about .3 V across the thylakoid membrane resulted in ATP formation.

In another type of experiment, not involving thylakoid membranes however, Racker and Stoeckenius (1974) prepared lipid vesicles containing purple membrane from H. halobium and bovine heart mitochondrial ATPase. When the vesicles were illuminated (which according to Racker and Hindle, 1974, and Kayushin and Skulachev, 1974, should induce both a membrane potential and pH gradient) ATP was formed. These experiments were repeated by Ryrie and Blackmoore (1976) with yeast ATPase, by Winget, Konner and Racker (1977) with ATPase from spinach chloroplasts and by Yoshida, Sone, Hirata and Kagawa (1975) with cristalline ATPase from the thermophillic bacterium PS 3.

Electrogenic potential generation is expected to occur in association with a charge separation across a membrane. It has been suggested (Junge and Witt, 1968) that such a charge separation takes place across the thylakoid membrane as a result of the excitation by light of the photosynthetic reaction centers present in the membrane. Some evidence that such a potential difference developes across the thylakoid membrane in response to illumination has been obtained. Several methods have been applied to demonstrate the primary electrogeneis at the thylakoid membrane upon light excitation. These include: 1) the fast light-induced absorbance change at 515 nm, first discovered by Duysens (1954), later observed also in photosynthetic bacteria (Amesz and Vrendenberg, 1966). The change in absorbance has been attributed to a shift in the absorption bands of the native pigments in the membrane in response to an electric field (electrochromic shift) by Junge and Witt (1968). The rise time of the change was found to be less than 20 ns (Wolff, Buchwald, Ruffel, Witt and Witt, 1969). Spectral shifts of carotenoid, similar to the ones observed in photosynthetic bacteria, were observed in valinomycin treated chromatophore preparations in response to a KCl-induced membranes potential (Jackson and Crofts, 1969). 2) Charge polarization as measured by external electrodes in a layer of stripped chloroplast which was illuminated by a beam of light perpendicular on the layer (Witt and Zickler, 1973). 3) Several attempts have been made to associate the delayed fluorescence first observed by Strehler and Arnold in 1951 with the generation of a membrane potential (Fleischmann, 1971; Crofts, Wraight and Fleischmann, 1971; Barber and Kraan, 1970). This delayed light emission is believed to be the reversal of the initial photochemical and thermochemical events associated with PS2 (Strehler _et al_. 1951) and arise as a result of charge recombination within that reaction center (Arthus and Strehler, 1957). It has been suggested that the magnitude and intensity of ms delayed light emission was an expotential function of the membrane potential (Barber and Kraan, 1970). Bell, Haug and Good, (1978) however, have questioned this interpretation based on the results of their experiments where they observed stimulation and not inhibition of delayed fluorescence by uncouplers when the latter caused an increase in the rate of electron transport. Their data suggested that the decrease in intensity of millisecond-delayed fluorescence was not due to the elimination of a membrane potential but "more likely a reflection of the rate of disappearance of some other electron transport-generated condition, a condition which is uncouplers-insensitive". 4) A more direct method has been employed by Bulychev, Andrianov, Kurella and Litvin (1972) who used microcapillary electrodes to measure the light induced potential difference in chloroplasts. This approach although the most desirable, has the dissadvantage that the position of the electrode inside the chloroplast is not known, and if inside a granumm stack, there is the possibility of inducing ionic leakage. The kinetics of the 515 nm absorption change in illumination periods of 1 s or more, which have been suggested to indicate a proportional change in the membrane potential (Junge _et_ _al_., 1968) are different from those measured with micro-capillary glass electrode. 5) One other method which has been applied successfully in the photosynthetic bacteria involves the reconsitution of the photoactive membrane or the reaction centers (RCs) from the membranes into artificial planar model membranes (Skulachev, 1979; Schonfeld _et_ _al_. 1979; Packham _et_ _al_., 1980). This method has the advantage that it allows for direct measurement of the potential and some control over the conditions under which the potential will be generated. The main disadvantage of this approach lies in the limitations imposed on the reconstitution by the characterisitics and properties of the artificial membrane system. It is expected that for successful reconstitution the functional activity of the system should be maintained and no artifactual behavior should be induced. This approach has not yet been applied successfully to the RCs of plants. In the experiments with bacterial RCs different reconstitution methods were used. Skulachev's group used the fusion approach, where vesicles containing bacterial RCs were added to one side of a thick ($\geq$ 1000 Å) planar membrane so that they fuse with it (for a review see Skulachev, 1979). Schoenfeld _et_ _al_., (1979) obtained a reaction center-lipid complex in hexane and formed planar bilayers by apposing two monolayers assembled from lipids and reaction center-lipid complexes in hexane. Packham _et_ _al_., (1980) formed planar membranes (thickness > 500 Å) by brushing into a teflon septum with a small aperture, a membrane forming solution containing bacterial RCs. The RCs were incorporated into the octane containing forming solution using phospholipids to carry the RCs into solution.

In all cases, photovoltages and photocurrents were obtained when the systems were supplemented with secondary donors and acceptors. The photoresponses all follow the absorption spectrum of the RCs.

CHAPTER 3

EXPERIMENTAL METHODS AND MATERIALS

MATERIALS

(a) Chemicals:

The chemicals used in these studies were obtained from chemical companies and used without further purification, with the exception of cholesterol which was recrystallized three times in ethanol. The chemicals include: n-butanol, calcium chloride ($CaCl_2$), carbonyl cyanide m-chlorophenylhydrazone (CCCP), chloroform, cholesterol, α-chymotrypsin, coenzyme Q_6 (CoQ_6), n-decane, n-dodecane, ethylenediaminetetraacetic acid (EDTA), ferric chloride ($FeCl_3$, hydroxylamine (NH_2OH), magnessium sulfate ($MgSO_4 \cdot 7H_2O$), N-bromosuccinimide (NBS), n-octane, o-phenanthroline, phenazine methosulfate (PMS), egg phosphatidylcholine (PC), bacterial phosphatidylethanolamine (PE), bovine phosphatidylserine (PS), potassium chloride (KCl), potassium carbonate (K_2CO_3), sodium acetate ($C_2H_3NaO_2$), sodium ascorbate, sodium borohydride ($NaBH_4$), sodium chloride (NaCl), sucrose, N-tris (hydroxymethyl)-methylglycine (Tricine), trisodium citrate, vitamin K_3 (Vit K_3, (Difco) Yeast Extract.

(b) Composition of solution used for forming BLM:

In most cases (except where specified) the BLM-forming solution consisted of a mixture of phospholipids and cholesterol dissolved in an n-alkane. The composition is as follows: 1.2% egg phosphatidylcholine, 2.2% bacterial phosphatidylethanolamine, 0.7% bovine phosphatidylserine and 0.8% cholesterol in n-octane or n-decane. All percentages are weight by volume. In the experiments on incorporation of thylakoid membrane fragments in BLM the cholesterol was omitted. In some experiments on photoelectrospectrometry of BLM (see Appendix A) a BLM-forming solution made-up of an extract from spinach leaves dissolved in a 1:1 mixture of n-butanol and n-dodecane was used.

(c) Purple membrane (PM):

The PM used in these studies was extracted (see Methods section d) from Halobacterium halobium cells strain R_1 grown in the laboratory. A sample of the cells was originally obtained as a generous gift from Professor Cassim of Ohio State University.

(d) Thylakoid membrane fragments (TMFs):

The TMFs used were obtained from spinach chloroplasts following published procedures (see Methods section g). The spinach was obtained from local markets.

(e) Experimental set-up for electrical measurements and formation of BLM:

A diagram showing the experimental arrangement used in the BLM experiments is shown in Figure 3.1 It consists of:

(1) Light source- 1000 W Hannovia Xenon gas lamp with Schoeffel Instruments power supply.

(2) Monochromator A Baush and Lomb 250 mm light path monochromator equipped with a 600 grooves/mm difraction grating was used together with the light source to illuminate

the membrane with light of different wavelengths.

(3) Motor - A dc variable speed motor used to drive the grating.

(4) Variable voltage source - A box containing two 1.5 volts size D batteries and a variable resistance were used as a variable source of power for the motor.

(5) Shutter - Functions to control the duration of illumination.

(6) Lens - Quartz lens to focus the light on the BLM.

(7) BLM outer chamber - Consists of a plexiglass block with two holds bored through it (one of them playing the role of outer chamber) with two windows, one for illumination (quartz) and the other for observation (glass).

(8) Teflon septum for BLM formation - A 10 ml Teflon beaker with a 1.5 mm in diameter hole punched through it.

(9) Stirrers - Two magnetic stirrers one in each chamber.

(10) Calomel electrodes - Two colomel electrodes were used to provide electrical contact with the membranes. The inner chamber electrode was anode (or high impedance electrode) and the outer chamber electrode was cathode (or reference electrode).

(11) Electrometer - Keithley 610BR electrometer used to measure the potential difference across the membrane.

(12) Picoammeter - Keithley 417 high speed picoammeter used to measure the current flowing through the membrane.

(13) External variable voltage source - A box containing a simple circuit consisting mainly of a 1.5 V size D battery and a 10 turn helipot used as external variable source of voltage and current for the BLM.

(14) Chart recorder - Baush and Lomb VOM6 recorder used to record the time course of the photovoltages and photocurrents.

(15) X-Y recorder - MFE Plotamatic 715 M X-Y recorder was used to record the action spectrum of the photoelectric effects.

(16) Capacitance meter - ICE/Electronic Model 1-6 low level capacitance meter used to measure the BLM capacitance.

(17) Flash unit - General Radio 1538A Strobotac flash light used to measure the photoresponse to short duration illumination.

(18) Oscilloscope - Tektronic R5031 Dual Beam Storage oscilloscope used to record the photoresponse to short duration illumination.

(19) Special circuit for measuring the photoresponse to short duration illumination.

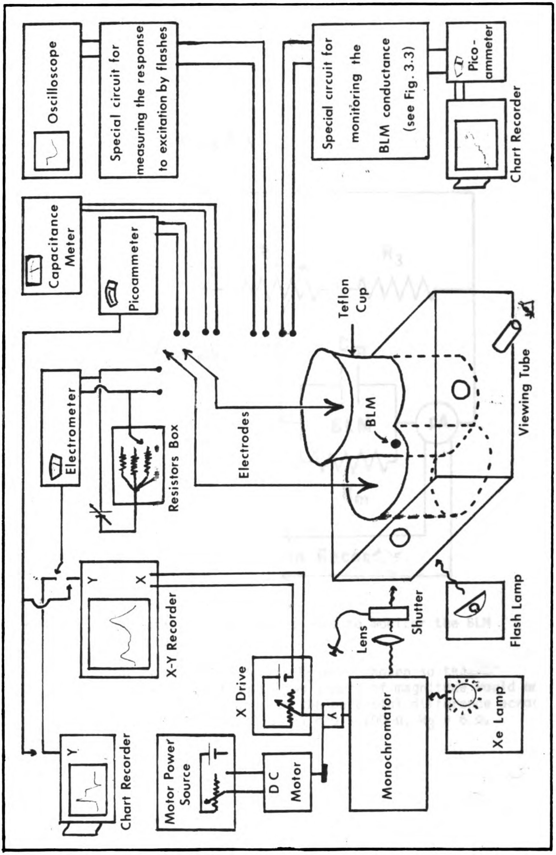
(20) Special circuit to monitor changes in BLM conductance (see Figure 3.2).

METHODS

(a) Formation of BLM:

BLMs were formed by the usual technique of injecting a small amount of "forming" solution with the aid of a Hamilton mycrosyringe, over a small (1.5 mm in diameter) hole located on a Teflon septum (see Tien, 1974). In this case the Teflon septum consited of a 10 ml beaker located in a plexiglass chamber with two mutually perpendicular windows, one made of quartz, for illuminating the membrane, and the other made of glass for observing the membrane. In all experiments addition of chemicals or membrane preparations was done after the membranes had reached the bilayer ("black") stage.

Figure 3.1 - Block diagram of the experimental arrangement used in the BLM studies.


Oscilloscope
Special circuit for measuring the response to excitation by flashes
Capacitance Meter
Picoammeter
Special circuit for monitoring the BLM conductance (see Fig. 3.3)
Pico-ammeter
Chart Recorder
Electrometer
Resistors Box
Electrodes
Teflon Cup
BLM
Viewing Tube
Y
X
X-Y Recorder
X Drive
λ
Lens
Shutter
Flash Lamp
Xe Lamp
Monochromator
Y
Chart Recorder
Motor Power Source
D C Motor

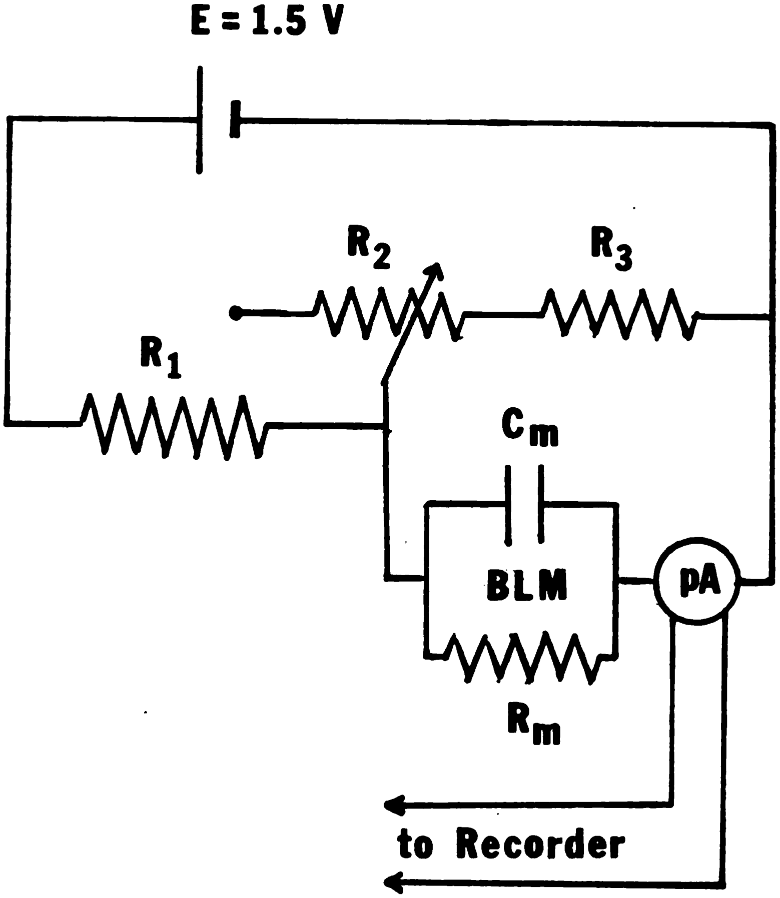


Figure 3.2 - Schematic of the circuit used to monitor the BLM conductance.

The value of R_1, R_2 and R_3 were chosen so that a change in R_m of up to two orders of magnitude would only cause a small change in the potential difference across the BLM. (R_1 = 820 Ω, R_2 = 0 - 100 Ω, R_3 = 6 Ω.

(b) Culture conditions for H. halobium:

The cells were grown under intense illumination (J ∿ 5 x 10^3 erg/cm^2-s) from a bank of fluorescent tubes at 37°C in two-liter flasks located inside an incubator. The growth medium per liter of culture consisted of: NaCl, 250·0 g; $MgSO_4 \cdot 7H_2O$, 20·0 g; KCl, 2·0 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; trisodium citrate·$2H_2O$, 3.0 g; Difco Yeast Extract (0127-03), 5.0 g. Each culture was inoculated with 200 ml of cells from a previous culture. The cells were aerated by bubbling air through the culture at a rate of 15 cubic feet of air per hour for 5 days or until the end of log phase. Then the cells were aerated at a rate of 8 cubic feet per hour for 3 days. After that the cells were harvested.

(c) Harvesting:

For harvesting the cells were spun down by centrifugation at 8000 rpm for 15 minutes. The pellet collected this way was then washed twice in basal salts (first 4 items listed above for growth medium) and resuspended in basal salts.

(d) Isolation of purple membrane:

The purple membrane (PM) was isolated by dialyzing H. halobium cells for 24 hours against 12 liters of 0.1 M NaCl (4 liter container change 3 times). The fraction from the dialysis was then centrifuged for 1 hour at 31,000 g. The supernatant was then decanted and 5 ml of 0.1 M NaCl were added to the pellets which were then gently shaken to resuspend the PM while keeping the muddy-colored cell-debris as a pellet. The resuspended PM was decanted and homogenize with a Thomas tissue grinder. This process was repeated until no muddy-colored cell-debris remained and the color of the supernatant changed from deep orange to faint purple. The final pellet was resuspended in 10 ml of double distilled water (dd H_2O) and then subjected to a high speed density gradient centrifugation. A continuous 30%-50% sucrose gradient, with a 1 ml 60% sucrose cushion, was prepared with 5 ml of each 30% and 50% sucrose added to a density gradient maker. Two ml of the pM sample were added to the gradient in each of six cellulose nitrate tubes. The gradients which were centrifuged to equilibrium for 18 hours at 35,000 rpm in a Beckman LB ultracentrifuge using an SWTi 41 rotor, yielded a dense purple band and a diffuse red band. The PM band in sucrose was collected with a disposable pipette after discarding the top layers. The PM in sucrose obtained from the centrifugation was washed twice in dd H_2O at 31,000 g for an hour and stored as pellets.

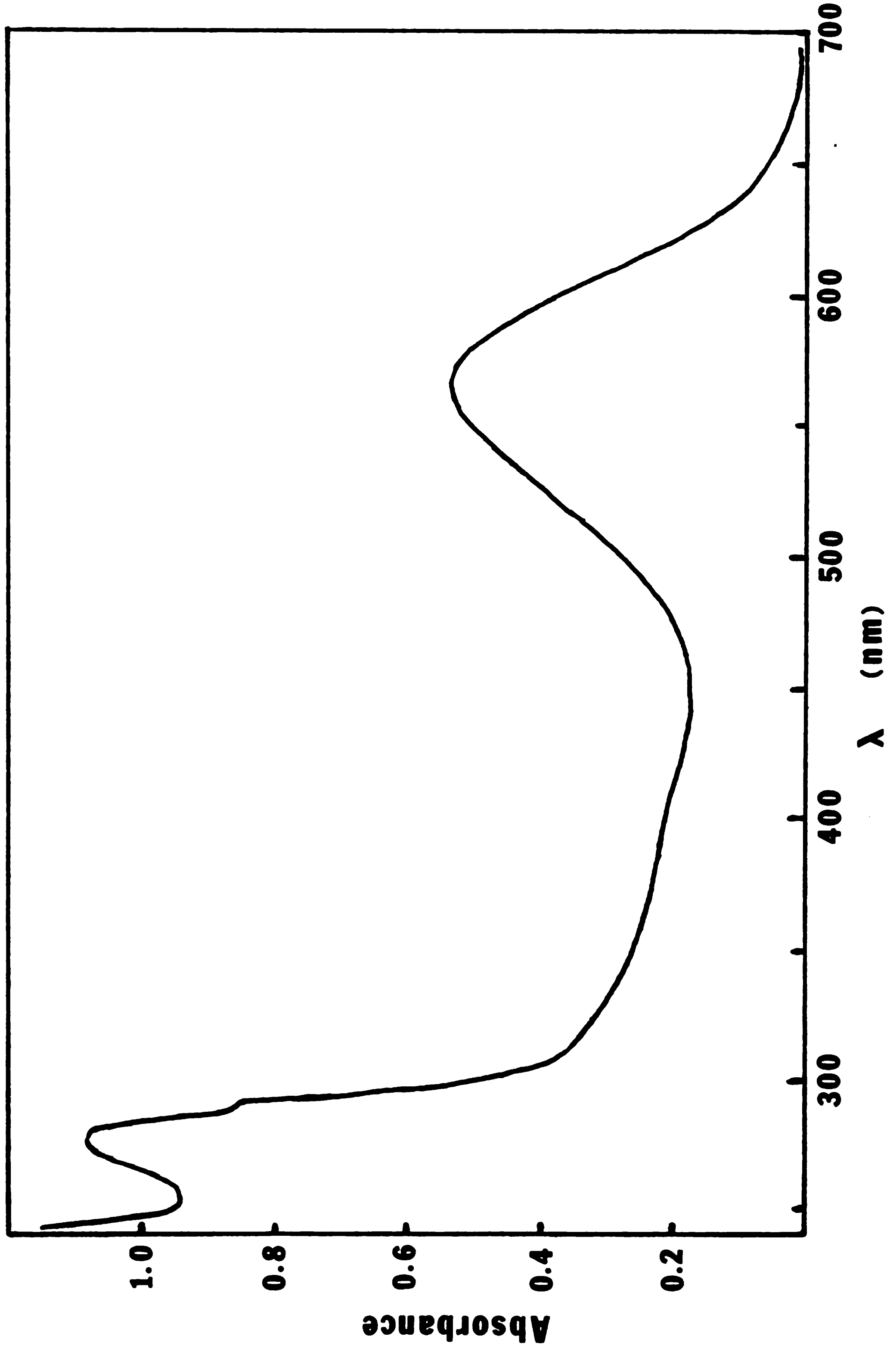
An absorption spectrum of the pM obtained with this procedure is shown in Figure 3.3. The spectrum is similar to the one reported in the literature (Oesterhelt and Stoeckenius, 1974), and exhibits a ratio of 280 nm (protein) peak to 560 nm (chromophore) peak of 2.0.

(e) Chemical modification and/or bleaching of PM:

Three different types of chemical modification and bleaching of PM were done: (1) chemical modification with N-bromosuccinimide (NBS), (2) bleaching with hydroxylamine (NH_2OH), and (3) bleaching with sodium borohydride ($NaBH_4$).

(1) Chemical modification (with bleaching) of PM with NBS was carried out following the method of Konishi and Packer (1977). Enough PM for a bacteriorhodopsin (bR) concentration of 0.2 g/l ($\sim$ 8 μM) was suspended in 10 ml samples of 0.1 M KCl and 0.02 M Na-acetate at pH 4.7. To each 10 ml sample, a different amount of NBS was added. The

Figure 3.3 - Absorption spectrum of purple membrane from bacteria grown in the laboratory.


Absorbance
1.0
0.8
0.6
0.4
0.2
300
400
500
600
700
λ (nm)

absorption spectra of the samples containing the different molar ratios of NBS to bacteriorhodopsin were measured and are shown in Figure 3.4. The spectra agree with the ones obtained by Konishi et al. (1977).

(2) The method used for bleaching PM with NH_2OH was a variation of the ones reported in the literature (Oesterhelt et al., 1974; Becher and Eberly, 1976; Bauer, Dencher and Heyn, 1976). The method is as follows. A fresh solution of 0.2 M NH_2OH at pH 7 was prepared and divided into 20 ml samples. An amount of PM containing 4 mg of bR protein was then added to each sample. Each sample was illuminated for a different amount of time with light from a 1000 W Xenon lamp. The light was filtered through 5 cm of a 1% $CuSO_4$. After the period of illumination for each sample had ended, the bleached PM was washed with 0.15 M KCl twice by centrifugation at 30,000 g for 1 hour and resuspended in 0.15 M KCl. The absorption spectrum for the bleached PM was measured (see Figure 3.5) and found very similar to the spectra published by other authors (Oesterhelt et al., 1974).

(3) The method used for bleaching PM using $NaBH_4$ is a variation of the method used by Peters, Peters and Stoeckenius (1976). PM was suspended in 10 ml samples of freshly prepared carbonate buffer, pH 10. The concentration of bR in each 10 ml sample was 0.2 mg/ml ($\sim$8 μM). 10 mg of $NaBH_4$ were added, the samples were then placed in an ice bath and illuminated with a bank of fluorescent tubes. Each vial was removed at a different time. After all the samples were collected they were washed several times by centrifugation and resuspension in 0.15 M Na-phosphate buffer at pH 7. The process was repeated until the residual $NaBH_4$ was removed. The absorption spectrum of the bleached PM was measured (see Figure 3.6) and found in agreement with the ones

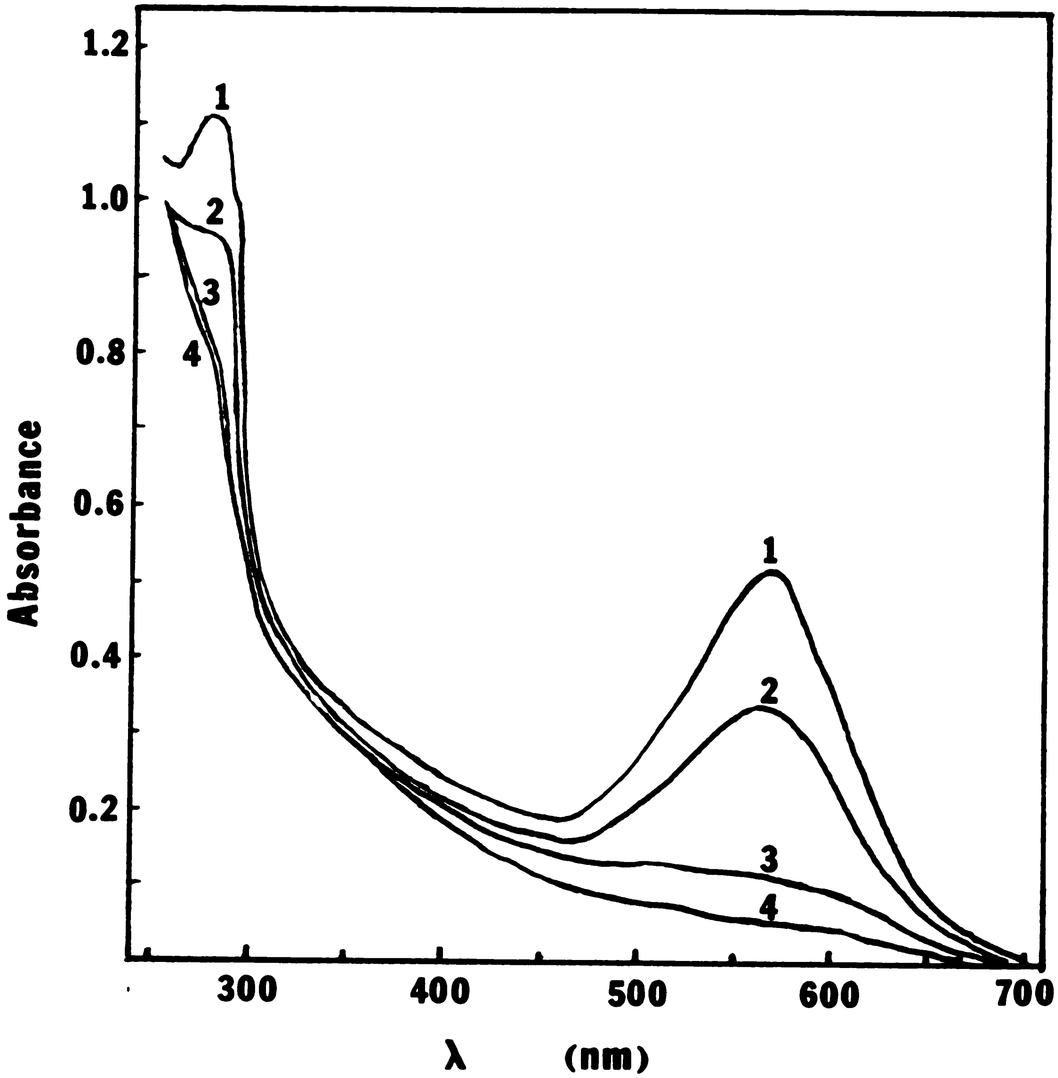


Figure 3.4 - Absorption spectra of purple membrane modified with NBS.

Each curve represents a different molar ratio of NBS to bacteriorhodopsin (bR). The molar ratio is equal to: 4 in curve (1), 10 in curve (2), 20 in curve (3), and 40 in curve (4).

Figure 3.3 - Absorption spectra of purple membrane bleached with NH_2OH in the presence of light.

Curve 1, purple membrane (PM) not illuminated; curve 2, PM illuminated for 2 hours; curve 3, PM illuminated for 28 hours.

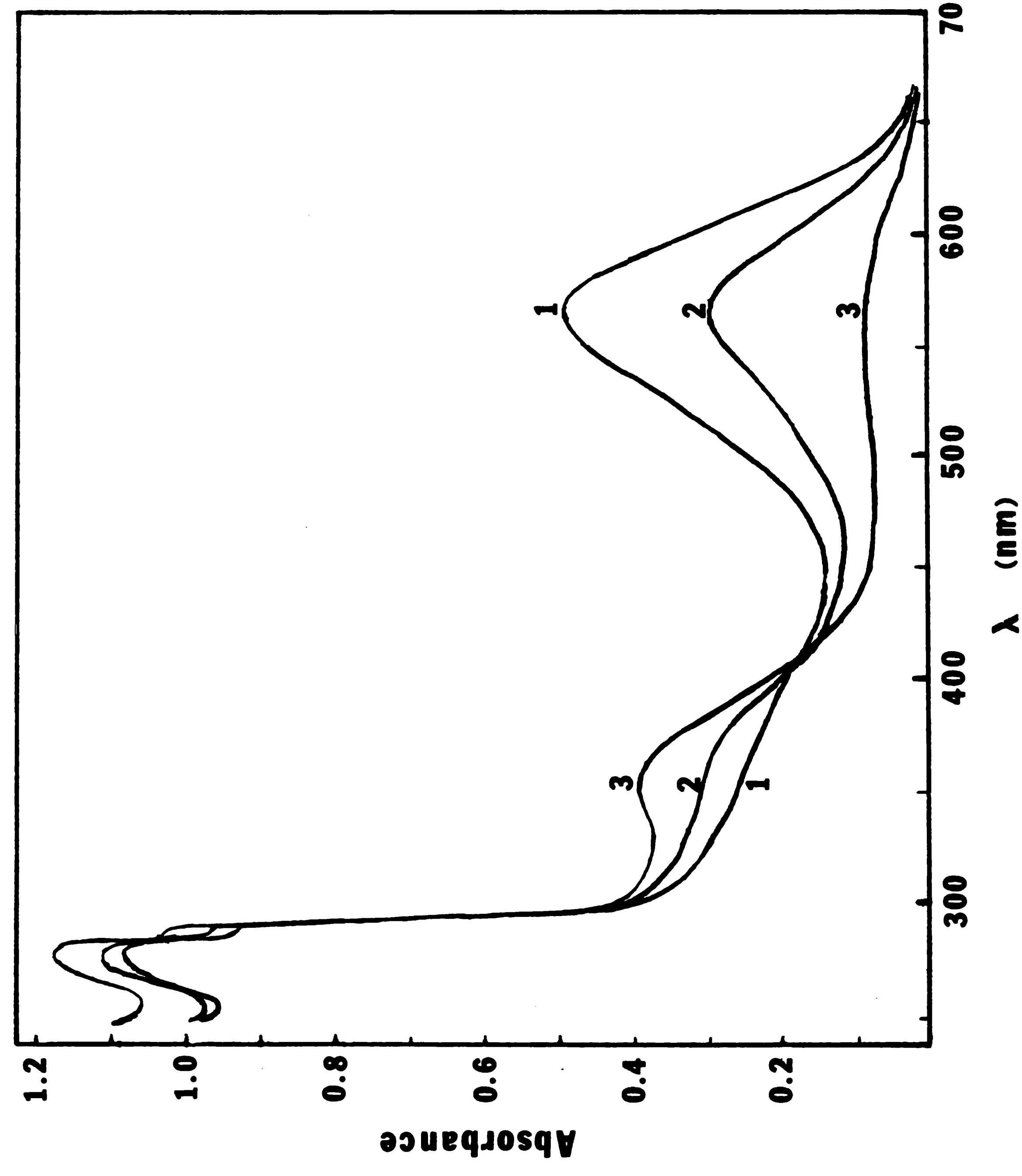
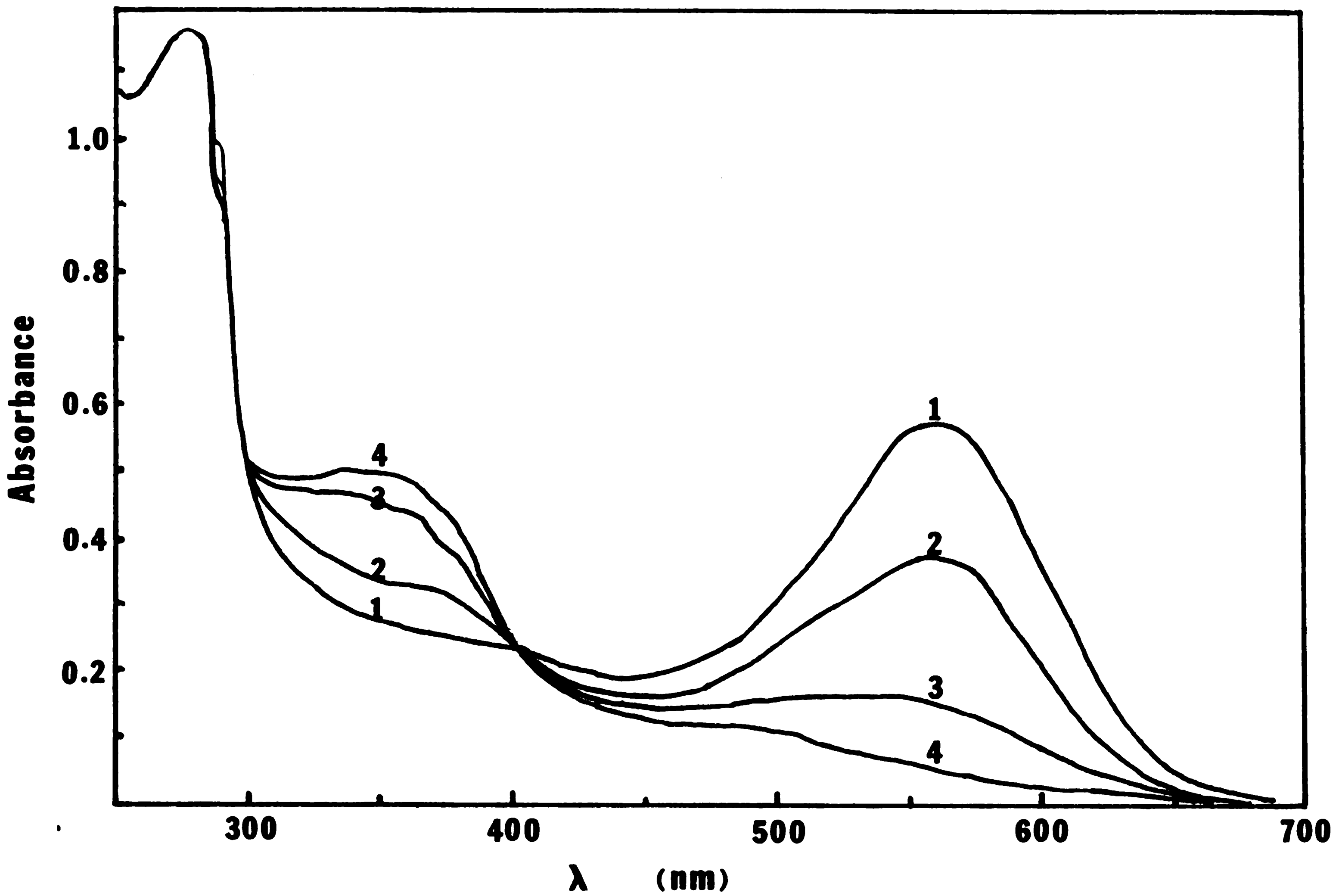

Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
300
400
500
600
700
λ (nm)
1
2
3
3
2
1

Figure 3.6 - Absorption spectra of purple membrane bleached with $NaBH_4$ in the presence of light.

The purple membrane was bleached (bacteriorhodopsin reduced) with $NaBH_4$ and light at pH 10. Curves 1 to 4 represent PM exposed to light for: 0 min (curve 1), 5 min (curve 2), 15 min (curve 3), and 75 min (curve 4).


