Electric Field Effects on Spectral Characteristics and Phototransformations of Bacteriorhodopsin (bR) in Self-Assembled Films

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The influence of an external electrostatic field upon self-assembled films of purple membranes (PM) from *H. salinarium* containing a unique protein bacteriorhodopsin (bR) was studied by making use of a variety of optical methods: absorption spectroscopy, differential pulse and steady state spectrophotometry, circular dichroism, resonance light scattering, non-linear spectroscopy, etc. The electrostatic field has several effects on bR spectral and photochemical characteristics in films, namely: (a) reversible changes of an overall bR absorption spectrum; (b) slowing down the bR photochemical cycle and decreasing the quantum yield of the primary photoinduced intermediate formation; (c) modification of bR non-linear optical properties, studied by the second harmonics (SH) generation. The effects are reversible and depend on the electric field direction. Self-assembled bR films adsorbed on solid conducting surfaces of various origins (metals, semiconductors, etc.) possess some more interesting properties (linear and non- linear photochromism, generation of a photopotential up to 10 V per a multilayer film, long- live field-induced polarization of membrane components, etc.). Such systems are considered promising in constructing microelectronic chips, memory devices and effective biosensors based on photo- and electrically induced optical effects, found by us and described in this work.

I. Introduction

Primary stages in functioning of light-transducing biological membranes (bacterial chromatophores, chloroplasts of higher plants, purple membranes, containing bacteriorhodopsin, etc.) include physical separation and transfer of charges (electrons, protons) accompanied by building up local and transmembane electric fields as high as $10^7 \div 10^8$ V m^{-1}. Such fields should affect considerably optical, structural, conformational properties of membrane components.

Electric field effects upon spectral characteristics of large molecules, in condensed phase in particular, are described by the theory of electrochromism[1]. Electric field can change directly the energetic state of the molecule due to differences in dipole characteristics of initial and final (Frank-Condon) states of the electron transition. The absorption (emission) bands appear shifted by the value:

$$\Delta\lambda = (\lambda^2/hc)(\Delta\vec{\mu} + 0.5\ \Delta\alpha\vec{E})\vec{E} \qquad (1)$$

where λ is the position of the absorption maximum, $\Delta\vec{\mu}$ - the change of the molecule dipole moment by excitation, $\Delta\alpha$ - the change of the molecule polarizability, $\vec{E}$ - external electric field.

When molecules are distributed isotropically in the medium, the shifts in both directions described by the first member are equivalent and the band appears broadened simmetrically. When molecules are oriented, the first tenn describes the shift, the sign of which depends on the mutual orientation of the molecular dipole moment and the field.

The second term describes the interaction of the molecule with the field through changes in polarizability and induces the spectral shift independent on their orientation.

Electrochromic spectral shifts are rather small. Indeed, for $\Delta\mu = 10D(3.3\times10^{-29}\text{C m})$ and $E = 2.5\times10^{6}$ V m^{-1} at $\lambda = 500$ nm the shifts are $\Delta\lambda \cong 1$ nm. The shifts described by the second member are by a decimal order smaller. That is why electrochromic spectral shifts are usually registered by differential spectroscopy as the changes in the absorption coefficient. The differential spectrum is the first derivative of the absorption one:

$$\Delta\varepsilon = \Delta\lambda(\delta\varepsilon/\delta\lambda) \qquad (2)$$

where $\Delta\varepsilon$ is the absortion coefficient change for the given absorption band. From (1) in the scalar form and (2) we get:

$$\Delta\varepsilon = (\lambda^2/hc)(\Delta\mu E\cos\theta + 0.5\Delta\alpha E^2\cos^2\theta)(\delta\varepsilon/\delta\lambda) \quad (3)$$

The first member equals zero in the isotropic medium and describes the shifts for oriented molecules only. The second one depends on the induced dipole moment and in all cases describes the long-wavelength shifts as in the excited state the molecule has higher polarizability than in the ground one[2].

The formation of the transmembrane electric field in the process of functioning of light-transducing biological membranes gave the possibility to explain some experimental facts, in particular, the nature of shifts of some absorption bands of membrane-bound pigments which may not even participate in photochemical reactions. This is the case of carotenoids in chloroplasts of higher plants and in chromatophores of phosynthesizing bacteria[3–11]. The analysis of their electrochromic spectra show that they can be presented as a superposition of components proportional to first derivatives of individual absorption bands of pigments. However, the shifts of the maxima are as large as 4-6 nm while the theory of electrochromism predicts smaller values for carotenoids (about 0.1 nm)[1]. The amplitude of these changes depends on the native state of the membrane and is decreased by preliminary heat denaturation. It means that the protein-lipid matrix is involved in forming the field-induced spectral changes due to its structural rearrangements[7–11]. The same conclusion can be derived from the presence of slow kinetic components in electrically induced changes.

