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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 $\approx$ 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

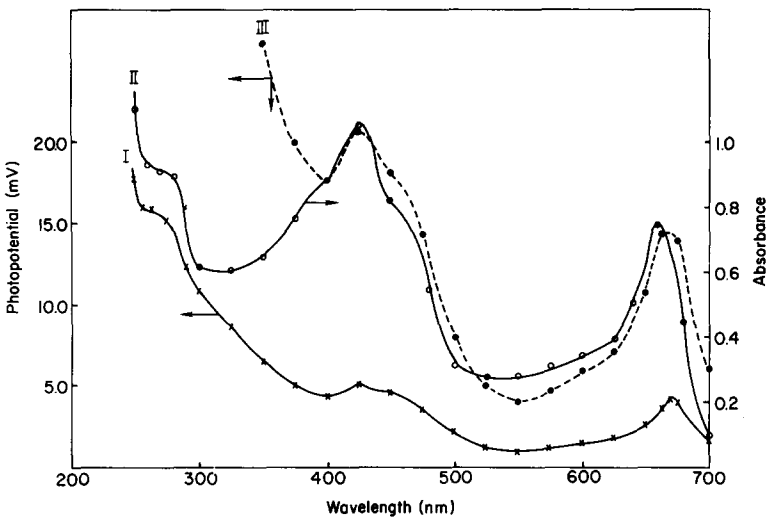

Fig. 1. Curve I (X———X): open-circuit photovoltaic action spectrum of a chloroplast-bilayer lipid membrane in the presence of $FeCl_3$. Curve II (○———○): absorption spectrum of chloroplast-bilayer lipid membrane-forming solution. Curve III: Curve I X4.

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.

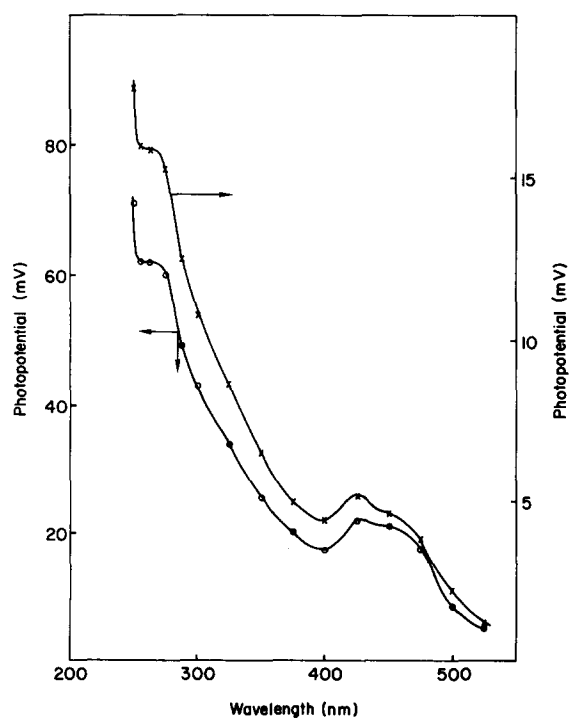

Fig. 2. Comparison of the action spectrum of a chloroplast-bilayer lipid membrane in the presence of ascorbate (X———X) and in the presence of $FeCl_3$ (○———○).

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. $1 \cdot 10^9$ Ω. 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