Absorbance
1.0
0.8
0.6
0.4
0.2
300
400
500
600
700
λ (nm)
1
2
3
4
4
3
2
1

previously published (Peters et al., 1976).

(f) Isolation of chloroplasts from spinach leaves:

Between 80 and 100 g of whole green leaves were washed with distilled water, had the stalks and ribs removed and were allowed to dry for 30 min. The leaves were then chopped in a blender in 150 ml of 0.4 M sucrose plus 0.05 M Tricine-OH (pH 7.5) at low speed first and then after all the leaves had been added, at high speed for 30 s. The homogenized mixture was then filtered through eight layers of cheese cloth and the filtrate was collected. The green suspension was then centrifuged at 200 g for 5 minutes and the supernatant was collected. The supernatant was then centrifuged again for 12 minutes at 1000 g and the pellet which contains the chloroplasts was collected. The chloroplasts obtained with this procedure were then used for thylakoid membrane extraction.

(g) Isolation of thylakoid membrane fragments (TMFs):

Two different procedures were used to isolate TMFs. The first procedure was developed by Douce, Holtz and Benson (1973) and it involves the rupture of chloroplasts by gentle osmotic shock. The procedure is as follows. Intact purified chloroplasts in 0.4 M sucrose 3.0 ml, about 8 mg chlorophyll per ml, were treated for 2 minutes at 4 °C in 70 ml of swelling medium (10 mM Tricine-OH buffer, pH 7.5 and 4 mM $MgCl_2$. Samples of 5 ml were then layered on top of a discontinuous sucrose gradients and centrifuged in a swinging bucket rotor for 60 minutes at 20,000 rpm in a Beckman model L3-50 ultracentrifuge. The gradients were prepared by layering 3 ml of sucrose solutions containing 5 mM Tricine-OH buffer at pH 7.5 and 2 mM $MgCl_2$ into six tubes in the following sequence: 1.5, 1.2, 0.93 and

0.6 M. The centrifugation resulted in a separation of the membranes into three distinct bands which formed at the top of the inerfaces of the sucrose layers. A dark green fraction (heavy fraction) which sedimented a top the 1.5 M sucrose was collected from all 6 tubes using a syringe. The collected material was combined, diluted four times with 10 mM Tricine-OH buffer at pH 7.5 mixed well, and spun for 1 hour at 27,000 rpm. The pellets were suspended in a medium containing 0.3 M sucrose and 10 mM Tricine-OH buffer at pH 7.5. The chlorophylls content was determined using the method of Arnon (1949), which is based on the specific absorption coefficients of chlorophylls a and b in 80% (V/V) acetone as determined by McKiney (1941).

The second method used is a variation of the one used by Becker, Shefner and Gross (1965) and makes use of the more drastic technique of sonication. The chloroplasts are resuspended in low ionic strength medium, 15 mM KCl and 5 mM Tricine-OH at pH 7.5 and stirred for one hour (low stirring) at 4 °C. The disrupted chlorplasts are then sonicated in an ice-water bath with a Branson model 140-D Sonifier Cell Disruptor for a total of 9 minutes (3 times for 3 minutes with a 1 minute rest between sonication periods) at 60 W. The sonicated chloroplasts were then centrifuged for 1 hour at 30,000 g. The supernatant which consisted of thylakoid membrane fragments (TMFs) was collected. As before, the chlorophyll content was determined by the method for Arnon (1949). It was found that the concentration of total chlorophyll ranged beween 0.8 and 1.6 mg/ml per 100 g of leaves. Although these numbers appear to vary considerably, it was also found that the relative amount of Chl a and Chl b before and after centrifugation were very similar from preparation to preparation.

(h) Preparation of liposomes or vesicles containing PM or TMF:

The method used to prepare liposome is essentially the method of Blok *et al*. (1978). 100 mg of PC in hexane or chloroform was poured into a round bottom flask and the solvent was evaporated to dryness by means of a Buchner flash evaporator. 10 ml of 0.15 M KCl and 0.01 M Tricine-OH (pH 7.5) or 0.1 M Na-acetate (pH 5.0) were added, depending on the particular experiment. The phospholipids were dispersed by adding a few glass beads and shaking in a vortex mixer for 10 minutes. The biological membrane to be incorporated was added and the mixture was sonicated at 80 W for 15 minutes (60 sonication period of 15 s, each followed by a 45 s rest period) in an ice bath under a stream of N_2. The vesicles were then centrifuged for 30 minutes at 10,000 g to remove heavy fragments and titanium particles.

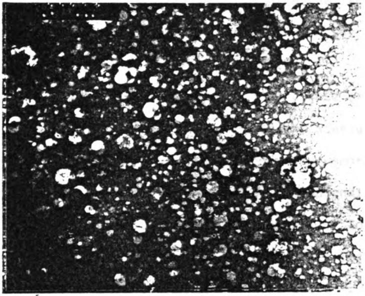
In the case of PM-vesicles, the concentration of bR in the vesicle suspension was 0.2 mg/ml ($\sim$8 μM). For TMF-vesicles enough sonicated broken chloroplast suspension was added to the phospholipid dispersion for a total Chl concentration of 0.1 mM. In experiments with TMF-vesicles containing CoQ_6 or Vit K_3 in the vesicular membrane, the concentration of the quinones in the vesicle suspension of 0.1 mM. In experiments involving incorporation of TMF in BLM, the TMF vesicles were prepared in a buffer solution supplemented with sucrose to a final concentration of 0.2 M sucrose. Electron microscopy of the vesicles revealed that they consisted of closed structures (see Figure 3.7).

(i) Fusion of vesicles with BLM:

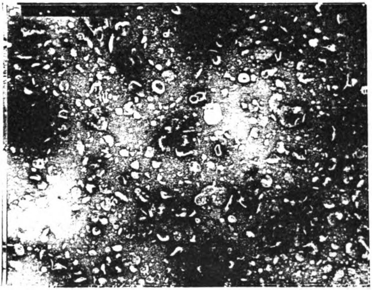
Two different vesicle preparations were used for fusion studies: PM-vesicles and TMF-vesicles. In both cases the same method was used to achieve fusion. The BLM was formed with a solution which contained

Figure 3.7 - Electron micrographs of the vesicles used in these studies. (34,560x) A) PM-vesicles. B) TMF-vesicles.

A.



B.



a negatively charged phospholipid, phoaphatidylethanolamine. A divalent cation, usually Ca^{2+}, was present in the bathing solution. With both of these conditions met, addition of either vesicle preparation resulted in fusion of the vesicles with the BLM.

(j) Incorporation of proteins into the BLM:

Two distinct approaches were used to incorporate proteins into BLMS. One approach was to add the protein to the aqueous phase at one side of the BLM and wait until it adsorbs onto the membrane. It was used in the experiments with α-chymotrypsin (see Appendix A). The other approach involves the incorporation by means of total fusion of the vesicle with the BLM. This approach was used to incorporate TMF, or more specifically, PS1 reaction center into the BLM. In order to obtain total fusion between the vesicle and BLM, Ca^{2+} (to a final concentration of 10 mM) had to be present on the side containing the vesicles. Also, and perhaps more important, the vesicle interior had to be hyperosmotic with respect to its surrounding aqueous environment. This was accomplished by preparing the vesicles in a medium containing 0.2 M sucrose, 0.1 M KCl and 5 mM Tricine-OH at pH 7.5. The aqueous phase on opposite sides of the membrane consisted of 0.1 M KCl and 10 mM Tricine-OH at pH 7.5 and 10 mM Ca^{2+} on the side to which the vesicles were added.

(k) Electrical measurements:

Electrical measurements were done as usual (see Tien, 1974). For the action spectra measurements, the diffraction grating was moved mannually in increments of 20-25 nm or continuously by means of a motor. For the experiments where the conductivity of the BLM was monitored, the circuit shown in Figure 3.2 was used. The circuit is by no means a voltage clamp circuit; however, the changes in voltage should be small as long as the membrane resistance stays high.

CHAPTER 4

PHOTOELECTROSPECTROMETRY OF BLM

INTRODUCTION

As mentioned previously, one approach used to study how the components of biological systems work is to reassemble them into model membranes, including BLMs and liposomes. Liposomes are very suitable for studies involving spectroscopic techniques. This is mainly because by being rather small they have a relatively large surface area to volume ratio. Light spectroscopy studies (e.g. fluoresence, absorption, etc.) on BLMs are rather difficult and usually require the use of sophisticated equipment (see for example Steinemann, Stark and Lauger, 1972). One way to overcome this difficulty is to do photoelectric spectroscopy studies on the BLM. These involve the measurement of the action spectrum of the photoelectric effect from a photosensitive BLM and usually consists of a photovoltaic or phoconductivity action spectrum. The technique itself is fairly simple and due mostly to the high electrical resistance of the BLM, rather sensitive.

In this chapter, the results of photoelectric spectroscopy studies on BLMs modified with certain photoactive species will be reported. The purpose of the studies is two-fold: First, to explore the possibility of extending such studies into the uv region, the region where most proteins absorb radiation, and second, to examine the kind of information which can be obtained from such studies.

The results are divided into two parts. The first part includes the results of studies with BLMs containing chlorophyll pigments, purple membrane (PM) from H. halobium and adsorbed chymotrypsin. Since these results have already been published, the reader is referred to Appendix A for details on the results and discussion. The second part deals with a more detailed study of the PM-BLM, namely photoelectric spectroscopy (or photoelectrospectrometry) of chemically modified PM reassembled into BLMs. Before going into the second part, however, some recent observations relevant to the first part will be discussed.

In the experiments discussed in Appendix A, it is shown that chymotrypsin molecules, when adsorbed onto one side of a BLM, can generate photovoltage if irradiated with uv light. Chymotrypsin is normally a non-photoactive protein, however the initial charge separation which leads to the observed photovoltages could be explained in terms of photoionization of the aromatic amino acids by the uv radiation. This is known to occur in biological compounds and is believed to be the major initial photoreaction in radiation damage processes (Grossweiner, Brendzel and Blum, 1981).

After it was found that the action spectrum followed the absorption spectrum of the aromatic amino acids, it was decided to see if the aromatic amino acids adsorbed onto one side of a BLM would also generate photovoltages. The experiments were done, but no photovoltages were observed. More recently, however, Huebner, Arrieta and Millar (1982) have reported the observation of transient photovoltages in response to light flashes of 5 μs duration from aromatic amino acids adsorbed on one side of BLMs. They have also observed transient photovoltages from the proteins aldolase, chymotrypsinogen A and ribonuclease A. They have also attributed the photoresponse to photoionization of the aromatic amino acids.

PHOTOELECTRIC SPECTROSCOPY OF CHEMICALLY MODIFIED PM RECONSTITUTED ON BLMS

(a) Background:

The purple membrane of the halophilic bacterium H. halobium is a specialized section of the plasma membrane which developes when the cell is growing under low O_2 conditions. The PM fragments, which occur in crystalline arrays, contain a single protein termed bacteriorhodopsin (bR) because of its resemblance to the visual pigment of animals (Oesterhelt and Stoeckenius, 1971). The protein bR contains retinal bound to a lysine (Oesterhelt et al., 1971) residue in the form of a Schiff base and exhibits an absorption maximum at 560 nm. Upon absorption of light, bR undergoes a cyclic photoreaction (Lozier, Bogomolni and Stoeckenius, 1975) accompanied by the translocation of protons across the membrane. For a more detailed review on the structure and function of bR and the PM, the reader is referred to a review article by Stoeckenius, Lozier and Bogomolni, (1978).

As mentioned previously, several membrane models containing PM incorporated into different ways have been studied by many investigators (for a review see Schreckenbach, 1979). In those model systems photovoltages and photocurrents whose action spectrum (in the visible region) follows the absorption spectrum of the chromophore were obtained. The photoelectric effects are attributed to the proton pumping activity of bR. In the experiments described in Appendix A, the measurement of the action spectrum from a PM-BLM model membrane was investigated in the visible and uv regions. The spectrum not only exhibited the usual retinal chromophore peak at 560 nm but also showed a peak at 280 nm. The peak is attributed to the protein part, however the nature of the photoeffects elicited by the uv illumination was not known. The experiments on this section were designed to try to answer this question. More specifically, if the photoelectric response is due to charge separation in the absorbing species itself (as in the case of chymotrypsin) or comes about through other mechanisms, such as energy transfer. With this in mind, it was decided to do experiments using chemically modified and/or bleached purple membranes.

(b) PM modified with N-bromosuccinimide (NBS):

NSB is a reagent which is known to react with tryptophan, tyrosine and cysteine (Spande and Witkop, 1967). For these studies however, the reaction with cysteine is not important since bR does not contain this amino acid (Bridgen and Walter, 1976). The reaction with tyrosine and tryptophan is pH dependent, at low pH NBS reacts exclusively with tryptophan and at neutral or alkaline pH it reacts mainly with tryptophan, but also with tyrosine (Spande et al., 1967).

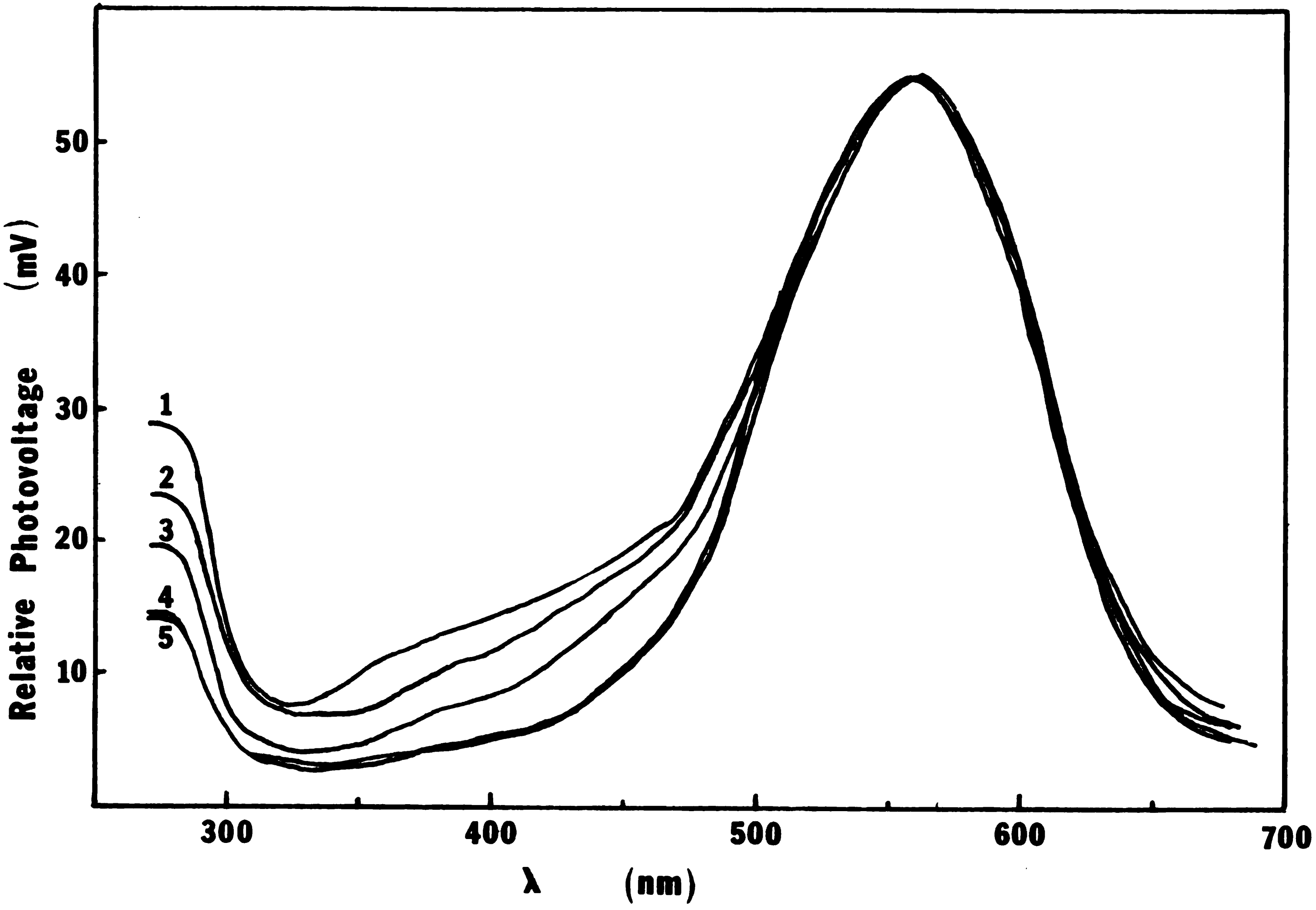
When PM was treated with NBS, it was noted that the purple color began to fade as the reaction progressed. This loss of purple color by the PM was also dependent on the concentration of NBS with more color fading the higher the NBS concentration. These results are consistent with those obtained by Konishi et al. (1977) and indicate that the interaction of the retinal chromophore with the rest of the protein is being disturbed.

The absorption spectrum of PM treated with NBS at pH 5 (see Figure 3.4) exhibits not only a decrease in absorbance of 280 nm (tryptophan and tyrosine absorption region) but it also shows a decrease in absorbance in the chromophore band at 560 nm.

Liposomes containing PM chemically modified with NBS were added on one side of a BLM in the presence of Ca^{2+}. There BLMs exhibited photovoltages when illuminated with visible and uv light, whose polarity and the electrical characteristics were similar to those exhibited by BLM with unmodified PM. The only significant effect was in terms of the magnitude of the photovoltage which decreased the higher the NBS concentration used. After measuring the action spectrum of the photoresponse for each preparation, it was observed that both action spectra, for modified and unmodified PM, were similar in shape (see Figure 4.1) both exhibiting a structure at 280 nm. In the case of modified PM, however, a decrease in the height of the 280 nm peak relative to the 560 nm peak was observed (see Figures 4.1 and 4.4A). As shown by Figure 4.4A, the decrease eventually reached a minimum value so that the relative height of the 280 nm to the 560 nm peak reached a constant value. Also, the magnitude of the photovoltage at all wavelengths decreased with increased NBS concentration. The results show that tryptophan is involved in at least part of the uv-elicited photovoltages and also suggest that the intact chromophore is required for their appearance. The results also suggest that intact tryptophan residues are required for the proton pumping activity of bR, since modification of these amino acids with NBS reduces the magnitude of the photovoltage. This last observation is in agreement with those of Konishi and Packer (1977). The results also give support to the

Figure 4.1 - Photovoltaic action spectra from BLM fused with vesicles containing purple membrane modified with NBS.

Each curve represents a different molar ratio of NBS to bR. The molar ratios are: curve 1, 0; curve 2, 4; curve 3, 10; curve 4, 20; curve 5, 40. The curves were normalized at 560 nm.


Relative Photovoltage (mV)
10
20
30
40
50
λ (nm)
300
400
500
600
700
1
2
3
4
5

idea that the bathocromic (red) shift in bR is due to some solvent effect caused by protein, i.e., various interactions with amino acid residues in the immediate vicinity of the chromophore.

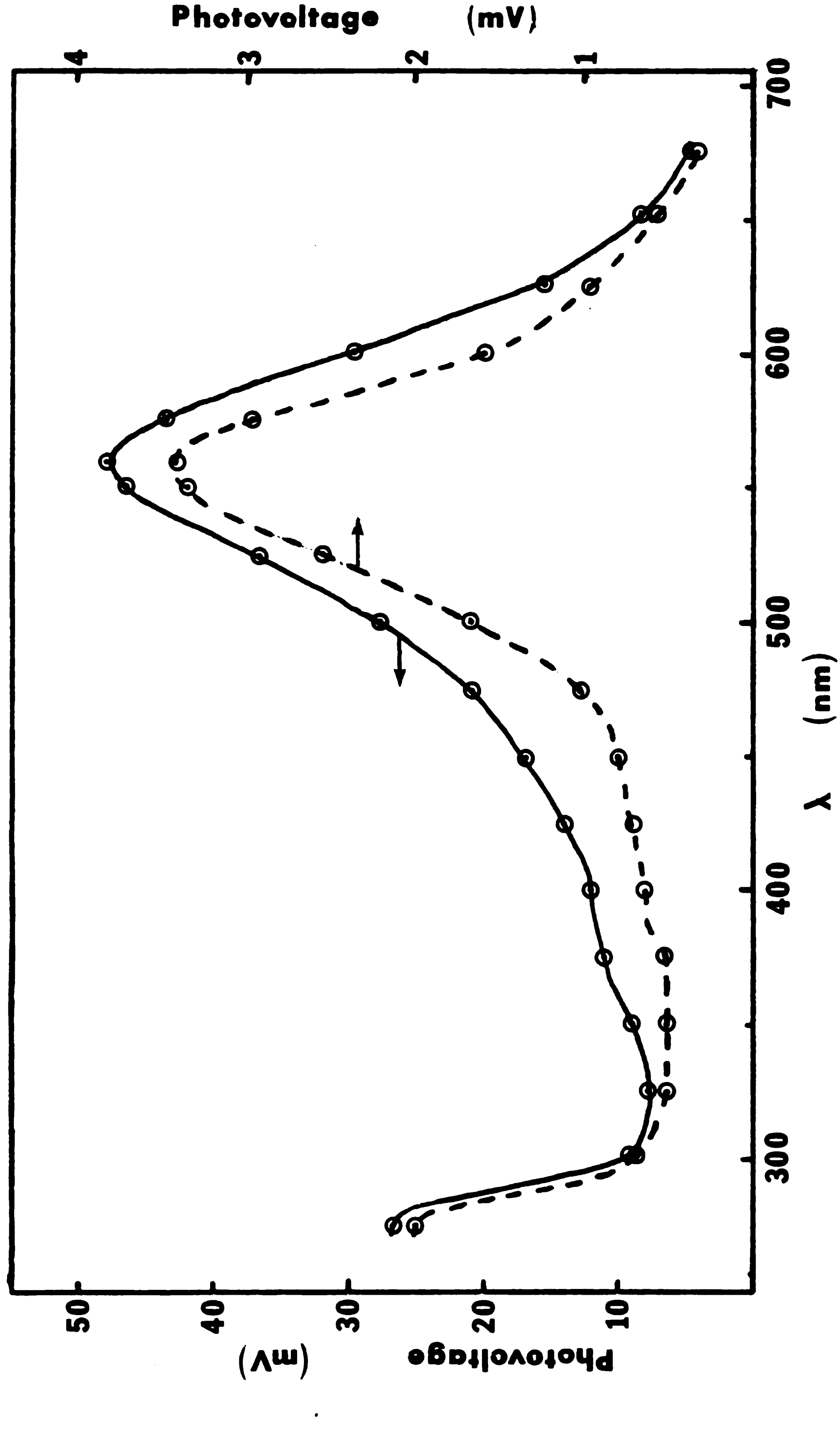
(c) Bleaching with hydroxylamine (NH_2OH):

The reaction of NH_2OH with bacteriorhodopsin in the presence of light breaks the Schiff base bond of the retinal, forming a retinal oxime. Once this covalent bond is broken, the interaction of retinal Schiff base with the protein, which is responsible for its absorbance at 560 nm diminishes and there is an equivalent increase in the retinal oxime peak at 355 nm (see Figure 3.5).

Liposomes containing pM bleached for different periods of time were added to one side of planar BLM. When the BLM was illuminated, a photoresponse could be detected which had similar polarity and characteristics as the one from unbleached PM. However, as in the case of PM treated with NBS, the magnitude of photoresponse decreased with increased bleaching of the PM. This result is not unexpected since bleaching of the PM should destroy its proton pumping activity and therefore destroy the photoresponse. The uv elicited photoresponse, however, need not decrease unless the intact chromophore is necessary for it to be generated. That this is actually the case was then found out when the action spectrum of the photoresponse for partially bleached PM was measured (see Figure 4.2). The action spectrum exhibited the same decrease in photovoltage at all wavelengths, including the 280 nm band. These results suggest that the uv elicited photoresponse is due to energy transfer presumably from tryptophan and tyrosine residues to the retinal chromophore. This interpretation is consistent with the results obtained by chemical modification with NBS.

Figure 4.2 - Photovoltaic action spectra from BLM fused with vesicles containing purple membrane bleached with NH_2OH.

The PM has been bleached using NH_2OH and exposure to light. Each curve represents illumination for a different amount of time. 0—0—0 no illumination, and 0--0--0 illuminated for 28 hours.


Photovoltage (mV)
4
3
2
1
700
600
500
400
300
λ (nm)
50
40
30
20
10
Photovoltage (mV)

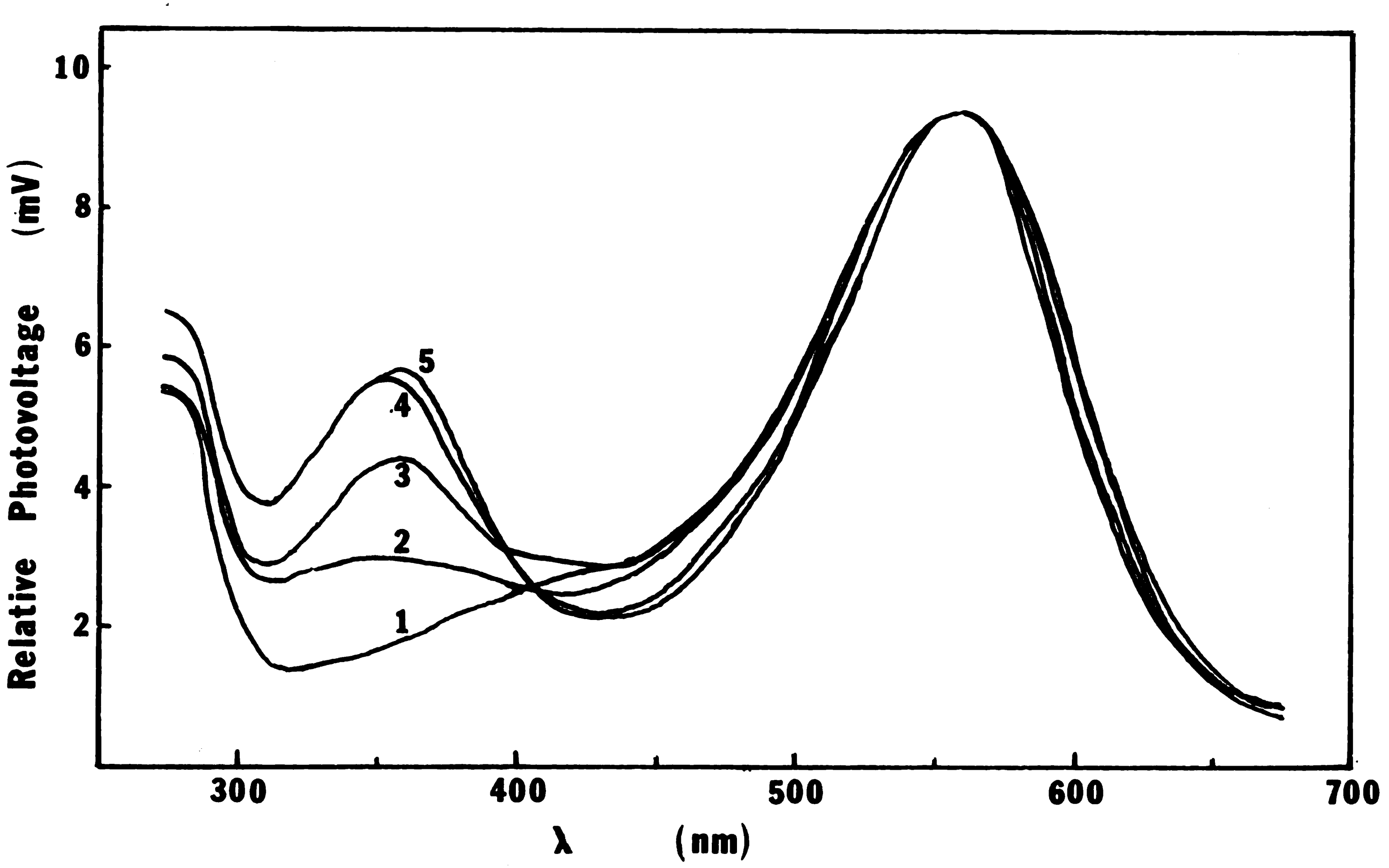
(d) Bleaching with $NaBH_4$:

Another series of experiments similar to the ones performed with NH_2OH and NBS were done using $NaBH_4$. The reaction of $NaBH_4$ with PM, which only takes place under illumination, results in reduction of the Schiff base with concomitant discoloring of the PM (Oesterhelt *et al.*, 1976). The discolored membrane exhibits the typical retinyl protein fluorescence and does not recombine with retinal to yield bacteriorhodopsin (Oesterhelt *et al.*, 1974). The resulting reduced form of bacteriorhodopsin shows absorption spectra with maxima at 360 nm and 280 nm. After a series of spectroscopic experiments Peters, *et al.* (1976) concluded that the $NaBH_4$ reaction in the light apparently modifies the covalent linkage between chromophore and protein while preserving existing or possibly establishing new non-covalent interaction(s).

Again, different liposome preparations were added to one side of a preformed BLM. Each liposome preparation contained PM bleached to a different extent by exposing it to $NaBH_4$ and light for different periods of time. As in the other two cases, the BLM exhibited photovoltages which were also similar in characteristics to that of unbleached PM. Another similarity was that the magnitude of the photovoltage decreased with increased bleaching of the PM. When the action spectrum was measured, however, a peak at around 360 nm together with the 560 nm and the 280 nm, was observed (see Figure 4.3). This peak at 360 nm, which corresponds to the reduced form of the bacteriorhodopsin as reported by Peters *et al.* (1976), was also observed in the absorption spectrum (see Figure 3.6). When the action spectra for several BLM-liposome preparations containing different

Figure 4.3 - Photovoltaic action spectra from BLM fused with vesicles containing purple membrane bleached with $NaBH_4$ and light.

Each curve represents exposure to light for a different time period. Curve 1: 0 min; curve 2: 3 min; curve 3: 15 min; curve 4: 45 min; curve 5: 90 min. The curves were normalized at 560 nm.


Relative Photovoltage (mV)
10
8
6
4
2
300
400
500
600
700
λ (nm)
1
2
3
4
5

amounts of bleached PM were measured and compared, the following results were obtained. The peak at 360 nm increased relative to the 560 nm peak from a small hump, for slightly bleached PM, to a maximum height and did not increase more even if the PM membrane was bleached more as could be seen from its absorption spectrum (see also Figure 4.4B). At the same time and similar to the two other cases previously mentioned (NBS and NH_2OH treatments), the magnitude of the photovoltage at all wavelengths decreases with increased bleaching.

These results can again be explained in terms of energy transfer. For the 360 nm band, however, the energy transfer is inter-molecular, from one chromophore to another in the trimer of bR molecules in the PM. For the 280 nm band, as in the other two cases, it should be intra-molecular energy transfer from the aromatic amino acids of the protein backbone to the retinal chromophore.

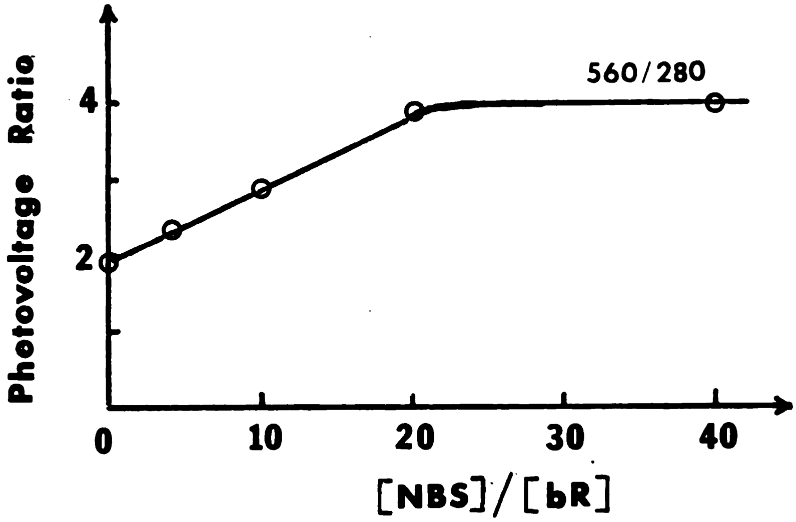
(e) Summary:

When the action spectrum of the photoresponse of a BLM containing PM from H. halobium is measured, a 280 nm band is observed together with the expected broad band peaking at 560 nm. Using bleaching and chemical modification of the purple membrane it was found that the uv elicited photoresponse was due to energy transfer from the aromatic amino acids of the bacterirhodopsin protein to the retinal chromophore, most probably from the four tryptophan residues close to the retinal moiety. This energy transfer is intra-molecular. There is not much overlap between the fluorescence emission spectrum of the aromatic amino acids (see Chen, 1967) and the absorption spectrum of the chromophore. However, the close proximity between the chromophore and the four tryptophan residues (see Packer and Konishi, 1978) suggests

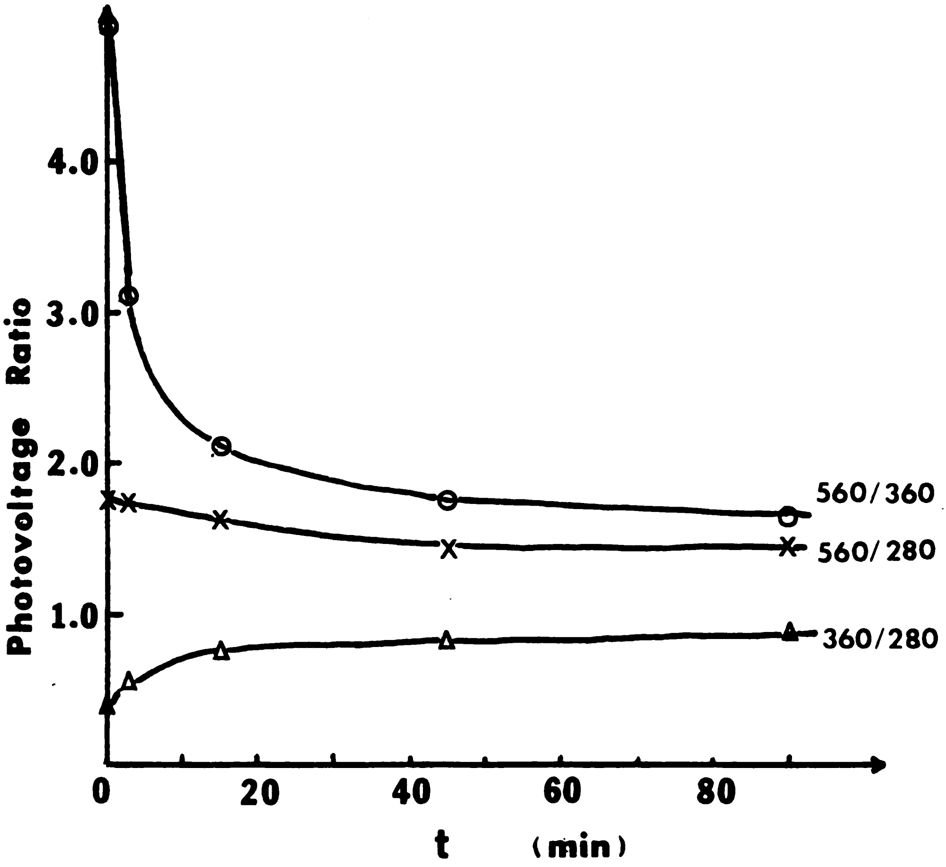
Figure 4.4 - A) Change in the height of the 280 nm peak relative to that of the 460 nm peak as a function of NBS concentration. The change is expressed as the ratio of photvoltage at 560 nm to photovoltage at 280 nm.

B) Change in the height of the 360 nm and 280 nm peaks relative to the 560 nm peak as a function of illumination time in the presence of $NaBH_4$. The changes are expressed as ratios of photovoltages at the given wavelengths.

A.