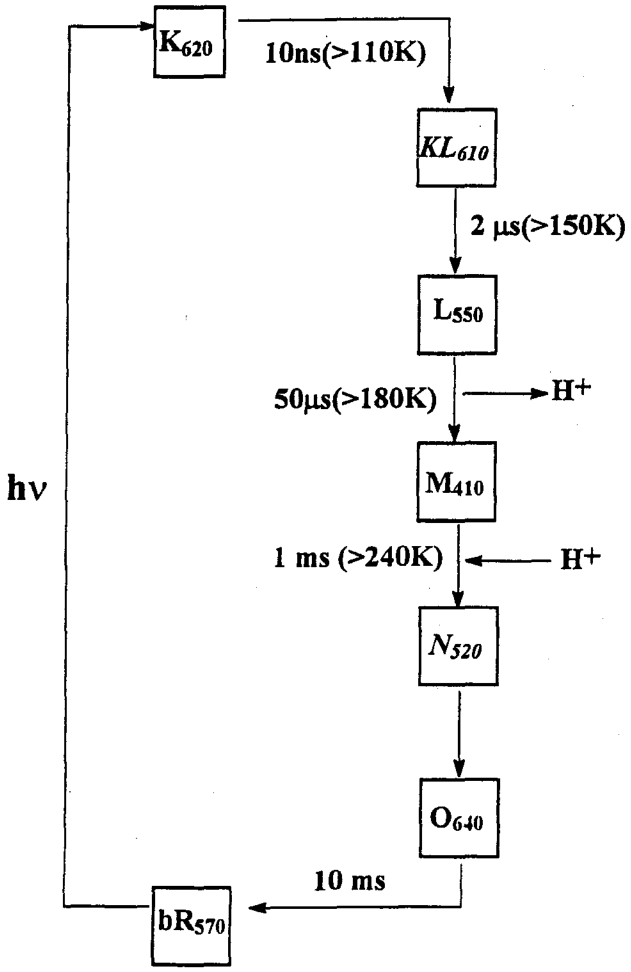

Figure 1. Photocycle of light-adapted bacteriorhodopsin (bR). Individual intermediates are labelled with latin letters and the numbers following the labels indicate the approximate absorption maximum in nanometers extrapolated to the ambient temperature. Species in italics are not observed under all experimental conditions. Relative free energies are related approximately to vertical position. Temperatures required for observing the formation of subsequent intermediates and formation times extrapolated to ambient temperature are shown. Approximate points of the Schiff base deprotonation and reprotonation are indicated.

All these effects are observed for purple membranes (PM) from extremely halophilic bacteria *Halobacterium salinarium* which contain a unique pigment-protein complex bacteriorhodopsin (bR) which is the simplest biological transformer of light energy[12,13]. A retinal bound to a Iysine residue of the protein molecule via a Schiff base is a chromophore group of the complex. At ambient temperatures under low-light conditions, the purple membranes contain a binary mixture of two forms of the complex: the first has the retinal in the 13-*cis* conformational state and the other - the all-*trans* retinal. The first form is called a dark-adapted bR and the second - a light-adapted one. As a result of light absorption the latter form undergoes a cycle of photochemical transformations manifested through changes of spectral characteristics of the complex (Fig.1). Further on we shall consider only this form and refer to it as bR. The photoinduced transformation of bR to K is the "primary event". The primary stable photoproduct, K, stores about 16 kcal/mol and involves an all-*trans* to 13-*cis* photoisomerization of the protonated Schiff base chromophore. All the following intermediate products are formed in the process of thermal relaxation. All the intermediates of the cycle can be stabilized by lowering the temperature (shown in Fig. 1) and the complete cycle can be traced as the temperature increases. At ambient temperatures all the intermediates are characterized by certain life-times (also shown in Fig. 1) and can be followed kinetically after excitation by a short light pulse. The key thermal intermediate in the photocycle is M. Its formation and relaxation is accompanied by the deprotonation and reprotonation of the Schiff base (Fig. 1). As a result during the photocycle a proton is transferred across the membrane, and the transmembrane electric field up to 10^7 V m^{-1} is generated. The effects of this field upon various bR characteristics were studied in self-assembled PM films.

II. Materials and methods

Purple membranes (PM) containing bacteriorhodopsin (bR) were isolated from *H. salinarium* cells (strain ET-1001) by the established procedure[14].

Self-assembled films of PM were prepared by two different procedures: **a**/ - by slow drying of their suspension in ultra pure water on the surface of a solid support[15] or **b**/ - by application of a low electric potential (about 1-3V) for 45 -60 s across a pellet of PM suspension 1 mm thick placed between two conducting plates, the lower one being the anode[16] (Fig.2A). Since PM possesses a high transmembrane dipole moment due to difference in the surface charge density on both sides, the latter procedure results in formation of the films with higher degree of orientation of PM fragments (Fig.2B) than the first one.

The bR molecules maintain their functional activity in such films as monitored by the spectral cyclic changes, though certain stages are slowed down due to dehydration[15]. The self-assembled films of PM containing bR could be stocked for months under ambient conditions without loss of their functional properties.

