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Oriented purple-membrane films as a probe for studies of the mechanism of bacteriorhodopsin functioning. I. The vectorial character of the external electric-field effect on the dark state and the photocycle of bacteriorhodopsin

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Oriented purple-membrane preparations from *Halobacterium halobium* were obtained by electrophoretic sedimentation of a purple-membrane suspension on a transparent current-conducting surface. Light exposure of orderly oriented purple-membrane films causes the generation of a photopotential amounting to several volts. The effects of external electric field on the dark state and photocycle of bacteriorhodopsin is studied in dry orderly oriented purple-membrane films. In contrast to nonuniformly oriented preparations (Borisevich, G.P., Lakashev, E.P., Kononenko, A.A. and Rubin, A.B. (1979) Biochim. Biophys. Acta 546, 171–174 and Lukashev, E.P., Vozary, E., Kononenko, A.A. and Rubin, A.B. (1980) Biochim. Biophys. Acta 590, 258–266), a specific feature of the field-induced phenomena observed in orderly oriented films is their vectorial character. The field-induced bathochromic shift of the maximum absorbance of bacteriorhodopsin is observed in an electric field, directed from the periplasmatic to cytoplasmatic side of the purple membrane and the field-induced rise of the photo-stationary M_{412} concentration in a field of opposite sign. This field-induced rise is a result of slowering of M_{412} decay. The observed effects seem likely to reflect the existence of the potential-dependent regulation of the bacteriorhodopsin photocycle in intact purple membranes.

Introduction

The transmembrane electric field is known to play an important role in the regulation of the energy-transduction processes in biological systems [1–3]. It would therefore be valuable to elucidate the effect of an external electric field on the photocycle of bacteriorhodopsin (BR), which seems to be a simplest biological energy transducer [4,5]. In our previous works we have described an experimental approach to study electric polarization phenomena in energy-transducing biological membranes and their fragments [6]. The method is to monitor changes in the optical and dielectric properties of film samples in response to external electric-field exposure. This approach makes it possible to simulate the influence of the transmembrane potential on the properties of bacterial chromatophores [7,8], chloroplasts and

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Abbreviation: BR, bacteriorhodopsin.

subchloroplasts particles [9,10,11], and purple membranes (bacteriorhodopsin sheets) [12–14]. It was found that the polarization of a BR molecule by an external electric field causes a reversible long-wave shift of the absorption maximum of the pigment-protein complex, and that this produces an intermediate which resembles spectrally the photoproduct K which is not involved in the subsequent BR photocycle [14]. An electric field applied to dry BR films was found to stimulate the accumulation of the M intermediate in the steady state as a consequence of its delayed decay [12–14]. It is remarkable that the field-induced absorption changes, in both dark and light, were found to be independent of the direction of the applied field. It is important that the bacteriorhodopsin films used by us were composed of two fractions of purple membranes, both parallel to the plane of the electrodes, but oriented in the opposite directions [12,15].

A question arises whether the electric field effects are 'scalar', i.e., independent on the orientation of the BR sheets with respect to the field, or the insensitivity of the effects to the field direction is due to the fact that the amounts of purple membranes with opposite orientations are approximately equal. In the latter case the field of any direction (from the two possible orientations) causes optical changes only in the 'corresponding' sheets population. To obtain convincing and well-documented data with regard to the electric-field effect on BR, experiments must be conducted with uniformly oriented preparations of BR sheets.

Several methods have been described in the literature for purple membrane orientation: by centrifugation [16]; by hydrophobic interactions in a hexane-water interphase [17]; by magnetic field, using the anisotropy of the diamagnetic susceptibility of protein segments [18]; by placing a purple-membrane suspension on a polylysine-coated plate [19]; by placing a purple-membrane suspension into the field of an air capacitor [20]; or by electrophoretic sedimentation [21]. Factors whose effects lead to the ordering of purple membranes have been discussed in ref. 16. The electrophoretic sedimentation of a purple-membrane suspension on a current-conducting plate and subsequent drying of the resultant sediment produce a preparation with a high degree of ordering. An indication of this is that the photopotentials generated by such preparations under continuous illumination are as high as 10–12 V [21,22], i.e., by two orders of magnitude higher that in dry purple-membrane films of randomly oriented purple membranes.

