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Photocurrent Kinetics of Purple-Membrane Sheets Bound to Planar Bilayer Membranes

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Summary. The kinetics of light-driven proton transport by bacteriorhodopsin has been studied in a model system consisting of a planar lipid bilayer membrane to which purple membrane fragments have been attached. After excitation with a 10-nsec laser flash a fast negative current-transient occurs, followed by a positive transient which decays to zero. The time course of the photocurrent may be represented by a sum of four exponentials with time constants $\tau_1 = 1.2\,\mu$sec, $\tau_2 = 17\,\mu$sec, $\tau_3 = 57\,\mu$sec, $\tau_4 = 950\,\mu$sec (at 25 °C). In a D_2O medium τ_2 and τ_3 are increased by a factor of 2.6 and 2.9, respectively, whereas τ_1 remains unaffected. The observed components of the photocurrent can be correlated to photochemical reaction steps inferred from flash-photometric experiments on the basis of the observed time constants, the activation energies, and the effects of pH and D_2O. From the photocurrent signals information may be obtained on the magnitude of the charge displacement associated with the elementary transitions of the bacteriorhodopsin molecule.

After light-excitation, bacteriorhodopsin returns to the ground state via a series of intermediates and thereby translocates protons through the membrane. The kinetics of this light-driven proton pump has been studied so far mainly with purple-membrane sheets in aqueous suspension (Stoeckenius, Lozier & Bogomolni, 1979). From these studies the spectral characteristics as well as the lifetimes of a number of intermediates in the photocycle could be obtained. In order to understand the pumping mechanism, the spectroscopic transitions have to be correlated with the elementary steps of proton translocation through the bacteriorhodopsin molecule. Information on proton transfer kinetics may be obtained by studying photoelectric transients in oriented purple-membrane samples. Orientation has been achieved by a number of different methods, for instance by attaching purple membranes or bacteriorhodopsin vesicles to lipid-impregnated filters (Drachev, Kaulen & Skulachev, 1978; Blok & Van Dam, 1979; Dancsházy et al., 1979) or to thin Teflon films (Trissl & Montal, 1977; Hong & Montal, 1979). In other experiments oriented purple-membrane multilayers have been formed using the Langmuir-Blodgett technique (Hwang, Korenbrot & Stoeckenius, 1978). Another method consists of orienting purple-membrane sheets in water by an electric field (Keszthelyi & Ormos, 1980).

In this paper we present an analysis of photocurrent transients observed after flash-excitation of purple membranes bound to planar lipid bilayers. The quasi-stationary properties of this system have been described previously (Dancsházy & Karvaly, 1976; Shieh & Packer, 1976; Herrmann & Rayfield, 1978; Bamberg et al., 1979; Seta, Ormos, d'Epenoux & Gavach, 1980). From the photocurrent response at steady illumination in the presence and absence of artificial proton channels in the bilayer it has been concluded that purple-membrane sheets become attached to the bilayer as shown in Fig. 1, with the extracellular face preferentially oriented toward the black film (Bamberg et al., 1979). With the experimental set-up described in the following, photocurrent transients could be analyzed in the time range between 1 μsec and about 1 msec. The lower limit is given by the bandwidth of the measuring system, whereas the upper limit results from the fact that slow photoelectric processes tend to have small current amplitudes. The observed photocurrent could be represented by four exponentials which could be partly correlated to known photochemical reaction steps on the basis of the time constants, the activation energies, and the effects of pH and D_2O.

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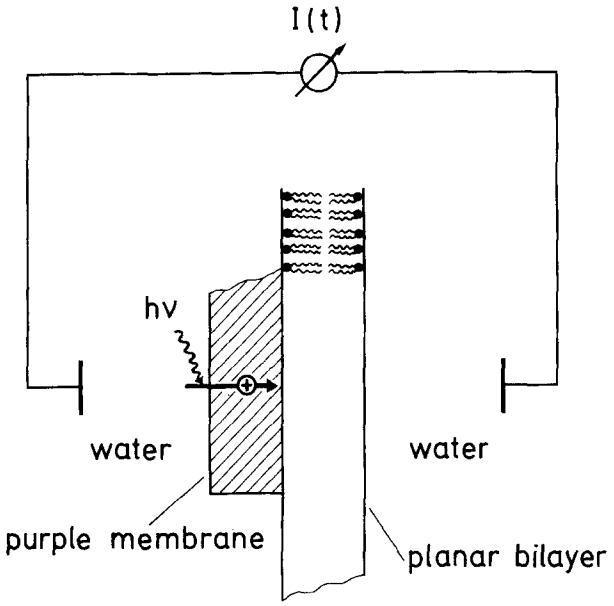

Fig. 1. Attachment of purple membranes to a planar bilayer after addition to the left-hand aqueous phase (Bamberg et al., 1979). The purple membrane sheets bind preferentially with the extracellular side facing the bilayer

Materials and Methods

Optically black lipid films were formed as described previously (Bamberg et al., 1979) in a membrane cell filled with aqueous electrolyte solution (0.1 M NaCl plus 5 mM Tris maleate, pH 7.0, if not otherwise indicated). The membrane-forming solution contained 1% (wt/vol) diphytanoyllecithin (L-1,2-diphythanoyl-3-phosphatidylcholine) and 0.025% (wt/vol) octadecylamine (Fluka, puriss.) in *n*-decane. The lecithin was synthetized by K. Janko (Janko & Benz, 1977); *n*-decane was from Merck (standard for gas chromatography). In a few experiments asolectin or di-O-oleylphosphatidylethanolamine have been used instead of diphytanoyllecithin; this did not affect the results. After addition of the purple membrane suspension, the black film was usually stable for many hours.

The membrane cell was made of black Teflon with a transverse septum inclined at 45° to the laser beam in order to allow optical control of the membrane formation process by a low-power microscope. The membrane was formed at a hole in the septum with a circular cross-section of 1.4 mm diameter. The Teflon cell was enclosed in a Peltier thermostat which could be operated between −20 and +60 °C. The temperature was measured in the aqueous solution close to the membrane with a thermistor.

Purple-membrane fragments were a gift of Dr. N.A. Dencher (Basel). The aqueous suspension of the membrane fragments was stored in frozen state at −20 °C. Before use for membrane experiments the suspension was sonified in the dark for 5 min at 10 °C (Bransonic, Mod. 12). Aliquots of 50 mm^3 of the suspension having an optical density of 4 at 570 nm ($l=1$ cm) were added under stirring to one compartment (volume approx. 7 cm^3) of the membrane cell. Photosensitivity slowly increased, reaching a half-maximum value after about 20 min. After stirring for about 1 hr the experiments were started. Under the conditions of these measurements, the bacteriorhodopsin was in the light-adapted form.

The membrane was excited by light pulses of about 10 nsec duration from a dye laser (Molectron DL 10/UV 12). In all experiments, rhodamine 6G was used as a dye and the emission wavelength was set at 575 nm. In order to obtain a homogeneous intensity distribution, only the central part of the laser beam was used; the beam diameter in the plane of the membrane was 0.8 mm, corresponding to an illuminated membrane area of 0.50 mm^2. The laser beam entered the cell from the bacteriorhodopsin-free front compartment. The energy per flash was monitored with a calibrated Joulemeter (Molectron, Mod. J3) using a semi-transparent mirror by which half of the beam intensity could be deflected into the Joulemeter. The maximum energy per flash falling onto the membrane was approximately 10 μJ, corresponding to an energy density of about 2 mJ/cm^2, or about $n \simeq 5.8 \times 10^{15}$ quanta/cm^2 (at 575 nm). Since the absorption cross-section of bacteriorhodopsin at 575 nm is about $\sigma \simeq 2.1 \times 10^{-16}$ cm^2, corresponding to an extinction coefficient of $\varepsilon \simeq 54000$ M^{-1} cm^{-1} (Oesterhelt, 1976), one has $\sigma n \simeq 1.2$, meaning that at the highest light intensity used in these experiments almost every chromophore becomes excited by a single flash. In most experiments, the light intensity was reduced with grey filters to 2–20% of the maximum value.