To apply an external electric field a second electrode (usually from aluminium) was evaporated on the top of the film. To perform optical measurements we used a glass plate covered with indium-tin oxide (ITO) as the down electrode and an evaporated aluminium film 70 Å thick as an upper one. This construction provided at least 75% transparency for visible light.

To study second harmonic (SH) generation in PM films the Nd^{3+}: YAG laser was used with semi-confocal resonator at $\lambda = 1.064$ μm (pulse duration 15 ns, the repetitive frequency 12.5 Hz, the diameter of the pumping beam about 1 mm). This pumping light is not absorbed by bR molecules and does not interfere with the photoprocess. The transmitted SH was measured. The intensity of the SH generated by the bR film was determined by substruction of the SH intensity from the Si substrate from the registered total SH intensity.

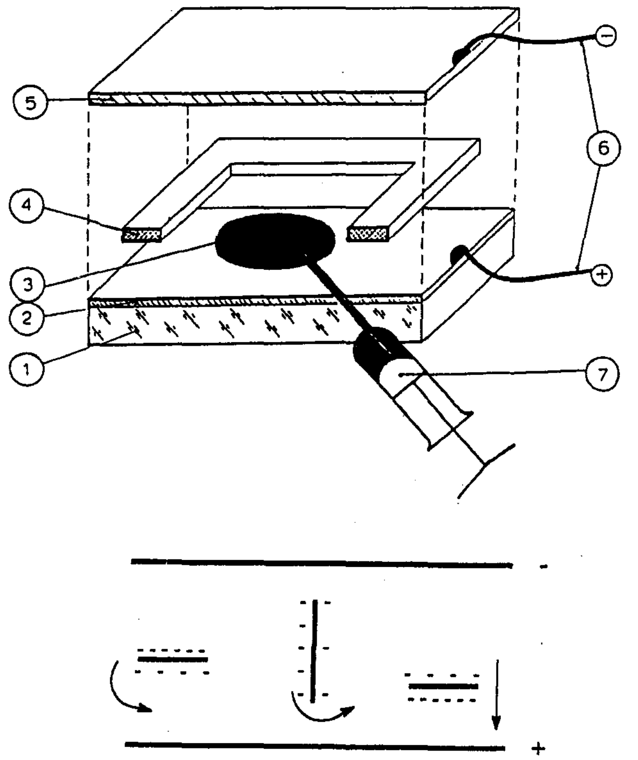

Figure 2A. Experimental set-up for preparation of uniformly oriented self-assembled films of purple membranes from *H. salinarium* 1 - glass substrate plate; 2 - SnO_2 or ITO (indium-tin oxide) conducting coating; 3 - pellet of purple membranes; 4 - teflon spacer; 5 - applied metal or glass/SnO_2 electrode; 6 - wires; 7 - syringe. B - The scheme of a PM orientation in the external electric field.

III. Results and discussion

Electrically induced changes of absorption spectra.

At ambient temperature the illumination of bR with a continuous light leads to an equilibrium which is characterized by a presence of two stable forms. One is the initial bR and the other is the M intermediate which appears accumulated at a certain concentration due to the difference of the kinetic characteristics of its relaxation and formation[12,13]. The differential spectrum has a minimum at λ = 570 nm and a maximum at λ = 410 nm (Fig. 3A, curve a). At low temperature (below 110 K) the continuous illumination induces another equilibrium characterized by the accumulation of the K intermediate - the bathochromic spectral shift is observed (the corresponding differential spectrum is shown in Fig. 3A, curve b).

Electric field applied to a PM film in the dark at ambient temperature induces a reversible shift of the bR absorption spectrum to longer wavelengths (bathochromic shift). The differential spectrum has a slightly structurized negative band in the spectral region 430-575 nm and a broad positive one with the maximum about 630 nm (Fig.3A, curve c). For non-oriented PM films the amplitude of the changes depends on the field quadratically (Fig.3A, inset) and is independent on its direction (Fig.4a). For oriented PM films the direction of the field is of importance and the ratio of the electrochromic signals for opposite field directions can serve as a measure of the PM orientation in the film (Fig.4b, 100% orientation). The amplitude of the signal depends linearly on the field strength.

