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Bacteriorhodopsin-Mediated Photoelectric Responses in Lipid/Water Systems

Lel A. Drachev, Andrey D. Kaulen, Vladimir P. Skulachev, and Vladimir M. Voytsitsky

Departments of Bioenergetics and of New Physical Methods, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, USSR

Summary. Bacteriorhodopsin-mediated photopotential generation has been studied in two kinds of lipid/water systems: (1) decane solution of asolectin was used as the lipid phase; (2) a mixture of bacteriorhodopsin sheets and hexane solution of phosphatidyl choline was applied onto a water surface to form a monolayer, and then the monolayer was covered with a 0.3-mm decane layer. In both cases, illumination was found to induce formation of an electric potential difference, with the bulk water phase being found negative when measured with a vibrating electrode. In the latter, but not in the former, system small amounts of a protonophorous uncoupler were found to stimulate the photoresponse. Large amounts of the uncoupler proved depressing in both systems. Phenyldicarbaundecaborane anion (PCB^-) was shown to substitute for the uncoupler, being much more potent both as an activator and as an inhibitor of the photoresponse. In both studied systems, gramicidin A inhibits the photoresponse, the effect being greatly potentiated by K^+, Na^+ or H^+ ions.

In the system "decane solution of asolectin/water," an Ag/AgCl electrode immersed into the lipid phase can be used instead of a vibrating electrode. All the measured features of the photoelectric responses observed with any of these electrodes were found to be quite similar to those inherent in a phospholipid-impregnated collodion film adsorbing bacteriorhodopsin sheets on one of its surfaces.

A scheme is discussed built on the assumption that photopotentials in all the studied systems are due to an uphill light-dependent transport of H^+ ions from the bulk water phase to a water cavity localized between a bacteriorhodopsin sheet and the surface of the bulk lipid phase. Thus, the above lipid/water systems containing bacteriorhodopsin are composed of four, rather than two, phases, as was supposed previously.

Bacteriorhodopsin-mediated photopotential generation has been studied also in the decane/water system without phospholipids. This system with bacteriorhodopsin sheets added to the water phase demonstrates a light-dependent photoelectric response reaching 1.5 V, which can be measured only by a vibrating electrode. The photoresponse starts after a lag period of several seconds. Switching off the light results in the reversal of the light-induced electric potential change. The off-effect also has a lag period. The action spectrum of the photoresponse shows at least two maxima: a smaller at 560 nm and a larger at <420 nm. Free retinal can substitute for bacteriorhodopsin in the studied system. All the above effects disappear if, instead of air, argon is used. In the system "decane solution of asolectin/water," a slow photoelectric response of this type can be demonstrated at neutral pH in the presence of gramicidin and at pH 4 without gramicidin. A suggestion is put forward that the slow photoelectric response is due to an interface Volta-potential change induced by a product of photooxidation of bacteriorhodopsin and/or free retinal released from bacteriorhodopsin.

Key words bacteriorhodopsin · proton pump · retinal · model lipid membrane · lipid/water interface · photoeffects

Introduction

In 1971 Oesterhelt and Stoeckenius described a protein from *Halobacterium halobium* that resembled the animal visual pigment, rhodopsin. The compound was found to be localized in special regions of the bacterial cytoplasmic membrane of crystalline structure (Blaurock & Stoeckenius, 1971). Studies on the biological functions of bacteriorhodopsin revealed that it operates as a light-driven electrogenic H^+ pump (Oesterhelt & Stoeckenius, 1973; Kayushin & Skulachev, 1974; Racker & Stoeckenius, 1974). In this group, a method of direct measurement of the bacteriorhodopsin-mediated electrogenesis has been elaborated (Drachev et al., 1974*a–c*, 1976; Drachev, Kaulen & Skulachev, 1977, 1978). The method consists of associating bacteriorhodopsin sheets with the surface of a planar phospholipid membrane, a membrane filter, or a collodion film impregnated with a decane solution of phospholipids. It was found that illumination of such systems results in charge separation across the planar membrane or filter, which can be measured by the orthodox electrometer techniques.

Boguslavsky et al. (1976) and Hwang, Korenbrot and Stoeckenius (1977*a, b*) reported that some photoelectric responses can be mediated by bacteriorhodopsin sheets in a biphasic system composed of lipid and water layers. Both groups of authors noted that addition of a protonophorous uncoupler is necessary to demonstrate a photoeffect. There was a difference

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in the data published by these groups: Hwang et al. (1977*b*) showed that there is an optimum in the photoeffect plotted against the uncoupler concentration, whereas Boguslavsky et al. (1976) observed a plateau with no photoeffect decrease at high concentrations of uncouplers. Both groups concluded that their systems are indeed biphasic with interface-linked bacteriorhodopsin pumping H^+ ions from water to the lipid phase. The uncouplers were postulated to play a role of lipid-soluble H^+ acceptors.

The aim of this work was to study the mechanism of bacteriorhodopsin photoeffects in lipid/water systems. We compared the behavior of bacteriorhodopsin associated with the lipid/water interface and that with planar membrane and collodion film. The existence of a bacteriorhodopsin-mediated photoelectric effect was confirmed, but the further study gave new information that proved inconsistent with the explanation of this photoeffect by H^+ transport from water to the lipid phase.

Materials and Methods

Bacteriorhodopsin sheets from *H. halobium* R_1 were isolated according to Oesterhelt and Stoeckenius' (1971, 1974) procedure. The bacteriorhodopsin quantity was measured spectrophotometrically using a 568 nm optical density coefficient equal to 63,000 M^{-1} cm^{-1} (Oesterhelt & Stoeckenius, 1973). Bacteriorhodopsin incorporation into a planar phospholipid membrane or a collodion film impregnated with decane solution of asolectin was made as described previously (Drachev et al., 1976, 1977, 1978).

The following materials were used: Tris, *m*-CCCP and soybean phospholipids (asolectin) from Sigma, MES from Merck, gramicidin A from Calbiochem; phosphatidyl choline was prepared from asolectin after Dawson (1963). Water was redistillated three times using a Pyrex glass distillator. One of the distillations was carried out with addition of $KMnO_4$ and H_2SO_4.

Light source OI-28 with KGM-24V-15-W quartz halogen lamp was used in the continuous illumination experiments. The light beam passed through a 1-cm water filter to prevent heating of the experimental cell and in most experiments through a filter of 300–820 nm light permittance. The light intensity on the lipid/water interface was about 50 $mW \cdot cm^{-2}$.

When the action spectra were measured, interferential and neutral filters were used. The latter were applied to vary light intensity.

The block-scheme of the device measuring electric potentials in the lipid/water system is shown in Fig. 1. The measurements were carried out in an experimental cell block (*1*) with a vibrating electrode (*c*), or an Ag/AgCl electrode (*d*) immersed into lipid phase (*b*). A reference Ag/AgCl electrode (*e*) was placed into water phase (*a*). Both Ag/AgCl electrodes were connected with the bulk phases via agar bridges with saturated water solution of KCl. When electrode (*d*) was used, the conditions of the measurement resembled those in the planar membrane or membrane filters since the second lipid/water interface has been formed in the place of contact of the agar bridge with the lipid phase.