Photovoltage Ratio
4
2
0
10
20
30
40
[NBS]/[bR]
560/280

B.


Photovoltage Ratio
4.0
3.0
2.0
1.0
0
20
40
60
80
t (min)
560/360
560/280
360/280

that the energy transfer may be by exciton migration. It was also found that $NaBH_4$-treated PM containing reduced bR protein, could also generate photovoltages when reconstituted in BLM model membranes. The action spectrum of the photovoltages exhibited a new band at ∿ 360 nm which corresponds to the absorption by the reduced form of bR. The appearance of this band was also attributed to energy transfer. However, in this case the energy transfer is inter-molecular and since there is considerable overlap between the fluorescence emission spectrum of the reduced bR (see Peters _et al._, 1976) and the absorption spectrum of the normal bR, most likely the energy transfer is also by exciton mechanisms. This is also supported by linear dichroism studies (King, Bogomolni, Hwang and Stoeckenius, 1977) from which a distance between chromophores of 18.6 A was estimated. Moreover, studies on circular dichroism of bR indicate that the interaction of the chromophore neighboring bR molecules produces exciton coupling between them (Becher and Ebery, 1976).

CHAPTER 5

PARTIAL RECONSTITUTION OF THE PS1 REACTION CENTER OF PLANTS ON BLM MODEL MEMBRANES

INTRODUCTION

(a) General:

As mentioned previously, one of the approaches used to study the mechanism of action of membrane components is that of reconstitution of the purified component, or of membrane fragments containing it, into membrane models.

The main purpose of the studies and experiments reported in this section is to try to reconstitute, as least partially, the PS1 reaction center (RC) of thylakoid lamellae into a planar BLM. It is hoped that from the final form of the reconstituted system, it will be demonstrated, by direct electrical measurements, if generation of a potential difference across the thylakoid membrane takes place as a result of absorption of light by the pigments in the RC. The specific method or technique which will be used to try to achieve reconstitution, is that of fusion or association of vesicles containing the PS1 RC with planar BLMs. This technique may or may not result in the eventual incorporation of the RC into the BLM.

(b) Background:

Several membrane components, including: photosynthetic RCs, visual transduction proteins, ion channels and others have been reconstituted into BLMs using the "fusion of vesicles" approach (reviewed in Chapter 2). Some results seem to indicate that when the vesicles are hyperosmotic with respect to the bathing solution and Ca^{2+} is present only on the same side as the vesicles, actual incorporation or insertion of the vesicular membrane or its components into the planar BLM, takes place (Miller *et al.*, 1976; Latorre *et al.*, 1982). In some studies, purified membrane components are used while in others, membrane fragments from physiological systems are used.

In previous studies using purified PS1 RCs isolated from pea chloroplasts Barsky *et al.* (1976) could not obtain direct measurement of light-induced electric effects. In their experiments, the generation of photoelectric effects was observed by means of the anion probe PCB^-. This was attributed to either lack of association between the vesicle and the thick (colored) planar membrane or to inactivation of the RCs by the solvent present in the membrane. In the experiments to be reported here, thylakoid membrane fragments will be used instead of isolated RCs.

RESULTS

(a) Association of TMF-vesicles with planar BLMs:

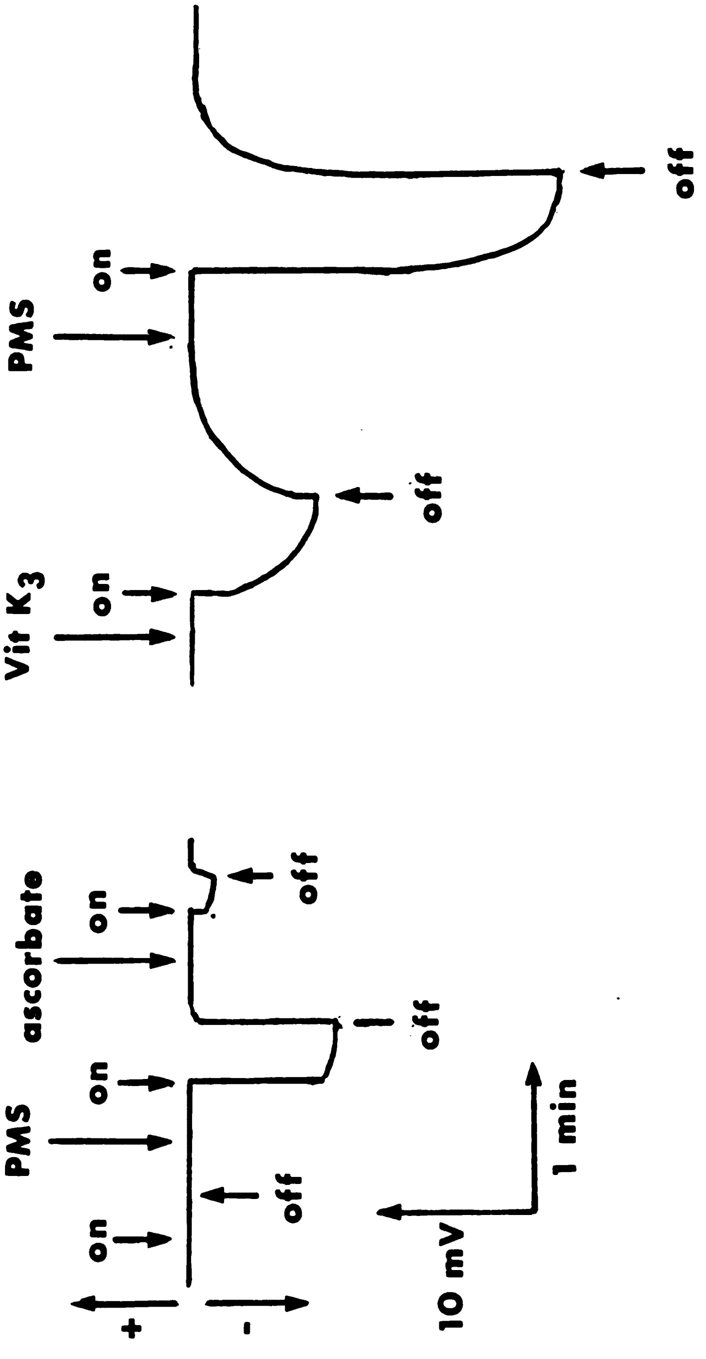
Addition of vesicles containing thylakoid membrane fragments (TMFs) to one side of a planar BLM resulted in the generation of light-induced membrane potentials. The generation of the photovoltage required the addition of artificial carriers of reducing equivalents (e.g., phenazinemethosulfate, (PMS)) to the same side as the vesicles or the presence of vitamin K_3 (Vit K_3), a quinone, in the vesicular membrane (see Figure 5.1). It also required the presence of Ca^{2+} (or Mg^{2+}) on the bathing solution. The polarity of the photovoltage indicated that the side containing the liposomes becomes negatively charged. Figure 5.1 also shows that addition of ascorbate to the same side as the PMS resulted in the decrease or disappearance of the photovoltage. This may be due to the reduction of PMS by ascorbate as evidenced by a change in the color of the suspension from light yellow to green. The reduced PMS (PMSH) will not be able to function then as electron acceptor. Addition of such chemical agents to the other side had no effect on the system, suggesting that the photoactive species had no access to the other side of the planar membrane. This plus the fact that no significant change in the BLM conductance was observed after addition of the vesicles, was interpreted as an indication that the TMFs were not becoming incorporated into the planar BLM. This means that only association or what has been termed partial fusion of the vesicles with the BLM was taking place.

(b) Kinetics of the association process:

The development of the open-circuit photovoltage as a function of incubation time was investigated. It was observed that the magnitude of the photovoltage increased with incubation time, rapidly at the beginning and then leveled off until a maximum was reached (see Figure 5.2). Assuming that the magnitude of the measured photovoltage is an indicator of the number of vesicles associated with the BLM the curve of Figure 5.2 shows the kinetics of the association process. In order to see if the maximum value was a function of the concentration of vesicles in the aqueous phase, the experiment was repeated for

Figure 5.1 - Open-circuit photovoltage generation by TMF-vesicles added to one side of a BLM.

The incubation mixture consisted of 0.1 M KCl, 0.01 M Tricine-OH buffer (pH 7.5) and 0.01 M $CaCl_2$. Additions: 0.5 mM PMS and 0.5 mM ascorbate to the bathing solution on the same side as the vesicles. Vit K_3 was mixed with the phosphatidylcholine at a molar ratio of 1:10 before vesicle preparation. The Chl a concentration on the bathing solution was approximately 1 μM.


+
-
on
off
PMS
on
off
ascorbate
on
off
10 mV
1 min
Vit K_3
on
off
PMS
on
off

different vesicle concentrations. It was found that approximately the same maximum value was reached except that at different times (see Figure 5.2), indicating that the maximum was not dependent on the vesicle concentration. This also suggests that the association may be an irreversible process.

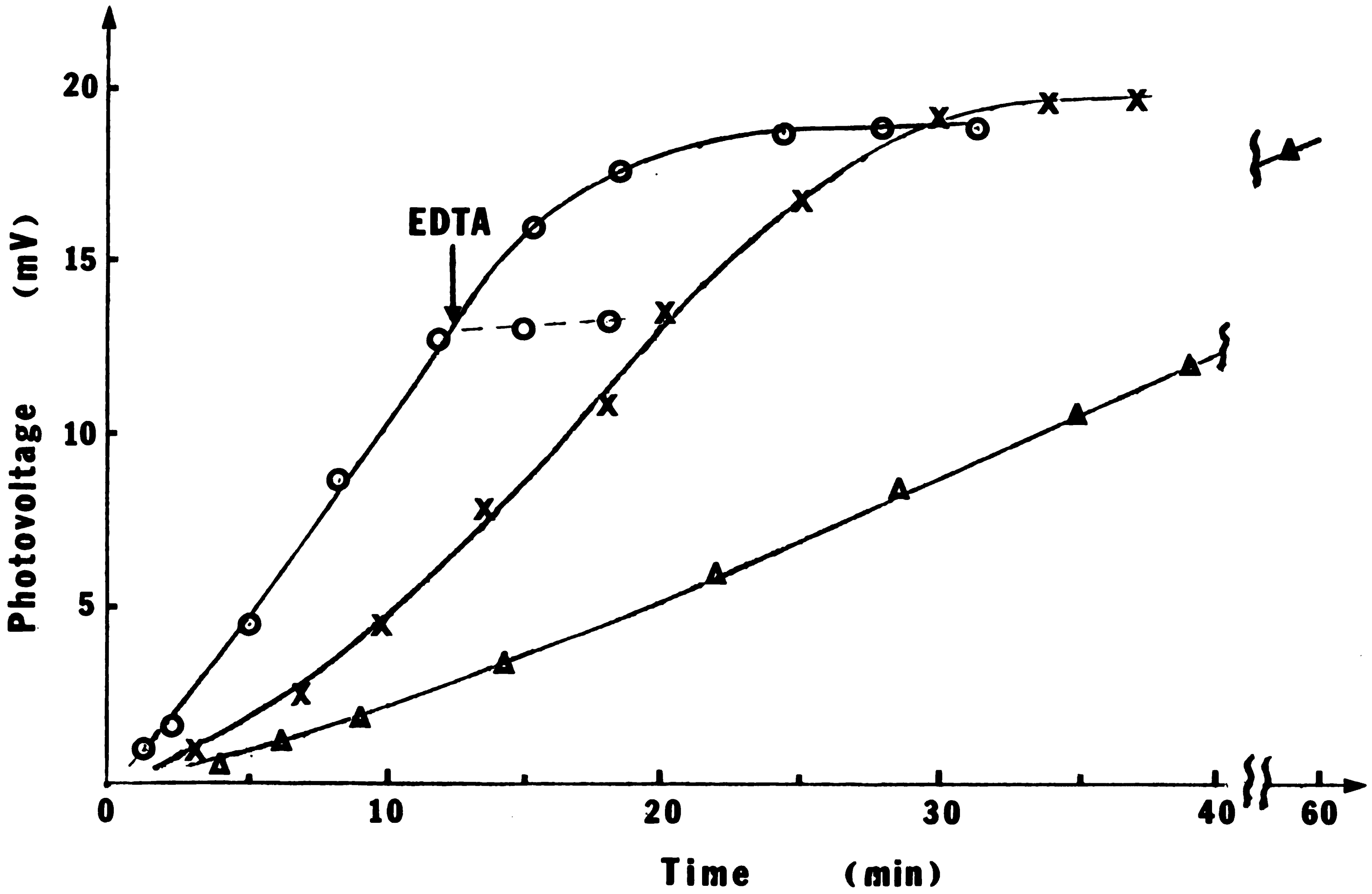
To test this possibility EDTA in excess of Ca^{2+} was added to the side containing the vesicles before the maximum had been reached. In this case it was found that the photopotential did not increase much more after the EDTA was added (see Figure 5.2). At the same time no decrease in the photovoltage was observed indicating that the vesicles did not become dissociated with the BLM. It was concluded therefore, that the process was irreversible.

The fact that a maximum value is not reached at longer incubation times could be due to inactivation of the vesicles in the aqueous solution. If this is the case, then addition of fresh vesicles to the incubation side could result in a sharp increase in the photopotential. However, when the experiment was done it was found that only a small increase took place. This suggests that the maximum value is due to the limited area of the membrane and the subsequent limited number of binding sites.

The data from Figure 5.2 was plotted again changing this time the independent variable from time to liposome concentration (see figure 5.3). From the figure, it appears that the photovoltage varies linearly with concentration. In another experiment liposomes containing different amounts of thylakoid membrane fragments (TMFs) were prepared and incubated on one side of a BLM. Figure 5.4 shows a plot of the time course of the photovoltage development. The

Figure 5.2 - Time course of the photovoltage development for different amounts of vesicles added to the aqueous phase.

The amount of vesicles added, expressed as mg phospholipid/ml were: 0.05 (Δ), 1.3 (X) and 2.5 (O). The Chl a concentration in the vesicle suspension was 80 μM. The bathing solution consisted of 0.1 M KCl, 0.02 M $CaCl_2$, 0.01 M Tricine-OH at pH 7.5. PMS at a concentration of 0.5 mM was present on the side containing the vesicles.


EDTA
Photovoltage (mV)
0
5
10
15
20
Time (min)
10
20
30
40
60

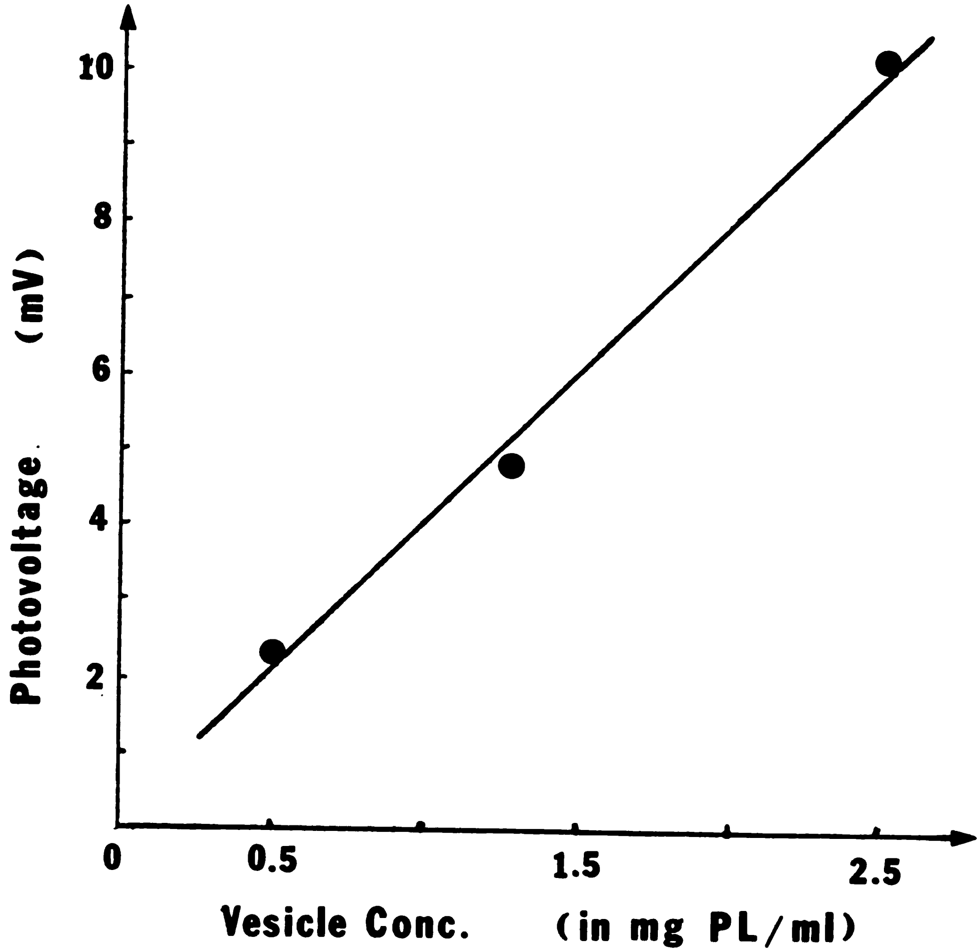
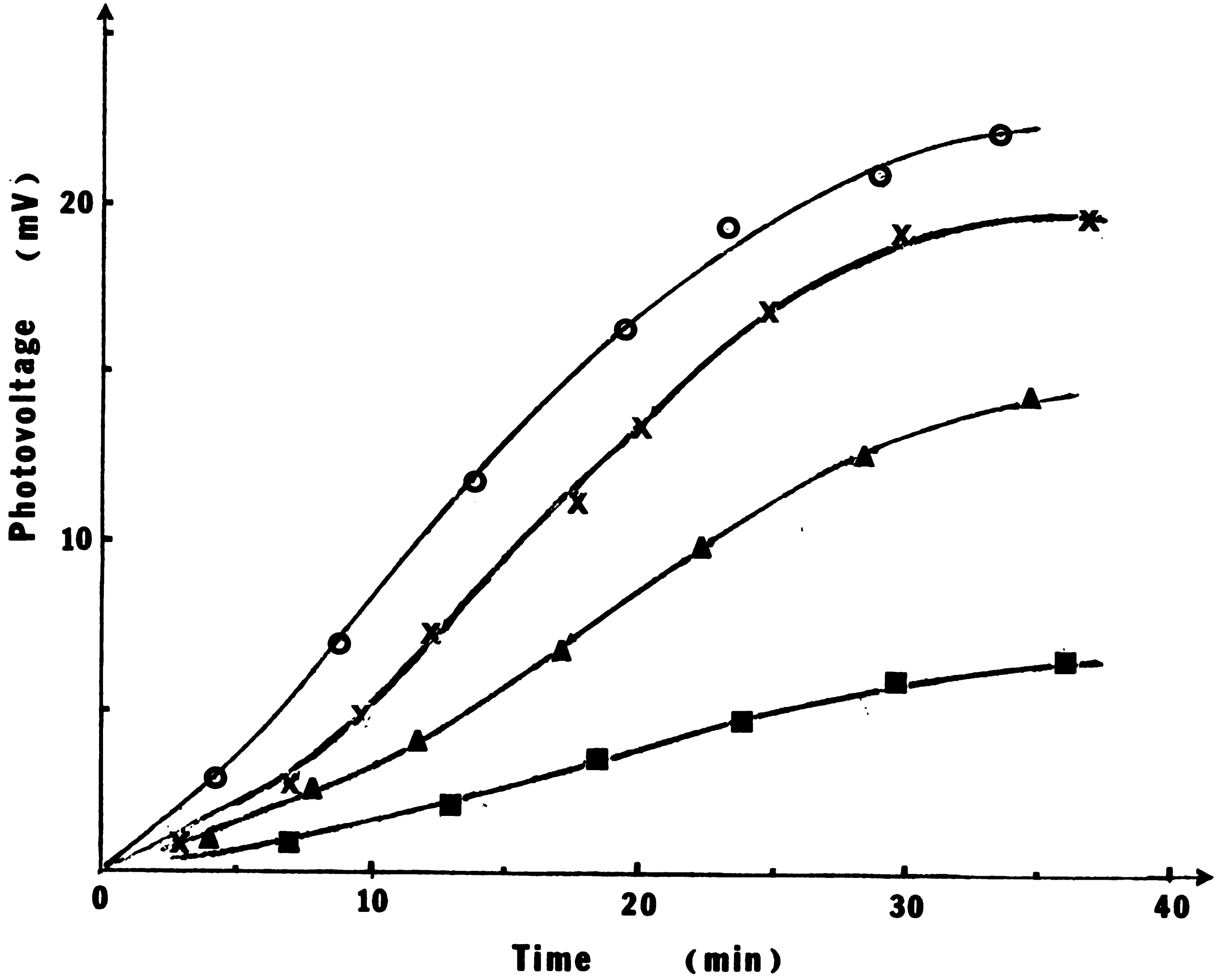


Figure 5.3 - Variation of the photovoltage as a function of the aqueous phase vesicle concentration.

Figure 5.4 - Time course of the photovoltage development for vesicles containing different amounts of Chl a.

The amount of vesicles added on all cases is the same, 1.3 mg PL/ml. The Chl a concentrations are: 0.2 μM (O), 0.5 μM (Δ), 1.0 μm (X) and 2.0 uM (O). Other conditions as in Figure 5.2.


Photovoltage (mV)
20
10
0
10
20
30
40
Time (min)

photovoltage as a function of the amount of TMF in the liposomes was also obtained from this data and is shown in Figure 5.5. As before the magnitude of the photovoltage varies linearly with the amount of TMF in the liposomes and hence, attached to the BLM. Deviations from linearity at high concentration of TMF may be expected due to the limited size of the vesicles and consequently, the amount of TMF that becomes incorporated into the liposomes.

(c) Action spectrum of the photoresponse:

In order to identify the species responsible for the photoeffect, the action spectrum was measured. Figure 5.6 shows the action spectrum of the photocurrent corrected for constant number of incident photons and also the action spectrum of the PS1 reaction center obtained from Ried (1972). The similarity between both curves provides evidence that the PS1 reaction center is directly involved in the phototransduction process.

(d) Open-circuit photovoltage:

The time course of the open circuit photovoltage for a typical membrane is shown in Figure 5.7. The photovoltage increased when coenzyme Q_6 (CoQ_6 or ubiquinone-30) or Vit K_3 were present in the vesicular membrane, and decreased sharply when o-phenanthroline was added to the side containing the vesicles (see Figure 5.7). The figure shows that the photoresponse when quinones are present in the vesicular membrane consists of the superposition of two components, one slow and one fast. The fast component resembles the photoresponse obtained in the presence of PMS only.

When the BLM is shunted with an external resistance of the same order of magnitude as that of the BLM, the photovoltage becomes

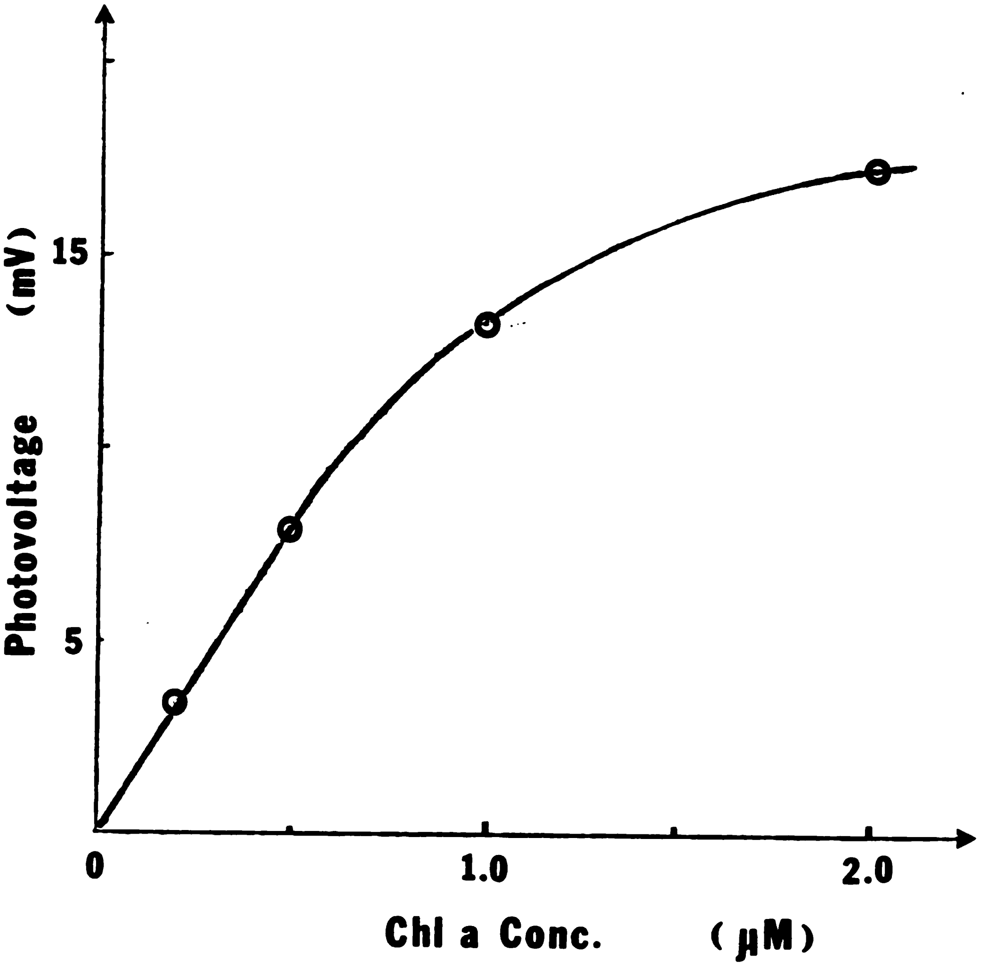


Figure 5.5 - Variation of the photovoltage as a function of the amount of Chl a in the vesicles.

Figure 5.6 - Action spectrum of the PS1 RC (from Ried, 1972) and photocurrent action spectrum of the photoelectric response for the TMF-vesicles associated with the BLM.

PMS at a concentration of 0.5 mM was present in the bathing solution and Vit K_3 was present in the vesicle suspension. The photocurrent was normalized to the PS1 action spectrum at 680 nm. The photocurrent was corrected for constant number of incident photons.

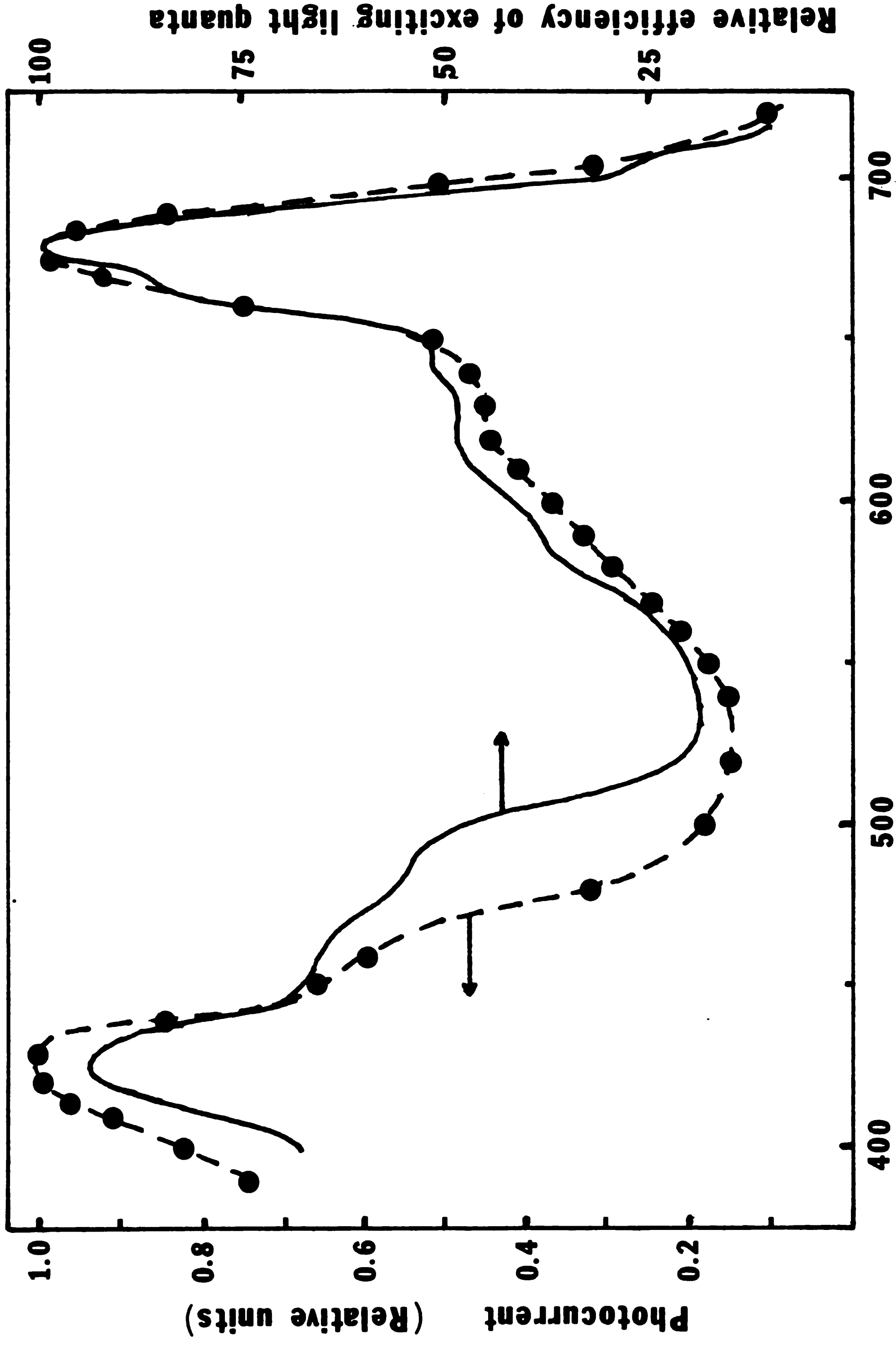
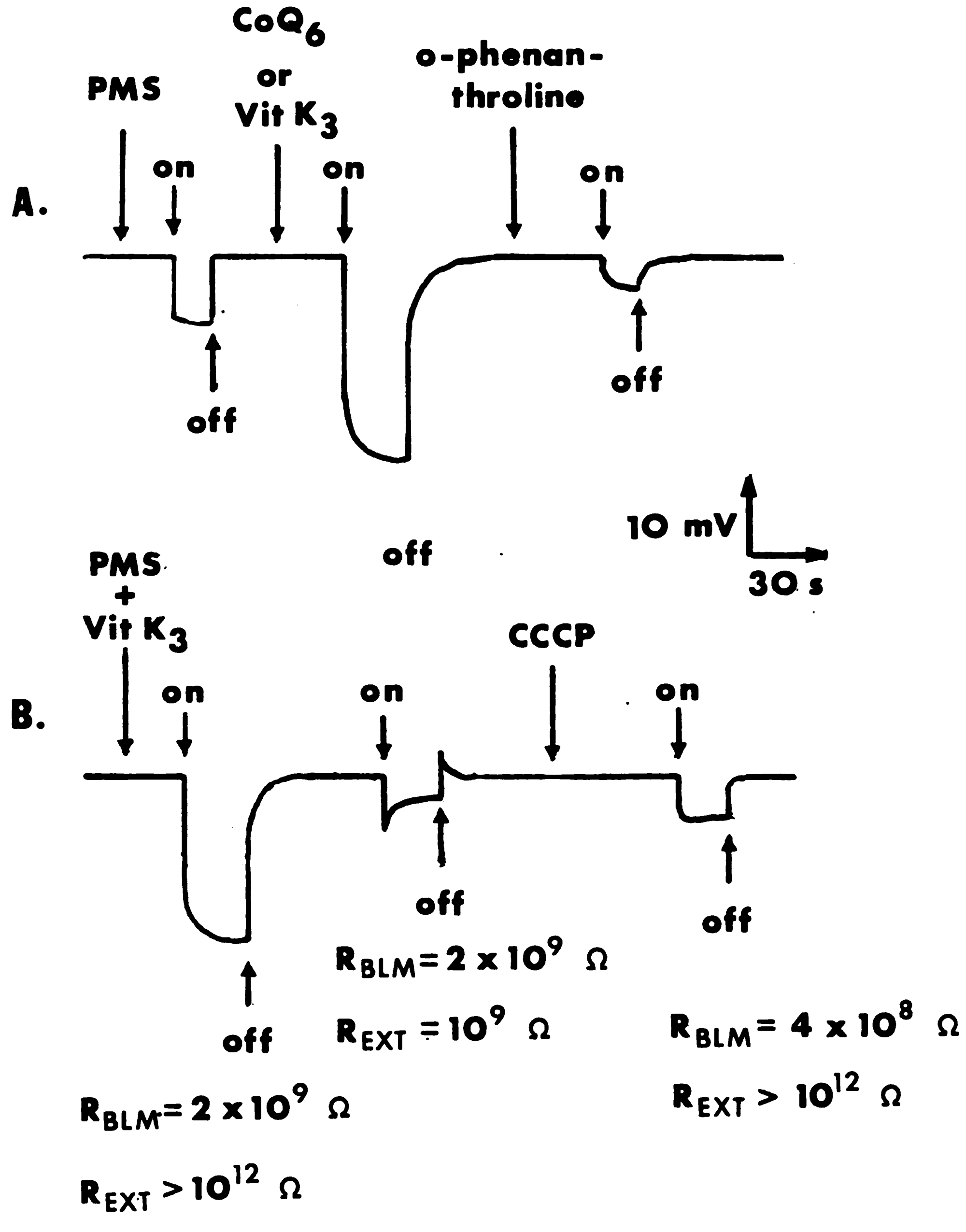

Photocurrent (Relative units)
1.0
0.8
0.6
0.4
0.2
400
500
600
700
λ (nm)
Relative efficiency of exciting light quanta
100
75
50
25

Figure 5.7 - Effect of CoQ_6, o-phananthroline, CCCP and of external shunt resistance on the open-circuit photovoltage.

A) Effect of quinones (Vit K_3 and CoQ_6) and o-phenanthroline on the photoelectric response of the TMF-vesicles associated with the BLM. The quinones were mixed with the phospholipid during vesicle preparation (molar ratio 1:10 quinone: phosphatidylcholine). The PMS and o-phenanthroline were both added to the same side as the vesicles to a final concentration of 0.5 mM and 2 mM respectively. The incubation mixture was as in Figure 5.1 The Chl a concentration in the aqueous solution was ∿1 μM.

B) Effect of shunting the open-circuit photovoltage with an external resistance R_{ext} and by additions of CCCP (0.5 μM). All other additions, the incubation mixture and the Chl a concentration were as in A.


A.
PMS
on
off
CoQ$_6$
or
Vit K$_3$
on
off
o-phenan-
throline
on
off
10 mV
30 s
B.
PMS
+
Vit K$_3$
on
off
$R_{BLM} = 2 \times 10^9$ Ω
$R_{EXT} > 10^{12}$ Ω
on
off
$R_{BLM} = 2 \times 10^9$ Ω
$R_{EXT} = 10^9$ Ω
CCCP
on
off
$R_{BLM} = 4 \times 10^8$ Ω
$R_{EXT} > 10^{12}$ Ω

differentiated. Addition of small amounts of the protonophorous uncoupler carbonylcyanide-m-chlorophenylhydrazone (CCCP), which is known to increase the permeability of membranes to protons (Le Blanc, 1971), cause a small decrease in the membrane resistance and a decrease in the amplitude of the photovoltage. However, no significant change in the shape of the photovoltage time course was observed, with the exception that if a quinone was also present the rise and decay time of the slower component decreased (see Figure 5.8). This observation suggests that the slower component may be associated with ion (probably H^+) diffusion across the membrane. The time constant of the slow component was calculated from the time course of the photovoltage shown in Figure 5.8 and found to be equal to 6 s. This value is in agreement with the value of 5 s calculated from the values of BLM resistance ($R_m = 1.2 \times 10^9\ \Omega$) and capacitance ($C_m = 4.1$ nF) for that membrane.