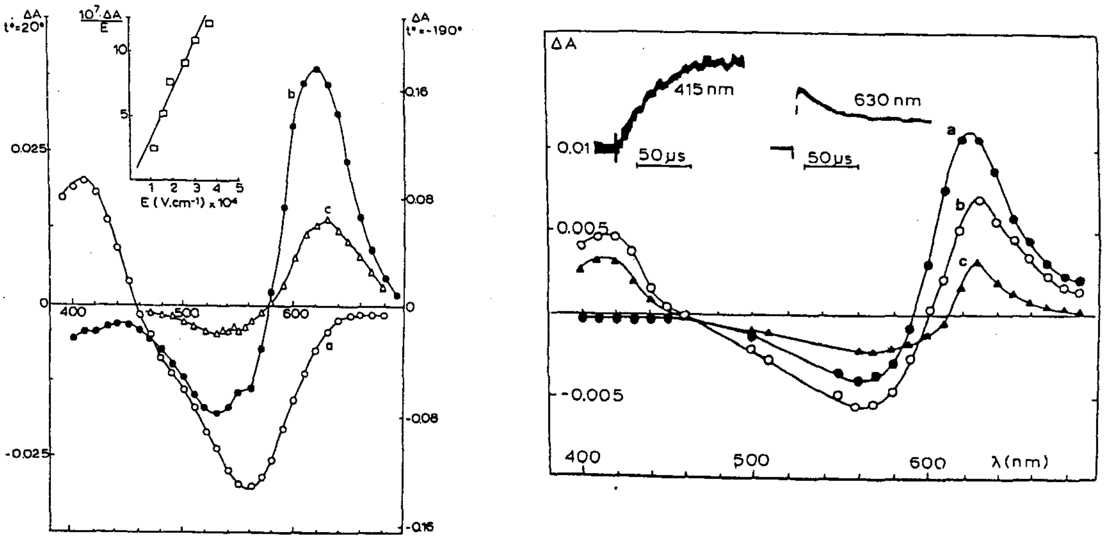

Figure 3. A - Spectra of the absorbance changes in self-assembled films of purple membranes from *H. salinarium,* strain ET-1001. a - absorbance changes induced by light with λ = 400-600 nm at 20°C; b - the same at $t^\circ = -190^\circ$C; c - electric field-induced absorbance changes at 20°C, E = 2x10^6 V m^{-1}. Inset: field dependence of $+\Delta A_{630}$. B - The bR absorption changes induced by a laser flash (530 nm; 15 ns; 3 mJ) in self- assembled PM films. a - immediately after the flash; b - 100 μs after the flash; c - 25 ms after the flash. Inset: kinetics of absorbance changes at 415 and 630 nm.

Electrically induced differential spectrum is very close to that describing the formation of the primary photoinduced intermediate product K at low temperature (Fig.3A, curve b) or in the experiments with flash photolysis (Fig.3B). However, the kinetics of electrically induced spectral changes contains slow components (Fig.4) and they are inhibited by low temperature and low humidity. These findings confirm our supposition that the external electric field affects the retinal both directly and through local conformational rearrangements of opsin groups in the retinal vicinity due to electrically induced changes in the positions of various charged, polar and polarizable groups. Low temperature and decreased humidity inhibit conformational mobility of the macromolecular matrix thus decreasing the local effective electric field.

From the similarity of the differential spectra of the electrically- and photoinduced bathoproducts we can conclude that the formation of the K intermediate in the normal bR photocycle sould be accompanied by the processes of dielectric polarization in the vicinity of retinal[15], and the external field in our experiments should model these processes.

With this in mind we have performed experiments on the mutual effect of the activating light and an external electric field.