The present paper reports an investigation of the electric-field effect on the dark state and photocycle of BR in dry films of oriented purple membranes obtained by electrophoretic sedimentation. It is found that the field-induced polarization of such membranes has a marked 'vectorial' character, in contrast to what is seen in non-orented purple membrane. We have already reported an observation of an electrically induced transition of a part of the BR population into a photochemically inactive state [14]. In the present experiments we found that the inactivation occurs only when the potential applied to the cytoplasmic side of the purple membrane is negative. The electric-field-induced changes of the steady-state concentration of the M intermediate and the delay of its decay are found to take place only when the potential applied to the cytoplasmic side of an oriented purple membrane is positive. The observed effects are probably a manifestation of the potential-dependent regulation of the BR photocycle in the intact membrane.

Materials and Methods

Samples used in the experiments were prepared from an aqueous suspension of purple membrane isolated from *Halobacterium halobium* strains R_1M_1 and 353-P. The fraction of purple membrane was isolated by a modification of the method of Oesterhelt and Stoeckenius [23]. After centrifugation in a sucrose gradient, the obtained membranes were purified by liquid chromatography on Sephadex G-50 (crude), equilibrated with distilled water. In this manner, the suspension can be freed completely from sucrose without aggregation of purple membranes. The specific conductivity of the obtained purple-membrane suspension (protein concentration 6 mg/ml, pH 6.8) was measured to be $10^{-3}\ \Omega^{-1} \cdot$ cm. Linear-dichroism (LD) measurements were made by the method described in Ref. 24 in the presence of the applied electric field. The LD data show evidence for the orientation of the induced dipole moments of the

purple membrane along the field lines, since the intrinsic dipole moments of the BR sheets are screened by the ions in the solution [22]. The vector of the induced dipole moment of the purple membrane is directed parallel to the plane of the membrane so that all the purple membranes in the sample appear to be oriented along the electric-field lines. With the wavelength of polarized excitation light being within the absorption band of the chromophore, the result of this is the so-called 'positive' dichroism, i.e., an increase of light absorbance, when the direction of light polarization coincides with that of the electric field (Fig. 1a). The purple membranes were then resuspended successively three times in bidistilled water, and then spinned at $80\,000 \times g$. The procedure is essentially the same as described in Ref. 21, with only minor modifications. In its course pH was not maintained strictly neutral, which eliminates the need for acidification or alkalization of the mixture during the subsequent washing. The advantage of this is that the suspension has a lower electric conductivity, i.e., it is less contaminated by ions. Conductivity measurements were made on a.c. bridge test set with a 1 kHz power supply. The conductivity of the obtained suspension was measured to be $10^{-4}\ \Omega^{-1} \cdot$ cm (pH 6.0–6.2), and its specific feature was a 'negative' electrically induced dichroism (Fig. 1b). In contrast to the positive dischroism observed in a non-purified suspension, this means that the electric-field exposure obviously causes the purple membrane to orient perpendicular to the field lines, the reason that the purple-membrane sheet possesses its own dipole moment, normal to the membrane plane [25]. To monitor the anisotropy of polarized light absorption induced by electric field, samples were exposed to voltage pulses. Practically the same electrically induced anisotropy was caused by the pulses of duration from 0.1 to 100 ms. Longer exposures might misrepresent the situation because of the additional absorbance due to the electrophoretic movement of the negatively charged membranes toward the anode. The above-described phenomenon – the highly ordered orientation of the purple-membrane sheets per-