The experimental cell (*1*) made of pyrex glass (15 mm high and 40 mm in diameter) has two branches, one for the reference electrode and the other for additions. Illumination of the cell was carried out through the water phase.

As a vibrating electrode, a 0.5-mm thick gold disc 10 mm in diameter was applied.

Oscillations (120 cps) of this electrode were supported by sinusoidal changes in the voltage of a G3-33 signal generator (block *2* in Fig. 1). The amplitude of oscillation was 1 mm; the distance between the lipid phase surface and the vibrating electrode 3 mm.

A signal of alternating current from the vibrating electrode was transmitted to operational amplifier (*3*), measuring amplifier (*4*), and phase detector (*5*). A transformed signal from the phase detector (*5*) reached the amplifier (*7*) and then the reversive asynchronous motor (*8*) of the recorder (*see* block *6*). To the same recorder, calibrated compensating voltage was fed. The compensation degree and noises were followed by oscillograph (*9*). The principle of electric potential difference measurement with a vibrating electrode was essentially the same as that of Boytsov and

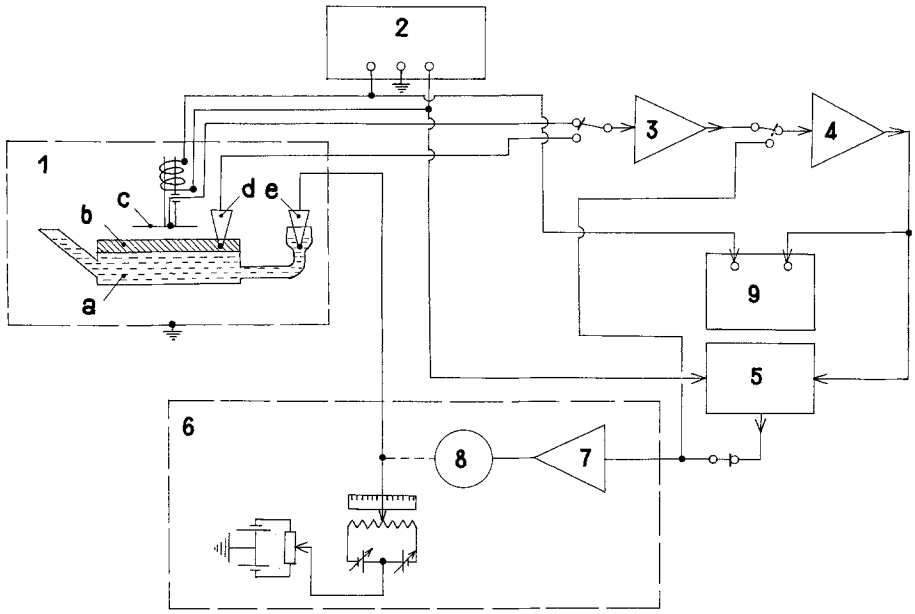

Fig. 1. Block-diagram for measurements of photoelectric responses (for explanation, *see* text)

Boguslavsky (1966) (*see also* Babakov, Mjachikov, Sotnikov & Terechov, 1972).

When an Ag/AgCl electrode contacting the lipid phase was used instead of the vibrating electrode, electric potential was transmitted via the operational amplifier to the amplifier of the reversible asynchronous motor.

Measurements with the vibrating electrode and with the Ag/AgCl electrode connected with the lipid phase, were carried out consecutively in the same sample.

Temperature in the experimental cell was monitored with a thermistor. Most of the experiments were performed at room temperature.

In the case of decane/water system, decane was layered on the surface of the water phase after addition of bacteriorhodopsin sheets into the water phase (the thickness of the decane layer was about 1 mm). Before addition, bacteriorhodopsin sheets were pretreated in one of the three ways: (i) 20 sec sonication of the water suspension of the sheets with a USDN-1 sonic desintegrator, (ii) grinding of the sheets with a glass homogenizer and (iii) 1 min treatment of the decane/water system with a vibrating electrode touching the decane surface (electrode oscillation frequency 120 cps, amplitude 1 mm). After the treatment, the vibrating electrode was washed. These procedures were proved necessary to obtain a photoeffect if bacteriorhodopsin sheets were stored for 3–5 days at 2–4 °C in water after isolation from bacteria. In the fresh-prepared sheets, no pretreatment was required.

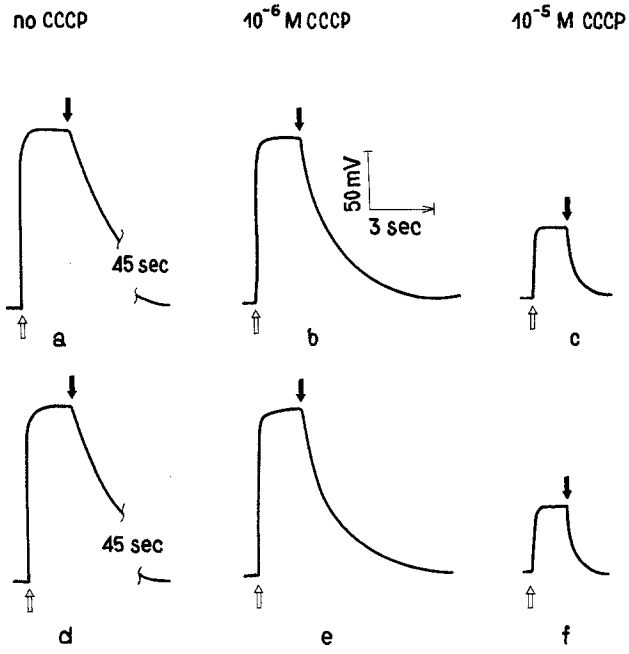

Fig. 2. Photoelectric responses of bacteriohodopsin in system "decane solution of asolectin/water" measured with a vibrating electrode (*a, b, c*) or an Ag/AgCl electrode connected with lipid phase (*d, e, f*). Here and below increase in the potential means positive charging of the lipid phase. White and black arrows are for switching the light on and off, respectively. Water phase: 0.05 M Tris-HCl (pH 7.4), 1.5 mM $CaCl_2$, and bacteriorhodopsin sheets, 50 μg protein/ml; Lipid phase: decane containing 2 mg of asolectin per ml

Results

Photoeffect in the System "Decane Solution of Asolectin/Water"

Comparison of photoresponses measured with vibrating electrode and with lipid-phase connected Ag/AgCl electrode. Effect of CCCP. Illumination of a biphasic system composed of decane solution of asolectin (2 mg/ml) and water with bacteriorhodopsin sheets added to the water phase has been shown to result in an electric potential difference being formed between the vibrating and reference electrodes. Small photoeffect could be observed soon after bacteriorhodopsin addition. To obtain maximal values of the photoeffect rather prolonged incubation was necessary, the process being accelerated by Ca^{2+} addition to the water phase. The highest potential differences observed after 4–6 hr of dark incubation were about 200 mV. Similar data were obtained when the vibrating electrode was replaced with an Ag/AgCl electrode connected with the lipid phase. An experiment of this type is shown in Fig. 2. One can see that switching on the light induced a potential difference formation whose rate was higher than the time resolution of the measuring system (< 1 sec). Switching off the light resulted in discharge of the photopotential that required about 1 min. Addition of a protonophorous uncoupler, *m*-chlorcarbonylcyanidephenylhydrazone (CCCP) greatly accelerated the dark decay and, at a higher concentration, lowered the amplitude of the photopotential. Both the kinetics and amplitudes of the above responses were the same regardless of whether the vibrating electrode or the lipid phase-connected Ag/AgCl electrode was used.