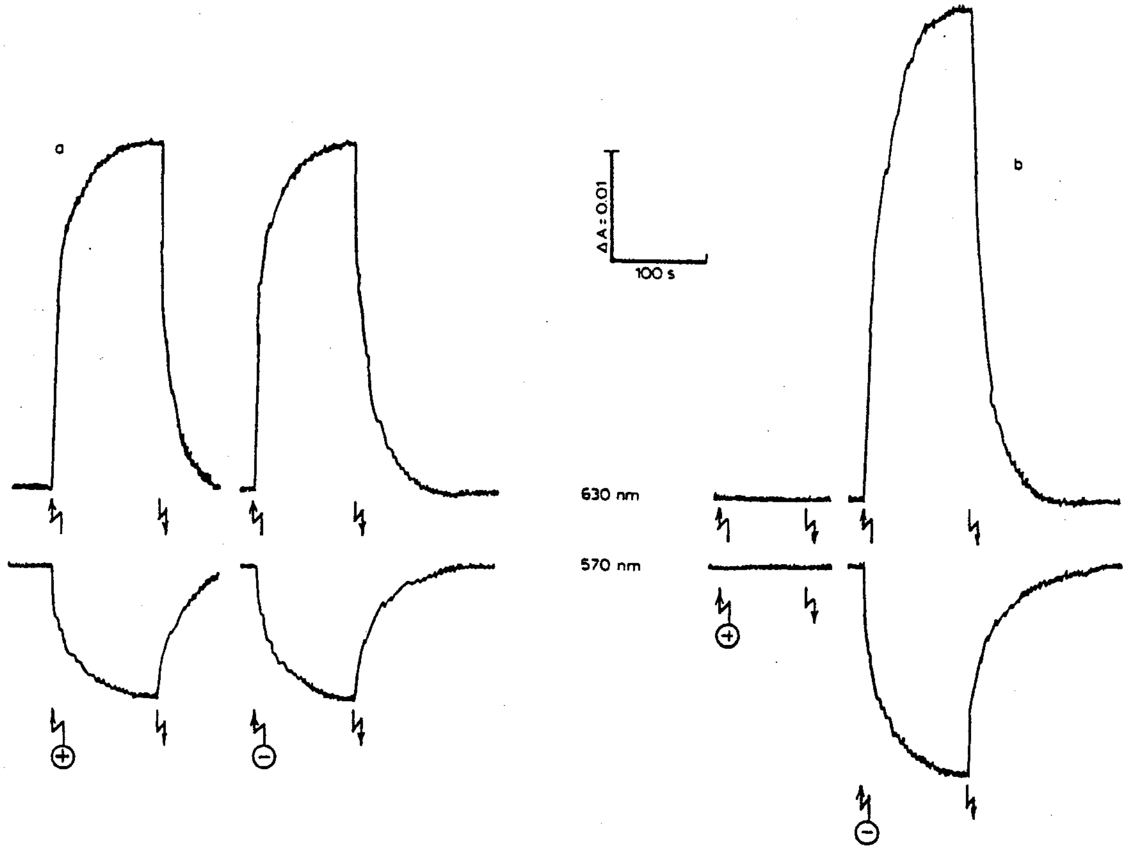

Figure 4. Kinetics of electrically induced absorbance changes of bR in purple membrane (PM) self-assembled films. Broken arrows show the moments of switching on (up) and off (down) the external electric field E = 10^7 V m^{-1}. a. Randomly oriented PM; b. - uniformly oriented PM. ⊕ - external field of such a direction that "+" is applied to the substrate plate (see Fig.2); θ "-" is applied to the substrate plate. Response time of absorbance measurements was about 1 s.

The amplitude of the laser-induced absorption changes at 630 nm reflecting the formation ofthe bathoproduct K is decreased by 30% in the field 5 × 10^6 V m^{-1} (Fig.5, top)[17]. At the same time the kinetics of its transformation into M-state (on microsecond time scale) remains practically unchanged. The formation of the electrically induced bathoproduct decreases the pool of bR molecules which can be involved in the normal photocycle.

This conclusion was also confirmed in experiments with the steady illumination at low temperatures where the light-induced bathoproduct is stable. The amplitudes of differential spectra at 630 nm characteristic for bathoform differ by 20% in the field 2 x 10^6 V m^{-1}.

Basing on these data we can suppose the existence of a mechanism of a negative feedback regulation in the bR photocycle. Activation of this factor should decrease the quantity of bR molecules involved in normal photocycle and hence decrease the specific activity of the system as a whole when a proton gradient across the membrane appears close to the value dangerous for its integrity.

One more mechanism of bR photocycle regulation follows from the dependence of the stationar concentration of the M intermediate on the electric field. The effect is of a vectorial character. When the positive potential is applied to the cytoplasmic side of PM (to the substrate in Fig.2) we observe the increase of the M stationary concentration (Fig.5, bottom). The opposite field induces a slight decrease of the stationary level of M. These changes are due to changes in the M relaxation kinetics in the photocycle. In the first case the relaxation is considerably slowed down and in the second slightly increased. The electric field affects the mutual position of proton-donating and proton-accepting groups in the bR structure. It is the reprotonation of the Schiff base that is affected as the kinetics of M formation is not changed by the electric field[17].

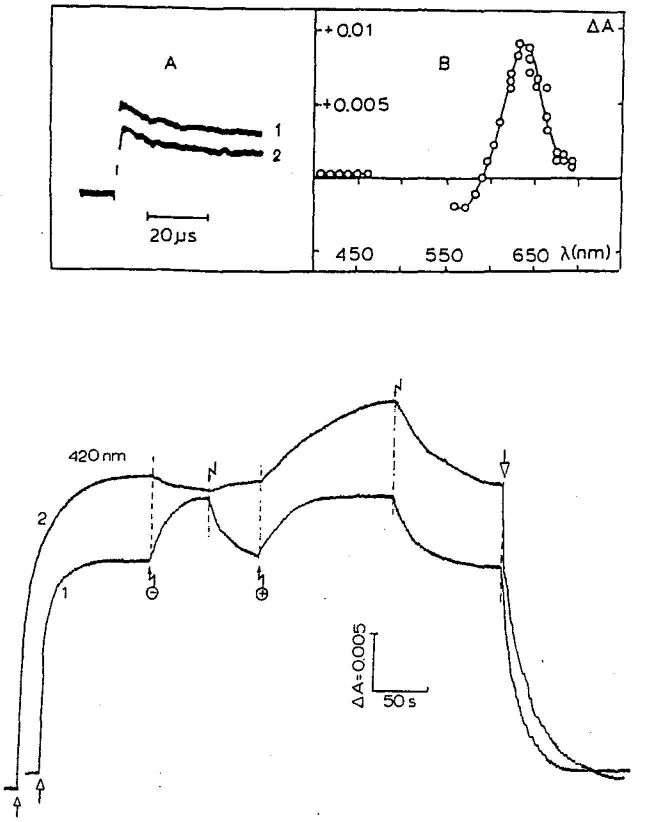

Figure 5. Top: Effect of external electric field (5×10^6 V m^{-1}) on laser flash-induced bR absorbance changes in self-assembled PM films. A - kinetics of absorbance changes at 630 nm (1 - in the absence of field; 2 -in the presence of field). B - the spectrum of the field- induced absorbance decrease measured as a difference between absorbance changes induced by the laser flash before and after application of the field. Bottom: Kinetics of light- and electric field-induced absorbance changes of self- assembled PM films at 420 nm. 1 - Randomly oriented PM; 2 - uniformly oriented PM. Straight arrows show the moments of the activating light on and off; broken arrows - field on and off, respectively. E = 10^7 V m^{-1}.