Open-circuit photovoltage measurements as a function of light intensity exhibited a saturation at high intensities (see Figure 5.9). The response of the membranes to excitation by short (8 μs duration) light flashes was also investigated. The results are shown in Figure 5.10. The rise time of the photopotentials was found to be of the order of 50 μs.

It was also found that the open-circuit photovoltage obtained in the presence of PMS and in the absence of any other exogenous substance, developed after some time, a slow component. The slow component, as in the experiments with quinones, had the same polarity as the fast component and was superimposed on it (see Figure 5.11). After doing some experiments it was determined that the development of

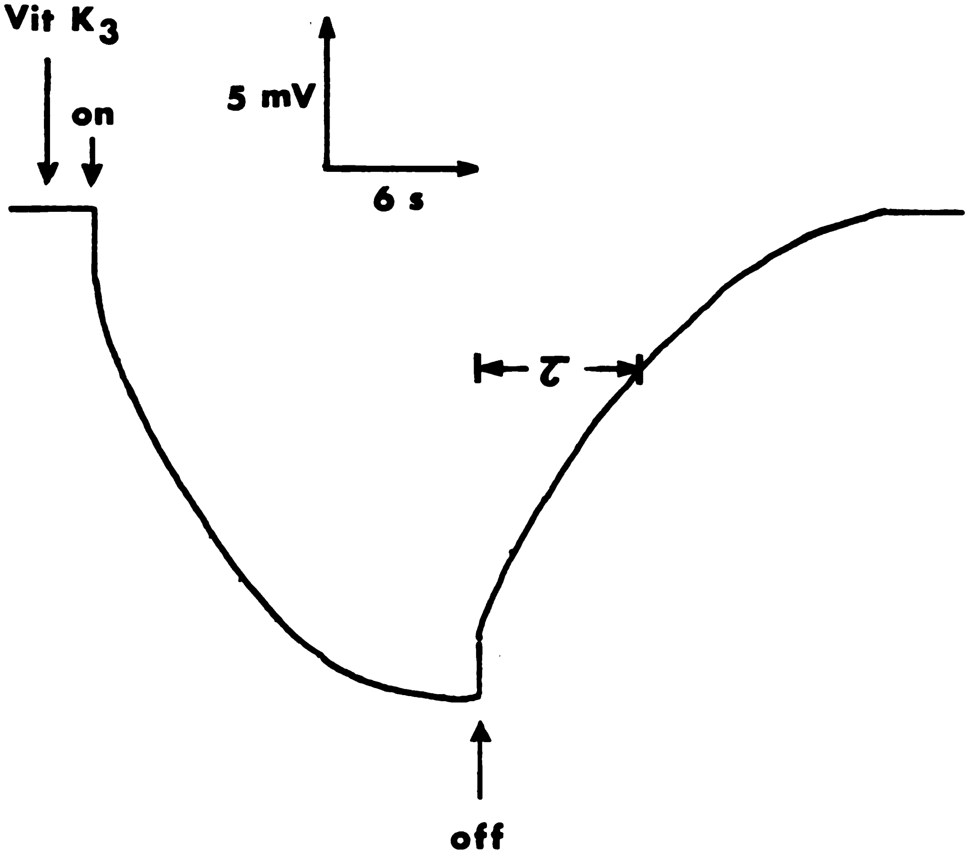
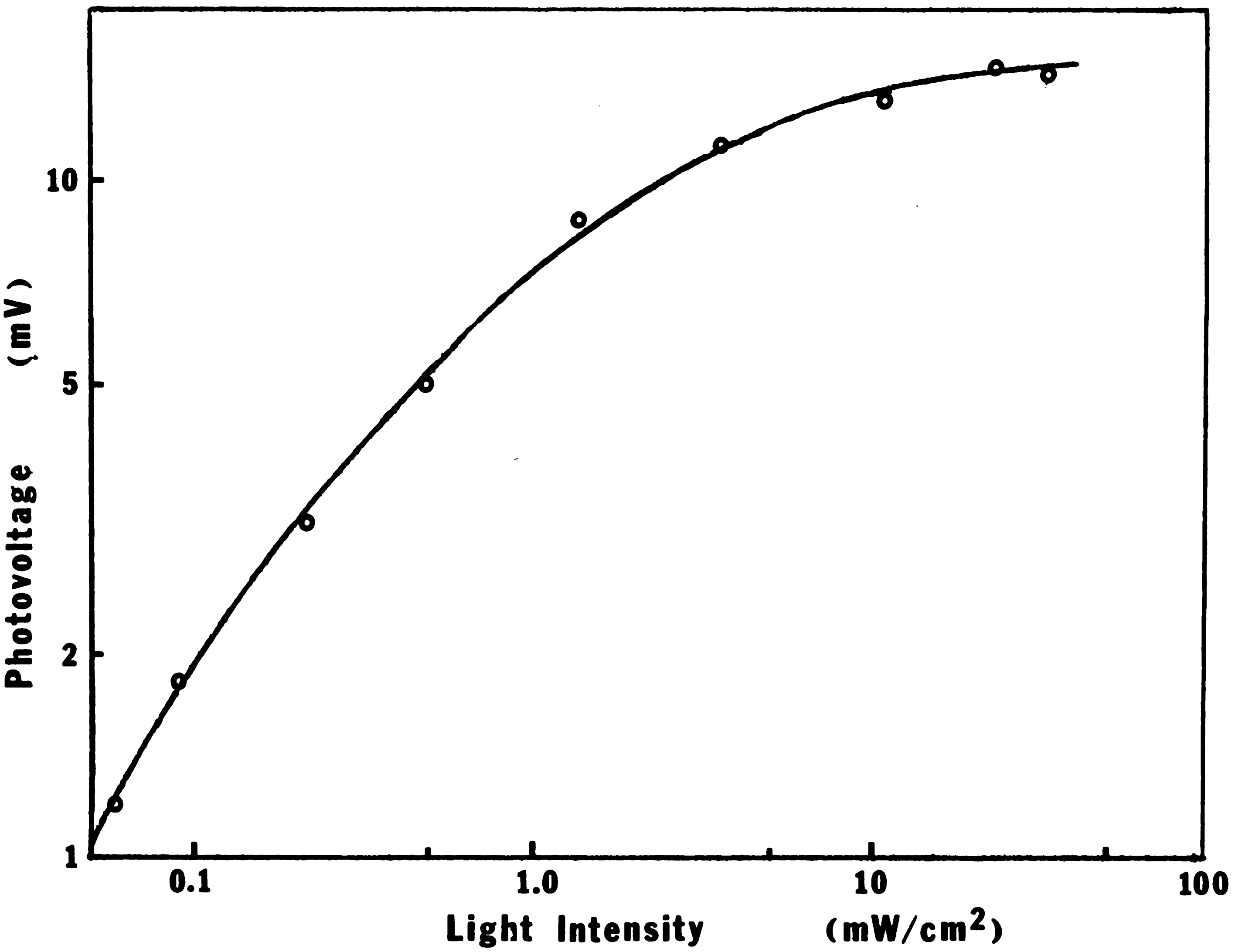


Figure 5.8 - Time course of the open circuit photovoltage in the presence of Vit K_3.

The amount of Vit K_3 and the incubation mixture are as in Figure 5.1

Figure 5.9 - Variation of the open-circuit photovoltage with light intensity.
Incubation mixture as in Figure 5.2.



Photovoltage (mV)
10
5
2
1
0.1
1.0
10
100
Light Intensity (mW/cm²)

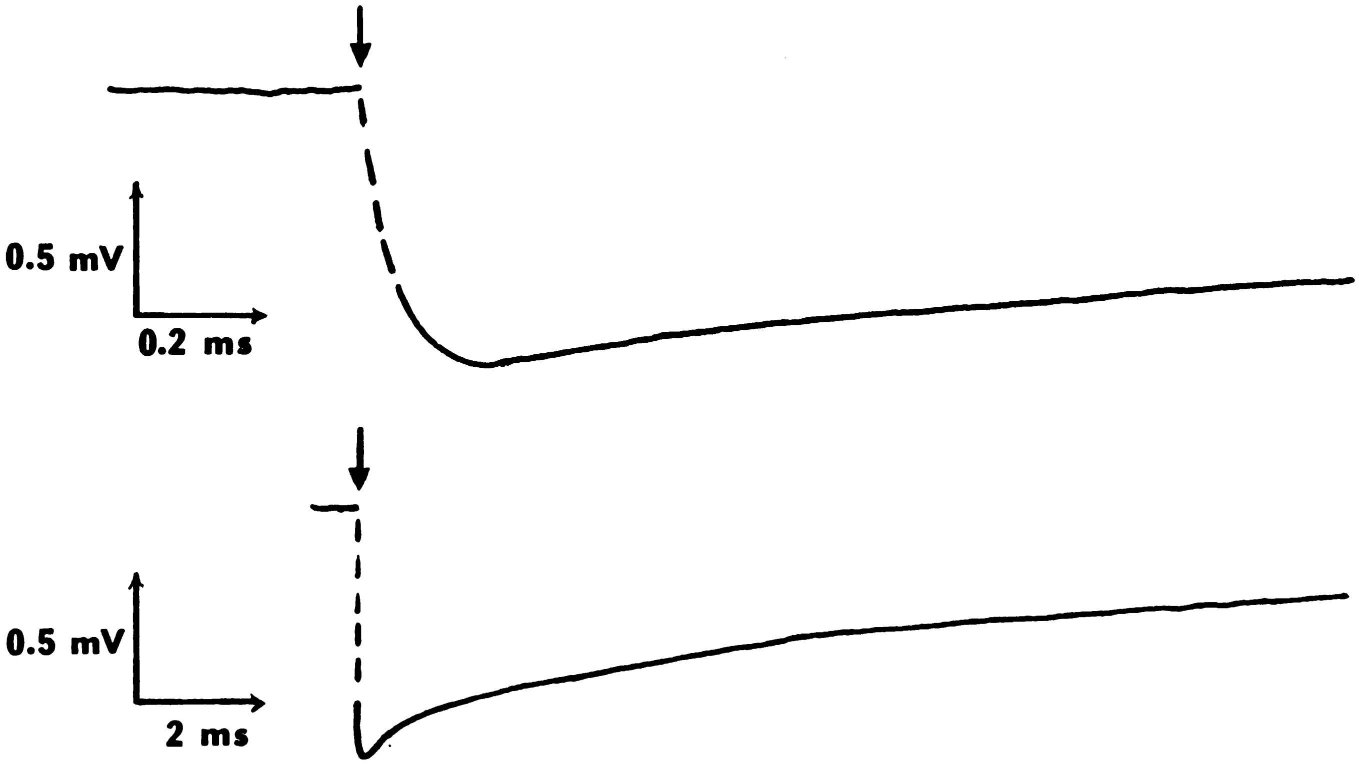


Figure 5.10 - Time course of the open-circuit photovoltage in response to illumination with an 8 µs in duration flash of light.

The arrow indicates the onset of the flash. The incubation mixture was as in Figure 5.1 . PMS to a final concentration of 0.05 mM was present on the same side as the vesicles.

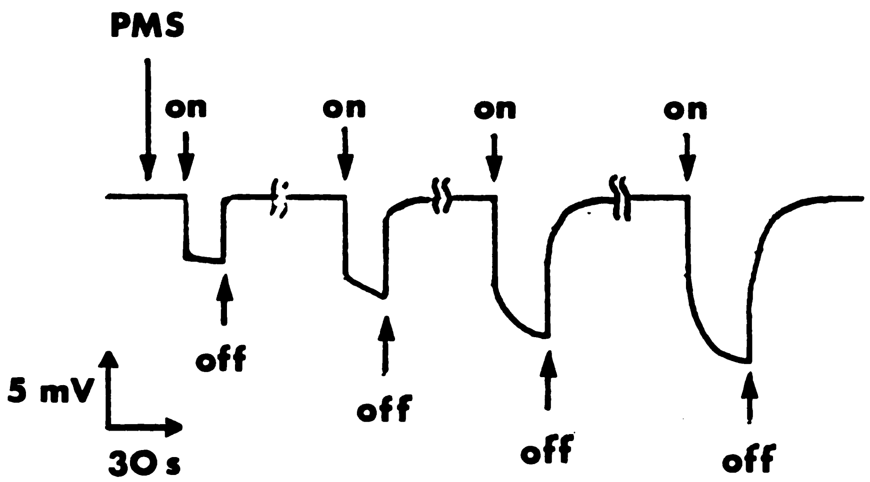


Figure 5.11 - Development of the low component of the photovoltage in the presence of 0.5 mM PMS. Incubation mixture as in Figure 5.1.

this slow component was dependent on the amount of time the membrane was illuminated. The rise and decay time of the component was found to be of the same order as the one observed for the quinones. It is suggested then that the component is ionic in nature, most probably due to H^+ diffusion. This idea is also based on the results of experiments with sonicated chloroplasts in the presence of PMS by Nelson, Nelson and Racker (1972). In their experiments it was observed that sonicated broken chloroplasts were capable of ATP synthesis in the presence of PMS. The interpretation given was that reduction of PMS was resulting in the net accumulation of PMSH, an amine, indside the sonicated chloroplast. The reduced PMS (PMSH) inside was then donating electrons to $P700^+$ with the concomitant release of H^+. The net result of the PMS-mediated cyclic electron flow being the lowering of the internal pH affecting the polarity of the membrane and creating the necessary conditions for cyclic photophosphorylation (Nelson _et_ _al_., 1972).

(e) _Short-circuit photocurrent_:

The time course of the short-circuit photocurrent for a typical membrane is shown in Figure 5.12. It consists of a large initial overshoot when the light is turned on, followed by decay to a smaller steady-state current and an undershoot when the light is turned off, followed by a return to the base line. When the conductance of the bilayer to protons was increased with CCCP the steady-state current also increased. These results taken together with the fact that no change in conductance was found for the BLM in the presence of the vesicles strongly suggest that the closed structure of the vesicles is maintained intact after fusion. The same model has been previously proposed for studies involving the fusion of planar membranes with

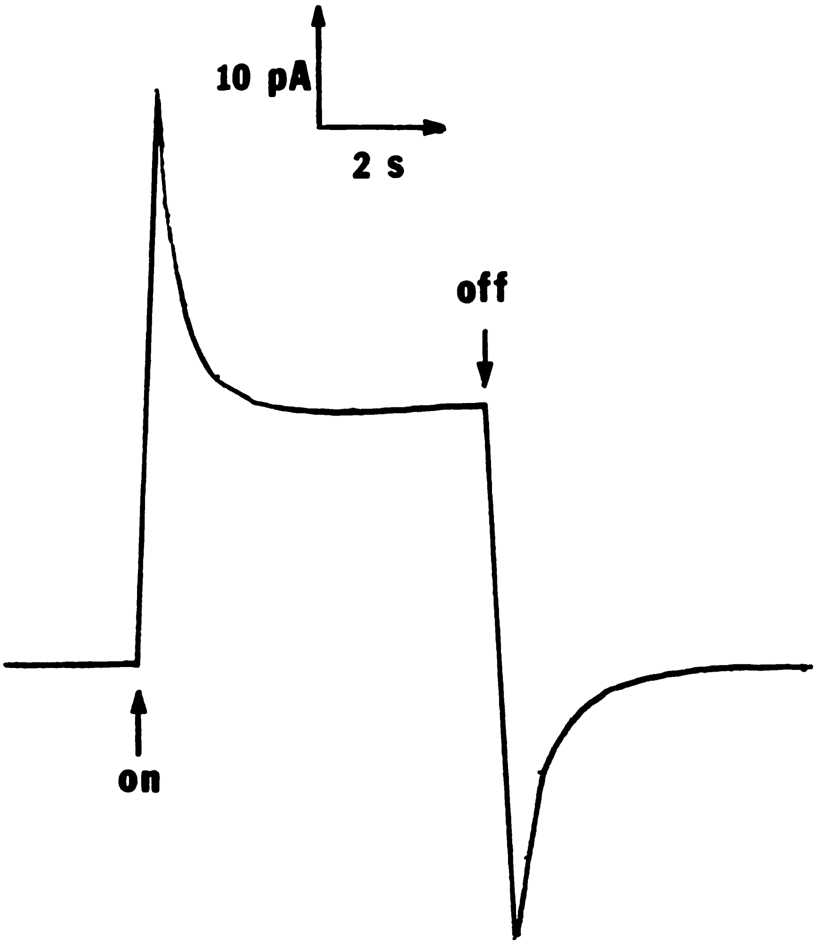


Figure 5.12 - Time course of the short circuit photocurrent for TMF-vesicles associated with a BLM.

PMS present at a concentration of 0.5 mM. The incubation mixture was as in Figure 5.1.

vesicles containing purple membrane from H. halobium (Herrmann et al., 1978) or bacteriochlorophyll complexes (Barsky et al., 1976).

A drawing showing the suspected configuration for the TMF-containing vesicles fused with a BLM is shown in Figure 5.13.

Assuming this configuration is correct, the polarity of the measured open-circuit photovoltage indicates that when the light is turned on, the outside of the vesicles charges negative and the inside positive. In order to test the validity of the proposed model, the system will be analyzed on the basis of an equivalent circuit similar to the one used by Herrmann and Rayfield (1978). This is justified by the similarities in the characteristics of the photoelectric response between this system and that of PM-vesicles fused with BLMs (Herrmann et al., 1978).

The equivalent electrical circuit for a single fused vesicle is shown in Figure 5.14. In the diagram R_v and C_v are the resistance and capacitance between the right chamber and the interior of the vesicle. R_f and C_f are the resistance and capacitance between the left chamber and the interior of the vesicle (fused portion). R_m and C_m are the resistance and capacitance of the planar BLM. All the equivalent current generators in one vesicle are designated by I and V_b represents the potential difference between the interior of the vesicle and either chamber. The shape of the observed transient short-circuit photocurrent as shown in Figure 5.12 is in agreement with this electrical circuit. The photocurrent is made up of two components, a displacement or capacitative current through C_f and a leakage current through R_f which starts to appear as the light is turned on. As the potential V_b reaches its steady-state value, the

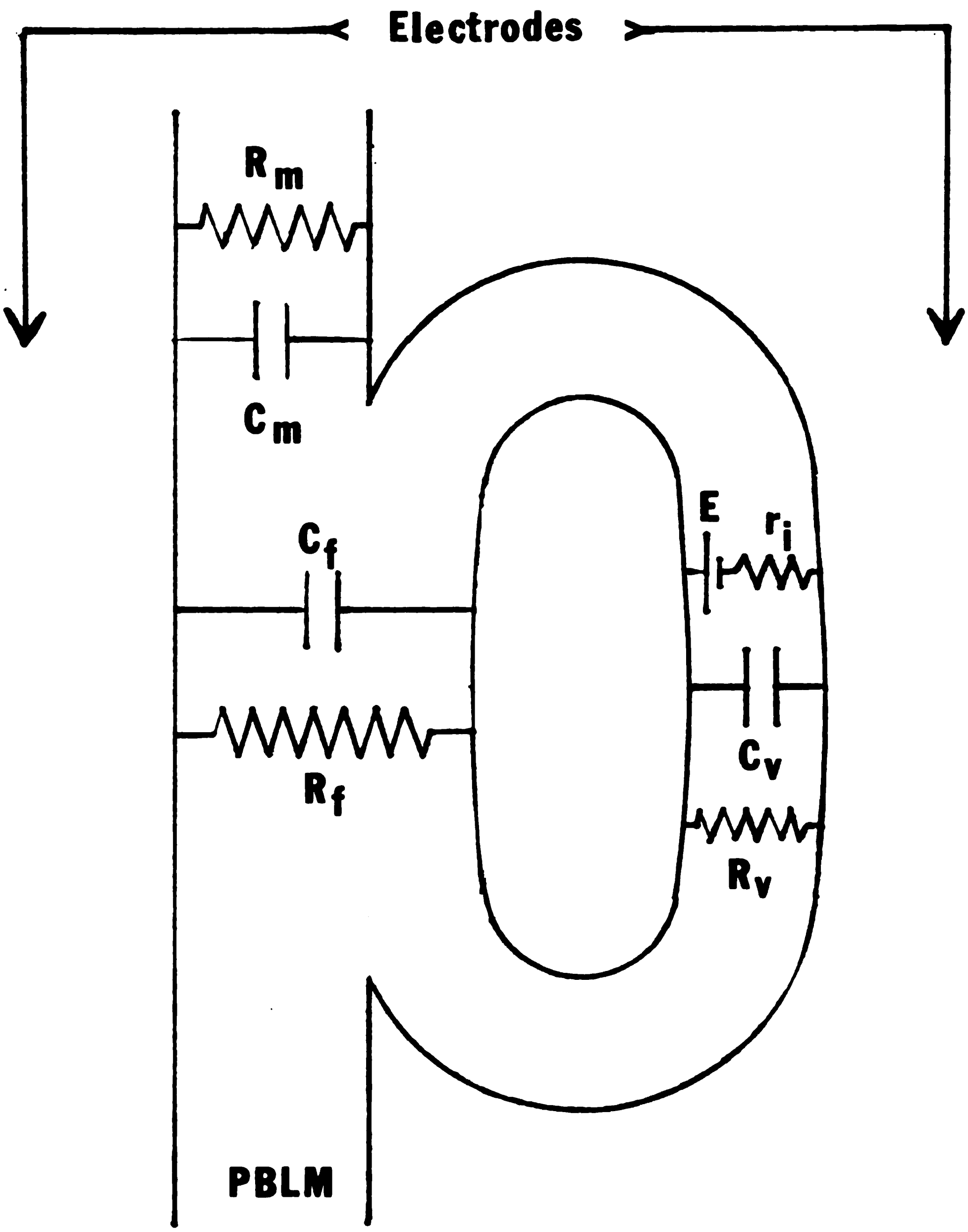


Figure 5.13 - Diagram representing the suspected configuration of the vesicle-BLM system after association of the two membraneous structures.

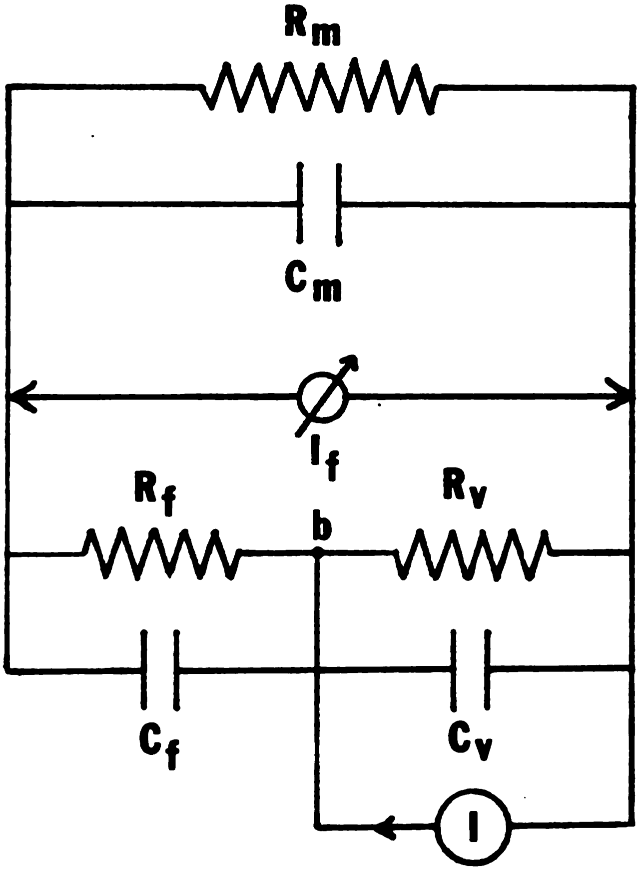


Figure 5.14 - Equivalent circuit diagram to represent the vesicle-BLM associated system as shown in Figure 5.13.

Under short-circuit conditions, the potential difference between a and b vanishes and I_f would represent the short-circuit current.

displacement current gradually decreases until only the steady-state current is left. When the light is turned off, the membrane begins to discharge (V_b decreases), this generates a displacement current of opposite sign. However, if the displacement current is larger than the leakage current, the short-circuit current reverses sign and gradually decays to zero.

In general, the current I depends on the voltage V_b and as a first approximation it may be presented as a linear function of V_b (Herrmann and Rayfield, 1978):

$$I\ (V_b,\ t,\ J) - I_o\ (t,J)\ (1-V_b/V_c), \qquad (1)$$

where I_o, which depends on the light intensity J, is the current for $V_b=0$ and V_c is a constant. Under short-circuit conditions the total voltage across the membrane vanishes and the part of the planar membrane with no vesicles attached may be omitted in the circuit analysis. From the circuit it follows that:

$$I=V_b/R + C\ dV_b/dt \qquad (2)$$

where $1/R=1/R_f + 1/R_v$ and $C=C_f + C_v$. Substituting for I from equation (1) into equation (2):

$$I\ =V_b\ (1/R + I_o/V_c) + dV_b/dt \qquad (3)$$

Since the light is turned on at t=0 and off at $t=t_o$, the following values for $I_o(t)$ can be set. $I_o(t)=0$ for $t<0$, $I_o(t)=I_o$ for $0<t<t_o$ and $I_o(t)=0$ for $t>0$. Using these values in equation (3) and solving the differential equation for $V_b(t)$, the following equation is obtained:

$$V_b(t)=I_oR'\ (1-e^{-t/\tau'})$$

where $1/R'=1\ R + I_o/V_c$ and $\tau'=R'C$. Since the measured short-circuit current I_f flowing through R_f is given by V_b/R_f +

CdV_b/dt, the equation for I_f is given by:

$$I_f = (I_o\ R'/R_f)\ (1-e^{-t/\tau'}\ ) + (C_f\ I_o\ R'/\tau')\ e^{-t/\tau'}. \tag{5}$$

Equation (5) can be written as

$$I_f = I^{\infty} + (I^o - I^{\infty})\ e^{-t/\tau'} \tag{6}$$

where $I^{\infty} = I_oR'/R_f$ represents the steady-state current and $I^o=C_fR'I_o/\tau' = I_oC_f/C$ is the short-circuit current at t=0.

Now, from the definitions of I^{∞} and R'

$$I/I^{\infty} = R_f/I_o\ R' = R_f/R\ I_o + R_f/V_c. \tag{7}$$

The current I_o is due to electron transfer through the PS1 reaction center and subsequent proton translocation across the vesicular membrane and should therefore depend on the light intensity J. Figure 5.14 shows a plot of $1/I^{\infty}$ versus 1/J. Since a straight line is obtained it follows that:

$$I^{\infty} = I^SJ/\ (J + J_{1/2}) \tag{8}$$

where I^S is a constant equal to the saturation current and $J_{1/2}$ is another constant equal to the half-saturation intensity. This equation follows from equation (7) if $1/I_o$ is proportional to the inverse of the light intensity 1/J. A plot of $1/I_o$ versus 1/J is shown in Figure 5.15 and it does show that a linear relationship exists between the inverse of both quantities. In other words:

$$1/I_o = K_1 + K_2/J \tag{9a}$$

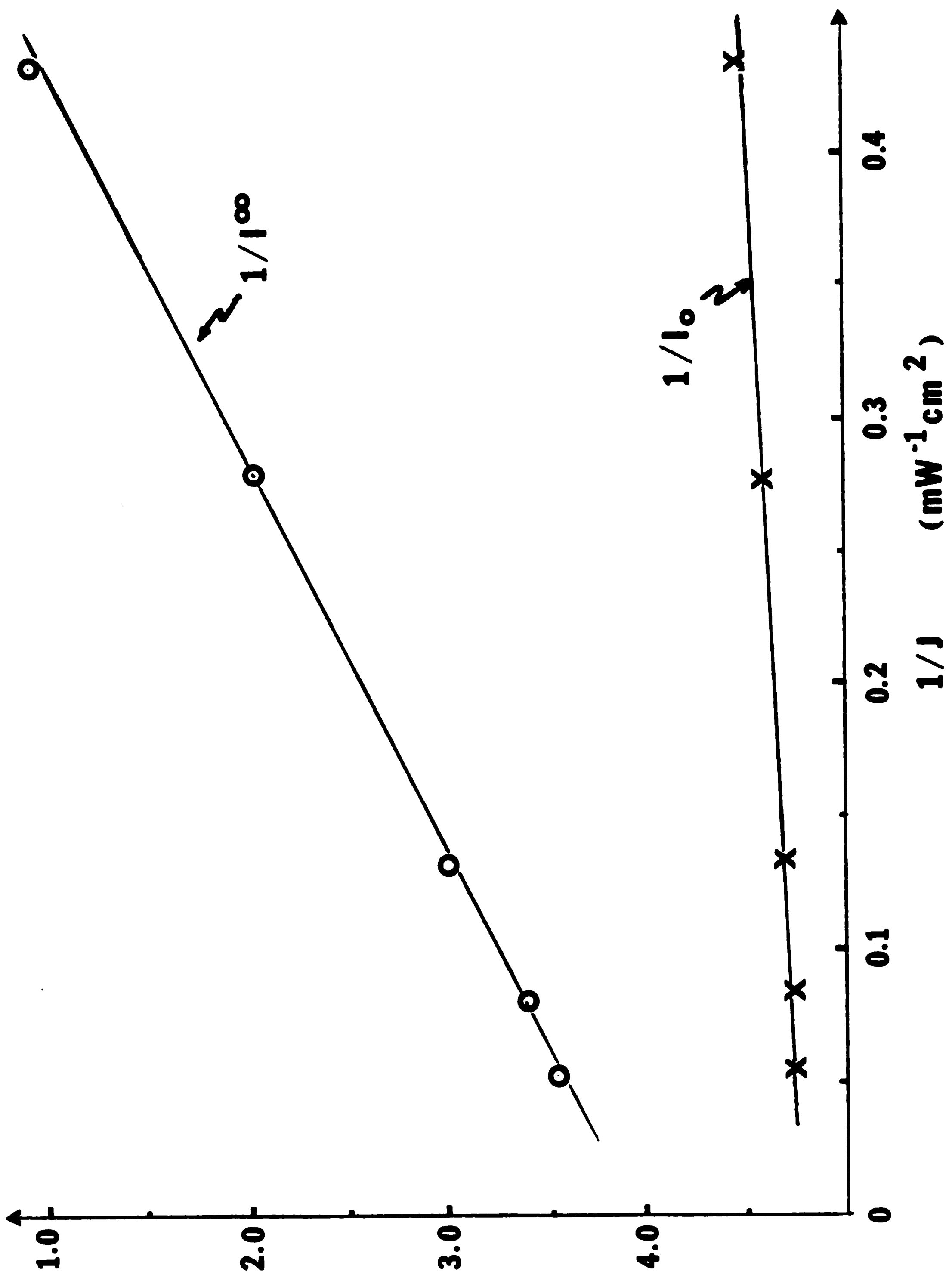
where K_1 and K_2 are constants. This equation can also be written as:

$$I_o = I_o^S\ J/(J + J_{1/2}) \tag{9b}$$

where, as before, I_o^S and $J_{1/2}$ are both constants equal to the saturation current and the half-saturation intensity respectively.

Figure 5.15 - Relationship between the short-circuit current and the intensity of illumination.

Incubation mixture as in Figure 5.12.


1/I (10^{11} A^{-1})
1.0
2.0
3.0
4.0
0
0.1
0.2
0.3
0.4
1/J ($mW^{-1}cm^2$)
1/I∞
1/I₀

Equation (9b) indicates that at low light intensities I_0 is proportional to J. From this and the definition of τ' it follows that $1/\tau'$ should vary linearly with J at low light intensities. At high light intensities the light generated photocurrent I_0 should saturate since there should be a limited number of light-driven reaction centers per unit area in the membrane. A plot of $1/\tau'$ versus J showing linearity at low values of J and saturation at high light intensities is shown in Figure 5.16.

It was mentioned previously that the protonophorus uncoupler CCCP caused an increase in the measured steady-state current by increasing the permeability of the membrane to protons. It has been found that the resistance of a bilayer varies with the concentration of CCCP according to the relation $1/R = 1/R_0 + \alpha c$ where R_0 is the resistance in the absence of CCCP, α is a constant dependent on the geometry and c is the molar CCCP concentration (LeBlanc, 1971). From the relation it follows that at low CCCP concentrations R_f/R is independent of CCCP concentration and that R_f varies inversely with c. Putting this information on equation (7) it follows that $1/I^\infty$ should vary linearly with 1/c. A plot of $1/I^\infty$ versus 1/c showing the linear dependency is shown in Figure 5.17.

The results mentioned in this section support the idea that under the conditions the above studies were done, the closed structure of the vesicles is maintained after fusion of the two membrane takes place. They also show by direct electrical measurements, that the TMFs reconstituted in such a way are capable of generating photovoltages and photocurrents which are directly related with the absorption of light by the PS1 reaction center present in the TMF.

Figure 5.16 - Variation of the measured time constant for the short-circuit current (') with light intensity.

Incubation mixture as in Figure 5.12.