Platinized platinum electrodes were used throughout. The photocurrent signals were amplified in a Burr Brown Mod. 3554 BM preamplifier which was mounted directly above the membrane cell inside the Faraday shield and which was adjusted to the actual values of the membrane capacitance and electrolyte resistance. The feedback resistance was 10 MΩ and the input resistance about 100 Ω. The preamplified signal was stored in a transient recorder (Biomation 805) and read out at an expanded time-scale into a signal averager (Nicolet 1074 or Tracor TN 1710). Depending on the signal amplitude, between 512 and 8000 single signals were averaged. The maximum repetition rate of flash excitation was 20 Hz, but in most experiments repetition rates of 1–5 Hz were used. The averaged signal was stored in digital form on tape (Kennedy 7000) and processed on a Telefunken TR 440 computer. For the computer analysis of the experimental transients the method developed by Provencher (1976*a*, *b*) was used. This method allows the analysis of data composed of a sum of exponential decay functions plus random noise plus an unknown constant "baseline." The fit program does not require an initial estimate of the number of exponential functions nor of the time constants and amplitudes.

A number of tests have been carried out with the experimental set-up prior to the purple membrane experiments. In order to check for effects of electromagnetic stray signals created by the laser discharge on the measuring circuit, a light shutter was placed before the membrane and data were collected with repetitive laser operation as in the actual experiments. It was found that any disturbance of the measuring circuit was below the limit of detectability after 1 μsec. The bandwidth of the amplifier and the performance of the data analysis system were tested in an experiment in which a squarewave generator (with a risetime of 0.1 μsec) was placed between electrodes and amplifier, whereas the black film with attached purple membranes was formed as usual. Application of a square-wave voltage resulted in a measured current signal with a risetime of 0.4 μsec which is determined by the bandwidth of the amplifier. The same result was obtained with a dummy circuit in place of the membrane, consisting of a capacitance of 4.7 nF in series with a resistance of 200 Ω.

The time resolution of the actual photocurrent measurement is limited by the time constant $\tau_s = R_s C \simeq 3$–5 μsec, where $R_s \simeq 500$–1000 Ω is the series resistance of the measuring circuit (electrolyte solutions plus electrodes plus input resistance of the amplifier), and $C \simeq 5$ nF is the total membrane capacitance (*see* below). In a number of experiments the external medium contained 1 M instead of 0.1 M NaCl; in this case the series resistance was $R_s \simeq 200$ Ω and the time constant $\tau_s \simeq 1$ μsec. Addition of a

further external series resistance (for instance 2 kΩ) resulted in an increase of risetime of the photocurrent signal of the expected magnitude (according to $\tau_s = R_s C$), without affecting the longer time constants (τ_2, τ_3, τ_4) of the photocurrent.

In order to check for possible light effects on the elctrodes, a photocurrent experiment with bound purple membrane was carried out as usual. Thereafter the photoactive membrane was destroyed, the lipid removed from the Teflon septum, and a second membrane formed with fresh lipid solution while the aqueous purple-membrane suspension still remained in the cell. Application of light pulses immediately after membrane formation gave no photosignal; the photoresponse then slowly developed within about half an hour which is the time required for adsorption of purple-membrane sheets to the bilayer.

In some experiments the purple membrane suspension in the rear compartment was exchanged for a bacteriorhodopsin-free solution after the membrane had become photo-active. After the exchange the photoresponse of the membrane remained unchanged. This suggests that the binding of the purple-membrane sheets to the planar bilayer is virtually irreversible.

Time and Amplitude Resolution of the Measuring Circuit

The electrical properties of the compound membrane may be described by the equivalent circuit shown in Fig. 2 in which G_p and C_p are the conductance and capacitance of the purple-membrane sheet and G_m and C_m are the corresponding quantities of the underlying bilayer membrane. Circuit elements corresponding to the uncovered part of the bilayer have been omitted, since all experiments to be discussed in the following have been carried out under short-circuit conditions where the voltage across the bilayer vanishes (apart from a small voltage drop across the series resistance $1/G_s$, *see* below). In order to analyze the relationship between the externally measurable current signal $I(t)$ and the primary photocurrent $I_p(t)$, we consider a hypothetical photosystem in which an infinitely short flash generates a primary photocurrent transient of the form

$$I_p(t) = I_{po} \exp(-t/\tau_i). \tag{1}$$

τ_i may be chosen to be one of the intrinsic time-constants of the photosystem to be measured. Assuming for the moment that the light-induced charge displacement occurs across the entire thickness of the photoactive membrane, the current generator may be placed in parallel to C_p and G_p (Fig. 2). Simple circuit-analysis then leads to the following expression for the current $I(t)$ in the external circuit (in the limit of zero series resistance of the cell, i.e., $1/G_s \simeq 0$):

$$I(t) = \frac{I_{po} C_m}{C_m + C_p} \cdot \frac{1}{\tau - \tau_i} \left[\tau \left(1 - \frac{\tau_i}{\tau_m}\right) \exp(-t/\tau_i) - \tau_i \left(1 - \frac{\tau}{\tau_m}\right) \exp(-t/\tau) \right] \tag{2}$$

$$\tau \equiv \frac{C_m + C_p}{G_m + G_p}; \qquad \tau_m \equiv \frac{C_m}{G_m}. \tag{3}$$

τ_m is the RC time constant of the lipid bilayer; for an undoped membrane; τ_m is of the order of 1–10 sec. The time constant τ of the compound membrane has been experimentally determined to be about $\tau \simeq 0.2$ sec (Bamberg et al., 1979). As the intrinsic time constants τ_i to be measured are in the range of microseconds to milliseconds, the relations $\tau_i \ll \tau_m$, $\tau_i \ll \tau$ hold. Under these conditions Eq. (2) reduces to

$$I(t) \approx \frac{C_m}{C_m + C_p} I_{po} \exp(-t/\tau_i). \tag{4}$$

This equation means that by capacitive coupling the primary photosignal $I_{po} \exp(-t/\tau_i)$ is attenuated by the factor $C_m/(C_m + C_p)$. With an estimated ratio of $C_m/C_p \simeq 0.2$ (Bamberg et al., 1979), the attenuation factor becomes about 0.17. It is seen from Eq. (4) that the use of a black film which has a high specific C_m value (around 0.5 μF/cm²) as a support for the purple membrane is favorable for a low attenuation of the photocurrent signal. (If the light-induced charge displacement occurs over only part of the purple membrane, then C_p in Eq. (4) has to be replaced by the capacitance of the dielectric layer across which the charge moves, whereas C_m now contains, in addition to the black film capacitance, contributions of those purple membrane layers which are not involved in charge displacement.)

At short times the influence of series resistance $R_s = 1/G_s$ can no longer be neglected. The existence of a series resistance leads to a small voltage drop across the entire membrane. For this reason the capacitance C'_m of the bilayer area not covered by purple membrane has to be taken into account, too. This can be done by adding a capacitance of magnitude C'_m in parallel to C_m and C_p. Since at short times the compound membrane exhibits a purely capacitative behavior, the conductance pathways across the bilayer and purple membrane sheets can be neglected ($G_m = 0$, $G_p = 0$). The photocurrent signal after a light flash at $t = 0$ is then obtained as (for times $t \ll \tau$ and $t \ll \tau_m$):

$$I(t) = \frac{I_{po} C_m}{C_m + C_p} \cdot \frac{\tau_i}{\tau_i - \tau_s} [\exp(-t/\tau_i) - \exp(-t/\tau_s)] \tag{5}$$

$$\tau_s \equiv \frac{C}{G_s}; \qquad C \equiv C'_m + \frac{C_m C_p}{C_m + C_p}. \tag{6}$$

τ_s is the time constant of the measuring circuit and C the total capacitance of the membrane. If the circuit time constant is small

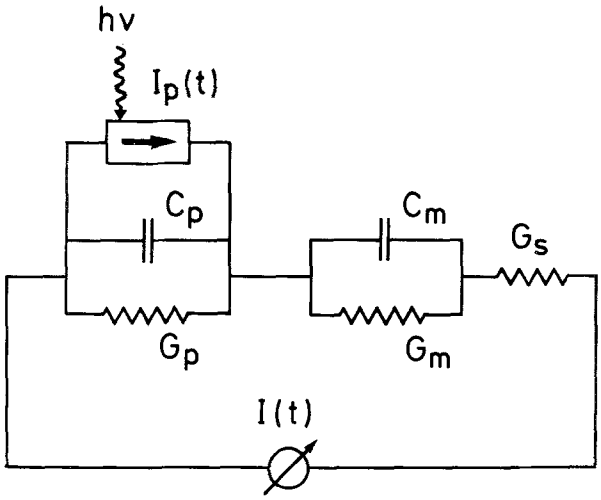

Fig. 2. Equivalent circuit of the compound membrane shown in Fig. 1. G_p and C_p are the conductance and capacitance of the purple membrane sheet, and G_m and C_m are the corresponding quantities of the underlying bilayer membrane. G_p includes contributions from leakage pathways in the space between purple membrane and planar bilayer. $1/G_s$ is the series resistance of the measuring circuit (electrodes plus solution plus input resistance of the amplifier). $I_p(t)$ is the photocurrent transient generated in the purple membrane by a flash of light. $I(t)$ is the externally measured short-circuit current