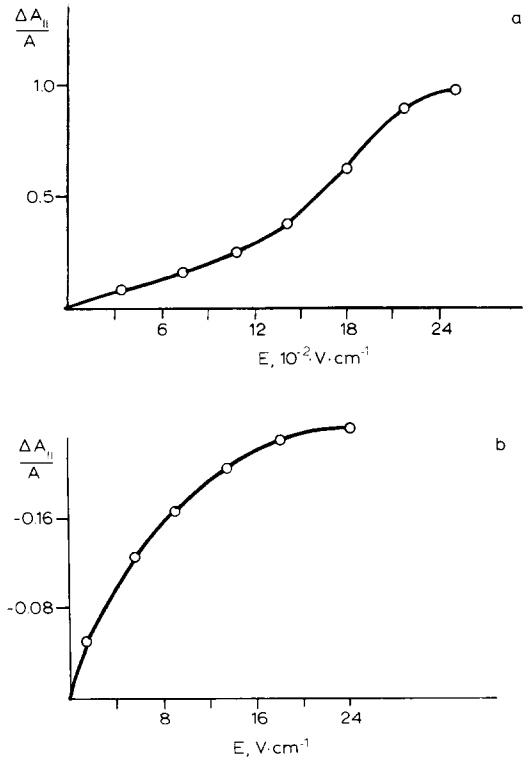

Fig. 1. Electrical pulse-induced absorbance changes in the suspension of purple membranes of *Halobacterium halobium* as a function of electric-field strength (E). Monitoring light was polarized in parallel to the vector of external electric field. (a) Stock suspension of purple-membranes, (b) Suspension of purple-membrane after washing with bidistilled water (see Materials and Methods). Wavelength of the measurements is 560 nm.

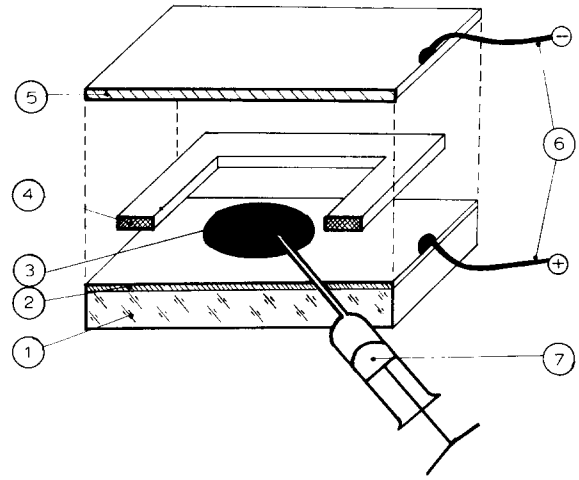

Fig. 2. Experimental set-up for preparation of uniformly oriented purple-membrane films. ① Glass plate ('substrate' plate); ② SnO_2 coating; ③ pellet of purple membranes; ④ teflon spacer; ⑤ applied metal, or glass/SnO_2-electrode; ⑥ wires; ⑦ syringe.

pendicular to the electric-field lines and the sedimentation of the oriented purple-membrane sheets on the anode – was used by us to prepare oriented purple-membrane films. The preparation procedure was the following.

A glass plate, $36 \times 18 \times 1.5$ mm was coated with a layer of tin dioxide and placed in a cell as shown in Fig. 2. The resistance of the coated surface, as measured with a d.c. meter, was 200–500 Ω. A Pt or Ni plate served as a second electrode. The gap between the two plates could be adjusted between 0.5 and 2 mm with teflon U-shape gaskets. A dark-adapted purple-membrane suspension with protein concentration of 15 mg/ml was injected by a syringe between the electrodes and an electric potential of 3–6 V was applied across the electrodes. After a short time delay (30–240 s), with the potential staying on, the portion of the suspension that had not been sedimented on the anode was removed by the syringe from the layer adjacent to the cathode. The membranes being sedimented on the substrate plate were air dried for 24 h at room temperature and at a relative humidity of 50–60%. The obtained planar films were about 0.5 cm^2 in area and 10–12 μm thick. The procedure of orientation did not markedly change the spectral characteristics of purple-membrane films in the 400–750 nm region. The optical density of the films at the absorption maximum of BR was about $A = 1.6$–2.2.

Dry oriented purple-membrane films were spray-coated in vacuo by aluminium or nickel to produce a semi-transparent layer upon which the electrode was attached. This arrangement was used to measure the potential generated by the BR preparation following the illumination. For optical measurements, it was convenient to use another type of sample. The purple-membrane film sedimented on a transparent glass plate was covered with another glass plate, about 100 μm thick, and a glass electrode coated with SnO_2 was applied upon it. It must be pointed out that the same samples were used to study the gross directions of the photo-induced charges transport in the purple-membrane and of the vector of the applied electric field in the spectral experiments. An electrometer with an input resistance of 10^{14} Ω, in combination with a d.c. voltage source, was used to measure the resistance of each sample. The resistance of dry purple-membrane films was measured to be $(0.5–8) \cdot 10^{12}$ Ω at room temperature and relative humidity of 50–60% The scatter in the resistance values may be due to the non-uniform thickness of the films after drying, or a variation in the contact area of the electrode and in the state of its contacting surface. Moreover, the resistance of a purple-membrane film depends strongly on its humidity. For instance, the incubation of purple-membrane films for 15–20 min under the saturated water-vapor conditions causes a reversible lowering of their resistance by more that two orders of magnitude.