The directions of the electric field were also identical: in the light, the bulk water phase, to which bacteriorhodoposin was added, charged negatively.

It was found that the depth of immersion agar bridge of Ag/AgCl electrode into the lipid phase was without measurable effect on the observed photopotential (not shown) as well as on the measured resistance of the lipid layer.

The action spectra of the photoeffect measured by both types of the electrodes were in good agreement with the absorption spectrum of bacteriorhodopsin (Fig. 3).

Figure 4 shows the mangitude of the photopotential plotted against CCCP concentration. Note that the protonophore, if it affects the photopotential, is always inhibitory and never stimulating.

Effect of phenyldicarbaundecaborane (PCB^-). It was found that a lipid-soluble anion PCB^- markedly increases the photopotential in the "decane solution of asolectin/water" system (Fig. 5). The increasing effect was observed at a low PCB^- concentration, namely, between 10^{-11} and 10^{-9} M if calculated per

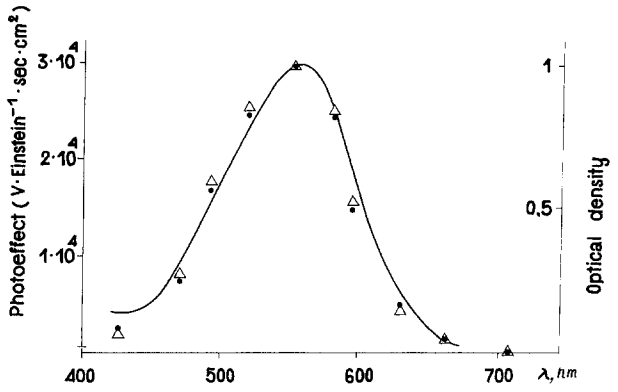

Fig. 3. Action spectra of photoeffects shown in Fig. 2. Solid curve shows optical density of the suspension of the bacteriorhodopsin sheets; (●) and (△), photoelectric responses measured with a vibrating electrode or with an Ag/AgCl electrode connected with the lipid phase, respectively

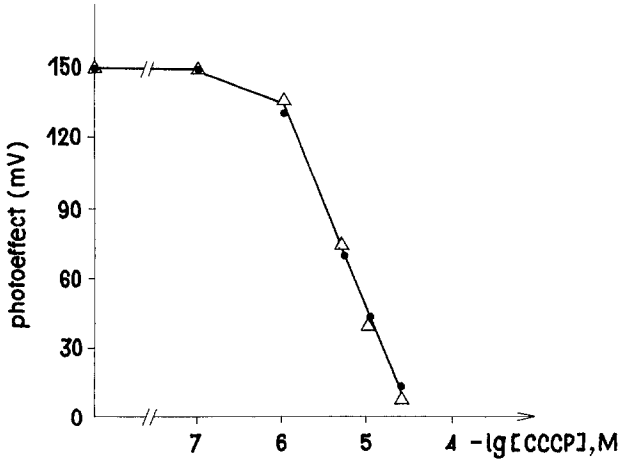

Fig. 4. Bacteriorhodopsin-mediated photoeffect in system "decane solution of asolectin/water" plotted against CCCP concentration. (●) and (△), measurements with a vibrating electrode or with an Ag/AgCl electrode connected with the lipid phase, respectively. Phase composition as in Fig. 2

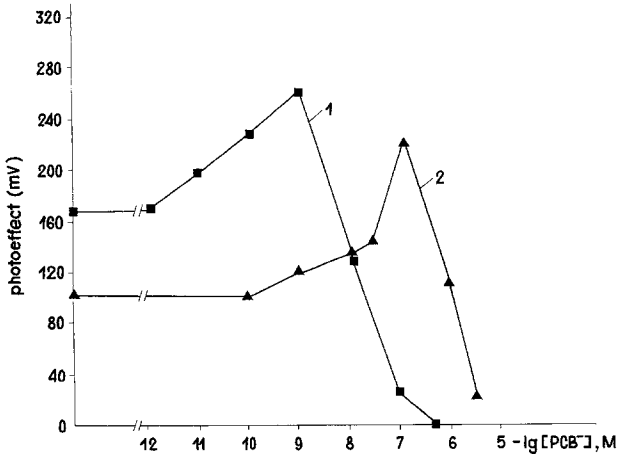

Fig. 5. Effect of a lipid-soluble anion, phenyldicarbaundecaborane (PCB^-), on the magnitude of the bacteriorhodopsin-mediated photoresponse in system "decane solution of asolectin/water." Measurement was with a vibrating electrode; water phase as in Fig. 2. Asolectin concentration: (■), 2 mg/ml; (▲), 60 mg/ml

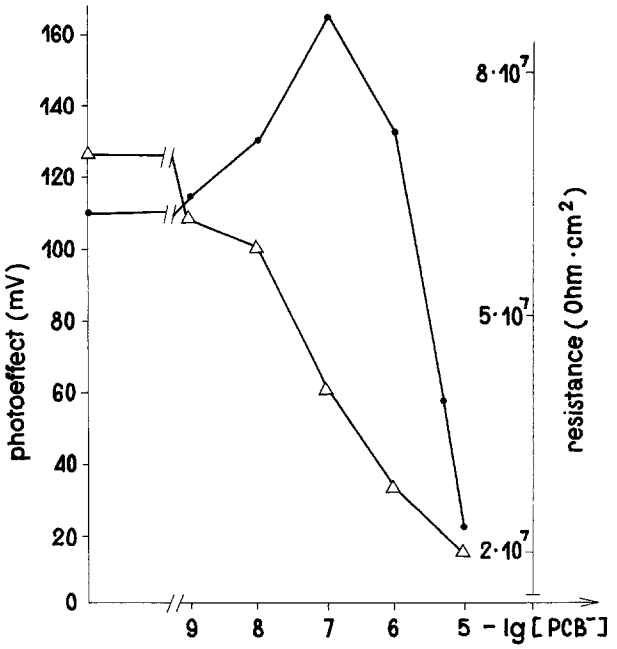

Fig. 6. The PCB^- action on the bacteriorhodopsin-containing collodion film impregnated with a decane solution of asolectin. (●), photoinduced electric potential difference across the film; (△), electric resistance of the film. Preincubation mixture: 0.1 M NaCl, 2.5 mM EDTA (pH 6.0) and in one of the film-separated compartments, bacteriorhodopsin sheets, 50 μg protein/ml. After completion of bacteriorhodopsin incorporation, 0.05 M Tris-HCl (pH 7.4) was substituted for pre-incubation mixtures in both compartments

ml of the water phase (certainly, its concentration in the lipid phase was much higher). At higher concentration, PCB^- was inhibitory. By increasing the asolectin concentration from 2 to 60 mg per ml of decane, we could shift the PCB^- concentration optimum from 10^{-9} to 10^{-7} M.