($\tau_s \ll \tau_i$), Eq. (5) reduces to Eq. (4). On the other hand, if the relaxation time of the photoprocess under study is much shorter than the time constant of the circuit ($\tau_s \gg \tau_i$), Eq. (5) assumes the form

$$I(t) = \frac{I_{po} C_m}{C_m + C_p} \cdot \frac{\tau_i}{\tau_s} \exp(-t/\tau_s). \tag{7}$$

This means that even for a very fast process ($\tau_i \to 0$) the measuring circuit responds with a signal of finite amplitude which is proportional to the charge $Q = I_{po}\tau_i$ displaced during that process.

Results

The time course of the photocurrent $I(t)$ after a 10-nsec laser flash at 575 nm is shown in Fig. 3. The polarity of the current is defined as indicated in Fig. 1, i.e., $I > 0$ corresponds to a movement of positive charge towards the supporting membrane. During the first 10 μsec after the flash a negative current is observed which rises steeply toward positive values and thereafter declines to zero. (The finite slope of the falling phase of $I(t)$ within the first 5 μsec is an artifact caused by the time constant of the circuit which was about 3 μsec in this case.) The shape of $I(t)$ for $t \geqq 5$ μsec can be represented with high accuracy by an expression consisting of a sum of four exponential terms:

$$I(t) = \sum_{i=1}^{4} a_i \exp(-t/\tau_i). \tag{8}$$

Using three instead of four exponential terms does not adequately fit the experimental $I(t)$ curves, i.e., with a three-exponential fit a deviation between theoretical and experimental curves was observed which was consistently greater than the experimental scatter. On the other hand, introducing further terms does not significantly improve the fit. The analysis was therefore carried out with four exponential functions throughout. In almost all experiments the calculated fit curve was identical with the experimental curve for times $t > 10$ μsec within the width of the drawing line in a plot such as Fig. 3*B*.

As discussed in the Appendix, a relation of the form of Eq. (8) is predicted for a photosystem in which the primary photoproduct P_1 decays in four consecutive monomolecular reaction steps with rate constants $k_i = 1/\tau_i$:

$$P_0 \xrightarrow{h\nu} P_1 \xrightarrow{k_1} P_2 \xrightarrow{k_2} P_3 \xrightarrow{k_3} P_4 \xrightarrow{k_4} P_5. \tag{9}$$

In reality the transition from the ground state P_0 to the product P_1 may proceed via intermediate steps too fast to be resolved at the given bandwidth; furthermore, the last step may be followed by other slow reactions having an undetectable current amplitude. If the condition $k_1 \gg k_2 \gg k_3 \gg k_4$ is fulfilled (each following reaction much slower than the pre-

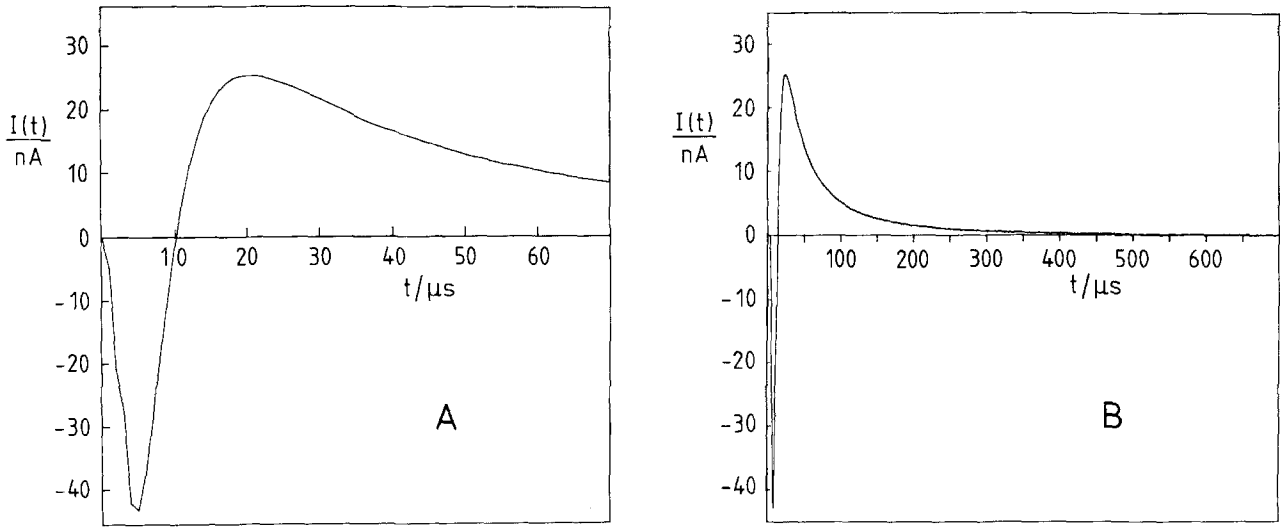

Fig. 3. Photocurrent $I(t)$ after a 10-nsec laser flash at 575 nm, represented at two different time scales. The curves have been directly reproduced from the original recorder plot. The polarity of the current is defined as indicated in Fig. 1., i.e., $I > 0$ corresponds to movement of positive charge towards the supporting membrane. The finite slope of the falling phase of $I(t)$ within the first 5 μsec is an artifact caused by the limited bandwidth of the amplifier. The signal represents an average over 3000 single excitations. A qualitatively identical signal but superimposed with considerable noise is obtained in a single-flash experiment. The light intensity was 2 μJ, corresponding (with an illuminated membrane area of 0.50 mm^2) to 4 mJ/cm^2. The temperature was $T = 22$ °C. Positive peak currents at light saturation reached about 50 nA (~10 $\mu A/cm^2$) at 22 °C. For times >10 μsec a highly accurate fit of the experimental $I(t)$ curve is obtained according to Eq. (8) with $\tau_1 = 3.9$ μsec, $\tau_2 = 21.0$ μsec, $\tau_3 = 64.4$ μsec, $\tau_4 = 420$ μsec, $a_1 = -708$ nA, $a_2 = 56.8$ nA, $a_3 = 17.6$ nA, $a_4 = 1.23$ nA. The fitted curve (not shown) is almost indistinguishable by eye from the experimental curve for $t > 10$ μsec (apart from the noise components)

ceeding one), the current amplitudes a_i are given by (*see* Appendix):

$$a_i = e_o \alpha_i N k_i \tag{10}$$

where e_o is the elementary charge and N the number of bacteriorhodopsin molecules which have been excited by the light flash. The dimensionless coefficients α_i describe the magnitude of charge displacement in the external circuit associated with the transition $P_i \rightarrow P_{i+1}$.

In order to test the performance of the data analysis system, a control experiment was carried out in which a square wave generator was interposed between membrane cell and preamplifier. When the falling phase of the voltage pulse was analyzed in the usual way, only one exponential process with a time constant of 1–5 μsec was found, corresponding to the product of membrane capacitance times the resistance of the cell (electrolyte plus electrodes), whereas at longer times (up to 50 msec) no further exponential process was detected even at the highest sensitivity.