Photoexcitation was given from a 300 W halogen lamp, coupled to a water heat filter. The flux density of the radiation used was 100 $W \cdot m^{-2}$. Direct sample temperature measurements in the course of illumination showed that the temperature change of the illuminated sample was not greater than 0.2 K. The 520–600 nm band used for excitation of BR was isolated by means of glass filters. A filter with a passband of 380–460 nm was used to prevent actinic light from getting into the photomultiplier.

Results and Discussion

An external electric field (10^7 $V \cdot m^{-1}$) causes a long-wave shift of the BR absorption band [12]. The present study of oriented purple-membrane samples showed asymmetric absorbance changes with respect to the direction of the applied electric field. The long-wave shift was observed only with a negative potential applied to the substrate plate (the electrode upon which the purple-membrane sheets were sedimented during the preparation). The field of opposite direction appeared to produce no effect on the spectral characteristics of BR in the darkness. The kinetics of the electrically induced absorbance changes of oriented purple-membrane films are shown on Fig. 3. Electrically induced absorbance changes in films of randomly oriented purple-membrane sheets are also shown in Fig. 3 for comparison. The increase of the 630 nm absorbance is accompanied by the bleaching of the main absorption band at about 570 nm. The differential spectrum of the electrically induced batho-product of bacteriorhodopsin, BR^e_{batho}, is shown in Fig. 4. There is an almost complete