Quite the same PCB^- action was demonstrated in a collodion film impregnated with decane solution of lecithin (60 mg/ml). The film-separated experimental cell was described elsewhere (Drachev et al., 1977, 1978). Two Ag/AgCl electrodes were immersed into the water solutions on both sides of the film. Bacteriorhodopsin sheets were added to one of the water compartments. In the several hours required for bacteriorhodopsin to be incorporated into the collodion film impregnated with the phospholipid solution in decane, a photoeffect of about 110 mV could be observed. It increased up to 170 mV after addition of 10^{-7} M PCB^-. Further increase in the PCB^- concentration strongly lowered the photoeffect. Both the stimulating and inhibitory PCB^- actions on the photopotential were accompanied by a decrease in the film electric resistance (Fig. 6).

Effect of gramicidin A. Figure 7 shows the effect of gramicidin A on the photoresponse in the system "decane solution of asolectin/water." It is seen that gra-

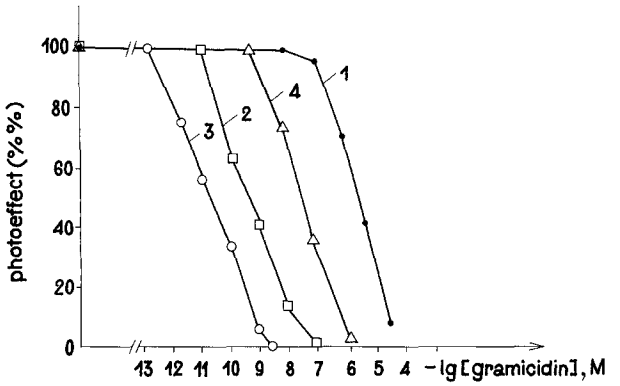

Fig. 7. Bacteriorhodopsin-mediated photoresponses in system "decane solution of asolectin/water." Effect of gramicidin A. Water phase containing 1.5 mM $CaCl_2$ and bacteriorhodopsin sheets, 50 μg protein/ml, was supplemented alternatively with (*1*) 0.05 M Tris-HCl (pH 7.4), (*2*) 0.05 M Tris-HCl (pH 7.4) and 1 mM KCl, (*3*) 0.05 M Tris-HCl (pH 7.4) and 0.1 M KCl, or (*4*) 0.05 M MES buffer (pH 6.0)

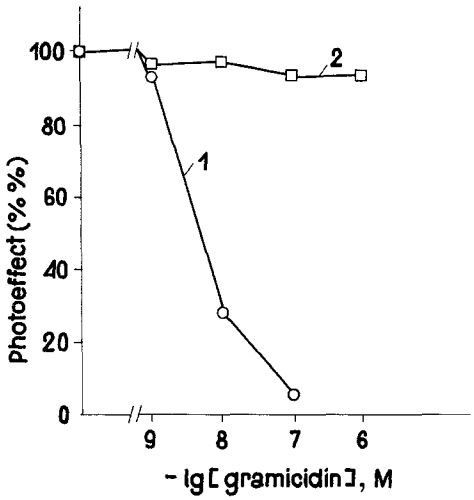

Fig. 8. Action of gramicidin A on the bacteriorhodopsin-containing collodian film impregnated with decane solution of asolectin. Curve *1*: gramicidin was added to the same compartment as bacteriorhodopsin. Curve *2*: gramicidin was added to the opposite compartment. Preincubation mixture: 0.1 M NaCl, 2.5 mM EDTA (pH 6.0), and in one of compartments, bacteriorhodopsin sheets, 50 μg protein/ml. After completion of bacteriorhodopsin incorporation, both compartments contained 0.1 M NaCl and 2.5 mM EDTA (pH 6.0)

micidin A decreases the light-dependent photoeffect. Addition of KCl or a decrease in pH strongly potentiates the gramicidin A action. For instance, at pH 7.4, gramicidin concentrations below 1×10^{-7} M did not decrease the photoeffect until KCl was added. In fact, addition of 100 mM KCl decreased K_i for gramicidin A by five orders of magnitude, so that measurable inhibition was obtained with 1×10^{-12} M gramicidin A.

Again, as in experiments with PCB^-, gramicidin A action in the lipid/water system was compared with that in the lipid-impregnated collodion film. As can be seen in Fig. 8, gramicidin A, if added to the same compartment as bacteriorhodopsin, completely inhibits the photoeffect. Its addition to the other compartment proves to be without any influence.

Higher K_i in this experiment as compared with lipid/water system is most probably due to the higher phospholipid content in the decane solution impregnating the collodion film. Increase in the phospholipid concentration in the lipid/water system was found to result in a rise of K_i (not shown).

It was also revealed that gramicidin A affects the collodion film electric resistance rather slightly when added to either the bacteriorhodopsin-containing or the bacteriorhopodsin-free compartment. The effect on the resistance could not be potentiated by univalent cations (not shown).

Photoeffect in the Lipid/Water System Prepared after Hwang, Korenbrot and Stoeckenius

In these experiments we used the system introduced by Hwang et al. (1977*a, b*). The principle of this method consists of the following: a mixture of water suspension of bacteriorhodopsin sheets and hexane solution of soybean phosphatidylcholine (0.16 mg/ml) is sonicated, and the upper fraction of the mixture is applied onto water surface to form a monolayer. Then decane is layered on top of this monolayer to form a 0.3-mm thick bulk lipid phase.

We repeated the procedure of Hwang et al. and confirmed the phenomenon of the photoeffect stimulation by low, and inhibition by high, concentrations of an uncoupler. As one can see in Fig. 9*A*, a manyfold increase in the photopotential (negative on the side of the water phase) can be achieved by CCCP addition at an optimal (1×10^{-7} M) concentration of this uncoupler. Further increase in the CCCP content strongly depressed the photopotential.

Further experiments showed that PCB^- can substitute for the uncoupler in the studied system. The optimal concentration of PCB^- proved to be two orders of magnitude lower than that of CCCP.

Figure 10 demonstrates gramicidin A effect on the photopotential in the system of Hwang et al. It is shown that addition of 10^{-7} M gramicidin A and 100 mM KCl abolishes the CCCP-stimulated photoeffect. The same amounts of gramicidin A and KCl, if added solely, do not influence the photopotential.

When phosphatidylcholine was replaced by asolectin, sometimes higher but fluctuating values of the photopotential were observed.