The dependence of photocurrent on light intensity J_l is represented in Fig. 4 where the current amplitudes a_i [Eq. (8)] are plotted as a function of J_l. It is seen that the amplitudes are linearly related to light intensity up to about 2 μJ. All determinations of kinetic constants were carried out in this linear range. Deviations from linearity at high light intensities probably result from saturation of the photosystem and/or from accumulation of hydrogen ions in the narrow space between the purple membrane fragment and the black film (Hellingwerf, Schnurmans & Westerhoff, 1978). A homogeneous intensity distribution over the illuminated part of the membrane was found to be important, otherwise deviations from linearity already occurred at lower intensities. It was also checked whether the time constants τ_i were independent of J_l. This condition was fulfilled within the linear range of the photocurrent signal, except for time constant τ_4 (*see* below). In a further series of control experiments the influence of repetition frequency of the light pulses on the time course of the photocurrent was studied. The values of the time constants τ_1, τ_2 and τ_3 were found to be independent of repetition frequency in the range of 1–5 Hz in which most experiments were carried out. The time constant τ_4 was found to increase with decreasing repetition frequency ν from a value of about 350 μsec at $\nu = 1$ Hz to 950 μsec at $\nu = 0.1$ Hz (25 °C, $J_l = 0.5$ μJ). A further reduction of ν to 0.01 Hz did not change τ_4 any more. A similar increase of τ_4 was observed when the light intensity J_l was reduced to about 50 nJ at fixed repetition frequency. The value of $\tau_4 = 950$ μsec represents the

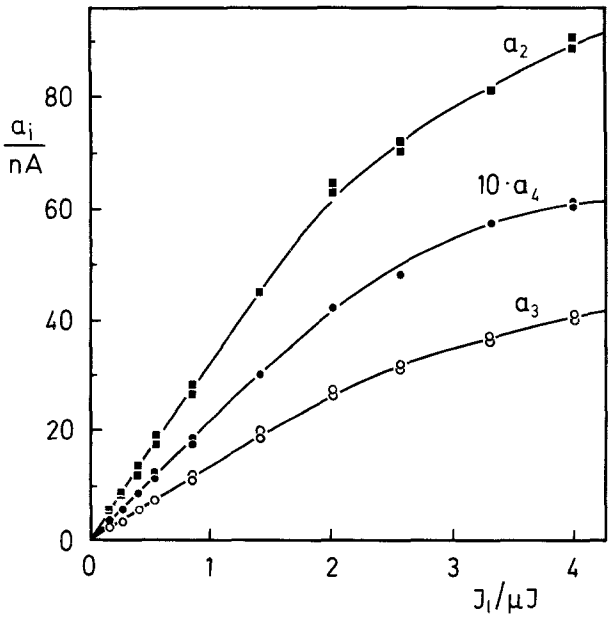

Fig. 4. Current amplitudes a_i [Eq. (8)] as a function of intensity J_l of the laser flash. J_l was varied using a series of calibrated grey filters. The repetition frequency was 5 Hz; $T = 25$ °C. All data have been obtained from a single membrane. The lines have been drawn to guide the eye.

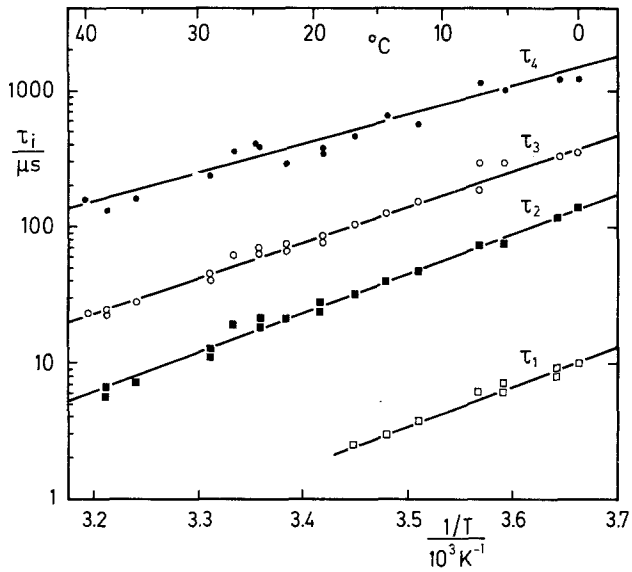

Fig. 5. Time constants τ_i of the photocurrent signal as a function of reciprocal temperature. The experimental points for τ_1 have been obtained with 1 M NaCl in the aqueous phase, corresponding to an RC time constant of the measuring circuit of $\tau_s \simeq 1$ μsec. The light intensity was 1.0–1.5 μJ and the repetition rate 5 Hz. τ_4 decreases to about 950 μsec at 25 °C in the limit of low light intensity and low repetition rate

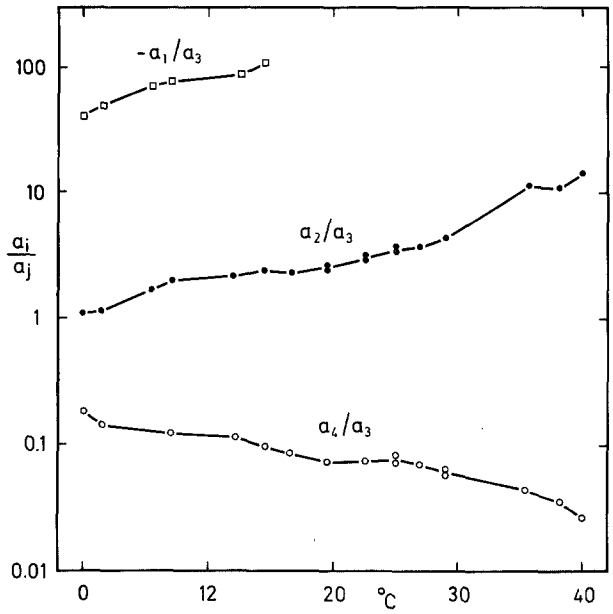

Fig. 6. Ratios of current amplitudes as a function of temperature. The absolute value of a_3 was about 9 nA at 25 °C

limiting value for low light intensity and low repetition frequency. The reason for this behavior of τ_4 is not clear so far; possibly τ_4 is particularly sensitive to changes of pH in the intermembrane space.

Varying the NaCl concentration in the range of 0.1–2.5 M or replacing 0.1 M NaCl by 0.1 M $MgCl_2$ had no influence on the photocurrent kinetics at times $t < 5$ μsec. Using as a buffer 0.1 M Hepes, pH 7, instead of 5 mM Tris maleate (as in all other experiments) had no effect either. The only influence of salt concentration was a change of the time constant $\tau_s = CR_s$ of the measuring circuit via the series resistance R_s (*see* above). Similarly, adding gramicidin or the lipid-soluble uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) to the system in order to increase the conductance of the planar bilayer (Bamberg et al., 1979) did not affect the photocurrent kinetics.

The kinetic parameters obtained at different temperatures in the range between −2 and 35 °C are summarized in Figs. 5 and 6. A linear relationship between $\ln(\tau_i)$ and $1/T$ in the Arrhenius plot of Fig. 5 was observed for all four processes. The corresponding activation energies E_i which were obtained according to the relation $\tau_i \propto \exp(E_i/RT)$ are given in Table 1 together with the values of the rate constants $k_i = 1/\tau_i$ at 25 °C. Because of bandwidth limitations the fastest process (process 1) could only be analyzed at low temperature (below 20 °C); the value of τ_1 given at 25 °C has been obtained by extrapolation. From Fig. 6 and Table 1 it is seen that the magnitudes of the current amplitudes a_i are ordered in the same sequence as the rate constants $k_i = 1/\tau_i$; according to Eq. (10) there is always a tendency for slow processes to have a small current amplitude, as a_i is proportional to k_i. Because of the small amplitude of process 4, the values of a_4/a_3 and τ_4 are less accurate than the other parameters. As the number N of excited bacteriorhodopsin molecules in Eq. (8) is not known and, furthermore, is likely to vary from experiment to experiment, only ratios of current amplitudes are given in Fig. 6.

The results of measurements at different pH values are represented in Figs. 7 and 8. It is seen that the pH dependence of the time constants it rather complicated and is likely to be determined by several protonable groups having different pK values. A prominent effect is the increase of the time constant τ_4 of the slowest process with pH. Furthermore, it is seen from Fig. 8 that the relative current amplitudes a_2/a_3 and a_4/a_3 show opposite pH effects.