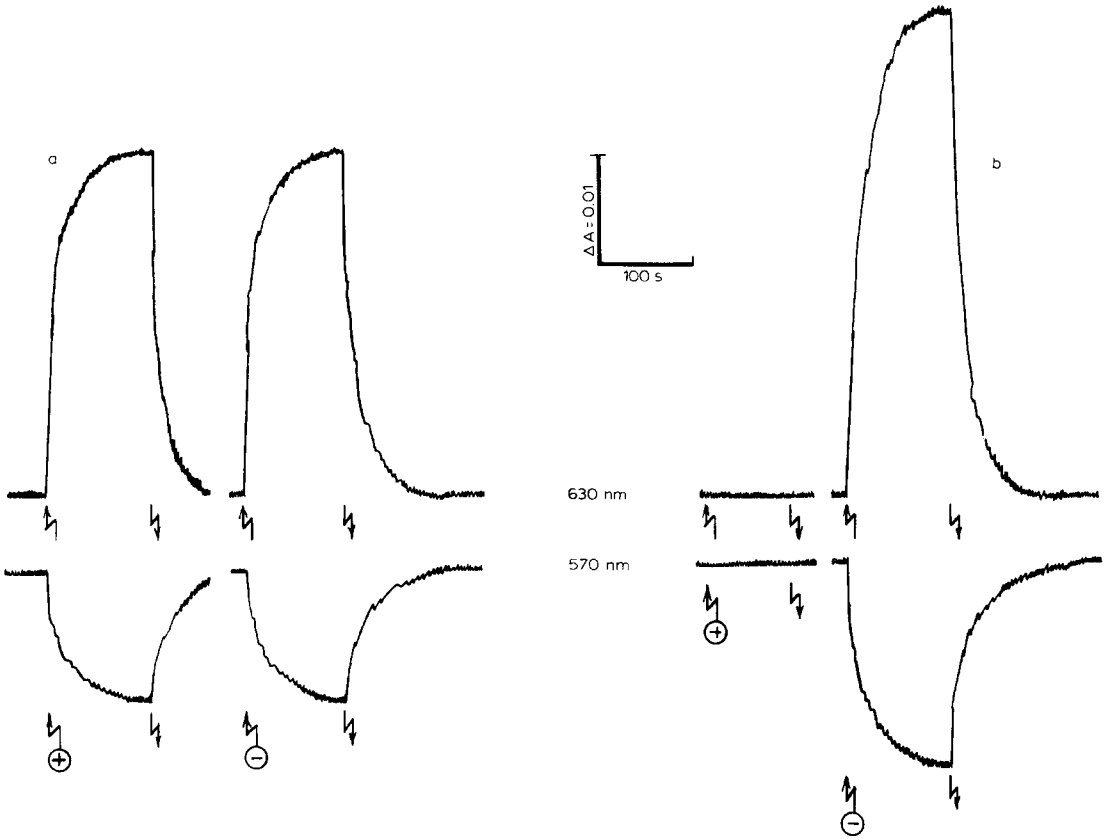

Fig. 3. Kinetics of electrically induced absorbance changes of air-dried films of purple membranes. External electric field of $E = 10^7$ $V \cdot m^{-1}$ on (↑) and off (↓). (a) Randomly oriented film. (b) Uniformly oriented film. ⊕, external field applied in such a direction that '+' corresponds to the 'substrate' plate (see Fig. 2). ⊖, idem, with '−' corresponds to the 'substrate' plate. Response time of absorbance measurements was about 1 s.

similarity between the spectra of the electrically induced BR^e_{batho} intermediate in oriented and non-oriented purple-membrane samples [26]. The increase in the absorbance at 630 nm occurs in proportion to the strength of the applied electric field, which was varied between $0.4 \cdot 10^7$ and $1.1 \cdot 10^7$ $V \cdot m^{-1}$ (Fig. 4, inset). The obtained results provide convincing evidence for a vectorial character of this effect.

The orientation of the purple membrane in a sample can be determined by measuring the photopotential generated by the sample in response to actinic light excitation. We observed a negative potential on the substrate plate under continuous illumination. This indicates that light excitation makes positive charges to move from the substrate plate to the applied electrode. It is known that an active purple membrane mediates a photo-induced translocation of protons from the cytoplasmatic to periplasmatic side [27]. Assuming that in a dry BR preparation the light-induced flux of charges flows in the same direction as in native conditions [17,22], we conclude that our purple-membrane preparations are all oriented with the cytoplasmic sides of the membranes facing the substrate. At pH higher that 5, the cytoplasmic side of a purple-membrane suspension has a negative potential relative to the periplasmic side [21]. Hence, the purple membranes in the preparations under study are oriented in such a way that their cytoplasmic sides face the substrate plate (the anode in the sedimentation procedure). In dry films, the degree of ordering of BR sheets depends on the sedimentation conditions during the preparation procedure. In the orientation by electrophoresis, the current flow through the purple-

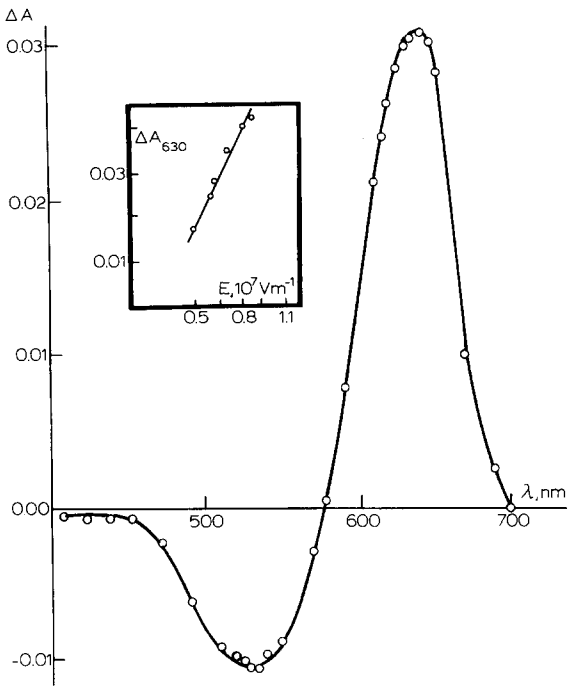

Fig. 4. The spectrum of electrically induced ($E = 10^7$ V·m^{-1}) absorbance changes of uniformly oriented films of purple membranes. Inset: absorbance changes at 630 nm as a function of electric-field strength.

membrane suspension causes the occurrence of chemical processes on the electrodes. Notably, at a fairly high voltage the surrounding water can undergo an electrolytic dissociation. Moreover, the orientation of BR sheets by sedimentation upon the anode may be influenced by the concomitant changes in pH in the nearest environment of the electrode. With a long exposure to current during the electrophoresis, the absorption peak of the films appears to be shifted toward longer wavelengths, perhaps due to the acidification of a portion of the bR moiety [30]. Under such conditions, BR sheets can be expected to be oriented with the cytoplasmic sides away from the substrate and, as a result, the degree of uniform orientation can be lower. The photopotentials registered on such preparations appeared to be no more than 1 V, whereas in samples with a high degree of ordering they were observed to be as high as 10 V. The results described in this paper were obtained on such highly oriented purple-membrane preparations.