Second harmonic generation. The influence of electric field

Photochemical reactions induce considerable changes of non-linear optical characteristics of bR molecules, in particular their quadratic hyperpolarizabilities $\gamma^{(2)}$ [18,19]. Bacteriorhodopsin is a non-centrosymmetric molecule and hence the value of its $\gamma^{(2)}$ is not zero. Thus the method of the second harmonic generation can be used for an effective investigation of the non-linear characteristics of bR.

The light wave characterized by the frequency ω absorbed by the media containing bR molecules induces the non-linear polarization $\vec{P}$ at 2ω given by

$$\vec{P}(2\omega) = \chi^{(2)} : \vec{E}(\omega)\vec{E}(\omega) = \Sigma N_i \gamma_i^{(2)} : \vec{E}(\omega)\vec{E}(\omega) \quad (4)$$

where $\chi^{(2)}$ is the effective non-linear susceptibility of the medium, $\gamma_i^{(2)}$ and N_i are the effective hyperpolarizability at 2ω and the concentration of i-th intermediate product in the bR cycle.

At constant activating light we observe the dynamic equilibrium between two states - the initial bR and the intermediate product M (see Fig 1). In this case in equation (4) $i = 1, 2$ and we have

$$\vec{P}2(\omega) = (N_1\gamma_1^{(2)} + N_2\gamma_2^{(2)}) : \vec{E}(\omega)\vec{E}(\omega) \qquad (4a)$$

It is supposed that the absorption bands of these components do not overlap[20].

As it was shown for PM in polyvinyl alcohol (PVA) matrix, the intensity of emission at 2ω, which is proportional to $P^2(2\omega)$, can give information about the non-linear properties of bR photocycle intermediates[18,19].

We have studied for the first time second harmonic generation in self-assembled PM films for bR in the initial state and in the course of its photocycle and the influence of the external electric field upon this process[20].

Fig. 6A shows the dependence of the SH intensity on the power of the constant activating light W at various wavelengths. For all wavelengths the SH intensity decreases and reaches a plateau at about 50% of the initial SH intensity which reflects the establishment of a dynamic equilibrium between bR and the M stage.

The first absorbs at 570 nm and is close to the double-photon resonance with the pumping light (1.064 μm). The M product is far from the resonance ($\lambda_{\max} = 412$ nm) and does not contribute to the SH generation.