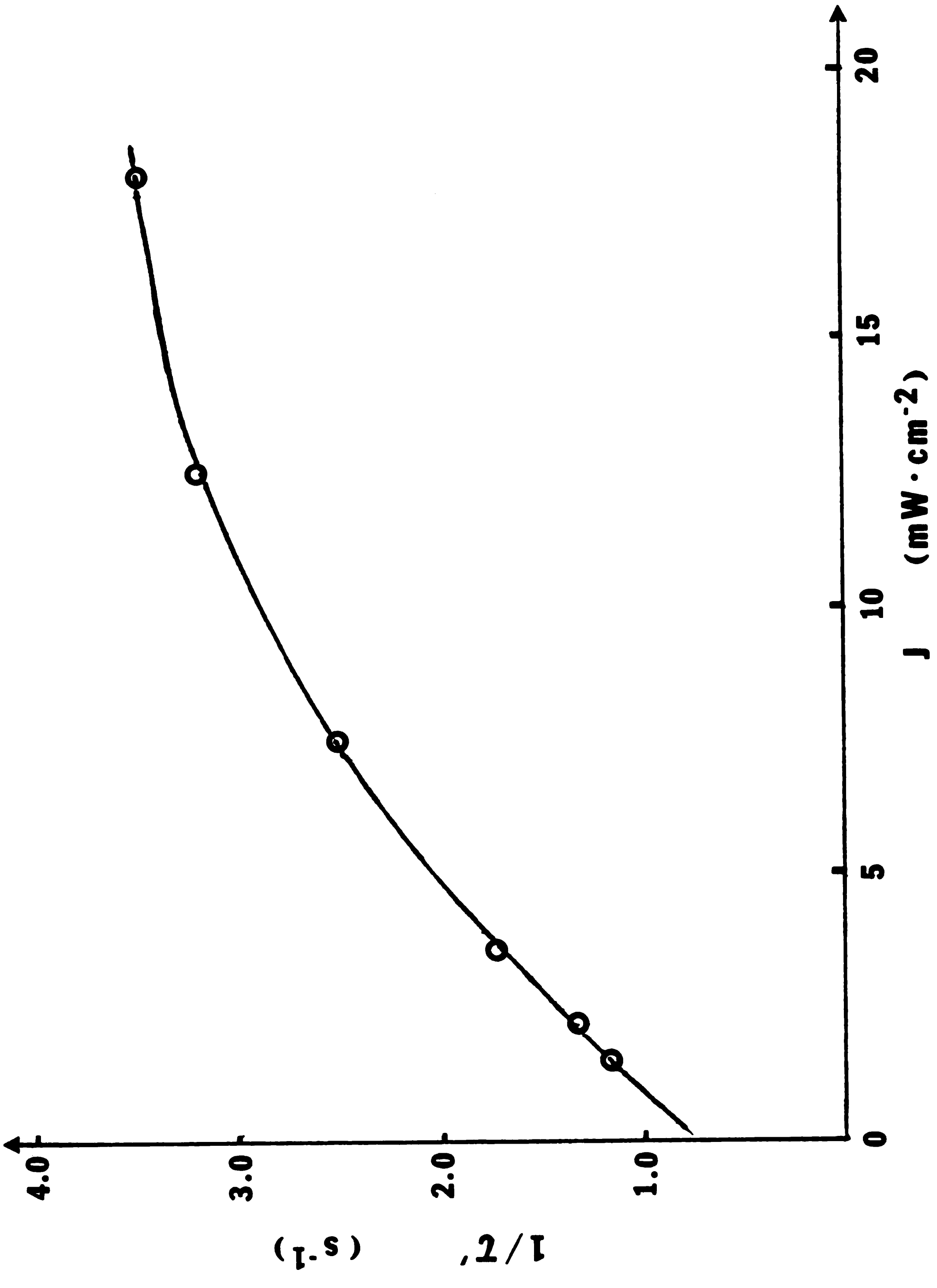
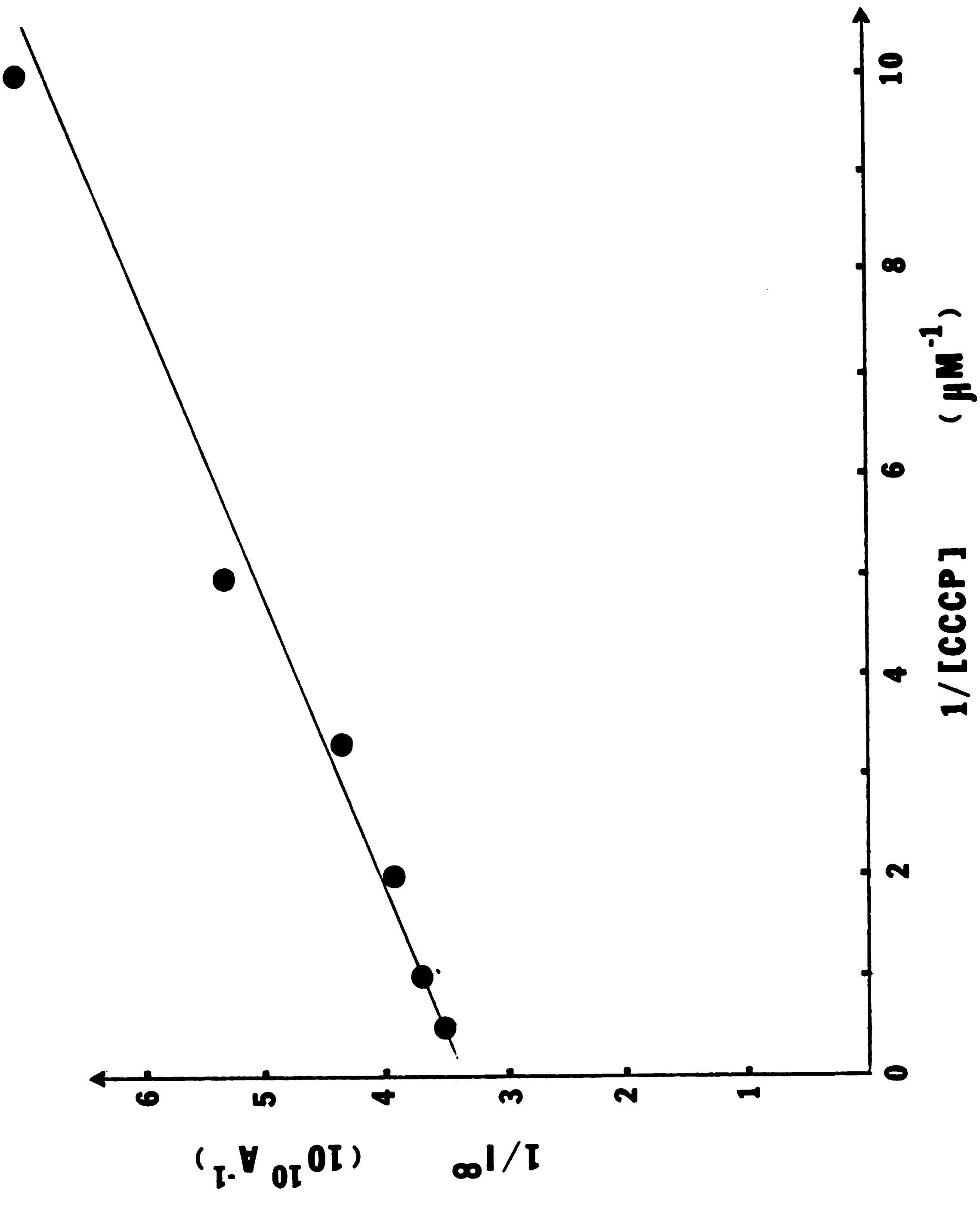

1/τ' (s-1)
4.0
3.0
2.0
1.0
0
5
10
15
20
J (mW·cm-2)

Figure 5.17 - Variation of short-circuit photocurrent with CCCP concentration. Incubation mixture as in Figure 5.12.


1/I∞
(10^10 A^-1)
1/[CCCP]
(μM^-1)
0
2
4
6
8
10
1
2
3
4
5
6

(f) Incorporation of RCs from thylakoid membranes into planar BLMs:

In this set of studies the successful incorporation of reaction centers from plants into planar membranes was pursued once again by means of fusion of vesicles containing TMFs with BLMs. This time, however, a procedure developed by other investigators for this purpose was used (already reviewed in Chapter 2; see also Miller et al., 1976; Zimmerberg, et al., 1980). It includes the use of negatively charged lipids, divalent ions and more importantly, the establishment of osmotic gradients across the membranes.

Vesicles containing TMFs and hyperosmotic with respect to the bathing solution were added to one side of a BLM. The conductance of the membrane was monitored continuously while applying a small external potential across the membrane. A short time after the liposomes were added sharp changes in conductance consisting in transient jumps of unequal duration were observed (see Figure 5.18). The conductance spikes were superimposed on a continuous step-wise increase in the background conductance of the BLM. It was found that the membranes became more unstable after the conductance changes started to appear and broke when the conductance became too high. To prevent this from happening and in order to be able to test the photoactivity of the BLMs, EDTA in excess of Ca^{2+} was added before the conductance increase had reached approximately two orders of magnitude. (The membranes lasted much longer in the absence of the vesicles.) These results indicate that incorporation of too much natural membrane has a deleterious effect on the BLM stability.

Figure 5.18 - Increase in the BLM conductance in the presence of hyperosmotic TMF-vesicles.

A potential difference of 50 mV was applied across the BLM. The up-ward arrow indicates the time of vesicle addition. The amount of vesicles added in μg PL/ml was about 65. The Chl a concentration in the aqueous phase was 1 μM. The incubation mixture consisted of 0.2 M KCl, 0.01 M Tricine-OH (pH 7.5) and 5 mM $CaCl_2$ present on the same side as the vesicles.

At this point, a small addition of PMS to the same side as the vesicles resulted in the generation of small photopotentials (up to 1 mv maximum). The photovoltage could be observed almost immediately after adding the PMS. The polarity indicated that the side containing the liposomes became negatively charged with respect to the other side. The response could not be observed in the absence of PMS and could be slightly increased by adding ascorbic acid to the opposite side. In this case, however, the photovoltages were transient, exhibiting a fast rise when the light is turned on with a concomitant slower decay to a steady-state value, followed by decay with a small undershoot when the light is turned off and a return to the base line (see Figure 5.19). This shape is reminescent of the photovoltages measured when the membrane is shunted with an external resistance (see Figure 5.1). It was also observed that the magnitude of the photovoltage decreased. At membrane resistance values below 10^5 Ω-cm^2 no photopotential could be measured. A small photovoltage was also observed when the conductance was low since only a small amount of TMFs had been incorporated into the BLM. (The above observations indicate some of the difficulties encountered in this set of experiments.) It was also found that if the PMS was added to the side opposite to where the liposomes had been added, a very small photovoltage of opposite polarity to the one observed before could be measured.

The photovoltage could be generated when the membranes were illuminated with red ($\sim$ 675 nm) and blue ($\sim$ 450 nm) light but not with green ($\sim$ 550 nm) light. These observations suggest that the photosynthetic pigments were involved at least in the initial light absorption process and partly responsible for the photopotential generation.

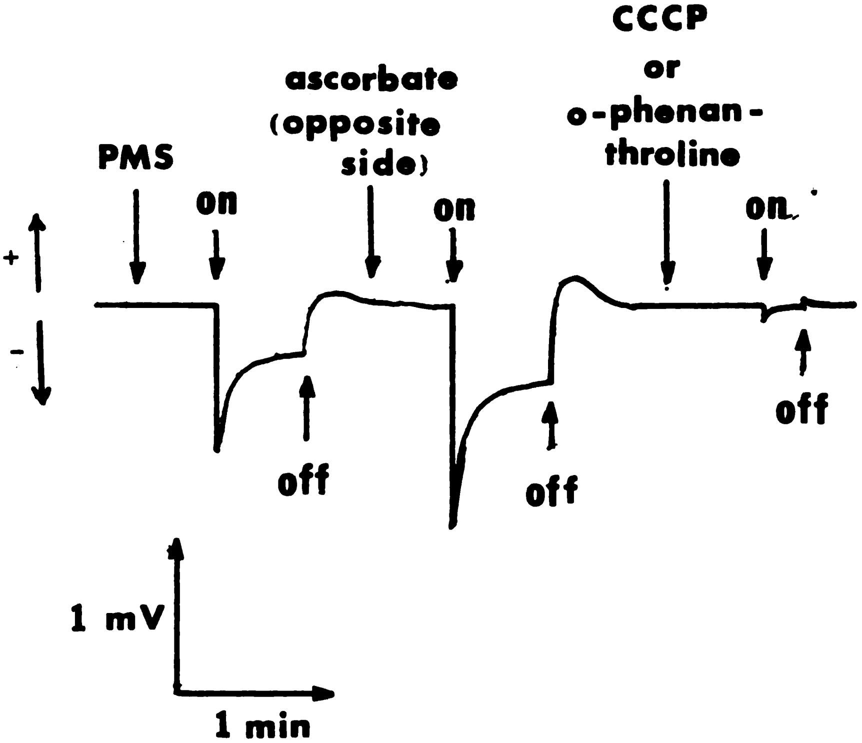


Figure 5.19 - Open-circuit photovoltage for a BLM with incorporated TMFs.

Time course of the open-circuit photovoltage after addition of hyperosmotic vesicles to one side of a BLM and after an increase in conductance had already been observed. The membrane resistance decreased from 2 x 10^9 Ω to 10^8 Ω. The final concentration of PMS and ascorbate after addition were 0.5 and 0.3 mM respectively. The interior of the vesicle contained 0.25 M sucrose. The incubation mixture for the BLM consisted of 0.1 M KCl, 0.01 M Tricine-OH (pH 7.5) and 5 mM $CaCl_2$ added only to the same side as the vesicles. After the resistance had reached that value, 10 mM EDTA was added to stop the fusion process. The polarity of the observed photovoltage indicates that the side containing the vesicles and PMS becomes negatively charged.

Attempts to measure the short-circuit photocurrent were especially difficult, mostly due to its expected low magnitude coupled with the large and noisy background (dark) current. Also attempts to measure the initial rise time of the photovoltage using short-duration (8 μs) light flashes failed. This was mostly due to the small magnitude of the photovoltage together with the relatively low light intensity of the flash.

The results obtained in this set of experiments demonstrate that a reconstituted system consisting of TMFs incorporated into a planar BLM is capable of generating photovoltages if suitable electron donors and acceptors are also present.

DISCUSSION

(a) Association of TMF-vesicles with BLMS:

Thylakoid membrane fragments when incorporated into phospholipid vesicles are capable of generating light-induced electric phenomena. Moreover, when such vesicles are added to the aqueous solution on one side of a BLM in the presence of divalent cations (e.g., Ca^{2+} or Mg^{2+}), interaction between the two membranes takes place. The interaction may result in association (also referred to as "partial fusion") or fusion of the two membranes. The above conclusions are based on the direct measurement by common electrical methods of photovoltages and photocurrents across the BLM when the TMF-vesicles and Ca^{2+} (or Mg^{2+}) are present. The photoelectric responses, whose action spectra followed very closely the action spectrum of PS1 activity, also required the presence of exogenous substances. These included PMS in the aqueous environment and/or Vit K_3 in the vesicular membrane.

It appears that in the absence of osmotic gradients, the TMFs are not becoming incorporated into the BLM. This stems from the observation that the exogenous substances only act when added to the same side as the vesicles, suggesting that the TMFs do not have access to the other side of the BLM. Moreover, no changes in the background conductance of the BLM were observed. The results of the short-circuit photocurrent measurements, in the absence and presence of CCCP, demonstrated rather conclusively, that the vesicles were associated with the BLM while still maintaining their closed structures. The kind of structure that exists at the region of contact between the two membranes is not really known and can only be speculated upon. Even though the association is irreversible, it does not seem to involve rearrangement or exchange of lipids between the structures. This is inferred from the fact that such processes should induce changes in the dark conductance of the BLM (Duzgunes and Ohki, 1980) and no such changes were observed. Therefore it seems that the region of association consists of the two opposed bilayers with both hydrophilic regions in very close contact with each other. The divalent ions seem to be responsible for bringing and/or keeping the two together by shielding the negative surface charges on the surface of both, the BLM and the vesicle. Diffusion of ions through this contact region from the interior of the vesicle to the other side of the BLM maintains electrical contact between the two.

The previous results are similar to the ones obtained with other membrane fragments and components using the same approach (Barsky et al., 1976; Herrmann et al., 1978).

(b) The photoelectric response:

The generation of the photoelectric effects from such reconstituted systems required the presence of PMS and/or Vit K_3. The polarity of the photovoltage indicates that the interior of the vesicle becomes positively charged. Both of these results are consistent with the results of experiments by Barsky _et al_. (1976) using vesicle containing purified PS1 RCs. In those experiments no association of the liposomes with the planar membrane took place and the photoelectric effects were detected by means of the probe PCB^-. The photoeffects required addition of exogenous substances (eg., PMS) and resulted in uptake of PCB^-, indicating that the inside was becoming positively charged. The polarity seems to indicate that the role of PMS is that of an oxidizing agent (electron acceptor). This was confirmed by the observation that addition of ascorbate to the same side as PMS, reduced dramatically the photoeffect, apparently by reducing the PMS. This is in contrast with the observation by Barsky _et al_. (1976) that addition of ascorbate to the vesicle suspension resulted in enhanced PCB^- uptake. This result in their experiments is difficult to explain based on the observed polarity of the photoelectric effects since ascorbate is a non-penetrating electron donor.

The need for addition of such agents in the experiments here described is most probably due to the fact that during the isolation of sub-chloroplast particles many of the compounds of the electron transport chain are destroyed or lost during the extraction procedure. In the case of isolation of sub-chloroplast particles by detergent treatment or sonication of chloroplasts, for example, the O_2 evolution capacity of chloroplast is destroyed (Spector and Winget, 1980; Schmidt, Radunz, Koening and Menke, 1978) while most plastocyanin (PCy), ferredoxin (Fd), and NADPreductase are lost (Hall, 1976).

In the studies described here PMS, Vit K_3 and ubiquinone-30 were required for development and/or enhancement of the light-induced electric effects. Ubiquinone-30 and Vit K_3 are both quinones very similar or related to the natural quinones found on the electron transport chain and function as electron transport intermediates (Williams, 1977). PMS (an amine) on the other hand, is an exogenous substance which is known to interact with the PS1 reaction center (Nelson, Nelson and Racker, 1972). When added to chloroplasts, PMS can pick up an electron from the reducing side of PS1, possibly from the secondary donor (A,B) (Clayton, 1980), move across the thylakoid membrane (Soha, Izana and Good, 1970) and donate its electron to $P700^+$. Since a proton is usually transferred across the thylakoid membrane the process results in "artificial" cyclic photophosphorylation and cyclic electron flow Nelson, et al., 1972). Both Vit K_3 and ubiquinone can act as intermediate electron donors to PS1 (Hall, 1976). The presence of either agent in the vesicular membrane resulted in the development (in the case of Vit K_3 together with PMS) of the observed photovoltage and photocurrent. These photoresponses could also be reduced or abolished by agents such as o-phenanthroline, an inhibitor of electron transport (Williams, 1977) and the proton carrier CCCP.

The above results together with the observation that the action spectrum of the photocurrent follows very closely the action spectrum of PS1 is taken as evidence that the reaction center (RC) is directly responsible for the photoeffects observed in these studies. Also, the results provide direct evidence in support of the idea that light absorption and subsequent charge separation in the RC results in the establishment of a potential difference across the thylakoid membrane of chloroplasts. Moreover, the observation that electron donors acting inside the vesicle donating electrons to PS1 and electron acceptors picking up electrons from PS1 from outside confirms the hypothesis that the RC spans the thylakoid membrane.

At the same time, however, the relatively long values obtained for the rise time of the open-circuit photovoltage argues against the idea that the initial charge separation in the RC results in the generation of a potential difference in the membrane. This point cannot be answered in the affirmative by means of electrical measurements, since the initial charge separation in the PS1 RC takes place in 10 ps (Fenton, Pellin, Govindjee and Kaufmann, 1979). If the potential difference does not arise as a result of initial charge separation, however, and takes longer time to develop it could be found out by electrical measurements.

However, the reason for the relatively long value for the rise time may have arisen from the configuration of the reconstituted system. In order to investigate this more thoroughly, the electrical circuit for the configuration shown in Figure 5.13 will be analyzed. The circuit is shown in figure 5.20. In the Figure, R_v, C_v, R_f, C_f, R_m, C_m have the same meaning as in Figure 5.13. E is the photopotential generator and r_i is its internal resistance. A represents the interior of the vesicle, B the side of BLM containing the vesicles, and D the opposite side. From the circuit it can be obtained that V_{AD}, the photovoltage generated across the vesicular membrane, will be given by:

$$V_{AD} = V_{AD} \left(1^{\infty} - e^{-t/RC}\right) \tag{10}$$

where: $V_{AD}^{\infty} = RE/r_i$ represents the steady-state photopotential, $1/R = 1/r_i + 1/R_v + 1/(R_f+R_m)$, and $C = C_v + C_fC_m/(C_f+C_m)$. Assuming the internal resistance r_i is much smaller than R_v, R_f and R_m (see also Drachev et al., 1976), the measured time constant τ_p, for the open-circuit photovoltage generation will be given by:

$$\tau_r \cong r_i \ (C_v + C_mC_f/(C_m+C_f)) \tag{11}$$

Equation (11) shows that the time constant of the photopotential generation is different from the expected value $\tau_p = r_iC_v$ due to the configuration of the system.

Skulachev and his collaborators reconstituted bacteriochlorophyll (BChl) RCs in a model system consisting of BChl-vesicles associated with a lipid-impregnated collodion film. Photovoltages with a rise time faster than 0.3 μs (limited by experimental conditions) were measured which were attributed to the primary charge separation events in the RC (Skulachev, 1979). It should be mentioned however that this matter cannot be resolved from their data since the transfer from P870* to the primary acceptor I and then to the secondary acceptor Q_a (a quinone) takes place in ∼ 200 ps (Clayton, 1980).

One last point to be discussed related to the configuration of the system is that of the magnitude of the measured photovoltage. From the circuit of Figure 5.20 it follows that the measured open-circuit photovoltage across the BLM is given by:

$$V_{BD} = V_{AD}R \ /(R_m+R_f) \tag{12}$$

From the suspected nature of the region of association between the two membranes it is most likely that $R_r > R_m$. As a consequence, the measured open-circuit voltage is not higher than 1/2 the photovoltage generated across the vesicular membrane.

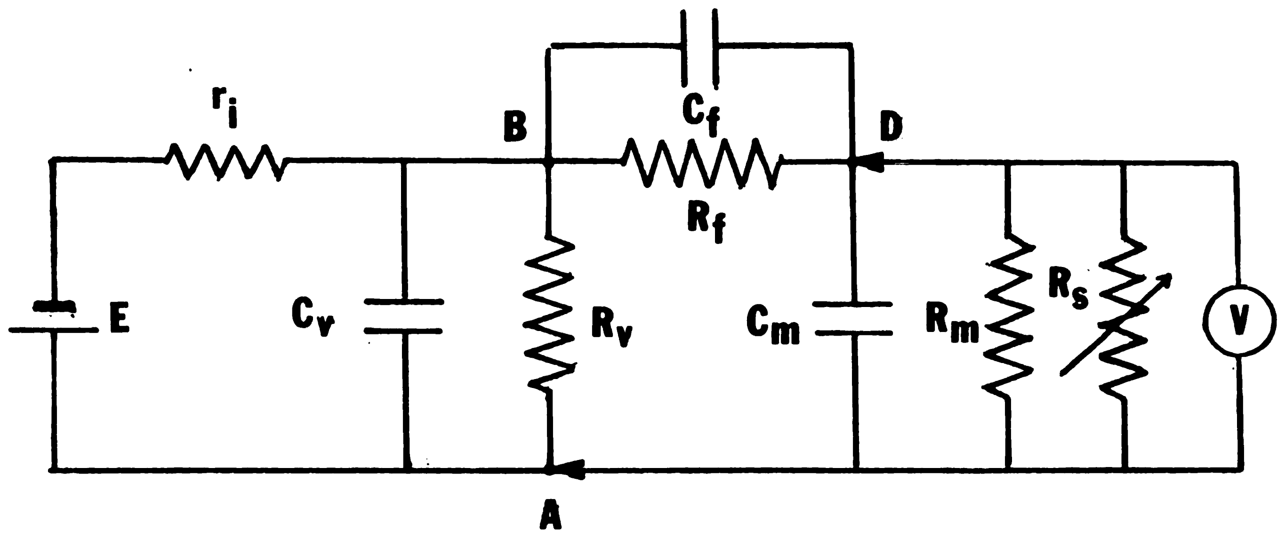


Figure 5.20 - Schematic of the electrical circuit which approximately represents the system consisting of vesicles associated with a BLM.

The circuit is similar to the one in Figure 5.14 with the exception of an external (shunt) resistance (R_S) introduced and the representation of the non-ideal current generator I, as an emf source E, in series with an internal reistance r_i.

(c) Incorporation of RC from thylakoid membranes into planar BLMs:

The results obtained in this set of experiments are interpreted as an indication that incorporation of the PS1 RC from thylakoid membranes into planar membranes takes place. This conclusion is based mainly on two observations. First, the step-wise increase in background BLM conductance suggesting that ion channels had been incorporated into it. Second, and more conclusive, the observation that the PS1 reaction center became accessible to non-penetrating agents from both sides of the planar membrane. Other investigators have also successfully incorporated proteins and membrane fragments using the same technique of fusion of hyperosmotic vesicles with planar BLMs (Miller *et al.*, 1978; Latorre *et al.*, 1982). The process seems to involve first, association of both bilayers aided by divalent cations (as in previous fusion studies) followed by mechanical stress (swelling of the vesicle induced by osmotic gradients across the vesicular membrane).

From the observation that photovoltages were generated in the presence of PMS and ascorbate by blue and red light (not by green), and by comparison with the results on partial fusion, it was concluded that the PS1 reaction center was directly involved in the process. The results are consistent with the previous experiments on TMF-vesicles associated with BLMS and provide more direct evidence that charge separation and electron transfer in the PS1 RC results in the generation of a potential difference across the thylakoid membrane. The results also provide strong evidence that the PS1 RC spans the thylakoid membrane of plant chloroplasts.

The results suggest that there are two populations of TMF-vesicles, inside-out and inside-in. This was concluded from the observation that photovoltages of opposite polarity could be obtained depending on the side to which PMS and ascorbate were added. There are several possible interpretations for this observation. One interpretation is that there is a preferred orientation of the membrane fragments in the vesicular membrane, as is the case of vesicles containing PM from <u>H. halobium</u> (Racker and Stoeckenius, 1974). Another possibility may be that since the membrane of thylakoids are asymmetric (Arntzen, Dilley and Crane, 1969; Coombs and Greenwood, 1976), more vesicles of one orientation associate with the membrane than those of the opposite orientation. Finally, it is also possible that intact vesicles may be contributing to the photoresponse increasing the magnitude of the photovoltage when PMS is present on the side containing the vesicles and staying inactive when PMS is present on the opposite side.

One last result to be discussed is that of the shape of the time course of the photopotential. The initial fast rise is attributed, as before, to the charging of the membrane due to charge separation in the RC. The decay of the photovoltage under illumination is most probably due to ionic leakage incorporated into the BLM as part of the TMF. The existence of such ion conductance pathways is evidenced by the changes in background conductance of the BLM as the TMFs become "incorporated" into the BLM. The time constant of the order of 1 s as measured from a typical response agrees with the values calculated from the measured values of membrane resistance and capacitance. The nature of the small undershoot with the subsequent return to the base line is not known at this time and more investigations will be needed to clarify this point.

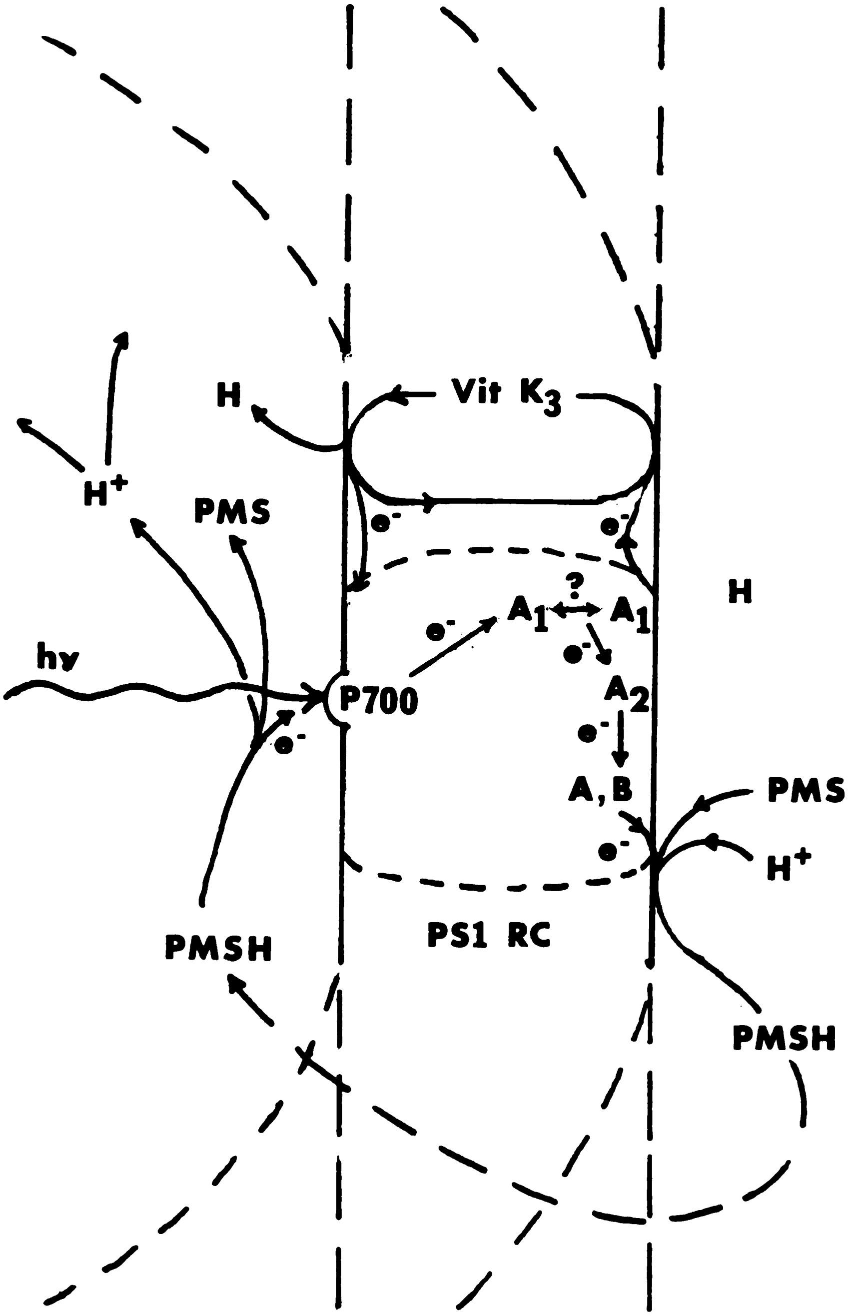
Finally, the small magnitude of the photoelectric effect in this set of experiments is probably due to the small numbers of active reaction centers incorporated into the BLM. One other possibility is that after incorporation some of the reaction centers become inactive due to their interaction with the solvent present in the membrane (Barsky _et al_., 1976).

(d) Proposed model for the photoelectric response of the two TMF-BLM-vesicle systems studied.

To explain the mechanism of the observed photoelectric response in both BLM systems studies, a model will be proposed (see Figure 5.22). It is based on the measurement of light-induced voltages generated across the TMF-BLMs in the presence of several suitable electron donors and acceptors. The nomenclature that will be used for the components of the electron transport chain in the PS1 RCs are the same used in Chapter 2.

The mechanism is as follows. When the membrane is illuminated the P700 pigment in the RC becomes excited and donates an electron to the primary electron acceptor A_1 (or X). The extent of this charge separation is not known and therefore it is not known if this first step results in the generation of a potential difference across the membrane. Subsequent electron transfer to A_2 and (A,B) takes place. From (A,B) the electron is transfered to PMS on one side of the membrane. At this stage a potential difference (first component) is already established across the membrane.

Figure 5.21 - Diagram of the proposed model to explain the possible sequence of events taking place across the thylakoid membrane responsible for the observed photoelectric response.


H
Vit K_3
H^+
PMS
e^-
e^-
H
hν
e^-
A_1
?
A_1
e^-
P700
A_2
e^-
e^-
A, B
PMS
e^-
H^+
PMSH
PS1 RC
PMSH

On the opposite side of the membrane electron donors (e.g., Vit K_3, ubiquinone-30, ascorbate, natural electron donors) reduce the oxidized P700 ($P700^+$) and protons are released on that side of the membrane. In the experiments on partial fusion these protons (H^+) diffusing from the interior of the vesicles across the planar BLM contribute to the slower component of the photovoltage. In the experiments on incorporation of the TMF into the BLM, the H^+ moving across the TMF-vesicle membrane are responsible for the decrease in the photovoltage during illumination.

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APPENDIX A

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PHOTOELECTROSPECTROMETRY OF BILAYER LIPID MEMBRANES

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Summary

Three different bilayer lipid membrane systems were studied under visible and ultraviolet illumination. The first system consisted of a bilayer lipid membrane formed with a mixture of phospholipids and cholesterol, to one side of which purple membrane fragments from *Halobacterium halobium* were added. The second system consisted of a membrane formed from spinach chloroplast extract. When either of these membrane systems was illuminated with ultraviolet and visible radiation, photopotentials were observed and photoelectric action spectra were recorded (the technique is termed photoelectrospectrometry). Each spectrum had a definite structure which was characteristic of each of the modified membranes. The third system studied consisted of an otherwise photoinactive membrane formed with a mixture of phospholipids and cholesterol, to one side of which chymotrypsin was added. When the membrane was illuminated with visible light no photoresponse was observed. On the other hand, a photopotential which increased with incubation time was observed when the membrane was illuminated with ultraviolet light. Since, in our systems, the photoresponses have been observed to be due to certain species incorporated into the membrane, it appears that photoelectrospectrometry is a useful tool for studying lipid-protein interactions, constituent organization and energy transfer in membranes.

Introduction

Pigmented membranes in living cells serve one of the two vital functions, energy transduction and signal detection. In the case of the former, the thylakoid membrane of chloroplasts is the best example, whereas the sac membrane of the rod outer segments in the vertebrate eye typifies the latter. The purple membrane of *Halobacterium halobium* appears to be capable of either function in response to environmental conditions [1]. Since biological membranes are a complex assembly of lipids, proteins and other constituents such as pigments and are difficult to study at the molecular level, model membranes have instead been resorted to by numerous investigators [2,3]. Of the many model membranes, the artificial bilayer lipid membrane of planar configuration is particularly pertinent as a model because its organization is similar to that of the lipid bilayer believed to exist in biological membranes [4].

In recent years, methods have been developed for studying pigmented bilayer lipid membranes separating two aqueous solutions [4,5]. Photoactive compounds such as chlorophylls [6—10], retinals [11—13] bacteriorhodopsin [14—16] and a variety of dyes [4,17—19] have been incorporated into these model membranes. To characterize this bilayer lipid membrane system, elegant spectroscopic techniques have been developed [4,6,8], which provide information on the composition and organization of constituent molecules in the bilayer lipid membrane. In this paper we report the study of bilayer lipid membranes in the ultraviolet and visible region, thereby extending the usefulness of membrane photoelectrospectrometry [4]: in particular, photoeffects from bilayer lipid membranes made of chloroplast extract, containing purple membrane and also bilayer lipid membranes to one side of which chymotrypsin has been added, when irradiated with ultraviolet light. In all three cases, open circuit photovoltage action spectra which followed the absorption spectrum of the modifier were obtained.

Materials and Methods

Chlorophyll-containing bilayer lipid membranes. A bilayer lipid membrane solution containing chlorophyll pigments in butanol/*n*-octane (1 : 1) was obtained from fresh spinach leaves by a procedure described elsewhere [4]. The aqueous solution consisted of 0.1 M acetate buffer at pH 5.

Bacteriorhodopsin-containing bilayer lipid membranes. The membrane-forming solution for the bacteriorhodopsin experiments consisted of either a 1 : 1.7 : 3.1 : 1.1 mixture of phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine/cholesterol in *n*-octane to a final concentration of 6.9% (w/w) or a 3.5% (w/w) *H. halobium*: phospholipids mixture in *n*-decane. The aqueous solution for these experiments consisted of 75 mM KCl + 25 mM $CaCl_2$ (unbuffered, pH ≈ 6.8).

Phospholipid vesicles containing purple membrane fragments were prepared from egg lecithin following a procedure described by Blok et al. [20]. After the membranes were formed, vesicles containing purple membrane were added to one side of the bilayer lipid membrane.

Bilayer lipid membranes with chymotrypsin in the aqueous solution. The membrane-forming solution consisted of either a 1 : 1.7 : 3.1 : 1.1 mixture of phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine/cholesterol in *n*-octane to a final concentration of 6.9% (w/w) or a 1 : 1 mixture of phosphatidylcholine/oxidized cholesterol in *n*-octane to final concentration of 2% (w/w).

The membranes were formed in $1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$ and $1 \cdot 10^{-1}$ M KCl. After the membranes had thinned, 200 μl of a 5% chymotrypsin solution in double-distilled water was added to one side of the membrane (vol. 8 ml).

Methods

The technique used in these studies is essentially the same as that described previously [4]. Bilayer lipid membranes were formed in a 1-mm aperture of a Teflon cup separating two aqueous solutions. The cup was placed in a plexiglass chamber with a quartz window. Electrical contact between the two aqueous solutions was made with a pair of calomel electrodies via salt bridges. The photopotentials were measured with a 610BR Keithley electrometer connected to a VOM 6 Bausch and Lomb chart recorder. Closed-circuit measurements were performed by applying a potential through an external resistance 10—20 times larger than the dark resistance of the membrane. The photovoltage action spectra in the ultraviolet and visible regions were obtained by illuminating the membranes with light from a 1000 W Xenon lamp after passing through a 250 mm Bausch and Lomb grating monochromator equipped with a 600 grooves/mm diffraction grating. The light from the lamp was focused onto the entrance slit of the monochromator by means of a quartz lens. The exciting radiation from the monochromator was focused on the 1 mm hole in the Teflon cup by means of a second quartz lens. The entrance and exit slits of the monochromator were both set at 3.0 mm. The curves were corrected for constant incident illumination with a Scientific Instrument Kettering radiant power meter. The absorption spectra were measured with a DB Beckman spectrophotometer. The photoresponses from the membranes were all recorded once the membranes had reached the 'black' stage and their resistance was constant. All of the experiments reported here were carried out at 22 ± 1°C.