While the orientation of the PM in the sample is known, it can be concluded that in the dark batho-product BR^e_{batho} is induced by an electric field directed from the periplasmic to cytoplasmic side of the BR sheet. There are some specific features in the dark polarization of purple membranes worth to be mentioned. Firstly, the bathochromic shift under the effect of the applied electric field occurs only when the electric field is applied from the periplasmic to cytoplasmic side of the purple membrane; no hypsochromic shift is observed with the field of opposite direction. Secondly, the spectral changes following the application or removal of the electric field are characterized by slow kinetics (half-time of about several minutes).

The direction and value of the external electric field inducing the BR^e_{batho} product coincides with that of the transmembrane field generated in the native purple membrane by the BR proton pump. This gives a good reason to believe that the long-wave batho-product BR^e_{batho} plays a regulatory role in the proton pump operation in the purple membrane, since in this state the pigment does not participate in the photochemical cycle [14]. It is possible that in this way a negative feedback mechanism is realized in the BR cycle: as soon as the transmembrane potential becomes close to the 'critical' level, the possible electric breakdown of the purple-membrane is prevented by reversible deactivation of a number of BR molecules.

It is worth mentioning that very similar effect has been recently observed when bound cations (Ca^{2+}, Mg^{2+}) were removed from BR molecule [28]. The correlation may imply the similarity of electrostatic mechanisms involved in realisation of both effects.

The exposure of dry-oriented purple-membrane films to an electric field of about 10^7 V/m under continuous illumination causes a change in the steady-state level of the M_{412} intermediate (Fig. 5). The effect is vectorial in character as well. When the potential applied to the cytoplasmic side of the purple membrane is positive, the concentration of M increases. When it is opposite, the steady-state level of M_{412} reduces negligibly. An investigation of the dark decay of the M intermediate after the cessation of continuous illumination has provided evidence that the change in the