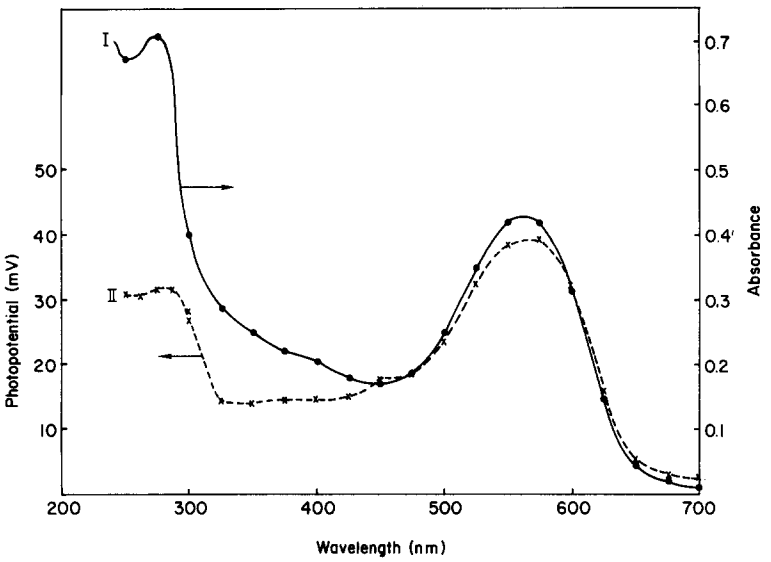

Fig. 3. Curve I (●———●): absorption spectrum of purple membrane from *Halobacterium halobium*. Curve II (X- - - - -X): open-circuit photovoltaic action spectrum of a bilayer lipid membrane-containing purple membrane.

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

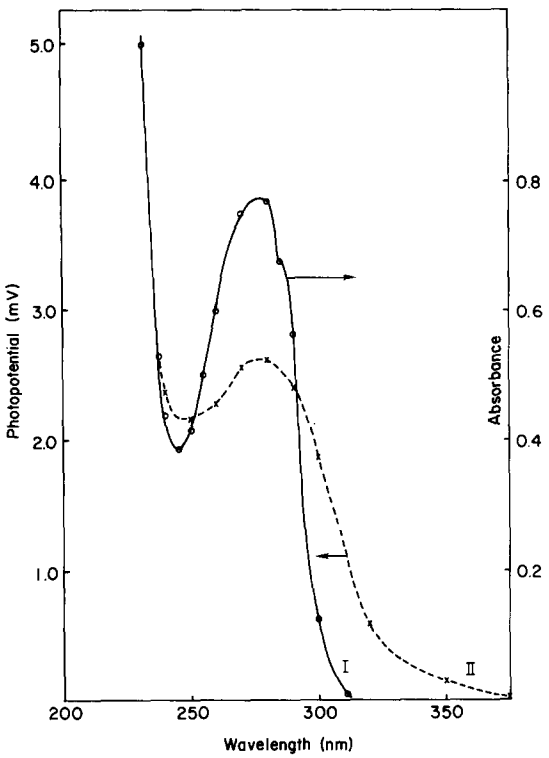

Fig. 4. Curve I (o———o): absorption spectrum of chymotrypsin in distilled water. Curve II (X- - - - -X): open-circuit photovoltaic action spectrum of bilayer lipid membrane to one side of which chymotrypsin was added.

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

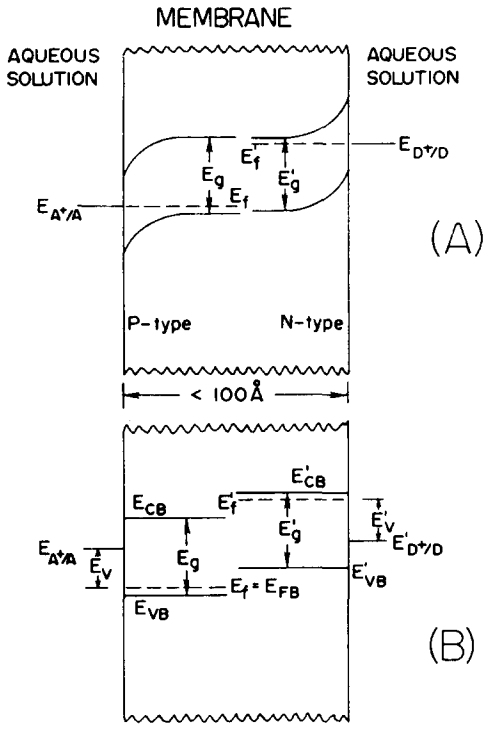

Fig. 5. Schematic diagram of a pigmented bilayer lipid membrane separating two aqueous solutions. (A) the membrane is in equilibrium with redox compounds in the bathing solutions in the dark. (B) the bilayer lipid membrane is under illumination.

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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