Results

Chlorophyll-containing membrane

After the membrane had thinned to the black state and its dark resistance has reached a steady value of approx. $1 \cdot 10^8\ \Omega$, it was irradiated with light of 300 nm wavelength. A small photopotential (0.5—2.0 mV) negative on the illuminated side was observed under open-circuit conditions. A similarly small photopotential was observed (as had been reported earlier [21]) when the membrane was illuminated with light of wavelengths 450 nm (1—4 mV) and 675 nm (0—5 mV). The characteristics of the response at the three different wavelengths were the same: a fast rise in the potential, reaching a maximum value under constant illumination and a relatively fast decay when the light was turned off. An open-circuit photovoltaic action spectrum was difficult to record due to the small values of the photopotential. The difficulty was increased by the bleaching of the chlorophyll pigments which became evident after eight or ten subsequent illuminations.

Since the photopotentials were very small, it was decided to impose some asymmetrical conditions across the membrane to see if the response could be enhanced.

A membrane was formed and after it had reached the 'black' state (membrane dark resistance approx. $1 \cdot 10^8\ \Omega$), a potential of 35 mV was applied across the membrane. When the membrane was illuminated with 300 nm radiation, a closed-circuit photoresponse of 14 mV with opposite polarity to the applied potential was observed. A spectrum of the closed-circuit photoresponse in the ultraviolet as well as in the visible regions was recorded. It should be mentioned that, under the conditions of externally applied potential, the bleaching of the chlorophyll pigments was much stronger than that under the open-circuit conditions. It was therefore difficult to record a complete spectrum for the whole region (250—700 nm) for a single membrane. The reproducibility of the photoresponses for different membranes at a particular wavelength was poor. On the other hand, if the photoresponses were obtained for a single membrane at three different wavelengths only (e.g., 300, 450 and 675 nm) the relative ratio of their values had good reproducibility. Calculating similar ratios for different wavelengths and comparing them with those obtained for the open-circuit measurements it was found that the two spectra were fairly similar.

Since addition of electron acceptors to one side of these membranes is known to enhance the photoresponse in the visible region [22], it was decided to investigate its effect under ultraviolet illumination. A bilayer lipid membrane was formed on the Teflon cup and after reaching the black state, 250 μl of 0.1 M $FeCl_3$ solution was added to one side of the membrane (final concentration $3 \cdot 10^{-3}$ M). It was observed that the value of the membrane resistance increased from $1 \cdot 10^8$ to approx. $5 \cdot 10^8\ \Omega$. When the membrane was illuminated with light of 300 nm wavelength, an open-circuit photopotential of up to 100 mV could be obtained. The photopotential was negative on the side containing the $FeCl_3$. It was observed that, when $FeCl_3$ was present on the side of the membrane facing the illuminating radiation, the ultraviolet response was almost zero whilst the visible response could still be observed. In photovoltaic action spectra measured this way the ultraviolet portion was completely removed, and at very high $FeCl_3$ concentrations (above $1 \cdot 10^{-3}$ M) the blue peak at 450 nm was observed to decrease relative to the 670 nm peak. This was found to be due to the strong absorption of $FeCl_3$ on the 200—400 nm region, thereby causing a reduction in the intensity of the ultraviolet light incident on the membrane. The results of control experiments involving the simultaneous presence of $FeCl_3$ on both sides of the membrane would then not be valid. No photoresponse is obtained when equal concentrations of $FeCl_3$ are present at the same time on opposite sides of the membrane under visible illumination [22]. By extension we expect the same to hold true under ultraviolet illumination.

A photovoltaic action spectrum in the range 250—700 nm of a chloroplast extract membrane in the presence of $FeCl_3$ is shown in Fig. 1.

The effect of electron donors was also investigated by adding 250 μl of 0.1 M ascorbate to one side of the membrane. When the membrane was illuminated by light with a wavelength of 300 nm an open-circuit photoresponse could be observed. An action spectrum was recorded (see Fig. 2) in the range 250—700 nm. The side containing the ascorbate was positive. Similar to the experiments involving $FeCl_3$, it was observed that if ascorbate was present in the outer chamber, the ultraviolet portion of the spectrum was much smaller and had a different shape to that when ascorbate was present in the

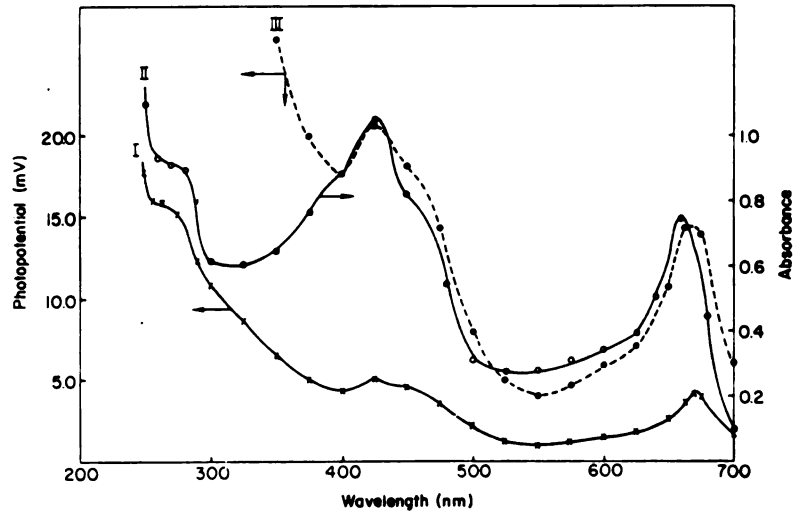

Fig. 1. Curve I (X———X): open-circuit photovoltaic action spectrum of a chloroplast-bilayer lipid membrane in the presence of $FeCl_3$. Curve II (○———○): absorption spectrum of chloroplast-bilayer lipid membrane-forming solution. Curve III: Curve I X4.

inner chamber. On the other hand, the visible photoresponses were similar in shape and magnitude when ascorbate was present in either chamber. Again this was observed to be due to the absorption of ultraviolet light by ascorbate.

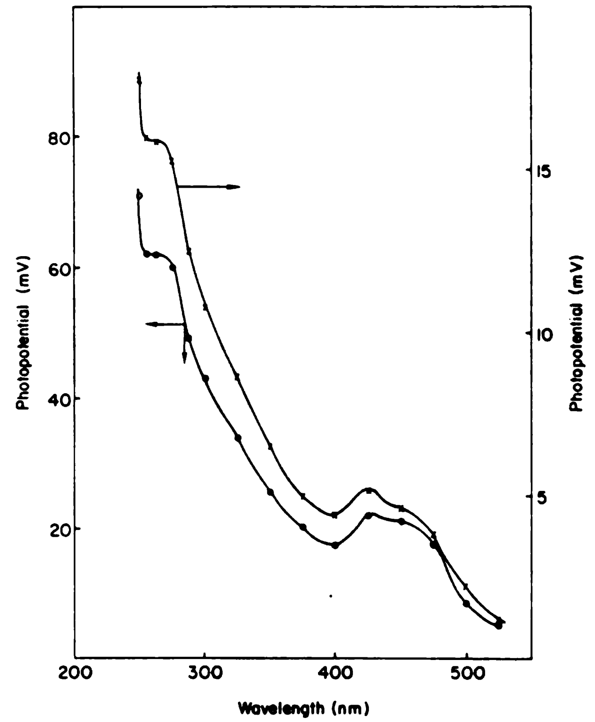

Fig. 2. Comparison of the action spectrum of a chloroplast-bilayer lipid membrane in the presence of ascorbate (X———X) and in the presence of $FeCl_3$ (○———○).

Therefore, the results of control experiments where ascorbate is present simultaneously on both sides of the membrane are not valid for ultraviolet measurements. However, these experiments have been performed for visible illumination and they show that under symmetric conditions there is no photoresponse [4]. We expect the same to hold true for ultraviolet illumination.

Since $FeCl_3$ and ascorbate both absorb in the ultraviolet, experiments could not be successfully performed with the two modifiers present simultaneously on opposite sides of the membrane under ultraviolet illumination. These experiments have already been performed for visible light and they show that a maximum photoresponse is obtained under these conditions [4]. For the same reason, all the results reported above are for $FeCl_3$ or ascorbate in the inside chamber.

In another set of experiments, membranes made up of a mixture of phospholipids and cholesterol in *n*-octane (see Materials and Methods) were formed in 0.1 M acetate buffer at pH 5. After the membrane had thinned to the black state, $FeCl_3$ or ascorbate was added to the inner chamber to a final concentration of up to $5 \cdot 10^{-3}$ M. Larger concentrations made the membranes unstable. The presence of $FeCl_3$ was observed to increase the dark resistance of the membrane from approx. $1 \cdot 10^8$ to approx. $1 \cdot 10^9\ \Omega$. Ascorbate did not show a definite effect on the dark resistance of the membrane. When these membranes were illuminated with ultraviolet or visible light in the presence of $FeCl_3$ or ascorbate, no photoresponse could detected. As mentioned earlier, we also observed an increase in the dark membrane resistance for the chloroplast membrane in the presence of $FeCl_3$. On the other hand, it has been reported that the dark membrane resistance of an oxidized cholesterol membrane can decrease by up to two orders of magnitude in the presence of $FeCl_3$ [23]. No experiment was performed with the non-pigment components of the chloroplast extract in the presence of $FeCl_3$ or ascorbate.

Bacteriorhodopsin-containing bilayer lipid membranes

Membranes were formed in a 75 mM KCl + 25 mM $CaCl_2$ aqueous solution and after the membranes had thinned to the black state, lipid vesicles containing purple membrane were added to one side of the bilayer lipid membrane. After 5 min, small photoresponses could be observed when the membranes were illuminated with visible light (560 nm). After 45—50 min, when the photoresponses had leveled off to their maximum values, the membranes were illuminated with ultraviolet light (300 nm) and photoresponses of approx. 25 mV were observed. An open-circuit photovoltage action spectrum was obtained in the range 250—700 nm. When the membranes were illuminated with ultraviolet light in the absence of the vesicles no photoresponse could be observed. A decrease in the membrane dark resistance of up to one order of magnitude was observed for the membranes in the presence of the vesicles.

Bilayer lipid membrane with chymotrypsin in the aqueous solution

Membranes were formed in $1 \cdot 10^{-4}$ M KCl. After the membrane had thinned to the black state, 200 μl of a 5% chymotrypsin solution were added to one side of the membrane (vol. 8 ml). After 10 min a very small photopotential was observed when the membrane was illuminated with ultraviolet light. After 40 min a maximum photoresponse of approx. 10 mV was observed. An open-circuit photovoltage action spectrum was recorded in the range 250—450 nm. No photoresponse was observed when the membrane was illuminated with visible light. A bilayer lipid membrane was formed in a 10 mM KCl solution and a smaller photoresponse than before was observed when the same amount of chymotrypsin was added. The photoresponse was still smaller in a 0.1 M KCl solution. No photoresponse could be observed in the absence of chymotrypsin.

Discussion

Bilayer lipid membranes formed from chloroplast extracts exhibit photoresponses when illuminated with ultraviolet light. Small open-circuit photoresponses were observed under chemically symmetric conditions presumably due to differences in the absorption of the incident light between the two layers of the membrane. The fact that these photoresponses are not due to thermal effects is supported by the fact that the light intensity incident on the membrane was very small and also by the fact that the 'thermoelectric power' of the chloroplast membrane was also very small, approx. 60 μV/K over the range 15—40°C [4]. Closed-circuit photoresponses were also obtained under an externally applied electric field. This photoresponse could be interpreted as being due to a photoconductive effect, however, since an open-circuit photoresponse had also been observed, the photoresponse cannot be totally due to a change in the conductance. Furthermore, the fact that both action spectra were very similar indicates that the photoconductivity effect should be very small.

The action spectra obtained under closed- and open-circuit conditions also followed the absorption spectrum of the chloroplast membrane in the range 250—700 nm. This indicates that the primary event involved in the production of the potential is the absorption of light by the pigments present in the membrane.

In the presence of $FeCl_3$ and ascorbate, the action spectra are very similar (see Fig. 2) and both showed an enhancement of the ultraviolet part relative to the visible part (see Fig. 1). The fact that the photoresponse in the ultraviolet can be enhanced by the addition of electron donors or acceptors suggests the possibility that the nature of the charge separation in the ultraviolet involves energy transfer and redox reactions on opposite sides of the membrane. Coupled redox reactions taking place on opposite sides of the membrane suggest the possibility of electronic conduction through the membrane. That electronic conduction takes place in these bilayer lipid membranes was definitively established [4,11] after experiments were performed in which the membranes were excited by light flashes of microsecond duration. Photosensitization experiments involving this particular chloroplast bilayer lipid membrane were also successively performed using different dyes [4,18]. In these experiments, both types of sensitization, intrinsic as well as extrinsic were observed.

In the case of the bilayer lipid membrane containing purple membrane, the fact that the action spectrum in the ultraviolet follows the absorption spectrum of the bacteriorhodopsin in that region (see Fig. 3) indicates that those species in the bacteriorhodopsin molecule which absorb ultraviolet light are involved in charge separation. Three possibilities are: (1) charge separation takes place by

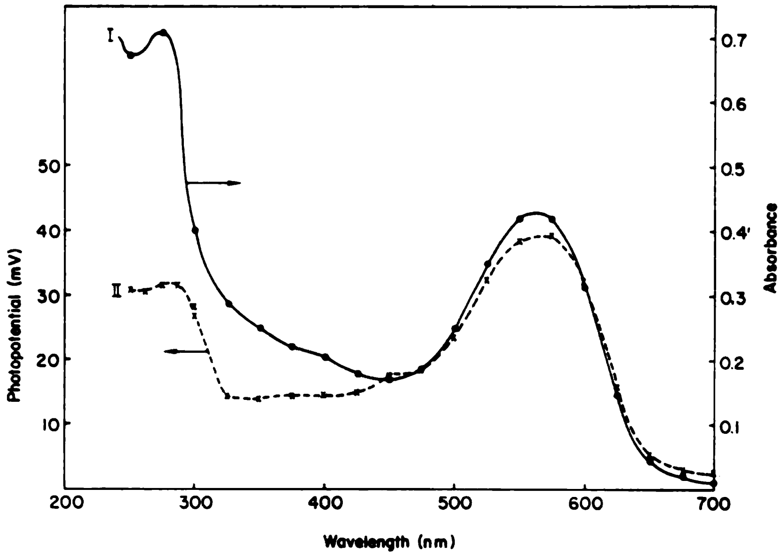

Fig. 3. Curve I (●———●): absorption spectrum of purple membrane from *Halobacterium halobium*. Curve II (X- - - - -X): open-circuit photovoltaic action spectrum of a bilayer lipid membrane-containing purple membrane.

direct action of the absorbing species; (2) charge separation is mediated by the absorbing species probably through energy transfer [26] or (3) both 1 and 2 are occurring simultaneously (some recent results give strong evidence for the second or third possibility). From Fig. 3 it can be seen that the relative intensity of the ultraviolet part of the spectrum is about half that of the visible. Therefore, not all the ultraviolet absorbing species in the protein can induce charge separation. This indicates that the nature, relative position, orientation and environment of the absorbing species may be involved.

The separation of charges across the membrane due to direct absorption of ultraviolet light by a protein was also observed in the case of the association of chymotrypsin with the bilayer lipid membrane. Again, in this case, the photoelectric action spectrum followed the absorption spectrum of the chymotrypsin (Fig. 4). The fact that the protein binds to the membrane is suggested by the fact that the photoresponse is not observed immediately after addition of the protein but after some time and that it increased with incubation time. This is also suggested by the fact that no photoresponse is observed at high salt concentrations (above 0.1 M KCl) which can be explained in terms of shielding of the charges present in the membrane and protein interface by counter-ions.

There are a number of questions that need to be addressed: (i) the number of photoactive species in the membranes; (ii) the orientation, location and conformation of constituent molecules in the bilayer lipid membrane, and (iii) the mechanisms by which energy and charges are transported. The answers for the first two questions may be found in a number of papers [4,6,8,22]. In the case of chlorophyll, for example, the maximum concentration is approx. $1 \cdot 10^{-10}$ mol/cm^2, with the porphyrin plate at an angle of approx. 45° with respect to the plane of the membrane. The hydrophobic phytol chain is anchored in the

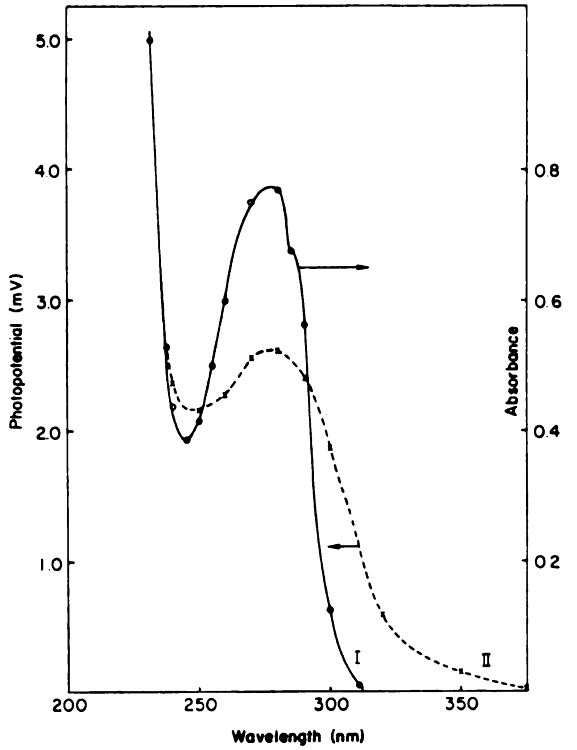

Fig. 4. Curve I (o——o): absorption spectrum of chymotrypsin in distilled water. Curve II (X- - - - - -X): open-circuit photovoltaic action spectrum of bilayer lipid membrane to one side of which chymotrypsin was added.

lipid bilayer. The porphyrin ring of the chlorophyll molecule is depicted to be among the polar head groups of lipid molecules in contact with the aqueous solution [24].

In the bacteriorhodopsin and chymotrypsin bilayer lipid membrane systems described here, no definite information on molecular organization as yet is available.

As mentioned earlier, in the chlorophyll-containing bilayer lipid membrane, the charge carriers are electrons [4,11] and the observed photoelectric phenomena are primarily due to the membrane/electrolyte biface [4,5,27]. The observed photovoltage is influenced by the redox compounds (electron acceptors and donors) present in the bathing solution [24,28]. To explain the observed photoelectric effects, the pigmented bilayer lipid membrane has been considered to be an organic semiconductor as shown in Fig. 5. The situation is quite analogous to that of a Schottky barrier except there are two interfaces [24]. That the observed photoelectric phenomena are primarily due to the bilayer lipid membrane/electrolyte biface and not the plateau-Gibbs border has been definitely established [4,5,27]. In Fig. 5, one side of the membrane is depicted as p-type, hence it acts as a photocathode and the other side is n-type (photoanode). In the bilayer lipid membrane/electrolyte interface, the aqueous solutions play the role of the metal. Thus, the energetics of the interface at

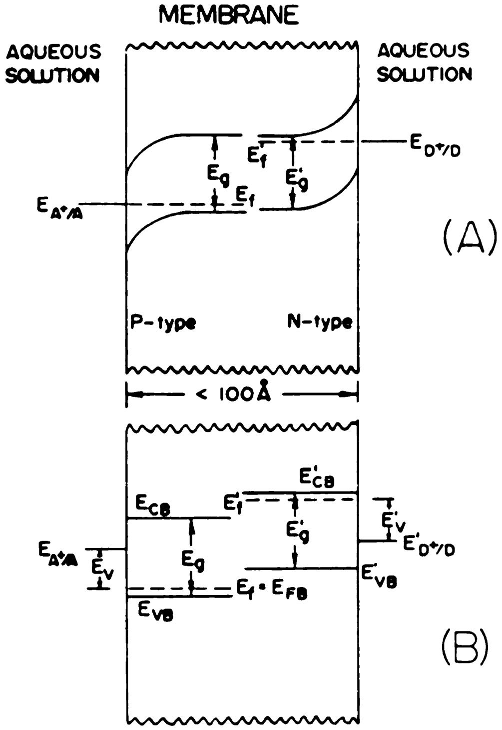

Fig. 5. Schematic diagram of a pigmented bilayer lipid membrane separating two aqueous solutions. (A) the membrane is in equilibrium with redox compounds in the bathing solutions in the dark. (B) the bilayer lipid membrane is under illumination.

equilibrium in the dark and in the light under open-circuit conditions are shown in Fig. 5A and Fig. 5B, respectively. E_g is the band gap, E_f is the Fermi level. $E_{A+/A}$ (or $E_{D+/D}$) is the electrochemical potential of the solution, E_{CB} and E_{VB} are the respective conduction and valence band positions. The symbols for the right-hand side are primed. The observed photovoltage, $\Delta V(E_V + E'_V)$ is obviously influenced by the redox compounds (electron acceptors and donors) present in the bathing solutions [24,28]. It should be pointed out that photogenerated electron-hole pairs in the bilayer lipid membrane result in current flow, except, unlike the metal in a Schottky-type cell, the aqueous solution is not an electronic conductor. Therefore, redox reactions must take place at the two interfaces in order to complete the circuit. A more detailed discussion on redox reactions and on the semiconductor model of charge separation and transport in pigmented lipid membranes has been published elsewhere [28,29]. It should be mentioned that in the case of bacteriorhodopsin-containing bilayer lipid membrane, photogenerated H^+ (and OH^-), owing to their small size, are unique and may act as primary charge-carriers in the membrane.

The major finding reported in this paper is that conventional ultraviolet as well as visible spectroscopy can be readily combined with photoelectric measurements in the bilayer lipid membrane system. The resulting technique, called bilayer lipid membrane 'photoelectrospectrometry' [30], should offer an approach to the study of energy transfer between lipids and proteins [31] and membrane reconstitution experiments. Some experiments of this type are in progress.

Conclusions

Charge separation can occur in pigments and proteins present in bilayer lipid membranes when they are illuminated with ultraviolet light. This allows for ultraviolet photoelectric action spectra to be recorded. The photoresponses obtained in the ultraviolet and visible regions for a chloroplast extract bilayer lipid membrane and bilayer lipid membrane-containing bacteriorhodopsin appear to be due to independent processes and exhibit different quantum efficiencies. Chymotrypsin can bind electrostatically to a bilayer lipid membrane and this association allows for charge separation across the membrane when it is illuminated with ultraviolet light. A photopotential action spectrum of the protein and bilayer lipid membrane follows the absorption spectrum of the protein. This result suggests the possibility of studying lipid-protein interactions in membranes through the extension of the spectroscopic studies of a bilayer lipid membrane into the ultraviolet region which is where most proteins absorb light.

The fact that the ultraviolet part of the spectrum, in the case of the chloroplast extract bilayer lipid membrane, is enhanced by the asymmetric addition of electron acceptors and donors suggests the possibility of redox reactions taking place on opposite sides of the membrane. The technique, termed photoelectrospectrometry, is at least four orders of magnitude more sensitive than absorption spectroscopy and is useful in the investigation of lipid-protein interactions and energy transfer studies in membranes.

Acknowledgements

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APPENDIX B

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284 - H. Halobium : I. *In vitro* Studies

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Summary

1. Purple membrane fragments (PM) from *H. halobium* were incorporated into lipid membranes either directly or *via* liposomes. A photoresponse was detected when liposomes containing PM were fused with lipid membranes. Low-resistance membranes or membranes shunted with an external resistor of 10^9 ohms showed decay of the initial light response to some equilibrium value in the light in both presence and absence of octadecylamine. The light response could be abolished by the addition of a sufficient amount of triethylamine to either side of the membrane.

2. The photovoltage action spectra of bilayer lipid membranes containing PM either directly or *via* liposomes were measured, and found to follow the absorption spectrum of bacteriorhodopsin.

3. Liposomes containing bacteriorhodopsin (BR) in PM extract of *H. halobium* were fused to one side of planar lipid membranes. The photopotential resulting from flash excitation rose and fell as the sum of three exponentials with time constants for the leading edge of 30 ± 10 μs and 35 ± 10 ms. The decay time constant of the photopotential was 840 ms, a value consistent with the membrane time constant given by the membrane dark resistance and capacitance.

Introduction

H. halobium, in which the so-called *purple membrane* is located, was first noted on dried salted fish in about 1900 in Scandinavia. This bacterium required a near-saturated salt solution for growth. The biochemical aspects of extreme halophilism displayed by these organisms have been reviewd by Larsen.[1] The purple color of *H. halobium* is due to a rhodopsin-like complex in the plasma membrane, termed bacteriorhodopsin, that develops when the cells are grown in the absence of a suitable source of nutrients or at low oxygen tension.[2] Bacteriorhodopsin, upon absorption of light, undergoes a photoreaction cycle which involves the translocation of protons, thereby generating a proton gradient that can be used as the driving force for ATP synthesis in accordance with the chemiosmotic hypothesis.[3] Recent work on *H. halobium* and its derivatives, purple membrane (PM) and bacteriorhodopsin (BR), have been comprehensively reviewed by OESTERHELT[4] and by SCHRECKENBACH.[5] Studies by spectroscopic techniques have elucidated the photochemistry of BR and PM.[6-8] Other studies include phase transitions by differential scanning calorimetry,[9] light-induced pH changes and ATP synthesis,[2,10-14] reconstitution experiments,[15-17] and incorporation of BR and PM into artificial lipid bilayers of both planar (bilayer lipid membranes or BLM) and spherical (lipid microvesicles or liposomes) configuration as well as millipore filters.[18-24]

Although much is known about the purple membrane and its derivative, bacteriorhodopsin of *H. halobium*, the mechanism of proton translocation by BR in the light remains obscure. However, it is generally accepted that the purple membrane (bacteriorhodopsin) acts as a light-driven proton pump and transports protons vectorially from *inside* the cell to the surrounding medium. That is, during the course of each photoreaction sequence, BR first releases protons on the extracellular side of the purple membrane and then picks them up on the cytoplasmic side.[4,5] This generally accepted direction of proton movement, however, has been recently questioned on the basis of published findings.[25] We have, therefore, carried out both *in vivo* and *in vitro* studies on *H. halobium*, regarding both the direction of proton movement and certain physical properties of BLM and liposomes containing the purple membrane hitherto unreported.[26]

Experimental

Materials

Halobacterium halobium was grown in two-liter flasks under intense illumination from fluorescent tubes using essentially the method described by BECHER and CASSIM.[27] The medium contained in 1000 cm^3 : NaCl, 250.0 g ; magnesium sulfate, 20.0 g ; KCl, 2.0 g ; $CaCl_2 \cdot 2\,H_2O$, 0.2 g ; sodium citrate, 3.0 g ; SIGMA peptone No. P–8388, 10.0 g or DIFCO Yeast Extract (0127–03), 5.0 g. Each culture was inoculated with 200 cm^3 of cells from a previous culture. The cells were grown with 15 cubic feet of air per hour bubbled through the culture for 5 days or until the end of log phase. Then the cells were aerated at the rate of 3 cubic feet per hour for 3 days. After that the cells were harvested. They were spun down and washed twice in basal salts (first 4 items listed above for the medium) before use.

The purple membrane (PM) was isolated by dialyzing *Halobacterium halobium* cells overnight against 0.1 *M* NaCl and then centrifuging the material from the dialysis bag at 31,000 g for 30 to 45 minutes. The red supernatant was then decanted and the pellets used as purple membrane.

The membrane-forming solution was prepared in the following manner : lipids were extracted from whole cells with 2 : 1 chloroform : metanol. The cells were homogenized with the chloroform : methanol in a blender for 3 minutes. The preparation was then filtered (No. 42 paper) and the filtrate flash-evaporated and redissolved in *n*-octane or *n*-decane. These lipids were combined in a 1 : 1 mixture with a 2 % solution of phosphatidyl choline in *n*-octane or *n*-decane to make the BLM-forming solution.

Incorporation of purple membrane (PM)

For planar bilayer lipid membrane (BLM) studies, either the PM was incorporated directly into the membrane-forming solution or lipid microvesicles (liposomes) containing PM were allowed to fuse with the BLM. The liposomes were made by drying enough egg lecithin (SIGMA) to form a 1 % solution on the inside of a round-bottomed flask. Purple membrane dissolved in 0.15 *M* KCl was then added and the solution dispersed on a vortex mixer in the presence of small glass beads. This suspension was then sonicated (BRANSON model W-140D) at high power (60-70 watts) under nitrogen atmosphere and at low temperature (ice bath) for 1 hour (15 seconds of sonication followed by 45 seconds coooling interval).

Standard bathing solution and optical cells[28] held the membranes for the other measurements. A planar membrane was formed and electrical and photoelectrical properties were measured in the usual manner.[28] Only for experiments in which the PM was incorporated into the membrane were light-induced voltages observed. Action spectra were obtained using a halogen lamp as a source of excitation. The white light from the lamp was focused by means of a lens onto a BAUSCH and LOMB high intensity grating monochromator. The monochromatic light was then focused upon the BLM in the teflon cup by means of a microscope lens. The photovoltage was measured with a KEITHLEY INSTRUMENTS Model 610 BR electrometer and the spectra were recorded of a KEITHLEY INSTRUMENTS Model 370 recorder. Flash illumination of 10^9 lumens/cm^2 from a xenon flash lamp and associated circuitry (GENERAL RADIO STROBOSLAVE, Type 1539-A and lamp) was used to study fast photoresponses. Electrical measurements were made through calomel electrodes connected by short leads to a high impedance (10^{12} Ohms), unity gain buffer amplifier of time contant 8 μs. Membrane potential was observed and recorded on a storage oscilloscope (TEKTRONIX R5031) and on a chart recorder (BAUSCH and LOMB VOM6). For all electrical measurements, the electrode immersed in the inner cup went to the high side of the buffer amplifier or electrometer, so that the sign of the voltages is that of the inner chamber.

When PM was incorporated by way of liposomes the usual procedure was to form the membrane, add the liposomes to the inner chamber, and wait until a stable photovoltage was reached. This waiting time depended upon the concentration of liposomes and stirring of the bath; it varied from 10 minutes at the highest concentration when the bath was stirred to a maximum of 250 minutes when the concentration was diluted 10–fold and the bath was not stirred.

Photoeffects in Purple Membrane – BLM

The observed photoeffect when liposomes containing PM are fused with BLM's can be understood in terms of protons being pumped from the inner chamber (to which liposomes were added) to the outer chamber in the presence of light. Fig. 1 shows the open–circuit photoresponse for a membrane containing 0.0025 % octadecylamine (ODA). Previously, Karvaly and Dancshazy[18] have reported that the presence of ODA in the bilayer lipid membrane resulted in a drastic increase of the protein binding to the BLM. Further, they reported that BLM containing the PM exhibited both a large–amplitude photovoltaic effect and photoconduction. The polarity of the compartment with the PM was negative upon illumination for all the systems investigated, indicating that at least for the PM–BLMs protons probably move across the membrane as suggested by several authors (for reviews, see Refs. 4 and 5). The am-

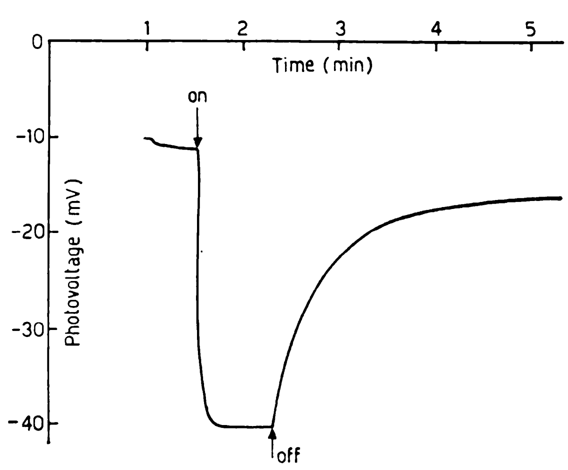

Fig. 1.

Photoresponse seen for BLM made from 1 : 1,2 % phosphatidyl choline and lipid extract from *H. halobium* with 0.0025 % octadecylamine in *n*–octane. R_m was 7×10^9 Ω. Bathing solution was 75 m*M* KCl and 50 m*M* $CaCl_2$, and 100 mm^3 (µl) liposomes with PM added to the inner chamber were allowed to fuse for 4 hours. The dark potential was —10 mV and the photoresponse was —30 mV. No light–decay or dark–overshoot were seen.

plitude of the photovoltaic response was of the order of 20 to 60 mV, depending upon the concentration of the incorporated PM and the intensity of the light. Fig. 2*B* shows that when the membrane of Fig. 1 is shunted by a $10^9\ \Omega$ resistor both light–decay (decay of voltage under illumination) and dark–overshoot potential (upon removal of illumination) can be seen. Also, the lower the resistance the lower was the maximum response. We have fully confirmed the observations of KARVALY and DANCSHAZY[18,22]. Further, Fig. 2 (*A* and *C*) shows the effect of an applied potential. Making the inner chamber, containing the PM liposomes, negative with respect to the outer chamber decreased the maximal response while enhancing the light–decay and dark–overshoot. Positive applied potentials enhanced the maximal response while reducing the light–decay and the dark–overshoot potentials.

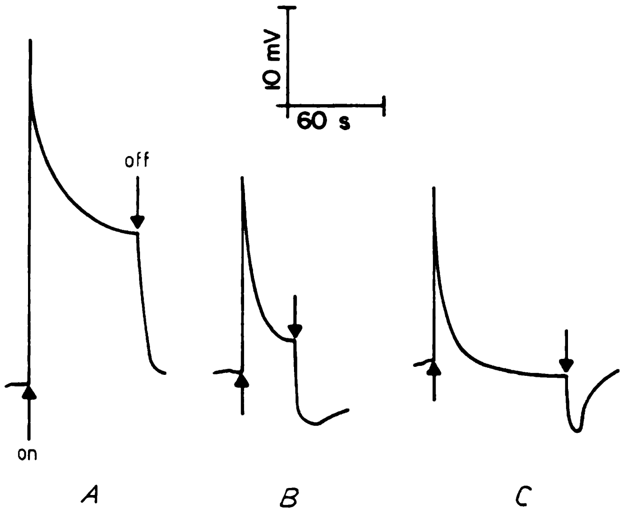

Fig. 2.
Photovoltages of shunted BLM. Same BLM and bath as described in Fig. 1 shunted by $10^9\ \Omega$ external resistor and subjected to applied potentials. The up arrows mean the light is turned on and the down arrows indicate the light is turned off. Three waveforms show the effects of (*A*) +54 mV (*B*) 0 mV, and (*C*) —54 mV applied potential on the photoresponse. All the photovoltages are negative in sign.

In the absence of octadecylamine both the open–circuit and shunted BLM gave photovoltage waveforms that also exhibited light–decay and dark–overshoot potential, with shapes like that of Fig. 2*B* (see also Fig. 5). Both effects were enhanced by lowering in either membrane resistance (R_m) or shunt resistant (R_s).

The effects of octadecylamine, resistance, and applied potential can be understood if the light–decay and dark–overshoot are due to the variable permeability of the membrane to protons and the possible presence of an external circuit for electrons. The electrons, attracted by the electrostatic charge of the increased proton concentration in one chamber, will flow *via* the external circuit (shunt resistor) into that chamber. In the absence of an electronic pathway (no external shunt), the ODA prevents light–decay by providing a positively charged membrane which prevents protons from flowing back across the membrane. The high resistance membranes tend to do the same, and the positive applied potentials also hinder the back proton flow. Triethylamine, an electron and proton donor, was found to short out the photoresponse when added to either the inner or the outer chambers. It is thought, according to the above scheme, that triethylamine could be transporting or releasing either protons or electrons to short out the response.