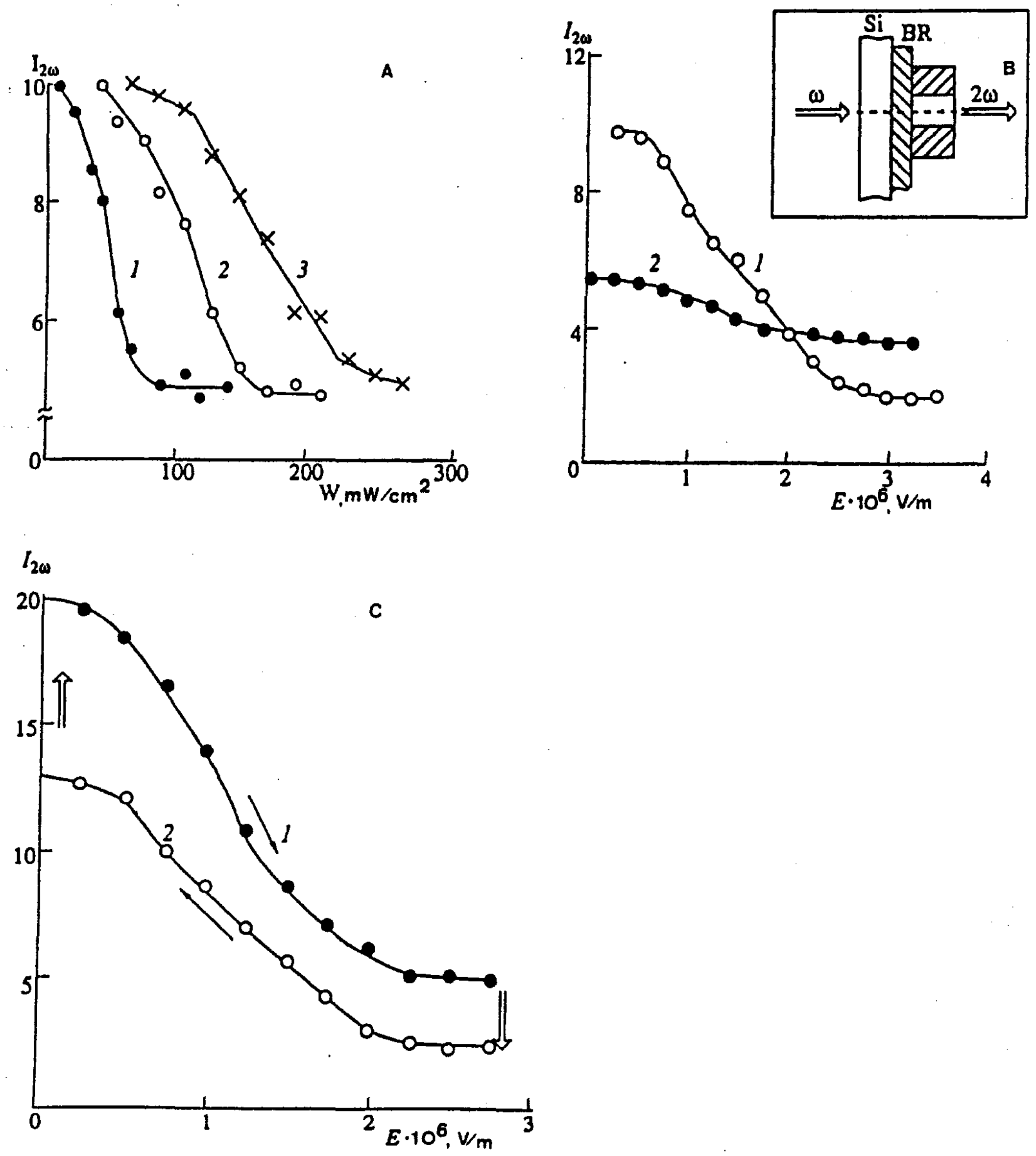

Figure 6. A - The dependence of the intensity of the second harmonics (SH) $I_{2\omega}$, transmitted through a uniformly oriented self-assembled PM film on the power of the actinic illumination W at different wavelengths: 1 - $\lambda_1 = 514.5$ nm; 2 - $\lambda_2 = 488.0$ nm; 3 - $\lambda_3 = 647.1$ nm. B - The dependence of the SH intensity $I_{2\omega}$ transmitted through the PM film on the external electric field strength: I - uniformly oriented PM; 2 - randomly oriented PM. Inset: the scheme of the sample: Si - polished Si(III) plate; BR - PM film, containing bR; ω and 2ω - the beams of the pumping illumination and the SH, respectively. C - The external electric field dependence of the SH intensity $I_{2\omega}$ transmitted through the film of uniformly oriented PM in the presence of the activating light with $\lambda = 514.5$ nm (1) and in its absence (2). The scanning direction is shown by thin arrows. Double arrows mark moments of the activating light switching on (up) and off (down).

To measure the SH changes due to the electric field the PM film was formed at the surface of a Si(III) plate of n-type 200 μm thick and the SH intensity transmitted by the support and bR was measured in its dependence on the field strength (Fig. 6B). The positive sign of the field corresponds to "+" at the upper metallic electrode made from In-Ga conducting paste. It looked like a circular mask with about I mm hole in the center (see inset on Fig. 6B). The thickness of the mask (0.1 mm) was enough to absorb the SH generated by itself.

The positive electric field $E \approx 10^6$ V m^{-1} decreases the intensity of SH generated by bR in oriented PM films down to a stationary level of about 20% from the initial value $I_{2\omega}$. It is worth mentioning that the field of the same direction and value induced the formation of the electrical bathoform which is inactive photochemically (Fig.5, top)[15]. It should have lower non-linear molecular hyperpolarizability at the SH frequency $\gamma_{2\omega}^{(2)}$ than bR in the initial state. As a result the non-linear polarization $P(2\omega)$ decreases manifested through the decrease of the SH intensity. Simple calculations by use of the equation (4a) show that about 30% of all the bR molecules in this case appear in the non-active electrically induced bathoform. This result coincides well with the evaluations made previously from the linear optical experiments on mutual action of the external electric field and the pulse of the activating light (Fig.5, top)[17].

As it was shown in the previous section (see also Fig. 5, bottom), the mutual action of the external electric field and the constant activating light forms a new dynamic equilibrium between the initial bR and the M intermediate state. Similar experiments have been done for the SH generation (Fig. 6C). Curve I shows the dependence of $I_{2\omega}$ on the electric field in the absence of the activating hght. The arrow shows the scanning direction. When a stationar level of $I_{2\omega}$ is reached (about 20% from the initial $I_{2\omega}$) the activating light is switched on (shown by a double arrow). It induces an additional double decrease of the SH intensity. In the second part of this experiment the electric field decreases down to zero in the presence of the activating light. The SH intensity 5-fold increases (curve 2). When at E = 0 the activating light is switched off, the SH intensity reaches the initial value. Thus the effects of the field and the activating light appear additive. The sequence of the light and the field action is not important for the final effect. The absence of any hysteresis means reversibility and non-destructive character of the processes observed in the PM films.