Spectral composition of the light proved critical for the demonstration of the described photoeffect. It was found that, in addition to a usual light filter transmitting 300–820 nm light, one more light absorbing filter shorter than 540 nm is required. Without

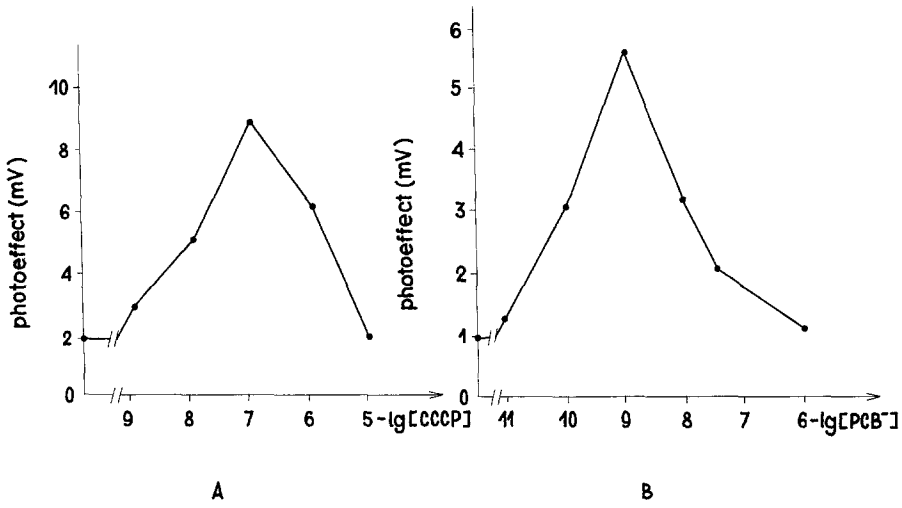

Fig. 9. Effect of CCCP and PCB^- on the photoresponse in a system prepared after Hwang et al. For lipid phase, *see* text. Water phase: water, redistillated three times, pH 5.8. Measurements were with a vibrating electrode

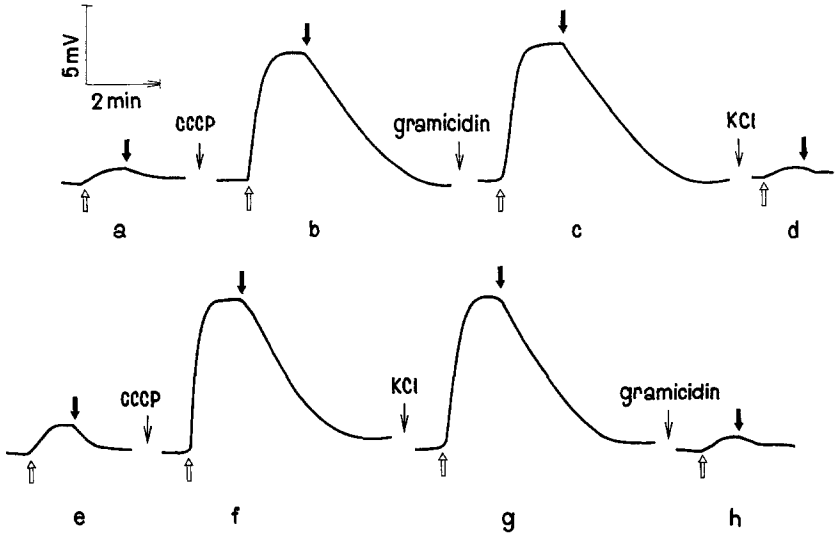

Fig. 10. Effect of gramicidin and KCl on the photoresponse in the Hwang et al. system. Data of two different experiments (traces *a–d* and *e–h*) are shown. For conditions, *see* Fig. 9. Additions: 10^{-7} M CCCP, 10^{-7} M gramicidin A, 0.1 M KCl

the latter, a slow photoeffect of the opposite direction developed, similar to that obtained in a decane/water system without phospholipids.

Photoeffect in the Decane/Water System

Illumination of the decane/water system with bacteriorhodopsin added to the water phase was shown to induce a large photopotential reaching sometimes 1.5 V, which can be measured by a vibrating gold electrode. The photoresponse could not be monitored with an Ag/AgCl electrode immersed into the lipid phase due to, first of all, very high resistance of the decane phase. The observed effect differs in many respects from those found in phospholipid-containing lipid/water systems. Its maximal magnitude is much larger; it requires neither uncoupler nor lipid-soluble anion (*cf.* Hwang et al., 1977*b*; Drachev et al., 1977); its kinetics and action spectrum has quite a different character as compared with both "decane solution of asolectin/water" and the Hwang et al. systems.

In Fig. 11 photoresponses of the decane/water system are measured in min (*A*) and sec (*B, C*) time scales. One can see that illumination induces generation of a potential difference between two bulk phases that disappears after switching off the light. It is also clear that there is a lag phase (about 5 sec) between the beginning of illumination and that of photoresponse rise as well as between switching off the light and the potential decrease (Fig. 11 *B*). There is some electric potential formation in the dark after 5 sec illumination (Fig. 11 *C*), indicating that illumination initiates a rather slow process(es) developing in the dark.

The polarity of the photoresponse varied from sample to sample. It was also found that addition of bacteriorhodopsin in the dark always induces formation of a small potential difference between the

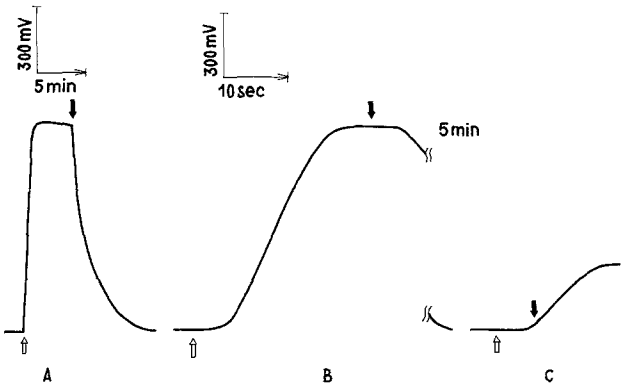

Fig. 11. Bacteriorhodopsin-mediated photoelectric responses in a decane/water system. Water phase: 0.05 M Tris-HCl (pH 7.4) and bacteriorhodopsin sheets, 50 μg protein/ml

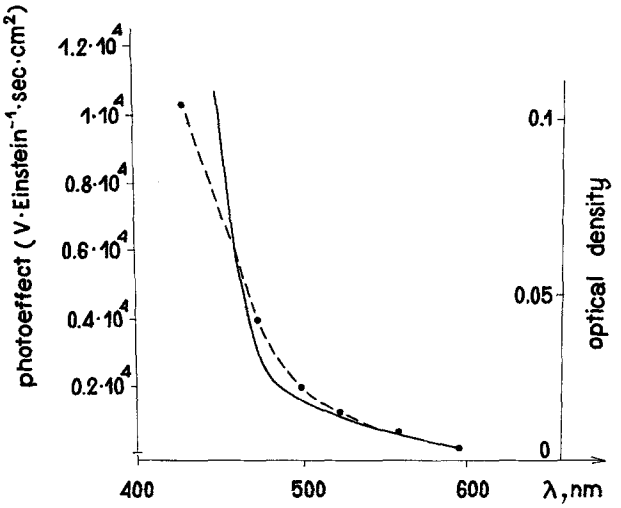

Fig. 13. Action spectrum of the retinal-mediated photoresponse in the decane/water system. Solid line, optical density; dashed line, photoresponse. Water phase: 0.05 M Tris-HCl (pH 7.4) and 10^{-4} M all-*trans*-retinal