From flash photolysis studies it is known that certain steps in the photocycle of bacteriorhodopsin are slowed down in D_2O (Korenstein, Sherman & Caplan, 1976; Lozier & Niederberger, 1977). Such isotope effects also occur in the photocurrent transients (Figs. 9 and 10). In D_2O the rate constants of processes 2 and 3 at 25 °C are decreased by a factor of 2.6 and 2.9, respectively, whereas the change of k_4

Table 1. Rate constants $k_i = 1/\tau_i$ at 25 °C and activation energies $E_i = R \cdot d \ln \tau_i / d(1/T)$, as obtained from Figs. 5 and 9[a]

	k_1 (sec^{-1})	k_2 (sec^{-1})	k_3 (sec^{-1})	k_4 (sec^{-1})	E_1	E_2	E_3	E_4
					(kJ/mol)			
H_2O	8×10^5	5.9×10^4	1.7×10^4	1.1×10^3	41	56	49	39
D_2O	8×10^5	2.3×10^4	5.9×10^3	0.64×10^3	41	54	54	60

[a] In the D_2O experiments the external medium was a solution of 1 M NaCl and 5 mM Tris maleate in D_2O. (A few D_2O experiments have been carried out with 0.1 M instead of 1 M NaCl, which gave almost identical results). The purple membrane was for at least 24 hr in contact with D_2O prior to the measurement. The value of k_1 at 25 °C have been obtained by extrapolation from lower temperatures.

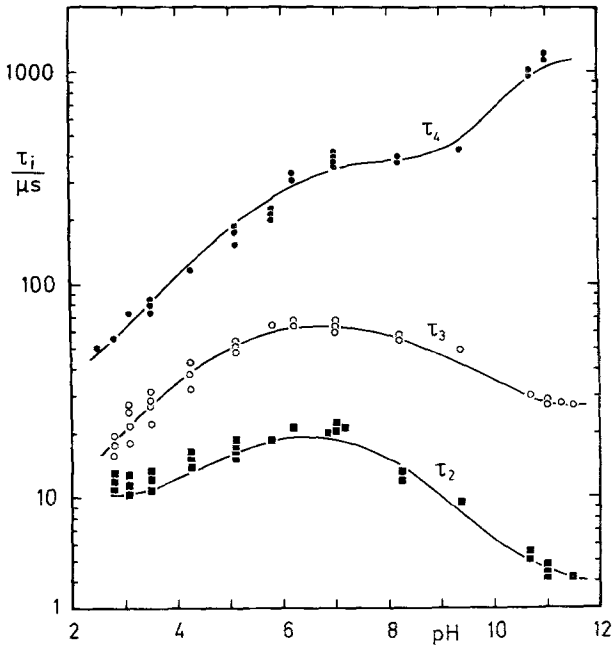

Fig. 7. pH dependence of the time constants τ_i at 23–25 °C. The membranes were formed in a solution of 0.1 M NaCl and 5 mM Tris-maleate, and the pH was adjusted by adding the appropriate amount of HCl or NaOH. In order to check the reversibility, an experiment was performed in which the pH was lowered from 7 to 3 and then readjusted to 7. The second measurement at pH 7 gave nearly identical values of the time constants and current-amplitude ratios as the first one, although the absolute amplitudes were reduced to about 40% of the original value, possibly due to desorption of purple membrane fragments. A similar check of reversibility at high pH could not be carried out because of membrane instability. The lines were drawn to guide the eye

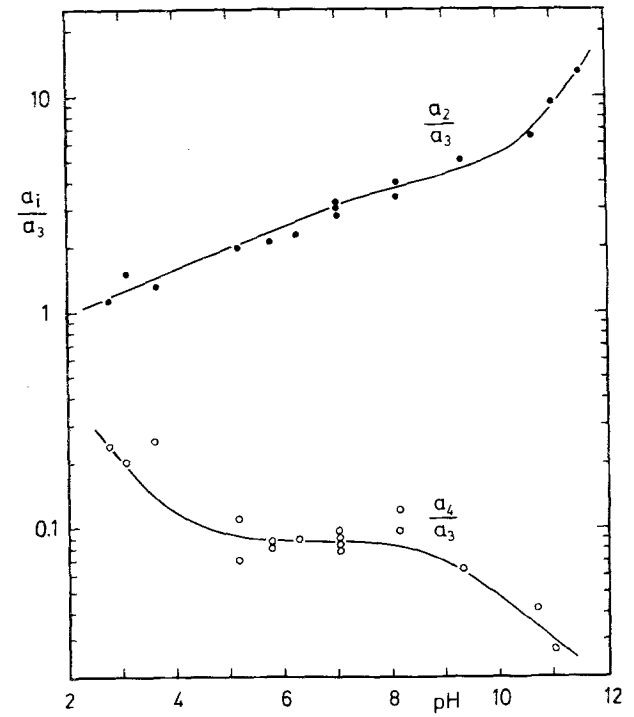

Fig. 8. pH dependence of current-amplitude ratios (experimental conditions as in Fig. 7)

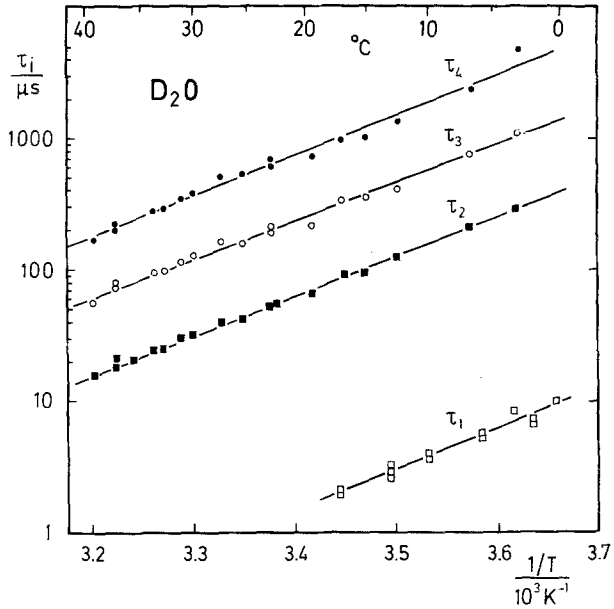

Fig. 9. Effects of D_2O on the time course of the photocurrent: time constants τ_i as a function of reciprocal temperature. The external medium was a solution of 1 M NaCl and 5 mM Tris maleate, $pD \simeq 7$, in D_2O. The purple membrane fragments were in contact with D_2O at least 24 hr prior to the measurement

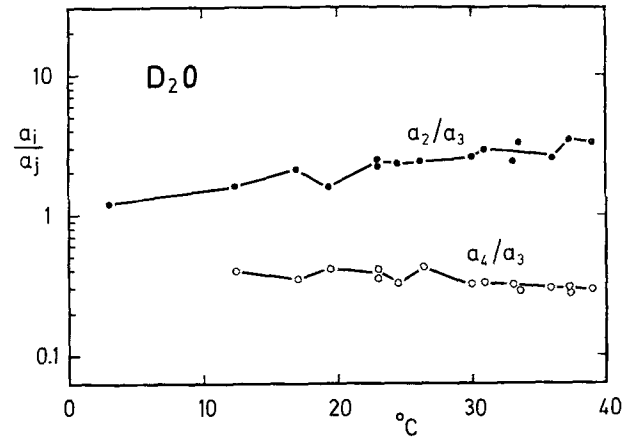

Fig. 10. Effects of D_2O on the time course of the photocurrent: current-amplitude ratios as a function of temperature. The experimental conditions were the same as in Fig. 9. The absolute value of a_3 was about 2.5 nA at 25 °C

is only about 1.5-fold and k_1 remains unchanged (Table 1).

Discussion

The flash-excitation experiments described here demonstrate that purple membrane fragments attached to a black lipid film represent a suitable model system for studying fast photoelectric signals associated with proton transport by bacteriorhodopsin. Previous quasi-stationary measurements have

shown that the purple membrane binds preferentially with the extracellular side to the black film (Bamberg et al., 1979). After excitation by a 575-nm flash, a fast inward photocurrent transient is observed corresponding to a movement of positive charge towards the cytoplasmic side of the membrane, opposite to the direction of stationary light-driven proton flow. This inward current is followed by a slower outward current transient. These observations qualitatively agree with previous photoelectric experiments with purple membranes bound to collodium or Teflon films (Drachev et al., 1978; Hong & Montal, 1979). A similar behavior (fast negative current transient followed by a slower positive transient) has also been observed with purple membrane fragments which have been oriented in free solution by an electric field (Keszthelyi & Ormos, 1980).