Possible applications of bR self-assembles films in microelectronics and biotechnology

As shown above, bR possesses a unique set of photochromic, electrooptic and other properties which makes it very promising for application in biotechnology and microelectronics. The creation of new systems of high-speed treatment and storage of information, of dynamic recognition of images appears one of the fields[12,21].

Highly organized membrane structures containing bR possess good adhesive properties and can be formed as thin films at surfaces of various solid optically transparent conducting technological supports, in particular used in microelectronics. Bacteriorhodopsin has some more advantages, namely: high functioning speed (in the range of ps), rather high quantum yield of the primary photoreaction (0.64), reversibility and cyclic pattern of transformations (more than 10^6 photocycles), high resistivity to photochemical and thermal actions, high stability (in dry films bR preserves its functional activity for years). In [12] one can find a detailed analysis of perspective and already realised projects using bR.

The comparative analysis of performance of optical and magnetooptical carriers of information shows that it is worth looking for new non-traditional materials for high-speed memory devices.

Thin films of PM were used to construct an optical disk carrier of information (Fig.7)[22]. The film was formed from the water-gelatine (5%) PM suspension. The suspension was distributed uniformly over the surface of a rotating glass disk warmed up to 45°C. The film was dried and covered by 0.5 nm thick light-reflecting metal layer. A focused laser beam (<2 μm in diameter) with λ = 560 nm and power enough to heat the protein up to the temperature where it was denaturated (>85°C) was used to form the separating tracks. Information tracks consisting from native bR were either concentric circles (random choice) or helices (succesive choice). Information was saved in a double-code form.

Two different modes of information saving were used. According to the first one tne information was saved by denaturating the protein by the laser beam (see above) ("I" of the information). The regions of native bR corresponded to "0" of the information. The signal- noise ratio reached 60 D.

According to the second mode the information was saved and read through reversible spectral bR changes. The 5 mW power laser beam with λ = 560 nm induced the photochemical cycle and the temperature was decreased down to -50°C to stabilize the bR photoinduced transitions at the stage of the M intermediate. Reading of the information (bR relaxation into the initial state) occurs when the information band is illuminated by the 6 mW power light with $\lambda = 420$ nm. The signal-noise ratio for this regime is 34 D.

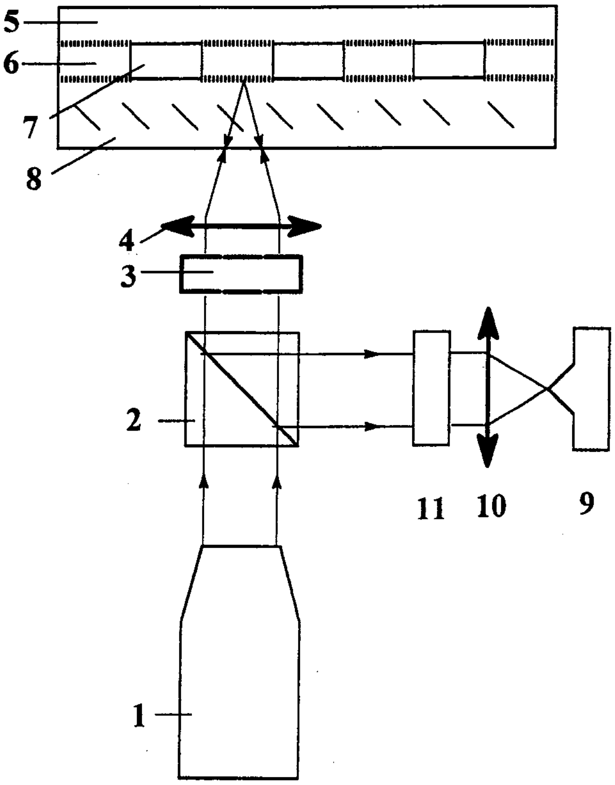

Figure 7. Optical scheme of the memory device with bR self-assembled film as the information carrier (optical disk): 1 - laser light source; 2 - separating block; 3 - 0.25λ plate; 4 - objective lense; 5 - passivating layer; 6 - information band; 7 - separating band; 8 - transparent support (glass, quartz); 9,10,11 - registration system (reading).

The described disk information carrier has the following characteristics:

- space resolution more than 100 lines per mm;

- high capability for reversible save ($> 10^6$ cycles of writing-erasing);

- exploitation period not shorter than for magnetic carriers;

- insensitivity to magnetic fields;

- saving in the spectral range 520-590nm, reading at 420 nm;

- the dynamic range equals 60 D for single and 34 D for reversible regime;

- high speed (equals to the lifetime of an appropriate intermediate).

To increase the effectivity of the disk carrier in the reversible regime it is necessary to increase the lifetime of the M intermediate. It is possible to slow down M relaxation by decrease of the temperature or hydration of the film, by genetic or chemical modifications, etc. Recently it has been shown that in the PM film pretreated with 1,2-cyclohexandione at high pH the M intermediate has the lifetime more than 30 hours[23].

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