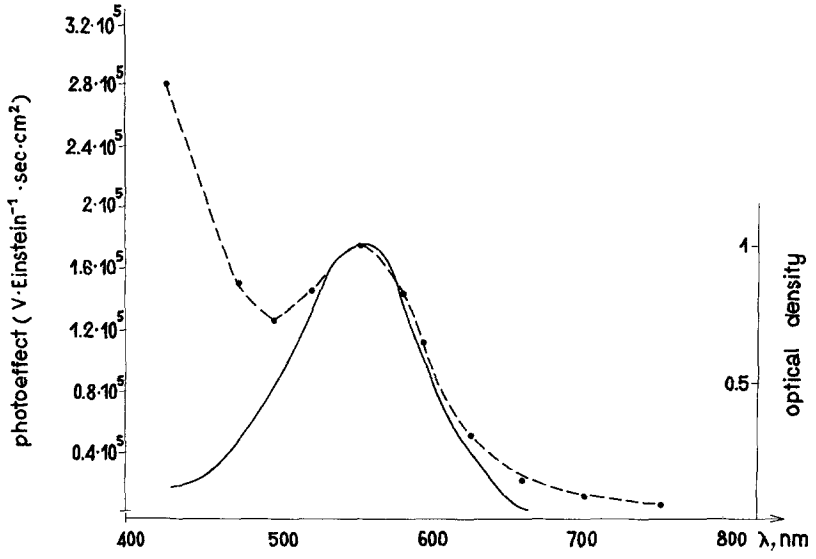

Fig. 12. Action spectrum of the bacteriorhodopsin-mediated photoresponse in a decane/water system. Solid line, optical density; dashed line, photoresponse. Water phase as in Fig. 12

decane and water phases. The direction of this change also proved to vary. Nevertheless, the responses induced by both bacteriorhodopsin addition in the dark and illumination of the bacteriorhodopsin-containing system were found always to be of the same direction (not shown).

The action spectrum of the bacteriorhodopsin-mediated photoelectric effect shows at least two maxima, a smaller at 560 nm and a larger at a wavelength shorter than 420 nm (Fig. 12).

Further experiments revealed that not only bacteriorhodopsin but also free all-*trans*- or 13-*cis*-retinals demonstrates a photoresponse in the decane/water system. The characteristics of the retinal-mediated photopotential were mostly similar to those mediated by bacteriorhodopsin, differing only in two respects: (i) there was no 560 nm maximum at the retinal action spectrum (Fig. 13) and (ii) a lag period after switching on the light was shorter with retinal than with bacteriorhodopsin.

A measurable photoelectric effect was observed at a retinal concentration as low as 10^{-9} M (bacteriorhodopsin concentration in the above experiments was usually about 10^{-6} M).

Treatments releasing retinal from bacteriorhodopsin were found to increase the photoresponse in the studied system. This could be achieved by prolonged sonication of bacteriorhodopsin sheets or their incubation with sodium dodecyl sulfate (SDS).

In Fig. 14 the effect of SDS is shown. Under the

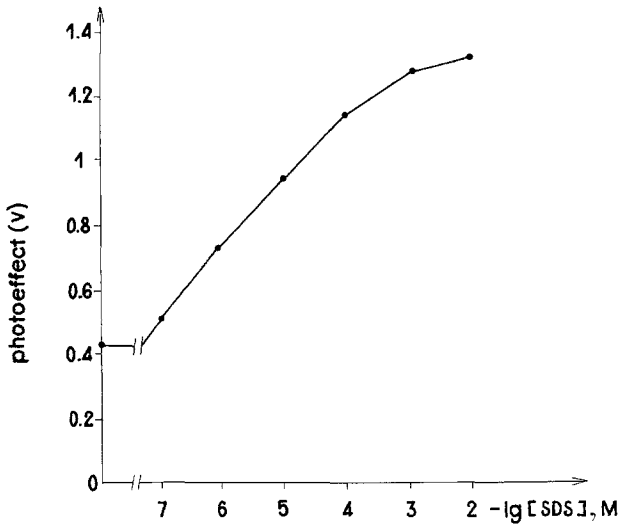

Fig. 14. Effect of sodium dodecyl sulfate (SDS) on the magnitude of bacteriorhodopsin-mediated photoresponse in a decane/water system. Water phase as in Fig. 12

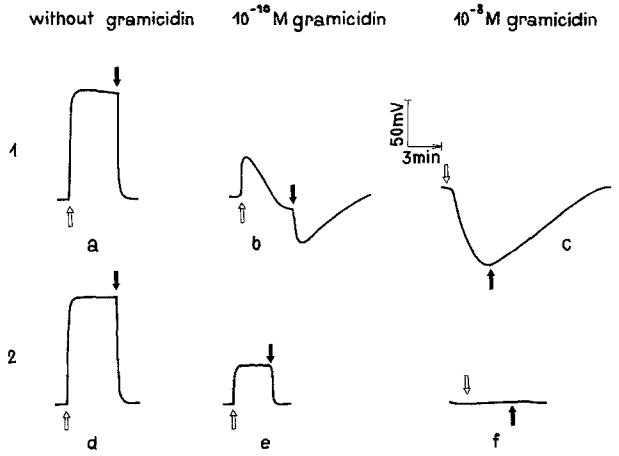

Fig. 16. Fast and slow photoresponses in system "decane solution of asolectin/water" at pH 4 in the water phase. Lipid phase was as in Fig. 8. Water phase: 0.05 M Tris-acetate (pH 4.0), 1.5 mM $CaCl_2$, and bacteriorhodopsin sheets, 50 μg protein/ml. *1* and *2*, measurements with a vibrating electrode or Ag/AgCl electrode connected with lipid phase, respectively

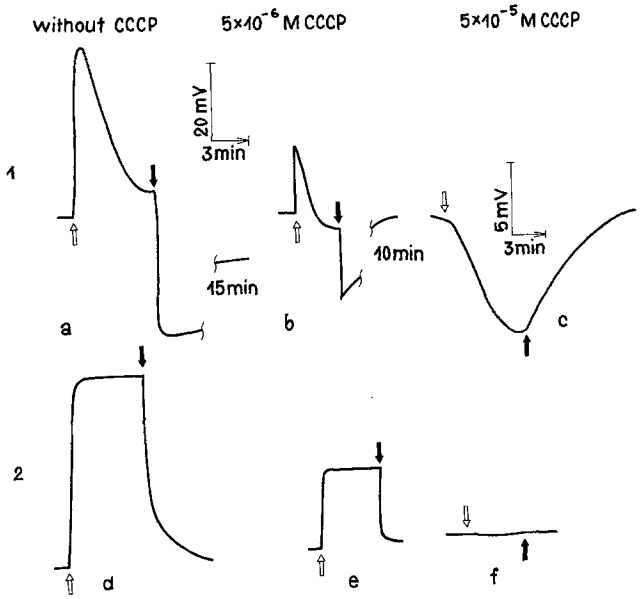

Fig. 15. Gramicidin A-induced appearance of a slow bacteriorhodopsin-linked photoresponse in system "decane solution of asolectin/water." Lipid phase: decane and asolectin (2 mg/ml). Water phase: 0.05 M Tris-HCl (pH 7.4), 1.5 mM $CaCl_2$, 0.1 M KCl, and bacteriorhodopsin sheets, 50 μg protein/ml. *1* and *2*, measurements with a vibrating electrode or an Ag/AgCl electrode connected with lipid phase, respectively

conditions used, the bacteriorhodopsin sheets demonstrated the photopotential of about 0.4 V. The SDS treatment resulted in a photopotential increase up to 1.3 V. This was accompanied by a shift of the absorption maximum from 560 to 390 nm (not shown). Similar data were obtained when sonic treatment was used instead of SDS (not shown). The photoconversion of retinal was shown to be dependent upon molecular oxygen since substitution of argon for oxygen prevented both the bacteriorhodopsin- and retinal-linked photoeffects (not shown).