As discussed previously (Bamberg et al., 1979), the model system used here possibly contains a fraction of purple membrane fragments bound to the black film with the reverse orientation; this would mean that the observed photosignal represents the difference between two opposite currents. This, however, should not affect the measured values of the time constants and current-amplitude ratios, as long as binding to the planar bilayer membrane does not modify the properties of the bacteriorhodopsin molecule. Such a modification is unlikely, since the photoelectric action spectrum of purple membranes bound to planar bilayers is virtually identical with the absorption spectrum in free suspension (Bamberg et al., 1979). For an orientation-independent kinetic behavior it is further necessary that any difference in pH or electrical potential between bulk solution and intermembrane space remains small. Changes of the electrochemical potential of H^+ in the intermembrane space are unlikely to affect the kinetic results (apart from a possible effect on τ_4, *see* above), since the addition of gramicidin A or FCCP had no effect on the phototransients. Both agents have been shown to strongly increase the proton permeability of the black film underneath the purple membrane (Bamberg et al., 1979).

Another problem in the analysis of the experimental results concerns the question to what extent the single components of the photocurrent transient originate from processes within the bacteriorhodopsin molecule or whether charge movements of a more unspecific nature contribute to the observed signal (Hong & Montal, 1979). In the course of the photocurrent measurement the voltage across the compound membrane (planar bilayer plus attached purple-membrane sheet) is clamped to zero (apart from a small potential drop across the series resistance $R_s = 1/G_s$, *see* Fig. 2), but any light-driven charge movement in the purple membrane creates a voltage across G_p and an equal but opposite voltage across G_m. This voltage may decay by unspecific conductance processes within G_m and G_p and thereby may give rise to an additional current signal in the external circuit. The possibility that this decay appears as a component in the recorded current-transient may be excluded, however, on the basis of the expected time constant of this process which should be of order of $\tau \simeq (C_m + C_p)/(G_m + G_p)$. τ has been determined to be about 0.2 sec (Bamberg et al., 1979) which is much longer than the time constants of the current transient. Even when the measurement was extended to the time range of 0.1–1.0 sec, the current signal from the voltage decay process could not be detected because of its small amplitude. Another possible source of signal distortion is the formation of multiple layers of purple membrane on the surface of the black film. While this possibility cannot be strictly excluded, we consider stack formation as rather unlikely under the given concentration conditions. We therefore assume in the following that the single components of the photocurrent transient result from processes within the bacteriorhodopsin molecule.

A tentative correlation between the components of the photocurrent transient and the photochemical reaction steps inferred from flash-photometric experiments can be established on the basis of the observed time constants, the activation energies and the effects of pH and D_2O. The fast negative component of the photocurrent with a (extrapolated) time constant of $\tau_1 = 1.2$ μsec at 25 °C is tentatively assigned to the $K \rightarrow L$ transition of the photocycle which has a spectrophotometrically determined half-time of about 1 μsec (Stoeckenius et al., 1979). The same assignment has been proposed by Keszthelyi and Ormos (1980) who observed a time constant of 4 μsec for the fast negative component of the photovoltage signal of field-oriented purple-membrane sheets.

The two intermediate processes have similar time constants, $\tau_2 = 17$ μsec and $\tau_3 = 57$ μsec (at 25 °C). When processes 2 and 3 are lumped together using a fit program with three (instead of four) exponentials, a time constant of 32 μsec is assigned to the compound process which is similar to the half-time of about 27 μsec for the $L \rightarrow M$ transition at 25 °C (calculated from data of Dencher & Wilms, 1975). The finding that the experimental photocurrent transients could not be fitted adequately with a sum of three exponential indicates that processes 2 and 3 are real. This assumption is consistent with spectrophotometrical observations indicating that the M product occurs in two different forms (Eisenbach, Bakker,

Korenstein & Caplan, 1976; Hess & Kuschmitz, 1977; Korenstein & Hess, 1977; Hoffmann, Graca-Miguel, Barnard & Chapman, 1978; Korenstein, Hess & Kuschmitz, 1978; Marcus & Lewis, 1978). It is not known so far whether the two forms of the M intermediate are part of a linear reaction sequence or whether a branching of the chain occurs somewhere before the M product (Hess & Kuschmitz, 1977; Korenstein et al., 1978). The notion that components 2 and 3 of the photosignal are associated with the $L \rightarrow M$ transition is further supported by the similarity between the observed activation energies, $E_2 = 56$ kJ/mol, $E_3 = 49$ kJ/mol and the spectrophotometrically determined activation energy $E = 59$ kJ/mol of the $L \rightarrow M$ reaction (Dencher & Wilms, 1975).

The slowest component of the photocurrent transient has a time constant $\tau_4 = 950\,\mu$sec (at 25 °C). This value is similar to the time constant for the $M \rightarrow N$ transition which has been determined spectrometrically to be about 1 msec at room temperature (Stoeckenius et al., 1979; *see also* Sherman, Korenstein & Caplan, 1976; Rosenheck et al., 1978). It cannot excluded that process 4 is involved in the rebinding of the proton to bacteriorhodopsin. This assumption is consistent with the observation that the time constant τ_4 increases and the relative amplitude a_4/a_3 decreases with increasing pH of the medium (Figs. 7 and 8).

The effect of D_2O on the photocurrent kinetics supports the interpretation of the time constants given above. From flash-photometric experiments it is known that the rate constant of the $L \rightarrow M$ transition is decreased by a factor of 4–5 and the rate constant of the $M \rightarrow 0$ transition by a factor of 2–3, whereas the $K \rightarrow L$ process is little affected (Korenstein, Sherman & Caplan, 1976; Lozier & Niederberger, 1977; Stoeckenius et al., 1979; Keszthelyi & Ormos, 1980). The time constant of component 1 of the photocurrent (which probably corresponds to the $K \rightarrow L$ transition) is found to be virtually the same in H_2O and D_2O (Table 1 and Figs. 5 and 9), in agreement with the spectroscopic observations. Components 2 and 3 are found to be slowed down in D_2O, τ_2 increasing 2.6-fold and τ_3 2.9-fold, whereas τ_4 changed only by a factor of about 1.6. If processes 2 and 3 are lumped together by using a fit program with three instead of four exponentials, the increase in time constant of the compound process is about fourfold in D_2O as compared with H_2O, similar to the results of spectroscopic studies (where the $L \rightarrow M$ transition has been analyzed in terms of a single exponential process). The marked isotope effect on τ_2 and τ_3 is consistent with the notion that processes 2 and 3 are associated with proton dissociation from the binding site.

The temperature dependence of the relaxation times in H_2O and D_2O could be represented by a linear relationship between $\ln \tau_i$ and $1/T$ in the Arrhenius plot of Figs. 5 and 9 in the whole temperature range between 0 and 40 °C. Thus, these experiments provide no evidence for a phase transition in the purple membrane in this temperature range (Sherman et al., 1976).

The photoelectric signals contain information on the charge displacement associated with the elementary transitions of the bacteriorhodopsin molecule. If Φ_i transitions from state P_i to state P_{i+1} take place per unit time in the membrane, a displacement current of magnitude $e_0 \alpha_i \Phi_i(t)$ is recorded in the external measuring circuit (e_0 is the elementary charge). The "displacement coefficients" α_i describe the magnitude of charge movement associated with the transition $P_i \rightarrow P_{i+1}$. If N bacteriorhodopsin molecules are excited by a flash at time $t = 0$, the total charge q_i transferred by process i in the external circuit is given by

$$q_i = e_o \alpha_i \int_0^\infty \Phi_i \, dt = e_o \alpha_i N. \tag{11}$$

If the membrane is considered as a homogeneous dielectric film of thickness d interposed between two conducting phases, and if one elementary charge is moved over the distance l_i during the transition, the displacement coefficient is simply given by $\alpha_i = l_i/d$ (Frehland & Läuger, 1974). Since a lipid membrane with embedded proteins represents a strongly inhomogeneous dielectric medium, this simple interpretation of α_i is not generally valid, however. As the number N of excited bacteriorhodopsin molecules in the membrane is not known, only ratios of displacement coefficients can be obtained (In principle, N can be evaluated from the time integral of the photocurrent; this method, however, cannot be used here since contributions to the time integral at long times remain undetected due to low current amplitudes). According to Eq. (A14) the ratios α_1/α_3, α_2/α_3 and α_4/α_3 can be expressed by the experimental values of the current-amplitude ratios $a_i/a_j = A_i/A_j$ and the rate constants k_i, assuming that processes 1 to 4 are arranged in a linear sequence. (In cases where $k_1 \gg k_2 \gg k_3 \gg k_4$ holds, the approximate relation (10) can be used). Values of α_1/α_3, α_2/α_3 and α_4/α_3 obtained in this way are given in Fig. 11. It is seen that α_2/α_3 and α_4/α_3 are virtually temperature-independent between 5 and 25 °C, while $|\alpha_1/\alpha_3|$ increases with temperature. The charge displacement by process 1 is about five times that of process 3 (at 15 °C) and in opposite direction. Processes 2 and 3 exhibit nearly equal charge displace-