Spectroscopy of purple membrane–BLM

Planar bilayer lipid membranes containing purple membrane (PM) incorporated by liposome fusion on one side of the membrane exhibited a large amplitude fast photovoltage response between 15 and 50 mV at constant monochromatic illumination, depending on the amount of li-

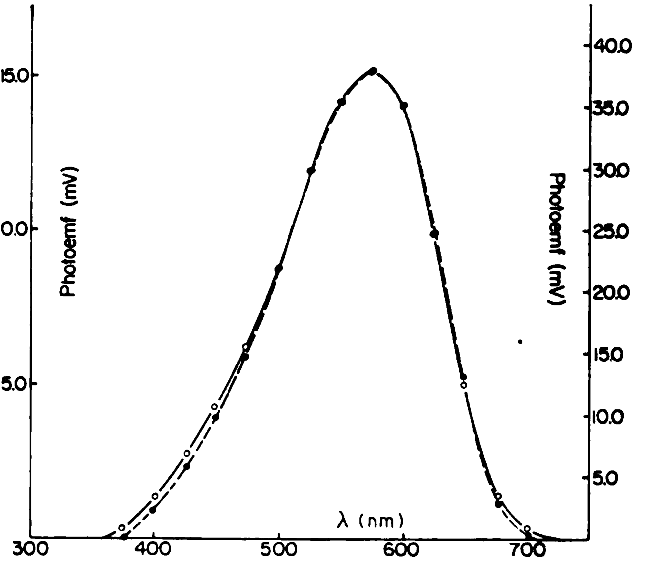

Fig. 3.

Open–circuit photovoltaic action spectra. PM has been incorporated into BLMs by two different methods : vesicle fusion (●, right–hand scale) and dissolving directly into the membrane forming solution (○, left–hand scale). All other experimental conditions were the same for both membranes : basic BLM forming solution of 1 : 1 2 % PC in decane : *H. halobium* extract, aqueous bath 75 m*M* KCl and 50 m*M* $CaCl_2$, and R_m range of 5–8 × × 10^9 Ω.

posomes added and on the wavelength used. The side containing the liposomes with PM was always negative upon illumination. The photovoltage action spectrum from this PM–BLM system is shown in Fig. 3. BLM containing PM which was incorporated directly into the membrane forming solution also exhibited a fast photovoltage effect upon illumination with monochromatic light. The photovoltage response was between 5 and 10 mV. The reason for a smaller photoresponse is probably due to the fact that the amount of PM incorporated by liposome fusion was larger than in the case of incorporating PM into the membrane forming solution, given that the PM is slightly soluble in organic solvent.

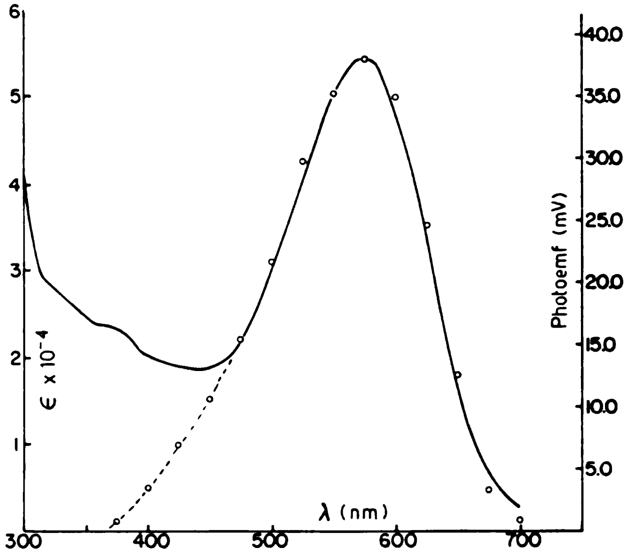

Fig. 4.

Comparison of spectra. One action spectrum of Fig. 3 for PM vesicle fusion (o, right-hand scale) is compared with the absorption spectrum of bacteriorhodopsin in aqueous dispersion (solid line, left-hand scale).

The photovoltage action spectra of both types of PM–BLM were similar (Fig. 3), and resemble very much the absorption spectrum of bacteriorhodopsin in aqueous solution except for the region between 350 nm and 450 nm (Fig. 4). This has been explained by Dancshazy and Karvaly[18] as being due to shielding or quenching by carotenoids, which absorb in this region. Recent spectroscopic investigations on purple membrane fragments layered on a glass surface possess definite shoulders or even maxima at the wavelengths indicated above.[29] This means that a certain amount of the absorbed light may be dissipated or emitted as fluorescence.

The time course of the PM–BLM photoresponse as a function of wavelengths was monitored. Fig. 5 (*A* and *B*) illustrate the light-induced voltage at 560 nm and at 411 nm, respectively. The complicated waveform shown in Fig. 5*A* will be discussed in conjunction with the flash experiments described in the next section.

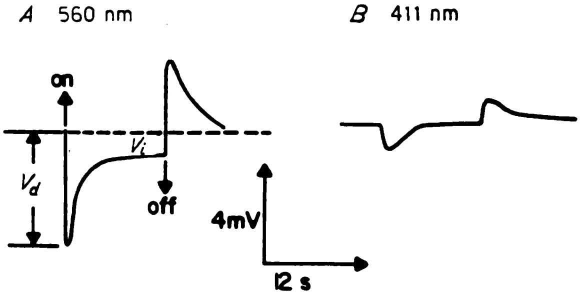

Fig. 5.
Open-circuit photopotential of PM–BLM. The membrane was illuminated at two wavelengths and equal intensity (about 5 mW/cm^2). Dark membrane potential is zero. PM incorporated by fusion of liposomes with PM to BLM. R_m was 5×10^8 Ω, and membrane and bath solutions as given in Fig. 3. Light on and off is indicated by arrows. Ordinate is the potential of the inner chamber.

All the above data taken together, it can be concluded that the resemblance between the photovoltage (action) spectrum and the absorption spectrum establishes that the observed photoeffect is due to the presence of PM in the BLM.

The successful incorporation of PM into BLM and the preliminary photoelectronspectroscopic investigations on them give impetus to incorporate charged proteins into lipid bilayers and may make even broader the application of the BLM technique in studying the function of proteins in membrane processes as well. The data on the wavelength dependence of photoresponses clearly demonstrate again the unique power of photoelectric spectroscopy in studying photobiological phenomena at the molecular level.[28,29]

Flash excitation of purple membrane–BLM

Typical photovoltages of PM–BLM to a 3 μs xenon flash are shown in Fig. 6. Purple membrane was incorporated by fusion of liposomes and BLM. The open-circuit photovoltage, designated as $V_p(t)$, appears to consist of two voltages. One component (V_d) is proportional to the polarization of the membrane by the net dipole electric field of the double layer produced by the finite lifetime of protonated and unprotonated forms of bacteriorhodopsin (BR) as it pumps protons. The other com-

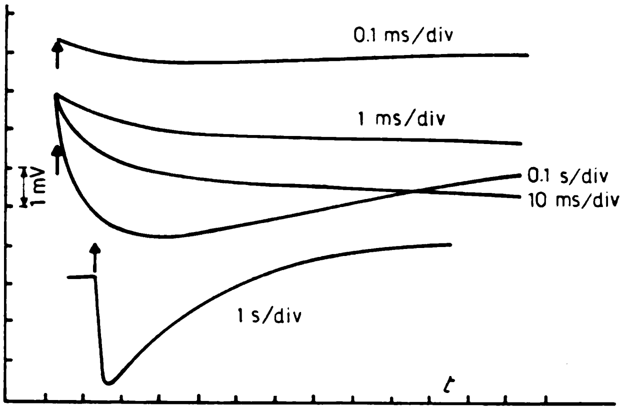

Fig. 6.

Photoflash potential. Tracings show the oscilloscope displays of the open–circuit photovoltage of PM–BLM following intense 3 μs xenon flash (indicated by arrows). One minute interval between flashes. PM incorporated by fusion of liposomes with PM to BLM. Solutions as in Fig. 3. R_m was 2×10^9 Ω. Ordinate is the potential of the inner chamber.

ponent (V_i) arises directly from the proton current, of which part establishes an ohmic potential drop across the membrane resistance (R_m) while the remainder charges the membrane capacitance (C_m). In similar and related pigmented bilayer lipid membrane systems it has been proposed that the observed photopotentials are best explained by treating the BLM together with its two co–existing solution |membrane interfaces as a parallel *RC* circuit and a generator, which is produced by light, connected across the membrane.[28,30-36] In the present case, owing to the fusion of liposomes to the membrane, the system is better represented by an equivalent circuit shown in Fig. 7*A*. The battery and resistor enclosed by dotted lines represent the PM proton pump, which acts as a non–ideal potential–generator.[24] The validity of a similar model circuit for the system of PM–liposomes fused to planar BLM has been demonstrated,[24] the only difference being the representation of the generator by a voltage–dependent ideal current generator. But this is electrically equivalent to the non–ideal potential–generator (one that has an internal resistance) shown in Fig. 7*D*.

The major portion of observed open–circuit photoresponse, $V_p(t)$, cannot be explained by the charging and discharging of membrane capacitances in Fig. 7 as the PM proton pumps turn on. First, the probable values of the circuit parameters, allowing for estimated density of liposomes on the planar membrane, give small dark–overshoot, defined as the ratio of V_d–V_i to V_i (see Fig. 5*A*). Second, the photovoltage rise time is limited by time constant $\tau_m = R_m C_m$. Typical values of τ_m are of the order of 1 s whereas the observed rise time of $V_p(t)$ was of order of 20 ms.

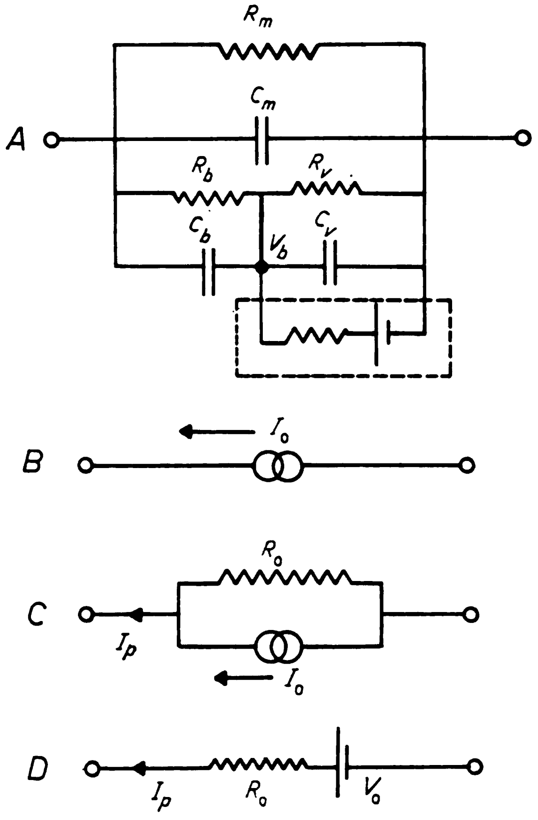

Fig. 7.
The equivalent electrical circuit for PM liposomes fused with BLM. (*A*) Linear circuit elements model the system. The generator (within dashed lines) simulating the pumping ability of PM is variously represented by circuit elements *B*, *C* and *D* in the literature. These are (*B*) ideal current generator with constant current I_0, (*C*) non-ideal current generator with finite shunt resistance R_0, short circuit current I_0, and open-circuit voltage $V_0 = I_0 R_0$, and (*D*) non-ideal potential generator with series resistance R_0, open-circuit voltage V_0, and short-circuit current $I_0 = V_0/R_0$. Generators (*C*) and (*D*) are equivalent.

We therefore look for an additional mechanism that could generate biphasic $V_p(t)$ of comparable voltage amplitudes and time constants, a mechanism not included in the equivalent circuit of Fig. 7. One way the PM differs from the electrical elements of current or potential generator is by the generation of an electrostatic dipole field as the bacteriorhodopsin molecule releases a proton. At the bath pH of about 7, there is evidence[18] bacteriorhodopsin has an overall negative charge in both protonated and unprotonated forms. Mutual electrostatic attraction may permit a short lived electric dipole before the proton is drawn away. In the remaining discussion in this section the rather complicated charge state after proton release will be denoted by BR^-; this is *not* to be taken as necessarily a *single* negative charge. Further, in the sequence of conformational changes preparatory to proton release, BR may attain a temporary dipole moment. Photovoltages of BLM with cyanine dyes have been attributed to redox generated dipoles[34] or to a photoinduced charge displacement.[37] We extend this concept to our system, and suggest that on illumination PM incorporated into BLM generates a dipole layer in addition to a proton current.

The proposed mechanism is as follows: Deprotonation of a BR molecule creates a dipole, which is subsequently annihilated by proton transport and diffusion away from BR^- and by proton uptake. A dipole layer, or double charge layer, is thus inherent in directional proton pumping by all the BR in a planar membrane. The strength of the dipole layer should depend on the proton release and uptake rates (*i.e.*, dipole lifetime), rates of diffusion, and applied fields (one polarity increasing V_d and the opposite polarity decreasing V_d). Generation of a dipole layer establishes immediately an electrostatic field which polarizes the medium quickly (about 1 μs), with a consequent rise V_d across C_m at the same rate. This rate is limited only by the rise time of the dipole layer, equivalent to the rise time of deprotonated BR, and would not be limited by the membrane time constant. The relative amplitude of the potential developed by the dipole layer to that by the proton current would increase with the dipole strength and decrease with the current associated with the creation and annihilation of one dipole.

How can the time course of $V_p(t)$ (Fig. 6) be interpreted in light of the above hypothesis? After the flash has extinguished, a number n^* of excited bacteriorhodopsin molecules BR^* remains. Each deprotonates to form a dipole and generate current. Later it takes up a proton to annihilate the dipole and either

(*a*) create more current, if the proton uptake is on the side opposite release, or
(*b*) annihilate current if the uptake is on the same side.

Assuming (*a*), the reaction is

$$I + \mathrm{BR} \xrightarrow{k_0} \mathrm{BR}^* \xrightarrow{k_1} \underset{\substack{\downarrow \\ \mathrm{H}^+}}{\mathrm{BR}^-} \underset{\substack{\uparrow \\ \mathrm{H}^+}}{\xrightarrow{k_2}} \mathrm{BR}, \tag{1}$$

where I is light intensity. The flash photopotential $V_p(t)$ is the sum of three potentials: $V_d(t)$ due to the polarization charge $Q_p(t)$ on the membrane in response to the dipole electric field, $V_1(t) = Q_1(t)/C_m$ due to the charging of membrane capacitance C_m by the proton current generated by proton release in equation 1 and $V_2(t) = Q_2(t)/C_m$, where Q_2 is the charge on C_m by that part of the proton current generated by proton uptake in equation 1:

$$V_p(t) = V_d(t) + \frac{1}{C_m}\,[Q_1(t) + Q_2(t)]. \qquad (2)$$

The time dependence of the separate terms is given by

$$V_d(t) \propto Q_p(t) \propto \left[\exp\left(-\frac{t}{\tau_2}\right) - \exp\left(-\frac{t}{\tau_1}\right)\right] \qquad (3)$$

$$V_1 = \frac{1}{C_m}\,Q_1(t) \propto \exp\left(-\frac{t}{\tau_m}\right)\left[1 - \exp\left\{-t\left(\frac{1}{\tau_1} - \frac{1}{\tau_m}\right)\right\}\right] \qquad (4)$$

$$V_2 = \frac{1}{C_m}\,Q_2(t) \propto \exp\left(-\frac{t}{\tau_m}\right)\left[1 - \exp\left\{-t\left(\frac{1}{\tau_2} - \frac{1}{\tau_m}\right)\right\}\right] \qquad (5)$$

where time constant and rate constant are related by $1/\tau_i = k_i$. These equations arise from the following physical considerations: C_m may be charged by protonic charge (Q_1 and Q_2) and by polarization charge (Q_p).

Discharge of protonic charge can occur only by ionic leakage current through R_m with time constant τ_m when there is no shunt resistance, whereas polarization charge can be discharged either by leakage current, or by collapse of the polarizing field, with time constants τ_m and τ_2, respectively.

The kinetics of reaction equation (1) show that theoretically an electrical double layer is present. The question is its importance; that is, is V_d of equation (3) comparable to or greater than V_1 and V_2 of equations (4) and (5)? All three components are rising and falling exponentials with fast rise times initially. V_d has τ_1, V_1 and V_2 have essentially τ_1 and τ_2 because τ_m is so large. Although the equivalent circuit of Fig. 7*A* correctly describes the current-generating properties of PM (and any potentials developed by the current), it does *not* include any possible dipole character of the PM (an electrostatic phenomenon). Consequently, this circuit may be used to examine V_1 and V_2, but not V_d. Clearly it shows that the rate of rise of V_1 and V_2 across C_m and R_m is limited by the time constant $\tau_m = R_m C_m$ (1 s or perhaps 100 ms), regardless of the values of τ_1 and τ_2. Therefore, $V_p(t)$ of Fig. 6 must include $V_d(t)$, the dipole potential.

Because the reaction time constants τ_1 and τ_2 are much smaller than the membrane time constant τ_m, they shape the leading edge of flash $V_p(t)$ as illustrated in Fig. 8. Proton release and uptake occur within the sequence of conformational changes of excited bacteriorhodopsin, BR*, K_{590}, L_{550}, M_{412}, N_{520}, I_{640}, and BR_{570}, with proton release shortly after the formation of M_{412} and proton uptake connected with the formation of O_{640}.[7,8,38,39]

$$\begin{aligned} &BR^* \rightarrow M_{412} + H^+, && \tau_1 = 30\ \mu s \\ &H^+ + M_{412} \rightarrow BR, && \tau_2 = 10\ ms \end{aligned} \qquad (6)$$

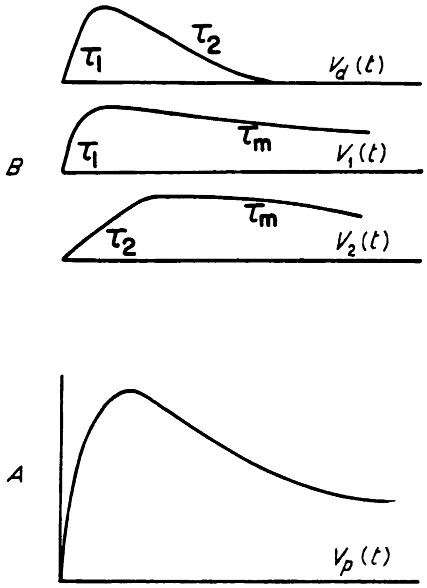

Fig. 8.

Sketch of theoretical photovoltage following flash illumination. Waveforms were determined by Equations. 3–5. (*B*) The three components, dipole voltage and two membrane capacitative voltages, sketched separately. Different time constant τ determine the rise and fall of each component. *A*. Sketch of the sum of components, *i.e.*, the total open-circuit photovoltage, under the condition $\tau_1 < \tau_2 \ll r_m$. Waveform (*A*) is the sum of (*B*) on the same scale.

For this case, $\tau_1 \ll \tau_2$, the waveform will look like Fig. 8*A*. Analysis of the scope trace (Fig. 6) yields

$$\begin{aligned} \tau_1 &= 30 \pm 10 \ \mu\text{s} \\ \tau_2 &= 30 \pm 10 \ \text{ms} \\ \tau_m &= 840 \ \text{ms} \end{aligned} \tag{7}$$

These values are comparable to those found by other methods,[38,39] with the assurance that they have not changed in the lipid–aqueous solution organized interface.

Under flash illumination, the decay of the photovoltage is due to the depletion of the excited molecules BR*. Under constant illumination, when sufficient ionic charge has passed through the membrane to compensate the initial polarization charge Q_p, then V_t is zero, and V_p is a constant determined by the proton current. Removal of illumination quenches the dipole layer and leads to a dark-overshoot until charge compensation again occurs. This effect is seen in other systems.[34,37]

The potential difference across a uniform dipole layer is given by the following equation

$$V = \frac{1}{\varepsilon_0 k} \int e\sigma S \mathrm{d}\omega \tag{8}$$

in mks units, where $\varepsilon_0 = 8.85 \times 10^{-12}$ Farad/meter, k is the dielectric constant of the medium between the double charge layers that constitute the dipole layer, $e = 1.60 \times 10^{-19}$ Coulomb (the proton's charge), σ is the number of deprotonated PM per m^2, S is the mean separation between the proton and PM$^-$, and ω is the solid angle subtended at the electrode by the planar membrane. Let the membrane diameter be 1 mm, the electrodes each be 1 cm away from the membrane, and $S = 100$ Å, and BLM dielectric constant $k = 2$. Then

$$\sigma/V \simeq 10^{-4}\ \text{Å}^{-2}\ \text{mV}^{-1}, \tag{9}$$

and the average separation between the PM$^-$ and H$^+$ is

$$S(\text{Å}) \simeq 10^{-3}\,[V_d(\text{mV})]^{-1/2}. \tag{10}$$

A typical V_d of our system is 25 mV, so S is 200 Å. This small separation is consistent with the rapid rise of V_p and with the diameters of our liposomes.

Summarizing, the existence of the dipole voltage, V_d, is demonstrated by the fast rise time of the flash photovoltage, V_p, and the fact that the equivalent circuit (Fig. 7*A*) does not completely explain its waveform (Fig. 6). (This circuit *does* completely explain $V_p(t)$ under near short circuit conditions, when the dipole field is shorted out. Then the shunting resistor permits much faster charge compensation). The rise time (much faster than $1/\tau_m$) suggests rapid polarization of the membrane by the generation of an electrical double layer inherent in the translocation of protons, and possibly the creation of temporary conformations of PM with electric dipole moment. The potential generated by the proton current through the membrane is also present. Thus from equation 2,

$$V_p(t) = V_d(t) + V_i, \tag{11}$$

where each term has different time constants. The proper circuit conditions can make one term dominant at any given time. Recognition of this resolution of photovoltage into two components is potentially important as a way to measure rate constants in the BLM environment, and as a consideration for future study of PM–BLM mechanisms.

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APPENDIX C

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PHOTOELECTRIC EFFECTS IN BILAYER LIPID MEMBRANES CONTAINING COVALENTLY LINKED PORPHYRIN COMPLEXES

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Summary

Complexes of retinal, carotene and quinone covalently linked to porphyrin were incorporated into bilayer lipid membranes (BLMs). The photovoltages and photocurrents in the BLMs were measured on continuous and flash (8 μs) illumination. The photovoltages obtained for tetraphenylporphyrin–carotene and porphyrin–quinone membranes are much larger than those obtained with simple porphyrin membranes. No pigmented BLMs exhibit any latency in their photopotentials when illuminated with a light flash of microsecond duration. The photovoltage and photocurrent action spectra were measured for these pigmented BLMs. The photovoltage and photocurrent observed for the pigmented membranes are explained in terms of a semiconductor model. The enhancement in these photoeffects is explained in terms of intramolecular charge transfer and energy transfer processes.

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1. Introduction

The formation of pigmented bilayer lipid membranes (BLMs) of planar and spherical configuration [1, 2] has allowed a variety of photophysicochemical studies to be carried out to investigate the effects of chemical composition, redox compounds and the wavelength of light. The most important feature of such studies may lie in the understanding they provide of the transformation of solar energy into electricity or the storage of this energy in

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chemical compounds as accomplished by green plant photosynthesis for example [3 - 6]. Some of the advantages of the planar BLM system reside in its relatively large size and its configuration which allows access to both its sides and makes it relatively easy for electrical measurements to be carried out. Studies on such pigmented membranes include investigations of membranes formed from chloroplast extract and from purified chlorophylls and their related compounds such as porphyrins with and without added modifiers such as quinones and carotenes [2, 7 - 14].

The photoeffects observed in such pigmented membranes are the result of charge separation and electron transfer reactions (redox reactions) from the excited pigment and suitable electron donors and acceptors at both interfaces. In photosynthetic reaction centers, charge separation and electron transfer take place between the excited chlorophyll molecules and the primary electron acceptors of the electron transport chain [3, 15]. There is evidence that quinones play the role of the primary electron acceptors in these reaction centers and that carotenes act as light-gathering accessory pigments [3, 15]. For these processes to take place efficiently it seems reasonable to assume that a relatively close proximity and proper orientation should exist between the donor species (*e.g.* excited chlorophyll or porphyrin) and the electron acceptor (*e.g.* a quinone).

To test these hypotheses several groups of workers have synthesized covalently linked porphyrin–quinone and porphyrin–carotene complexes [16 - 20]. These investigators have studied the photophysical and photochemical properties of these compounds in bulk solutions or on surfaces. Quenching of the porphyrin fluorescence in the presence of either quinones or carotenes was observed. A similar effect was observed in chlorophyll liposomes containing β-carotenes [21]. The quenched fluorescence of chlorophyll was ascribed to a static process of electron transfer or chlorophyll–chlorophyll interactions brought about by β-carotene.

The effect of quinone and β-carotenes on some membrane model systems has also been investigated. Barsky *et al.* [22] have reported an increase in photovoltages generated across a planar membrane containing reaction centers from photosynthetic bacteria when 1,4-naphthaquinone is added to the aqueous phase and when ubiquinone 30 is added to the membrane. The presence of certain quinones in BLMs containing chlorophyll a has been found to enhance both dark conductivity and photoconductivity [10]. Similarly, an increase in the photovoltage was found in the presence of β-carotene in the chlorophyll BLM [2].

In all these previous studies dealing with the effect of quinones and carotenes in pigmented BLM, however, compounds of porphyrins and/or carotenes were either dissolved in the membrane-forming solution or added to the aqueous phase, and the increase in the photoeffect was relatively small. We report here the photoelectrochemical properties of BLMs containing retinal, carotene and quinone compounds covalently linked to porphyrin. Preliminary results for quinone complexes have been communicated previously [23].

2. Materials and methods

Anhydrous sodium acetate (AnalaR grade), $FeCl_3$ (AnalaR grade; Mallinckrodt Inc.), ascorbic acid, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine (Sigma Chemical Co.) were used without further purification. However, cholesterol (Fisher Scientific Co.) was purified by repeated crystallization. The porphyrins with ester side chains were prepared by the condensation of dipynomethene. The amines were obtained from alcohols and mesylates [24]. The quinone compounds were prepared following the published methods [16, 17]. However, in our case silver oxide was used to oxidize the dihydroxy groups. Porphyrin–retinyl was obtained by the condensation of porphyrin amine and retinal. All these compounds were prepared under minimum illumination to protect them from photodecomposition and were stored at a low temperature. The purities of the final compounds were checked by thin layer chromatography and mass and optical spectroscopy. The tetraphenylporphyrin (TPP)–carotene complex was a generous gift from Professor Gust, Arizona State University. The synthesis and characterization of this complex has already been published [19]. *n*-octane (Fisher Scientific Co.) and doubly distilled water were used as solvents.

A solution of phosphatidylcholine, phosphatidylethanolamine, cholesterol and phosphatidylserine in the ratio 3.1:1.7:1.1:1.0 by weight was prepared in *n*-octane to a final concentration of approximately 7%. A saturated solution of each porphyrin compound in the above solution was used as the membrane-forming solution.

The membranes were formed by ejecting a small amount of membrane-forming solution over the orifice (1 mm in diameter) of a 10 ml Teflon cup separating two aqueous solutions (0.1 M sodium acetate buffer, pH 5.0) using a Hamilton microsyringe. The Teflon cup was placed in a Plexiglas chamber. The two faces of the Plexiglas chamber, one for illuminating and the other for viewing the membrane, contained optically flat glass windows. Calomel electrodes with salt bridges were used in the usual manner for electrical contact [2]. The aqueous solutions were continuously agitated by magnetic stirring bars in each of the chambers. The thinning of the membrane to the secondary black state was observed with a binocular microscope. $FeCl_3$ and ascorbic acid were added to the inner and outer chambers respectively to a final concentration of 10 mM after the membranes had thinned and reached the black state.

The photopotentials and photocurrents were measured using a Keithley 610 electrometer and a Keithley 417 picoammeter connected to a Keithley 370 or Bausch and Lomb recorder. In some cases the signal was amplified through a high impedance amplifier and recorded on a Tektronix R5031 dual-beam storage oscilloscope. The closed-circuit measurements were performed by applying a potential through an external resistance which was nearly equal to the membrane resistance in the dark. The membranes were illuminated with white light from a 250 W tungsten–halogen lamp or a

1000 W xenon lamp. Microsecond flash (8 μs) illumination experiments were performed using a General Radio Strobotac 1538A xenon flash lamp assembly. All the other experimental arrangements were the same as described elsewhere [2].

The experimental set-up for recording the action spectra consisted of a 1000 W xenon lamp assembly (Schoeffel), a Bausch and Lomb 250 mm grating monochromator, a pair of calomel electrodes, a Keithley 610 electrometer and a Bausch and Lomb or Plotamatic 715M recorder. The spectra were measured by rotating the diffraction grating with a slow speed motor or manually point by point in increments of 10 nm. The action spectra were corrected for constant illumination intensity with a calibration curve obtained for the experimental arrangement using a Scientific Instrument Kettering radiant power meter. The absorption spectra for BLM-forming solutions (saturated solution of pigment) were recorded on a Varian Cary 219 spectrophotometer by sandwiching the solution between two glass plates. All the experiments were performed at 23 ± 1 °C.

3. Results

The membranes reached the black state 5 - 15 min after their formation. The membrane resistance R_m in the dark was measured by applying an external voltage through a high resistance ($R_s \approx 10^8\ \Omega$) in series. The values of R_m for BLMs containing various porphyrin compounds are given in Table 1. These values were measured after 3 - 4 h of membrane formation when they were almost constant.

Open-circuit photo-e.m.f.s for various BLMs in the presence of $FeCl_3$ and ascorbic acid in the aqueous solutions are given in Table 1. For most of the compounds a small and slow photo-e.m.f. was observed initially; however, with time the photopotential showed an increase in magnitude and a decrease in the response time and finally after 3 - 4 h it became almost constant. This behavior was observed in BLMs containing porphyrin, porphyrin-C_{12} and TPP–carotene. However, the response for the BLMs containing porphyrin–quinone and porphyrin–retinyl was fast initially and became much faster with time with a simultaneous increase in the magnitude. The rise and decay of the open-circuit photo-e.m.f. with continuous illumination is shown in Fig. 1(a) for BLMs containing porphyrin, porphyrin–quinone and porphyrin–retinyl. All the values of the open-circuit photo-e.m.f.s given in Table 1 for BLMs containing various compounds were recorded after 3 - 4 h of membrane formation when the photo-e.m.f. was almost constant. The values of the photo-e.m.f. given in Table 1 clearly indicate that it is enhanced in all BLMs containing monosubstituted compounds except the BLM containing porphyrin-C_{12}. The BLMs containing TPP–carotene and porphyrin–quinone showed more than threefold enhancement compared with the BLM containing porphyrin. In BLMs containing disubstituted complexes the photo-e.m.f. was almost equal to or lower than that of the BLM containing porphyrin.

TABLE 1

Photovoltage, photocurrent and membrane resistance for bilayer lipid membranes containing porphyrin complexes

Compound	*Photovoltage* (mV)		*Photocurrent with continuous illumination* (nA)	R_m ($\times 10^8\ \Omega$)
	Continuous illumination	*Flash illumination*		
Porphyrin	83	0.4	0.10	4
Porphyrin–retinal	154	20	1.3	3
Porphyrin–diretinal	70	3.0	0.6	8
Porphyrin–quinone	302	42	22	8
Porphyrin–diquinone	92	1.0	1.2	3
Porphyrin–dodecyl	83	0.25	0.15	8
TPP–carotene	300	4.0	1.2	11

The intensity of white light in continuous illumination was 250 mW cm^{-2}. The values of the photo-e.m.f.s with flash illumination were measured 100 μs after the flash. The structural details of the compounds are given in Appendix A.

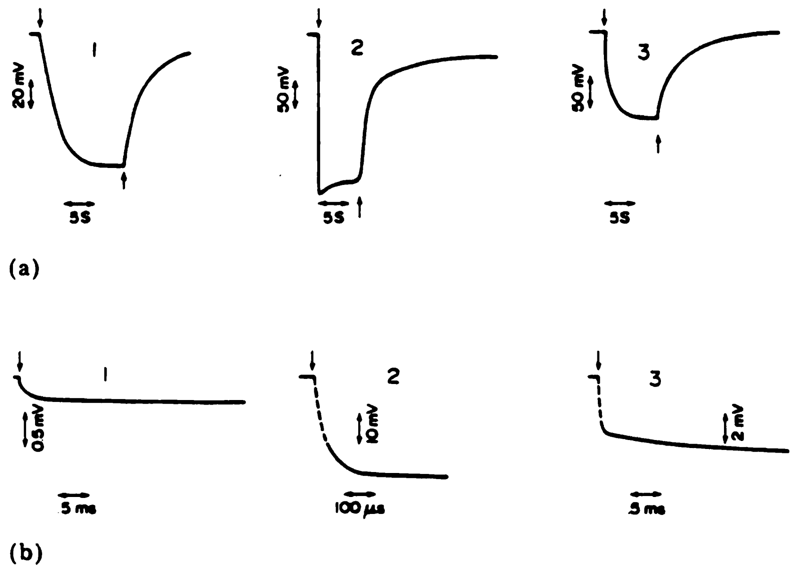

Fig. 1. (a) Development of the photovoltage under continuous illumination with white light for BLMs containing porphyrin (curve 1), porphyrin–quinone (curve 2) and porphyrin–retinyl (curve 3) (↓, light on; ↑, light off); (b) development of the photovoltage under flash illumination (8 μs duration) for BLMs containing porphyrin (curve 1), porphyrin–quinone (curve 2) and porphyrin–retinyl (curve 3) (↓, onset of flash illumination). The pigmented BLMs were formed in 0.1 M sodium acetate buffer (pH ≈ 5.00) with the outer solution containing 10 mM ascorbic acid and the inner solution containing 1 mM $FeCl_3$. The intensity of the white light was 250 mW cm^{-2}.

The membranes containing TPP–carotene, porphyrin–retinal and porphyrin–quinone all exhibit a small photovoltage in the absence of a donor or an acceptor in the bathing solution. The photopotential was found to increase after the addition of $FeCl_3$ to the inner chamber and showed further enhancement after the addition of ascorbic acid to the outer chamber. However, if only ascorbic acid was added to one side with no $FeCl_3$ on the other side, the photopotential observed was smaller than that observed in the presence of $FeCl_3$ only.

All photovoltage and photocurrent measurements were carried out in the presence of 1 mM $FeCl_3$ in the bathing solution in the inner chamber and 10 mM ascorbic acid in the bathing solution in the outer chamber. However, for some membranes, the open-circuit photo-e.m.f.s were also measured for different concentrations of $FeCl_3$ while the ascorbic acid concentration was kept constant (10 mM). The dependence of the photo-e.m.f. on the $FeCl_3$ concentration for a BLM containing porphyrin–quinone is shown in Fig. 2.

Open-circuit photo-e.m.f. measurements were also carried out on BLMs containing some of these compounds at higher pH values (sodium acetate (0.1 M) buffer, pH ≈ 7.5) in the presence of $FeCl_3$ and ascorbic acid or of ethylenediamine tetraacetic acid and anthraquinone-2-sulfonate but the magnitude of the photo-e.m.f. was lower at this pH. Our results are similar to those reported by Jimbo *et al.* [25] for TPP-coated electrodes in the presence of some quinones. These workers have suggested that the protonation of the porphyrin film surface plays an important role in the charge transfer process.