Addition of phospholipids somehow inhibits the development of the phenomenon described in this section. However, addition of gramicidin A was found to remove this inhibition. As seen in Fig. 15, gramicidin A addition to the system "decane solution of asolectin/water" results in the appearance of slow (with a lag period) negative charging on the lipid side instead of the fast positive one observed in the absence of gramicidin. This slow response has all features of that described above for the decane/water system.

It is interesting that the slow photoeffect can be measured with a vibrating gold electrode connected with the lipid phase, whereas the fast one is monitored with both types of electrodes.

In Fig. 16 the photoresponses of the system "decane solution of asolectin/water" were measured at pH 4 in the water phase. One can see that both the fast and slow photoelectric effects are present. Addition of CCCP completely abolishes the fast response, so that the slow response proves to be the only one still observed. No gramicidin A was found to be necessary at pH 4. Again, the slow photoeffect could be measured with a vibrating but not with a lipid phase-connected Ag/AgCl electrode.

Discussion

Analysis of the bacteriorhodopsin-mediated photoeffect in the system "decane solution of asolectin/

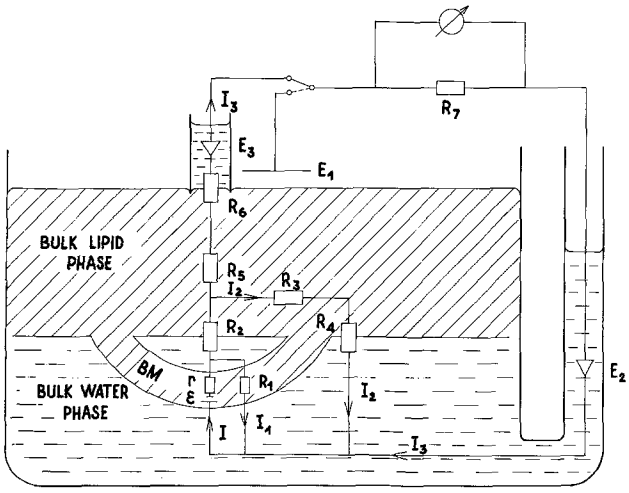

Fig. 17. Tentative electrical scheme for photoresponses in the lipid/water systems containing phospholipids and bacteriorhodopsin sheets. For explanation, *see* text

water" reveals its striking similarity to that in planar membranes, or in lipid-impregnated membrane filters and collodion films. These systems have the following common features:

1) The amplitude of photopotential is about 150–200 mV, the bacteriorhodopsin-containing water phase being charged negatively.

2) Bacteriorhodopsin incorporation is greatly accelerated by cations such as Ca^{2+}.

3) Protonophorous uncouplers strongly decrease the photoeffect.

4) Lipid-soluble anions, like PCB^-, at low concentrations increase, and at higher concentrations decrease, the photopotential.

5) Gramicidin is also inhibitory, the effect being potentiated by K^+ and Na^+.

The latter property is especially important for the understanding of the mechanism of the observed light-dependent responses. It is known that gramicidin forms channels permeable for Na^+, K^+ and H^+ only in thin membranes. The gramicidin channel is too short to transpierce membranes thicker than a phospholipid bilayer (Ovchinnikov, Ivanov & Shkrob, 1974). As it was shown, gramicidin A only slightly decreases the resistance of the lipid-impregnated collodion film, the effect being cation-independent. In black membranes, the same amounts of gramicidin lower the resistance by many orders of magnitude, and the acting gramicidin concentrations can be strongly decreased by the addition of univalent cations. So, the effect of gramicidin on the overall collodion film resistance does not correlate with gramicidin-induced inhibition of photopotential, which is very strong and cation-dependent.

To explain this fact, we suggested that the bacteriorhodopsin sheet does not fuse with the planar membrane (or lipids in pores of the film), but is rather attached to the planar membrane surface in such a way that a water cavity is formed between the planar membrane and the sheet membrane (Drachev et al., 1974, 1976).

Assuming such a scheme, we may account for the gramicidin action in terms of the formation of a cation-permeable channel through the sheet membrane which is as thick as 50 Å. Thus, H^+ ions pumped by bacteriorhodopsin from the bulk water phase to the cavity, cannot charge the sheet membrane if there is a gramicidin-mediated electrophoretic movement of K^+, Na^+ or H^+ in the opposite direction.

The similarity of the gramicidin effects in the planar membrane or collodion film and in the lipid/water system suggests that the above reasoning can be also applied to the latter case. Figure 17 illustrates a tentative electric scheme explaining the results obtained with the system "decane solution of asolectin/water." It is assumed that bacteriorhodopsin generator having emf and internal resistance r, charges the membrane of a bacteriorhodopsin sheet (BM) by means of the light-dependent H^+ current I directed from the bulk water space to a water cavity which is localized between the sheet membrane and the bulk lipid phase. Then current I is divided into currents I_1 and I_2. Current I_1 flows via resistance R_1 of the sheet membrane. In this way, H^+ (or other cations) leaves the cavity for the bulk water phase (or anions move in the opposite direction) down an electrical gradient. Current I_2 includes interface resistance R_2, bulk lipid phase resistance R_3 and interface resistance R_4. There are only these currents in the system if the measurement is carried out by vibrating electrode E_1 and reference Ag/AgCl electrode E_2. If another Ag/AgCl electrode (E_3) connected with the lipid phase, is used instead of E_1, one more current (I_3) should be taken into account. It includes the following series of resistances: R_2 (*see* above), bulk lipid phase resistance (R_5), interface resistance in the place of contact between the bulk lipid phase and the water phase of the agar bridge of the Ag/AgCl electrode (R_6), and resistance of a chain to which a voltmeter is connected (R_7).

As was mentioned above (*see* Results), in the system "decane solution of asolectin/water," the photovoltage proves to be of the same values regardless of whether E_1 or E_3 electrode was used. It was also found that the depth of the immersion of E_3 electrode into the lipid phase does not affect the measured photovoltage. Therefore we can conclude that I_3 current is much smaller than I_2, and the resistance of the bulk lipid phase (R_3 and R_5) is much lower than the interface resistance (R_2, R_4 and R_6). So, E_3 elec-

trode in fact measured the electric potential difference across the interface between the bulk lipid and the bulk water phases, resembling in this respect vibrating electrode E_1.

Gramicidin specifically decreases the R_1 resistance since, in this system, the sheet membrane is the only structure that is sufficiently thin to be crossed by the gramicidin channel.

The PCB^- effect is of a complex character. It decreases R_1 (as well as R_2, R_3 and R_4) as a charged penetrant and must therefore decrease the photopotential. This occurs when the PCB^- concentration is higher than 10^{-7} M. However, lower PCB^- levels markedly increase the measured photovoltage. It may be explained by the PCB^- effect on the interface resistances. It was found that PCB^- added both with and without bacteriorhodopsin changes the potential difference between the lipid and water phases. The polarity of this potential difference is indicative of the PCB^- accumulation in the lipid phase. In this case, PCB^- must shunt R_2 more effectively than R_4.