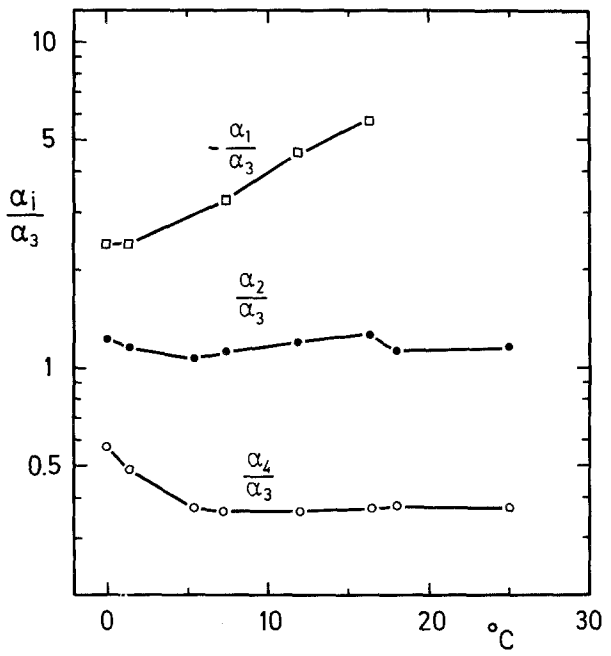

Fig. 11. Displacement coefficients α_i of processes 1, 2 and 4 (referred to α_3) as a function of temperature. $\alpha_i e_o$ is the amount of charge moving in the external circuit during the transition from state P_i to state P_{i+1} of the protein (e_0 is the elementary charge). The values of α_i/α_3 have been calculated from the data represented in Figs. 5 and 6 using Eq. (A14)

ments ($\alpha_2/\alpha_3 \simeq 1$), whereas the ratio α_4/α_3 is about 0.4. Varying the pH between 3 and 11 has little influence on α_2/α_3; α_4/α_3 increases from 0.38 (at pH 3) to about 1.5 (at pH 11). In D_2O at 3 °C the following values are obtained: $\alpha_1/\alpha_3 = -9.1$, $\alpha_2/\alpha_3 = 0.69$, $\alpha_4/\alpha_3 = 0.69$.

The displacement coefficients α_i not only contain contributions from proton transfer along the pumping pathway, but also account for any movements of polar groups during conformational transitions of the protein. Thus, the finding that α_1 is negative does not necessarily mean that in process 1 the proton moves backward (with respect to the direction of overall proton transport). Rather, the large negative value of α_1 could mean that the $K \rightarrow L$ transition involves a major conformational change. In any case, the analysis of photoelectric signals yields information on the pumping process not available from optical studies, and any mechanistic model of the pump must account for the observed values of the displacement coefficients.

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Appendix A

Current Transients Associated with the Photochemical Reaction Cycle

We consider the following reaction sequence in which a molecular species P_0 is transformed by light absorption into an excited species P_1 which decays in the dark via intermediates $P_2, P_3, \ldots$ to the final stable state P_{n-1} (in the case of a cyclic reaction, P_{n+1} is identical with P_0):

$$P_0 \xrightarrow{h\nu} P_1 \xrightarrow{k_1} P_2 \xrightarrow{k_2} P_3 \ldots P_n \xrightarrow{k_n} P_{n-1}. \tag{A1}$$

$k_1, k_2, \ldots$ are the reaction rate constants. We assume that all reactions are virtually irreversible so that back reactions may be neglected. We denote the number of molecules in state P_i by $N_i(t)$ and assume that at time $t<0$ all molecules have been in state P_0 and that at $t=0$ N molecules are transformed into state P_1 by a flash of light. The boundary conditions then are

$$N_1(0) = N \tag{A2}$$

$$N_i(0) = 0 \quad (i=2, 3, \ldots, n+1) \tag{A3}$$

$$N_1(t) + N_2(t) + \ldots + N_{n+1}(t) = N \quad (t \geqq 0). \tag{A4}$$

The rate of change of the N_i is given by

$$\frac{dN_1}{dt} = -k_1 N_1 \tag{A5}$$

$$\frac{dN_i}{dt} = -k_i N_i + k_{i-1} N_{i-1} \quad (i=2, 3 \ldots n) \tag{A6}$$

$$\frac{dN_{n+1}}{dt} = k_n N_n. \tag{A7}$$

The solution (for $n \geqq 2$) may be written in the form

$$N_1(t) = N \exp(-k_1 t) \tag{A8}$$

$$N_i(t) = N k_1 k_2 \ldots k_{i-1} \sum_{\nu=1}^{i} \frac{\exp(-k_\nu t)}{a_{i\nu}} \quad (i=2, 3, \ldots n) \tag{A9}$$

$$N_{n+1}(t) = N \left[1 - k_1 k_2 \ldots k_n \sum_{\nu=1}^{n} \frac{\exp(-k_\nu(t)}{k_\nu a_{n\nu}}\right] \tag{A10}$$

$$a_{i\nu} \equiv (k_1 - k_\nu)(k_2 - k_\nu) \ldots (k_{\nu-1} - k_\nu)(k_{\nu+1} - k_\nu) \ldots (k_i - k_\nu) = \prod_{\substack{j=1 \\ j \neq \nu}}^{i} (k_j - k_\nu). \tag{A11}$$

In general, each transition $P_i \rightarrow P_{i+1}$ is associated with a displacement of electric charge in the membrane dielectric which, in turn, leads to a charge displacement in the external circuit. The magnitude of charge displacement in the external circuit associated with a single transition $P_i \rightarrow P_{i+1}$ may be denoted by $\alpha_i e_o$ (e_o is the elementary charge). For instance, if the photoreactive membrane is a homogeneous dielectric of thickness d interposed between two aqueous electrolyte solutions, and if an elementary charge is moved over the distance l_i within the dielectric during the transition $P_i \rightarrow P_{i+1}$, then $\alpha_i = l_i/d$ (Frehland & Läuger, 1974). If m protons are transferred from one aqueous phase to the other during the photocycle, then $\alpha_1 + \alpha_2 + \ldots + \alpha_n = m$. The current $I(t)$ in the external circuit (at zero voltage) is the sum of the contributions of all transitions:

$$I(t) = e_o \sum_{i=1}^{n} \alpha_i k_i N_i(t). \tag{A12}$$

Introduction of Eqs. (A8)-(A10) yields

$$I(t)=e_o N \sum_{\nu=1}^{n} A_\nu \exp(-k_\nu t) \tag{A13}$$

$$A_1=\alpha_1 k_1+\alpha_2 \frac{k_1 k_2}{a_{21}}+\alpha_3 \frac{k_1 k_2 k_3}{a_{31}}+\ldots+\alpha_n \frac{k_1 \ldots k_n}{a_{n1}}$$

$$A_2=\alpha_2 \frac{k_1 k_2}{a_{22}}+\alpha_3 \frac{k_1 k_2 k_3}{a_{32}}+\ldots+\alpha_n \frac{k_1 \ldots k_n}{a_{n2}}$$

$$\vdots$$

$$A_n=\alpha_n \frac{k_1 \ldots k_n}{a_{nn}}. \tag{A14}$$

According to Eqs. (A13) and (A14) the current transient $I(t)$ contains the following information: the relaxation times yield the rate constants $k_1, k_2, \ldots, k_n$, whereas from the amplitudes A_ν the "displacement coefficients" $\alpha_1, \alpha_2, \ldots \alpha_n$ can be determined which describe the elementary charge displacements associated with the individual reaction steps.