Short-circuit photocurrent measurements with continuous light were also made for BLMs containing porphyrin compounds in the presence of

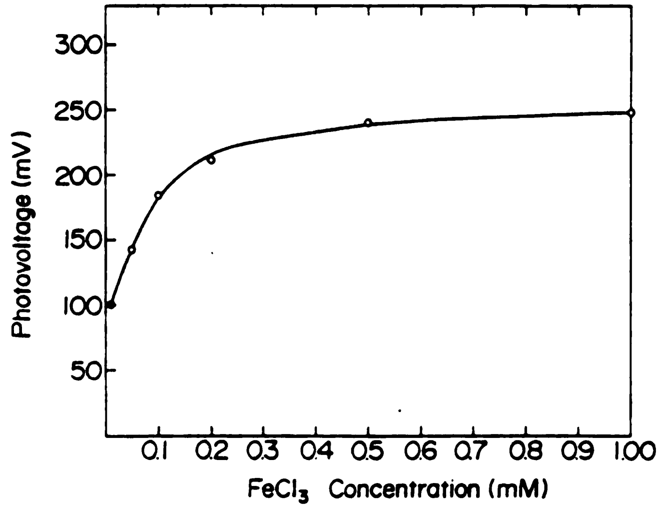

Fig. 2. Dependence of the photovoltage on the $FeCl_3$ concentration in the inner chamber of the bathing solution for a BLM containing the porphyrin–quinone complex. The bathing solution was 0.1 M sodium acetate buffer (pH ≈ 5.00) and the outer chamber contained 10 mM ascorbic acid. The intensity of the white light was 250 mW cm^{-2}.

$FeCl_3$ and ascorbic acid and the results are given in Table 1. For BLMs containing porphyrin–quinone compounds the short-circuit current is about 200 times higher than for the BLM containing porphyrin.

The open-circuit photo-e.m.f. and the short-circuit photocurrent for the BLMs were also measured for different intensities of white light. These results are shown in Fig. 3 for the BLM containing porphyrin–quinone. Both photovoltage and photocurrent are almost linear initially (*i.e.* for low light intensities) but the saturation point is reached much earlier for the photovoltage. Our results are similar to those previously observed for the chloroplast extract BLM and for photovoltaic cells [1, 2]. Similar experiments were also performed for light of wavelength 400 and 500 nm using interference filters and the results are similar to those obtained for white light illumination.

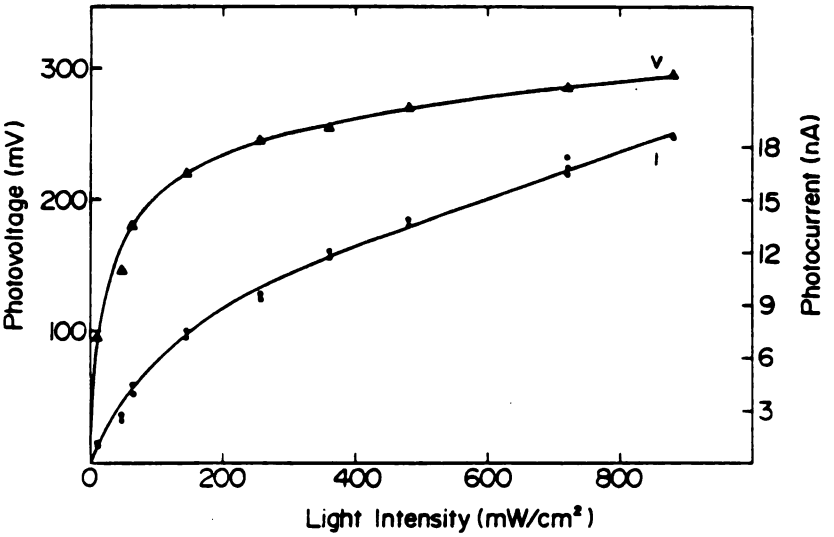

Fig. 3. Dependence of the photoeffects on the intensity of the illuminating light for a BLM containing porphyrin–quinone: ▲, photovoltage; ●, photocurrent. The bathing solution was 0.1 M sodium acetate buffer (pH ≈ 5.00) with 1 mM $FeCl_3$ in the inner chamber and 10 mM ascorbic acid in the outer chamber.

Apart from the continuous illumination, the open-circuit photovoltages were also measured under flash illumination (8 μs) for BLMs containing different porphyrin compounds and the photoresponses were observed. The magnitudes of the photovoltage 100 μs after the flash are listed in Table 1. The development of the photovoltage for BLMs containing porphyrin, porphyrin–quinone and porphyrin–retinyl is shown in Fig. 1(b). No delay was found from the flash to the generation of the photovoltage, which was limited by our 8 μs flash unit. This behavior is similar to that observed earlier for a chloroplast extract BLM [26].

The action spectra for the photoeffects of the porphyrin-containing BLMs were obtained. In general these spectra follow the absorption spectra of the specific porphyrin complex present in the membrane, *i.e.* the positions of the maxima in the action spectra match the positions of the peaks in the absorption spectra. Moreover, the positions of the maxima in the action spectra and in the absorption spectra for the different porphyrin complexes (including simple porphyrin) are almost identical. These results indicate that the species responsible for the photoeffects is the porphyrin ring. However, the relative intensity of the peaks in the action spectra for each porphyrin-containing membrane is different from the relative intensity in their corresponding absorption spectra. The difference is observed in the relative ratio of the band at 400 nm (Soret band) to the bands in the lower energy region beyond 450 nm (maxima at 500, 534, 574 and 624 nm). To investigate this in more detail the photocurrent action spectrum for the porphyrin–quinone membrane was measured (Fig. 4). A difference in the ratio of the bands in the photocurrent spectrum compared with the absorption spectrum was also observed. These results suggest that the quantum efficiency for the photocurrent may be different for the different absorption bands. A calculation of the relative quantum efficiency at the different bands suggests that the quantum efficiency at the lower energy bands is about twice the quantum efficiency at 400 nm. A higher quantum efficiency for red light compared

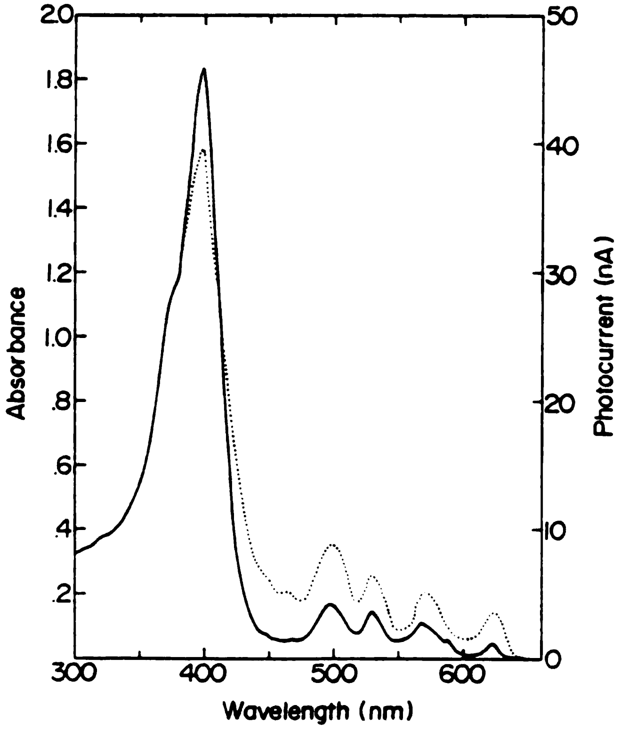

Fig. 4. Absorption spectrum of a saturated solution of the porphyrin–quinone-3 complex in the BLM-forming solution sandwiched between two thin glass plates (——); photocurrent action spectrum of a BLM containing the porphyrin–quinone-3 complex corrected for constant intensity of illumination (·······).

with blue light has been reported for electrodes coated with manganese(III) porphyrin [27]. One possible explanation for this difference may be the decrease in absorbance at the Soret band for higher pigment concentrations. However, in our studies the absorption spectrum used for comparison is that of a saturated solution. The only way to be certain would be to measure the absorption spectrum of the membrane itself. Owing to the thinness of the BLM, this is a very difficult task. This is also the reason why we have not measured the absolute quantum efficiency for any of the porphyrin complexes.

4. Discussion

The photoelectric effects observed for BLMs containing porphyrin complexes can be explained in the same way as for membranes made from chloroplast extracts [2]. The observed photoelectric phenomena are due to redox reactions occurring at both membrane–electrolyte interfaces with electrons acting as the charge carriers across the membrane [1, 2, 7 - 14]. In this respect the pigmented BLM is assumed to be an organic semiconductor [2, 4] which is analogous to a Schottky barrier except that the BLM system has two interfaces. Bearing this assumption in mind, one side of the membrane acts as a photocathode (p-type junction) and the other side acts as a photoanode (n-type junction) as shown in Fig. 5. The aqueous solution plays the role of the metal at the BLM–electrolyte interface. The photovoltages observed are influenced by the redox compounds (electron donors and acceptors) present in the bathing solution and/or externally applied voltages. It should be pointed out that current flow in the BLM is due to photogenerated and dissociated electron–hole pairs (excitons); however, unlike the metal in a Schottky-type cell, the aqueous solution is not an electronic conductor. Therefore redox reactions must take place at both interfaces in order to complete the circuit. A more detailed description of the semiconductor model of charge separation in BLMs has been discussed in detail elsewhere [2, 28].

The larger photoeffects observed in these experiments for the porphyrin complexes as compared with simple porphyrin can be explained in terms of two processes taking place between the porphyrin and the covalently linked compound: (i) charge transfer and (ii) energy transfer. In the case of the complexes consisting of benzoquinone molecules covalently linked to a porphyrin molecule, the enhancement in the photoresponse is believed to be due to the first process, *i.e.* intramolecular charge transfer between the donor (porphyrin) and the acceptor (quinones). This intramolecular charge transfer depends on the overlap between the highest filled donor orbitals and the lowest filled acceptor orbitals and is determined by the interaction between the π electrons in the donor–acceptor pair. This interaction should depend on the distance and orientation between the donor and acceptor parts of the covalently linked complex. Evidence for this is given by the fact

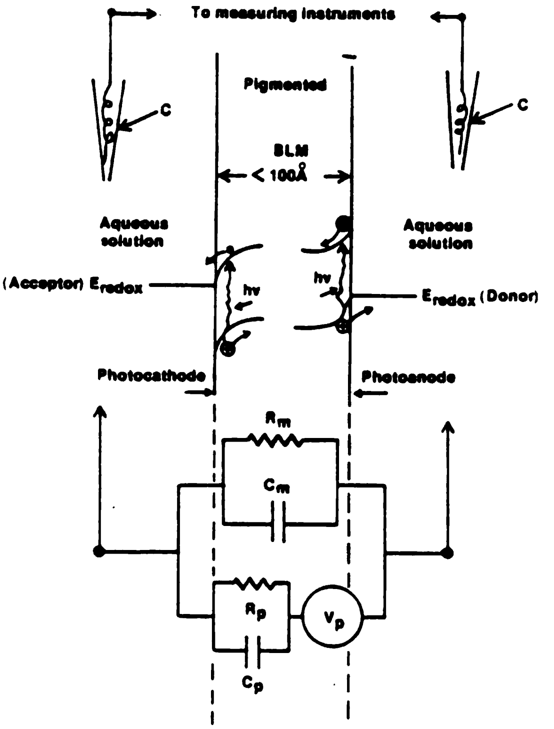

Fig. 5. Schematic diagram and equivalent circuit of a pigmented BLM under illumination with one membrane–electrolyte interface acting as the photocathode and the other as the photoanode: c, calomel electrode; E_{redox}, standard redox potential; R_{m}, membrane dark resistance; C_{m}, membrane capacitance; R_{p}, membrane photoresistance; C_{p}, membrane photocapacitance; V_{p}, membrane photopotential (see refs. 8, 12 and 27).

that the magnitude of the photoeffects varies for the quinones linked by different chain lengths to the porphyrin (see ref. 23). The largest enhancement is observed for the quinone linked by the chain with the largest number of atoms. The smaller photoresponse for the shorter chain may be due to improper orientation between the two groups or too short a distance between the two moieties. A smaller photoresponse for a closer proximity could be due to a higher probability of the back reaction regenerating excited porphyrin and the oxidized acceptor competing strongly with the charge separation reaction. A possible explanation for the smaller photoresponse in the double quinone complexes is related to the number of pigment molecules in the membrane. It was observed that the solubility of the double quinone complexes was much lower than that of the single quinone complexes. Our results on the enhancement of the photoeffects in these quinone–porphyrin complexes is further supported by some recent studies on fluorescence quenching in similar types of complexes [16 - 20]. Quenching of the porphyrin fluorescence would indicate that there would be more excited molecules available for competing non-radiative processes including charge transfer to take place. It should be mentioned, however, that the probability for non-radiative processes may be increased more than that for charge transfer since total quenching of fluorescence has been observed for a complex containing four quinones covalently linked to a porphyrin [19].

In the case of the covalently linked porphyrin–retinyl and TPP–carotene the enhanced photoeffects can be explained in terms of intramolecular energy transfer processes as well as intramolecular charge transfer processes. Here we believe that the stacked conformation in which the π electron of the retinyl or carotene chromophore resides lies just above the mean porphyrin plane. The retinyl or carotene moiety performs an antenna function by transferring the energy of the absorbed light to the singlet excited state of the porphyrin moiety as was observed by Moore *et al.* [19] in their optical and nuclear magnetic resonance bulk phase studies. These workers have also observed a substantial quenching of porphyrin fluorescence by the carotenoid moiety which suggests the possibility of the formation of a charge-separated state and results in the enhanced photoeffect across the BLMs in the present experiments. The quenching of chlorophyll fluorescence by carotenoids was explained similarly by electron transfer from carotenoid to chlorophyll by Beddard *et al.* [29]. Thus pigmented BLMs containing porphyrin–retinyl or TPP–carotene complexes may be very useful from the photosynthetic point of view because they can act both as antennae for gathering light energy and as efficient charge separators. Again it is interesting to note that the photoeffects in covalently linked porphyrin–diretinyl complexes were smaller than in the single retinyl complexes. This can be explained in the same way as in the case of covalently linked porphyrin–diquinones.

Finally in the case of the porphyrin–dodecyl complexes, no enhancement of the photoeffects was observed relative to simple porphyrin. The covalently linked moieties in this case cannot act as light-gathering entities nor can they act as electron donors or acceptors. This is additional evidence in support of our hypothesis of intramolecular charge transfer and energy transfer in the quinone–porphyrin and retinyl–porphyrin complexes. We propose that a porphyrin ring containing both covalently linked quinone and β-carotene would probably generate a very large photovoltage and photocurrent. This type of compound may prove to be very useful in solar energy conversion [3, 4, 6].

Acknowledgments

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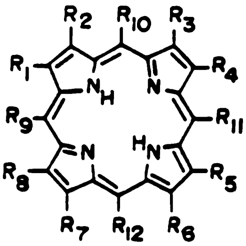
Appendix A



Porphyrin: $R_1 \equiv R_3 \equiv R_5 \equiv R_7 \equiv CH_3$; $R_2 \equiv R_6 \equiv C_5H_{11}$; $R_4 \equiv R_8 \equiv (CH_2)_2COOCH_3$; $R_9 \equiv R_{10} \equiv R_{11} \equiv R_{12} \equiv H$.

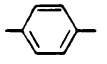
Porphyrin-retinal: $R_1 \equiv R_4 \equiv R_5 \equiv R_7 \equiv CH_3$; $R_2 \equiv R_3 \equiv R_6 \equiv C_5H_{11}$; $R_8 \equiv (CH_2)_2{-}COOC_{20}H_{29}$; $R_9 \equiv R_{10} \equiv R_{11} \equiv R_{12} \equiv H$.

Porphyrin-diretinal: $R_1 \equiv R_3 \equiv R_5 \equiv R_7 \equiv CH_3$; $R_2 \equiv R_6 \equiv C_5H_{11}$; $R_4 \equiv R_8 \equiv (CH_2)_2{-}COOC_{20}H_{29}$; $R_9 \equiv R_{10} \equiv R_{11} \equiv R_{12} \equiv H$.

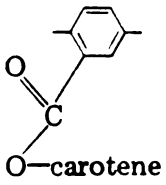
Porphyrin-quinone: $R_1 \equiv R_4 \equiv R_5 \equiv R_7 \equiv CH_3$; $R_2 \equiv R_3 \equiv R_6 \equiv C_5H_{11}$; $R_8 \equiv (CH_2)_3{-}N(Bu){-}CO{-}C{=}C{-}C_6H_3O_2$; $R_9 \equiv R_{10} \equiv R_{11} \equiv R_{12} \equiv H$.

Porphyrin-diquinone: $R_1 \equiv R_3 \equiv R_5 \equiv R_7 \equiv CH_3$; $R_2 \equiv R_6 \equiv C_5H_{11}$; $R_4 \equiv R_8 \equiv (CH_2)_3{-}N(Bu){-}CO{-}C{=}C{-}C_6H_3O_2$; $R_9 \equiv R_{10} \equiv R_{11} \equiv R_{12} \equiv H$.

Porphyrin dodecyl: $R_1 \equiv R_4 \equiv R_5 \equiv R_7 \equiv CH_3$; $R_2 \equiv R_3 \equiv R_6 \equiv C_5H_{11}$; $R_8 \equiv (CH_2)_2{-}CONHC_{12}H_{25}$; $R_9 \equiv R_{10} \equiv R_{11} \equiv R_{12} \equiv H$.

TPP-carotene: $R_1 \equiv R_2 \equiv R_3 \equiv R_4 \equiv R_5 \equiv R_6 \equiv R_7 \equiv R_8 \equiv H$; $R_9 \equiv R_{10} \equiv R_{12} \equiv$  ;

$R_{11} \equiv$



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PHOTOELECTROCHEMICAL PROPERTIES OF BILAYER LIPID MEMBRANES CONTAINING COVALENTLY LINKED PORPHYRIN—QUINONE AND OTHER COMPLEXES

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SUMMARY

Several examples of a new class of covalently linked porphyrin—quinone complexes have been incorporated into planar bilayer lipid membranes (BLM) and shown to exhibit light-induced charge separation as demonstrated by photoelectrospectrometry and electrical measurements. The photoelectric effects obtained on some of these new BLM systems are three times larger — the highest values hitherto reported — than those of chlorophyll-containing BLMs.

INTRODUCTION

To elucidate the mechanisms of quantum conversion in algal and green plant photosynthesis as well as to mimic Nature's photochemical system for possible solar energy utilization, a variety of model membranes has been investigated [1—3]. One of the extensively studied model systems has been the pigmented bilayer lipid membrane [4—12] of planar configuration, commonly referred to as BLM, whose usefulness lies in its being an appropriate analogue of the thylakoid membrane [8,13]. For efficient charge separation and electron transfer in the thylakoid membrane, a close proximity between a donor species (e.g. chlorophyll or porphyrin) and an electron acceptor molecule (e.g. quinone) is a prerequisite. To test this hypo-

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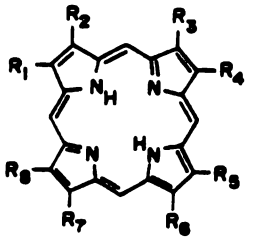
thesis several groups of workers have synthesized covalently linked porphyrin—quinone and porphyrin—carotene complexes [14—19]. We report here the photoelectrochemical properties of BLMs containing quinones and other compounds covalently linked to porphyrins. Our results support the idea that covalently linked porphyrin—quinone compounds are more efficient in charge separation and electron transfer than simple mixtures.

Previously, pigmented BLMs were formed from chloroplast extracts, purified chlorophylls and their related compounds such as porphyrins with and without added modifiers such as quinones and carotenes [8]. The addition of quinones and/or carotenes is owing to the well-documented evidence that quinones serve as the primary electron acceptor and carotenes as effective light-gathering accessory pigments [20]. Incorporation of the newly available porphyrin—quinone compounds (see Fig. 1), as a model for the initial photophysicochemical event in reaction centers of photosynthesis, into artificial BLMs is of obvious interest. The photoemfs obtained, for example, on some of these new BLM systems are indeed interesting in that they are larger by at least a factor of three — the highest values hitherto reported — than those of chlorophyll-containing BLMs.

EXPERIMENTAL

Pigmented BLMs were formed by our standard technique [4] at room temperature (23 ± 1°C) in which a 5 μl of membrane-forming solution is injected via a Hamilton syringe (Model PB-600) onto an aperture (diameter ≃1 mm) in the wall of a 10 ml Teflon beaker separating two aqueous solutions (0.1 M sodium acetate at pH 5). After the membrane formation, $FeCl_3$ (1 mM) on one side and ascorbic acid (10 mM) on the other side were added. The membrane-forming solution consisted of a 3.1 : 1.7 : 1.1 : 1.0 mixture (w/w) of phosphatidylcholine, phosphatidylethanolamine, cholesterol, and phosphatidylserine, respectively, in *n*-octane saturated with different porphyrin compounds. The different porphyrin compounds include: simple porphyrin, and porphyrin with covalently linked quinone, retinal, cholesterol, and dodecyl, etc. (Fig. 1). In the synthesis of the porphyrin—quinone (PQ) complexes, we started with the coupling of porphyrin amino and corresponding acid chlorides. The PQ complexes were then obtained following the published methods [14—19]. However, in our case, silver oxide was used to oxidize the dihydroxy groups. The purity of the final compounds was checked by TLC, mass and visible spectroscopy. Electrical properties of pigmented BLMs were measured using high impedance electrometers (Keithley Instruments, Inc.). To observe photoinduced effects, light from either a 250 W tungsten-halogen lamp or a 1000 W xenon lamp was used. For flash excitation a General Radio xenon lamp of 8 μs duration was the light source [8]. The action spectra of pigmented BLMs were obtained with the aid of a Bausch and Lomb grating monochromator and a VOM chart recorder [22].



I. $R_1 = R_4 = R_5 = R_7 = CH_3$, $R_2 = R_3 = R_6 = C_5H_{11}$
A. $R_8 = O\ (CH_2)_3 - N(Bu) - CO\ C_6H_3O_2$ (Q-1, P-BENZOQUINONYL)
B. $R_8 = -\ (CH_2)_3 - N(Bu) - CO\ (CH_2)\ C_6H_3)_2$ (Q-2)
C. $R_8 = -\ (CH_2)_3 - N(Bu) - CO - C = C -,\ C_6H_3O_2$ (Q-3)
D. $R_8 = -\ (CH_2)_2 - COO\ C_{20}H_{29}$ (VA, RETINYL)
E. $R_8 = -\ (CH_2)_2 - COO\ C_{27}H_{45}$ (CHOLESTEROL)
F. $R_8 - -\ (CH_2)_2 - CONH\ C_{12}H_{25}$ (C_{12}, DODECYL)

II. $R_1 = R_3 = R_5 - R_7 = CH_3$, $R_2 = R_6 = C_5H_{11}$
A. $R_4 = R_8 = Q\text{-}1$
B. $R_4 = R_8 = Q\text{-}2$
C. $R_4 = R_8 = Q\text{-}3$
D. $R_4 = R_8 = -CH_2 - COO\ C_{20}H_{29}$
E. $R_4 = R_8 = -(CH_2)_2 - COO\ C_{27}H_{45}$
F. $R_4 = R_8 = -(CH_2)_2\ COO\ CH_3$

III. $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = C_2H_5$, $R_7 = CH_3$
$R_8 = -(CH_2)_2\ CONH\ (C_9H_{17}NO)$, FREE RADICAL

IV. $R_1 = R_3 = C_2H_5$, $R_2 = R_4 = R_6 = R_8 = CH_3$
$R_6 = R_7 = -\ (CH_2)_2 - CONH\ C_{12}H_{25}$

Fig. 1. Structural formula and notations of compounds used.

RESULTS AND DISCUSSION

The effect of light on the pigmented BLMs can be easily detected by electrical methods, which may be combined with optical techniques to yield additional information concerning the pigmented BLM system [22]. The open circuit photovoltage and short circuit photocurrent for BLM containing different porphyrin compounds are given in Table I. The values were measured after 3—4 h of membrane formation when they were almost constant. The results presented in Table I clearly indicate that quinone-substituted complexes are most efficient in charge separation. Among the quinone-substituted complexes the PQ-3 membrane shows nearly 3-fold increase in photovoltage and 200-fold increase in photocurrent as compared to simple porphyrin membrane. In disubstituted PQ complexes, however, the magnitude of the photoeffect was lower or almost equal to that observed for simple porphyrin membrane. The time course of the photoresponse for PQ-3

TABLE I

TYPICAL RESULTS OF PIGMENTED BLM AS A FUNCTION OF PORPHYRIN COMPLEXES

Compound [a]	Photo-emf (mV)	Photocurrent (nA)	R_m (10^8 Ω)
(IV) P-di C-12	42	0.01	9
(II A) P-di Q-1	68	0.16	9
(II D) P-di VA	70	0.6	8
(II B) P-di Q-2	71	0.06	11
(I F) P-C-12	83	0.15	8
(II F) Porphyrin	83	0.10	4
Chloroplast extract	86	0.75	5
(I E) P-Chol	88	0.15	5
(II C) P-di Q-3	92	1.2	3
(III) P-Spin	112	0.18	6
(I D) P-VA	154	1.2	3
(II E) P-di Chol	175	0.84	6
(I B) PQ-2	190	12	4
(I A) PQ-1	194	15	7
(I C) PQ-3	302	22	8

[a] For abbreviations used see Fig. 1.

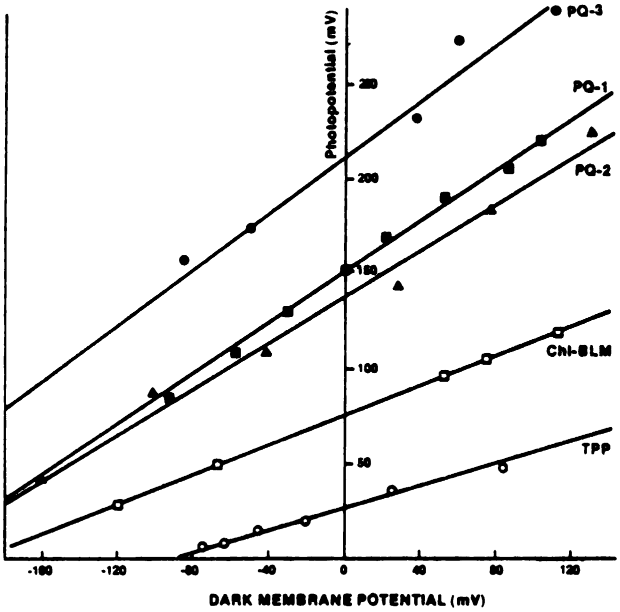


Fig. 2. Effect of externally applied voltage on the photopotential of different pigmented BLMs. The pigmented BLMs were formed in 0.1 M sodium acetate at pH 5 with outer solution containing 10 mM ascorbate and inner solution containing 1 mM ferric chloride. The external potential was applied under closed circuit conditions by means of a battery. Each datum point represents the maximum potential developed at each value of externally applied potential when the BLM was illuminated. The illumination period for each datum point was ~15 s. The maximum photopotential was reached in <10 s. See Table I for abbreviations used.

membrane for two different intensities of illuminating light is given in Fig. 4B. For PQ-3 membrane the photo-emfs were also measured for different concentrations of $FeCl_3$ while keeping ascorbic acid concentration constant. The magnitude of the photo-emf followed the stoichiometry of Fe^{3+} and ascorbic acid.

In Fig. 2 the effect of an externally applied electric field on the photoresponse of five different pigmented BLMs is shown. The external field was established by applying a potential difference by means of a battery under closed circuit conditions. As can be seen from the figure, the presence of the electric field has the same effect on the photoresponse for the five BLMs containing different pigments. Depending on the polarity of the externally applied field the photopotential can be either increased or decreased. The greater the applied voltage, positive on the side containing the electron acceptor (i.e. Fe^{3+}), the larger was the observed photopotential and vice versa. The results can be explained in terms of the ultra-thinness of the BLM (<10 nm) and its very high resistance ($R \simeq 10^8\ \Omega \cdot cm^{-2}$, several orders of magnitude larger than the aqueous solution), which means that the applied

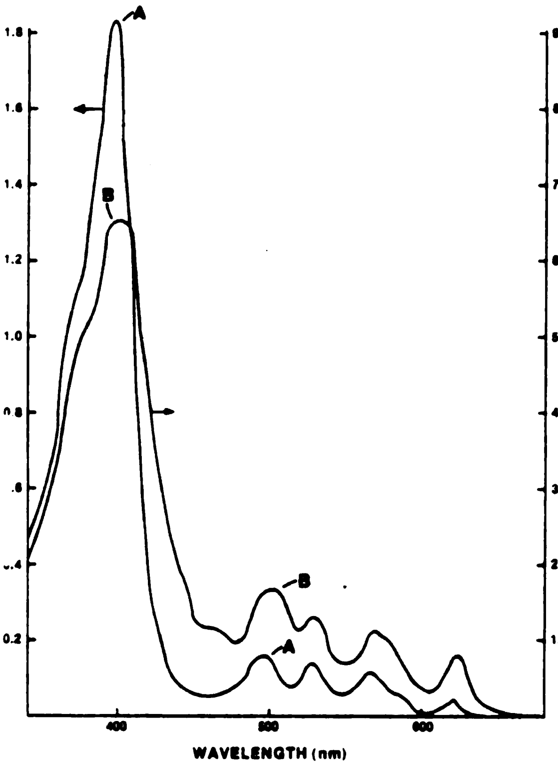


Fig. 3. A) Absorption spectrum of porphyrin—quinone complex PQ-3 in 1 : 10 methylene chloride: *n*-octane solution (PQ-3 concentration: 10 mM). B) Photovoltage action spectrum for BLM containing PQ-3 with 0.5 mM $FeCl_3$ on one side of the bathing solution, 0.1 M sodium acetate at pH 5. The spectrum has been corrected for incident energy. The arrows indicate the *y*-axis for each spectrum.

voltage will produce a very high electric field across the BLM. This large field strength (~10^5 volts/cm) will increase or decrease, depending on its polarity, the number of light-generated charges moving to opposite sides of the BLM.

Fig. 3 shows the wavelength dependence of a PQ-3 containing BLM. The action spectrum follows closely the absorption spectrum (curve A) of the membrane-forming solution. This provides strong evidence that the photons absorbed by the pigment only are responsible for the initial separation of electronic charges in the BLM and the observed light-induced effects. To obtain information on the fast photoprocesses and light-induced effects on the pigmented BLM system, the membranes were excited by light pulses of 8 μs duration. The photopotentials generated were recorded with different time scales. The results for a BLM containing PQ-3 are shown on Fig. 4A. When observed in this way, the photoresponse shows to be composed of several components with different rate constants. Since the pigmented BLM possesses an appreciable capacitance ($C_m \simeq 5 \times 10^{-9}$ farad) and resistance ($R_m \simeq 1$—$9 \times 10^8\ \Omega$), the membrane is electrically equivalent to a capacitor shunted with a resistor. The voltage transients appear across the effective

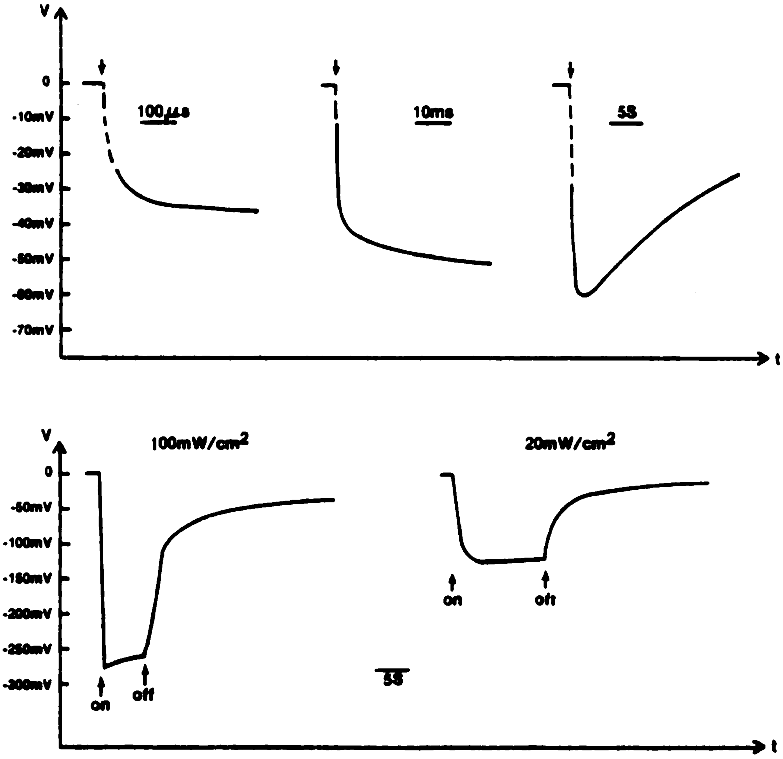


Fig. 4. A) Time course of the photoresponse from a PQ-3-containing BLM excited by short light flashes (8 μs duration). The time course recorded with three different time scales is shown. The downward arrows indicate the onset of the flash. B) Time course of the photoresponse with continuous illumination for a PQ-3-containing BLM with two different light intensities in mW · cm^{-2}.

membrane R_m—C_m combination as previously described [5—10]. The initial phase commences with about less than 1 μs delay and grows during the flash (Fig. 4A, upper left and middle curves). The final phase (Fig. 4A, right) is the decay of the flash-induced voltage lasting several seconds. The fast appearance of the photopotential across the membrane in the μs range (resolution limited by our apparatus) strongly suggests the generation and separation of electronic charges. The continuing growth of the voltage *after* the flash excitation implies the existence of long-lived species (e.g. triplet state excitons or free radicals) which upon oxidation and reduction by electron acceptors and donors, respectively, in the aqueous solution contribute further to the potential difference across the BLM.

A more detailed description of the possible sequence of events taking place at both interfaces can be expressed in terms of the following reactions:

$$\mathrm{P-Q} + h\nu \rightarrow [\mathrm{P-Q}]^* \rightarrow \mathrm{P^+-Q^-} \tag{1}$$

$$\mathrm{P^+-Q^-} + \mathrm{A} \rightarrow \mathrm{P^+-Q} + \mathrm{A^-} \tag{2}$$

$$\mathrm{P^+-Q^-} + \mathrm{D} \rightarrow \mathrm{P-Q^-} + \mathrm{D^+} \tag{3}$$

$\mathrm{P^+-Q^-}$ is, as mentioned previously, a long-lived species whose nature is at present not possible to identify exactly. The species $\mathrm{P^+-Q}$ and $\mathrm{P-Q^-}$ can then be reduced and oxidized, respectively, either by electrons and/or holes from donors (D) and acceptors (A) on opposite sides of the BLM in the bathing solution. The net reactions for these two cases are:

$$\mathrm{P^+-Q} + \mathrm{D} \rightarrow \mathrm{P-Q} + \mathrm{D^+} \tag{4}$$

$$\mathrm{P-Q^-} + \mathrm{A} \rightarrow \mathrm{P-Q} + \mathrm{A^-} \tag{5}$$

Mechanistically, a pigmented BLM/aqueous interface has been likened to that of a Schottky barrier [23], with the aqueous solution playing the role of the metal. In this way the membrane is considered to be capable of electronic conduction.

From our results obtained using P—Q complexes, it is clear that the enhanced photoeffect in the BLM system can be explained in terms of intramolecular charge—transfer processes between donor and acceptor moieties in these covalently linked compounds. The only difference between the three P—Q complexes is the chain length between the porphyrin and quinone moiety (see Fig. 1). Therefore, we attribute the difference in the photoresponses to the difference in the distance and orientation between the two moieties. The interaction between the π electron in the two chromophores, which depends on the distance and geometry of these chromophores in the covalently linked complexes, should be maximum. It appears that by covalently attaching these compounds, a more favorable orientation and closer proximity is attained between the donor and acceptor pairs which result in a maximum overlap between highly filled donor orbitals and lowest filled acceptor orbitals. This provides more favorable conditions for eventual charge separation rather than being dissipated by other pathways such as fluorescence. In fact, a quenching of the fluorescence emission for porphyrin—quinone and other complexes has already been reported [15—19]. Our results are consistent with those obtained by other workers on fluorescence quenching. In the presence of such quenching the fluorescence quantum yield will be low because of the utilization of all energy in charge separation which results in net enhanced photoeffect. Thus, our findings provide support to the idea that a more efficient charge separation can take place when the donor/acceptor pairs are in close proximity to each other. These covalently linked compounds, in particular those typified by the PQ-3 type, may be very useful in certain biomimetic solar energy transduction systems [1—3,23,24].

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