Uncouplers, such as CCCP and DNP, were found to change, like PCB^-, the dark potential difference between the two bulk phases. It is not surprising, therefore, that low ("subuncoupling") amounts of the protonophores can also stimulate photopotential formation, e.g., in the system of Hwang et al. (*see* Hwang et al., 1977, and Fig. 9*A* of this paper). We suggest that in this case the anion form of uncoupler plays the same role as PCB^-.

The possibility of a direct action of low concentrations of PCB^- and uncouplers on the bacteriorhodopsin molecules in the purple membranes looks unlikely. We have not observed any change in the parameters of the bacteriorhodopsin photochemical cycle in the presence of such low concentrations of PCB^- and uncouplers.

It is not clear why the stimulating effect of an uncoupler cannot be observed in the system "decane solution of asolectin/water." This system differs from that of Hwang et al. in the mode of the lipid phase preparation, as well as in the amount and type of a phospholipid used. The latter may be especially important. In the system of Hwang et al., phosphatidyl choline is used instead of asolection which represents the total mixture of soybean phospholipids containing, besides phosphatidyl choline, some acidic phospholipids. It should be emphasized that the stimulating PCB^- effect is much more pronounced in the system of Hwang et al. than in the asolectin system (*cf.* Figs. 5 and 9*B*). Both the rate and the amplitude of the photopotential generation were always much lower in the system of Hwang et al. as compared to the asolectin system. Maybe the resistance of the interface between the bulk lipid and water phases (R_2 and R_4) prove higher in the system of Hwang et al., so that decrease of R_2 by PCB^- or by an uncoupler anion proves necessary to observe a measurable photoeffect.

As to the decrease in the photopotential caused by high uncoupler concentrations, it can be easily explained by the sheet membrane shunting, i.e., by lowering of R_1 besides that of R_2, R_3 and R_4.

There is no reason to postulate an H^+ transport from the bulk water space to uncoupler anions in the bulk lipid phase in the system of Hwang et al., as was assumed by these authors in their two-phase model (Hwang et al., 1977*b*). PCB^- is an anion of a strong acid, so it cannot serve as a protonophore and can hardly be an H^+ acceptor in the lipid phase. If H^+ acception were a function of uncouplers in the system of Hwang et al., PCB^- should not substitute for uncouplers. However, experiments showed that PCB^- is even more effective than so potent a protonophore as CCCP (*see* Fig. 9).

Another observation that cannot be accounted for in terms of a two-phase model is the effect of gramicidin. At low gramicidin concentrations, its inhibiting effect on the photoresponse can be demonstrated only in the presence of K^+ (Fig. 10). It is very typical of a transmembrane channel function of gramicidin, and rather surprising if one assumes that this antibiotic changes the pathway of the proton transfer inside the bacteriorhodopsin molecule.

Moreover, the two-phase model fails to explain qualitative resemblance of the data obtained using (i) a vibrating electrode, (ii) a lipid phase-containing Ag/AgCl electrode, and (iii) techniques of planar membranes and lipid-impregnated films.

It seems to be quite reasonable to conclude that in all the mentioned systems the primary electrogenic event is bacteriorhodopsin-mediated transmembrane H^+ movement from the bulk water phase to the water space in the cavity between the bacteriorhodopsin membrane and the bulk lipid phase. So, "biphasic" lipid/water system supplemented with bacteriorhodopsin sheets, is composed of not two but four phases.

As to the slow photoelectric response in the decane/water system, it cannot be defined in simple terms, such as, e.g., being a result of the bacteriorhodopsin-mediated translocation of H^+ ions from the bulk water to bulk lipid phase (*see* Boguslavsky et al., 1976; Hwang et al., 1977*a, b*). Our results suggest that such a photoresponse is due, at least partially, to some light-dependent conversion of retinal which is released from bacteriorhodopsin under illumination in the decane/water system. The mechanism of the retinal-linked photoresponse is obscure. It is hardly due to the retinal molecule polarization which is known to occur in the light (Mathies & Stryer, 1976).

Polarization *per se* cannot explain the slow kinetics and a lag phase in the light effect. For the same reason, the described phenomenon cannot be accounted for by re-orientation of the interface-bound retinal. Photooxidation of retinal to a surface-active product seems to be a more plausible explanation.

As mentioned above, the slow photoeffect in the "decane solution of asolectin/water" system can be measured with a vibrating electrode but not with an Ag/AgCl electrode connected with the lipid phase. This observation indicates that the slow (retinal-linked) photoresponse is due to a change in the Volta potential difference across the interface, rather than to the charge translocation processes which are to be measured with both the vibrating and lipid phase-connected electrodes.

It is known that addition of low amounts of gramicidin A in the presence of high KCl (or NaCl) concentration transduces the electric potential difference to Δ pH across a coupling membrane due to electrophoretic movement of the monovalent metal ion. These very conditions existed in the experiment shown in Fig. 8. Therefore, one may think that the water cavity between the sheet membrane and the surface of the bulk lipid phase was acidified in the light if gramicidin was added. The acidification might facilitate the light-induced hydrolysis of the retinal-bacteriorhodopsin Schiff's bond and the release of free retinal, which is, apparently, competent in a slow photoresponse. If so, the slow photoresponse should not require gramicidin, provided pH of the water phase is sufficiently low. The relevant experiments (*see* Fig. 16) confirmed this suggestion.

Thus, the following chain of events seems to result in a slow photoresponse in the system "decane solution of asolectin/water" at neutral pH if the bulk water phase contains gramicidin A and KCl.

1) Acidification of the water cavity between the sheet and bulk lipid phase due to cooperation of the light-dependent H^+ pump of bacteriorhodopsin and gramicidin-facilitated K^+ electrophoresis.

2) Light-dependent release of free retinal from bacteriorhdopsin, which is facilitated by acidic pH in the cavity.

3) Reversible photoconversion of retinal into a product changing the Volta potential in the lipid/water interface.

It is not clear whether stage *1* is present in the decane/water system described in this paper.

In this system there is no fast photoresponses linked with electric potential formation by a bacteriorhodopsin H^+ pump. Apparently, topologically closed water cavity is absent in this case or the borders of the sheet attached to the decane surface are leaky. In agreement with this statement, it was found that gramicidin A does not affect the photoresponse in a decane/water system up to 10^{-3} M concentration.

Perhaps the sheet-formed cavity in the decane/water system is leaky for ion(s) other than H^+, so that acidification of the cavity in the light takes place even without gramicidin A. ΔpH dissipation by an uncoupler prevents the Schiff's base hydrolysis and the retinal release.

At any rate, one should take into account the possibility of existence of the above slow photoelectric responses in lipid/water systems containing bacteriorhodopsin. Such responses are side effects or very indirect consequences of the bacteriorhodopsin electrogenic H^+ pump activity and must be distinguished from photoelectric responses directly originating from the bacteriorhodopsin-mediated H^+ transfer.

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