A simple situation is given when each following reaction is much slower then the preceding one, i.e., when $k_1 \gg k_2 \gg k_3 \ldots \gg k_n$ holds. Under these conditions Eq. (A13) reduces to

$$I(t) \approx e_o N[\alpha_1 k_1 \exp(-k_1 t)+\alpha_2 k_2 \exp(-k_2 t)+\ldots + \alpha_n k_n \exp(-k_n t)]. \tag{A15}$$

In this case the current amplitude of each individual reaction is proportional to its rate constant k_i. This means that the slower reactions (k_i small) are difficult to observe in the current transient.

In the experimental analysis of photocurrent transients the situation may occur that some reactions are too fast to be detected at the given time resolution of the measuring device, whereas other reactions are very slow and have an amplitude below the resolution limit. It may be shown that in this case only the rate constants belonging to the reactions of intermediate rate enter into the expression for $I(t)$. In order to illustrate this point, we consider the specific case of a photoprocess with $k_1 \gg (k_2, k_3, k_4) \gg k_5, k_6, \ldots k_n$. In this case Eq. (A13) reduces to (for times larger than $1/k_1$):

$$I(t)=e_o N[A_2 \exp(-k_2 t)+A_3 \exp(-k_3 t) + A_4 \exp(-k_4 t)] \tag{A16}$$

$$A_2=\alpha_2 k_2+\alpha_3 \frac{k_2 k_3}{k_3-k_2}+\alpha_4 \frac{k_2 k_3 k_4}{(k_3-k_2)(k_4-k_2)}$$

$$A_3=\alpha_3 \frac{k_2 k_3}{k_2-k_3}+\alpha_4 \frac{k_2 k_3 k_4}{(k_2-k_3)(k_4-k_3)}$$

$$A_4=\alpha_4 \frac{k_2 k_3 k_4}{(k_2-k_4)(k_3-k_4)}. \tag{A17}$$

Thus, the parameters $\alpha_1, k_1, \alpha_5, k_5, \ldots$ of the undetectable reactions do not appear in the expressions for the amplitudes A_2, A_3 and A_4. By change of notation ($2 \to 1$, $3 \to 2$, $4 \to 3$), Eqs. (A16) and (A17) become identical with the corresponding expressions for the case $n=3$.

References

Bamberg, E., Apell, H.-J., Dencher, N.A., Sperling, W., Stieve, H., Läuger, P. 1979. Photocurrents generated by bacteriorhodopsin on planar bilayer membranes. *Biophys. Struct. Mechan.* **5**:277

Blok, M.C., Van Dam, K. 1979. Association of bacteriorhodopsin with lipid-impregnated filters. Evidence for fusion of bacteriorhodopsin-containing vesicles with the lipid phase of the filter. *Biochim. Biophys. Acta* **550**:527

Dancsházy, Zs., Drachev, L.A., Ormos, P., Skulachev, V.P. 1979. Kinetics of the blue-light induced inhibition of photoelectric activity of bacteriorhodopsin. *FEBS Lett.* **96**:59

Dancsházy, Z., Karvaly, B. 1976. Incorporation of bacteriorhodopsin into a bilayer lipid membrane: A photoelectric-spectroscopic study. *FEBS Lett.* **72**:136

Dencher, N., Wilms, M. 1975. Flash photometric experiments on the photochemical cycle of bacteriorhodopsin. *Biophys. Struct. Mechan.* **1**:259

Drachev, L.A., Kaulen, A.D., Skulachev, V.P. 1978. Time resolution of the intermediate steps of the bacteriorhodopsin-linked electrogenesis. *FEBS Lett.* **87**:161

Eisenbach, M., Bakker, P., Korenstein, R., Caplan, S.R. 1976. Bacteriorhodopsin: Biphasic kinetics of phototransients and of light-induced proton transfer by sub-bacterial *H. holobium* particles and by reconstituted liposomes. *FEBS Lett.* **71**:228

Frehland, E., Läuger, P. 1974. Ion transport through pores: Transient phenomena. *J. Theor. Biol.* **47**:189

Hellingwerf, K.J., Schnurmans, J.J., Westerhoff, H.V. 1978. Demonstration of coupling between the proton-motive force across bacteriorhodopsin and the flow through its photochemical cycle. *FEBS Lett.* **92**:181

Herrmann, T.R., Rayfield, G.W. 1978. The electrical response to light of bacteriorhodopsin in planar membranes. *Biophys. J.* **21**:111

Hess, B., Kuschmitz, D. 1977. The photochemical reaction of the 412 nm chromophore of bacteriorhodopsin. *FEBS Lett.* **74**:20

Hoffmann, W., Graca-Miguel, M., Barnard, P., Chapman, D. 1978. Evidence for conformational transitions in bacteriorhodopsin. *FEBS Lett.* **95**:31

Hong, F.T., Montal M. 1979. Bacteriorhodopsin in model membranes. A new component of the displacement photocurrent in the microsecond time scale. *Biophys. J.* **25**:465

Hwang, S.-B., Korenbrot, J.I., Stoeckenius, W. 1978. Transient photovoltages in purple membrane multilayers. Charge displacement in bacteriorhodopsin and its photointermediates. *Biochim. Biophys. Acta* **509**:300

Janko, K., Benz, R. 1977. Properties of lipid bilayer membranes made from lipids containing phytanic acid. *Biochim. Biophys. Acta* **470**:8

Keszthelyi, L., Ormos, P. 1980. Electrical signals associated with the photocycle of bacteriorhodopsin. *FEBS Lett.* **109**:189

Korenstein, R., Hess, B. 1977. Hydration effects on the photocycle of bacteriorhodopsin in thin layers of purple membrane. *Nature (London)* **270**:184

Korenstein, R., Hess, B., Kuschmitz, D. 1978. Branching reaction in the photocycle of bacteriorhodopsin. *FEBS Lett.* **93**:266

Korenstein, R., Sherman, W.V., Caplan, R. 1976. Kinetic isotope effects in the photochemical cycle of bacteriorhodopsin. *Biophys. Struct. Mechan.* **2**:267

Korenstein, R., Sherman, W.V., Caplan, S.R. 1977. Kinetic isotope effects in the photochemical cycle of bacteriorhodopsin. *Biophys. Struct. Mechan.* **2**:1805

Lozier, R.M., Niederberger, W. 1977. The photochemical cycle of bacteriorhodopsin. *Fed. Proc.* **36**:1805

Marcus, A.M., Lewis, A. 1978. Resonance Raman spectroscopy of the retinylidene chromophore in bacteriorhodopsin (bR_{570}), bR_{560}, M_{412}, and other intermediates: Structural conclusions based on kinetics, analogues, models, and isotopically labeled membranes. *Biochemistry* **17**:4722

Oesterhelt, D. 1976. Isoprenoids and bacteriorhodopsin in *Halobacteria. Prog. Mol. Subcell. Biol.* **4**:133

Provencher, S.W. 1976*a*. A Fourier method for the analysis of exponential decay curves. *Biophys. J.* **16**:27

Provencher, S.W. 1976*b*. An eigenfunction expansion method for the analysis of exponential decay curves. *J. Chem. Phys.* **64**:2772

Rosenheck, K., Brith-Lindner, M., Lindner, P., Zakaria, A., Caplan, S.R. 1978. Proteolysis and flash photolysis of bacteriorhodopsin in purple membrane fragments. *Biophys. Struct. Mechan.* **4**:301

Seta, P., Ormos, P., d'Epenoux, P., Gavach, C. 1980. Photocurrent response of bacteriorhodopsin adsorbed on bimolecular lipid membranes. *Biochim. Biophys. Acta* **591**:37

Sherman, W.V., Korenstein, R., Caplan, S.R. 1976. Energetics and chronology of phototransients in the light response of the purple membrane of *H. halobium*. *Biochim. Biophys. Acta* **430**:454

Shieh, P., Packer, L. 1976. Photo-induced potentials across a polymer-stabilized planar membrane in the presence of bacteriorhodopsin. *Biochem. Biophys. Res. Commun.* **71**:

Stoeckenius, W., Lozier, R.M., Bogomolni, R.A. 1979. Bacteriorhodopsin and the purple membrane of halobacteria. *Biochim. Biophys. Acta* **505**:215

Trissel, H.-W., Montal, M. 1977. Electrical demonstration of rapid light-induced conformational changes in bacteriorhodopsin. *Nature* (*London*) **266**:655

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