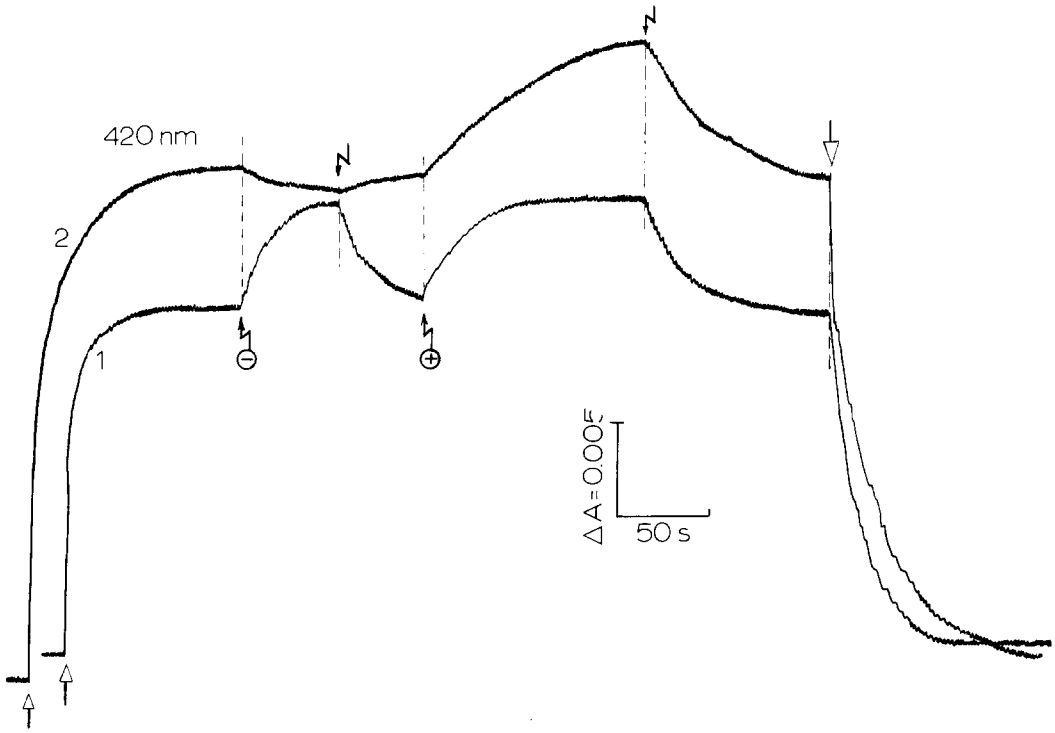

Fig. 5. Kinetics of light- and electrically induced ansorbance changes of films of purple membranes at 420 nm. (1) Randomly oriented films. (2) Uniformly oriented films. ↑, Light on; ↓, light off; $E = 10^7$ V·m^{-1}; Other symbols as in legend to Fig. 3.

amount of the M intermediate in the applied electric field is associated with a change in the kinetic of the $M_{412} \rightarrow BR_{570}$ reconversion. The application of the positive potential upon the cytoplasmic side of the purple membrane results in a significant prolongation of the M decay. The negative potential on this side makes the M-BR_{570} recovery somewhat faster. The kinetics of the M decay after switching the light off can be resolved into three first-order kinetic components, both in the absence and presence of electric field of any sign. The most strong effect of the electric field is on the slowest decay component. The application of the external electric field is practically without effect on the relative proportion of the contributions of each component in the M decay kinetics.

TABLE I

THE ELECTRIC-FIELD EFFECT ON THE DARK DECAY KINETICS OF THE M_{412} PHOTOPRODUCT IN PURPLE-MEMBRANE FILMS COMPOSED OF HIGHLY ORIENTED BR SHEETS (FIELD STRENGTH, 10^7 V/m)

A and τ are the relative contribution and time constant of the kinetic components, respectively. Marks '+' and '−' correspond to the sign of potential applied to the substrate plate of the sample.

	A_1 (relative units)	τ_1 (s)	A_2 (relative units)	τ_2 (s)	A_3 (relative units)	τ_3 (s)
In the absence of external electric field	0.16 ± 0.02	48 ± 10	0.26 ± 0.01	140 ± 20	0.58 ± 0.02	340 ± 130
Applied external electric field '+'	0.19 ± 0.01	46 ± 14	0.28 ± 0.02	190 ± 50	0.58 ± 0.02	1100 ± 100
Applied external electric field '−'	0.17 ± 0.03	35 ± 3	0.26 ± 0.03	130 ± 10	0.57 ± 0.06	160 ± 40

The observed effects of external electric field on the kinetics of the $M_{412} \rightarrow BR_{570}$ reconversion are summarized in Table I.

In an earlier investigation made on purple membranes incorporated into liposomes electric field effects on the BR photocycle have been suggested [29]. Further investigations showed that the transmembrane potential built up through electrogenic proton transport makes the bR photocycle shower [31]. This disagrees with our present observations that the decay of the M intermediate becomes faster when its negative sign is on the cytoplasmic side of the membrane. It is probable that these phenomena reflect the fact that in reconstructed purple-membrane sheets and in dry purple-membrane films the electrogenic processes occur in different ways. In purple membranes incorporated into liposomes, a system with normal proton transport activity, the Schiff base is reprotonated, during the $M \rightarrow BR_{570}$ recovery, from the cytoplasmatic side of the membrane. In dehydrated films, in which proton translocation activity seems to be restricted to local scales, H^+ transport does not go beyond its initial stages and, therefore, can affect only the proton-binding groups in the closest vicinity to the Schiff base. Under these conditions the nitrogen atom can be reprotonated from the periplasmatic side by the proton that was initially localized on the Schiff base.

From the results obtained here with highly oriented purple-membrane films it is concluded that the regulation of the BR cycle by electric field is at the stage of the reconversion of M into BR_{570}. It is also made clear that electric field blocks in part the formation of photoproduct K due to the transition of some bacteriorhodopsin molecules into photochemically inactive form BR^e_{batho}.

Along with investigations of effects of applied electric field on the BR photocycle, the availability of highly ordered purple-membrane preparations makes possible detailed studies of the mechanisms of the photoelectric processes in purple membranes of halobacteria. These problems are the subject of our next paper of this series.

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