KINETIC ANALYSIS OF DISPLACEMENT PHOTOCURRENTS ELICITED IN TWO TYPES OF BACTERIORHODOPSIN MODEL MEMBRANES

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ABSTRACT Fast displacement photocurrents have been reported in bacteriorhodopsin model membranes by several groups of investigators since 1977. A fast component (B1) is associated with positive charge displacement in the direction opposite to that of a physiological proton translocation. A slower component (B2) of opposite polarity is associated with positive charge displacement in the same direction as the proton translocation. Using two slightly different methods for model membrane formation, we observed photosignals with or without a significant B2 component under appropriate conditions. By means of the tunable voltage clamp method of measurement (Hong, F.T., and D. Mauzerall, 1974, *Proc. Natl. Acad. Sci. USA*, 71:1564–1568) we demonstrated that the time course of the B1 signal is completely predictable by an equivalent circuit containing a chemical capacitance. From the equivalent circuit analysis, we obtained a first-order relaxation time constant of 12.3 ± 0.7 μs at room temperature. We also found a slight temperature dependence of the B1 relaxation with an activation energy of 2.54 ± 0.24 kcal/mol. We found no pH dependence of the B1 component in the range of 0 to 11, whereas the B2 component is diminishing in a graded manner when the pH is varied from 0 to 10. These results are diametrically different from what reported previously (Drachev, L.A., A.D. Kaulen, L.V. Khitrina, and V.P. Skulachev, 1981, *Eur. J. Biochem.*, 117:461–470). Our results support the interpretation that the B1 component is generated by an intramolecular charge displacement accompanying the light-induced reactions of bacteriorhodopsin and that the B2 component is generated by a process of proton uptake from the intracellular aqueous phase and subsequent release into the same aqueous phase. The impact of the present results on the conventional practice of identifying photointermediates of bacteriorhodopsin by spectroscopic means is discussed.

INTRODUCTION

A fast photoelectric signal can be observed in a reconstituted bacteriorhodopsin model membrane when it is illuminated with a brief light pulse (Trissl and Montal, 1977; Hwang et al., 1977, 1978; Drachev et al., 1978, 1981; Hong and Montal, 1979; Bamberg and Fahr, 1980; Keszthelyi and Ormos, 1980; Fahr et al., 1981; Trissl, 1985; Dér et al., 1985; see also reviews: Skulachev, 1982; Trissl, 1982; Bamberg et al., 1984; Keszthelyi, 1984; Hong and Okajima, 1986). This photosignal belongs to a distinct class of bioelectric signals known as displacement currents, of which the gating current in squid axon (Armstrong and Bezanilla, 1974) and the early receptor potential (ERP) in the visual photoreceptor membrane (Brown and Murakami, 1964) are the best known examples. This class of bioelectric signals is generated by a transient and rapid charge separation in the membrane rather than by flow of ions through the membrane (Hagins and Rüppel, 1971; Armstrong and Bezanilla, 1974). The fast rate of the charge displacement is reflected in the lack of detectable latency of this type of signals after light stimulation (Cone, 1965, 1967). This peculiarity demands specific recording techniques as well as specific analytical methodologies. A method of analysis of light-induced displacement currents was developed by Hong and Mauzerall (1974, 1976), using a tunable voltage-clamp method and based on a concept of chemical capacitance. Using this approach, they were able to predict the time course of the displacement photocurrent generated by a microsecond-laser pulse in a lipid bilayer membrane containing a membrane-bound magnesium porphyrin coupled to an aqueous redox gradient in the two electrolyte solutions. In an attempt to generalize the concept of chemical capacitance in the description of displacement photocurrents, Hong (1980) demonstrated that it is possible to explain qualitatively data reported in the literature on a variety of pigment-containing membrane systems with a single equivalent circuit model. In particular, Hong was able to explain the peculiar differentiating waveform observed Drachev et al. (1974) when their bacteriorhodopsin model membranes were shunted by either ionophore CCCP or by a parallel shunt resistor. Their explanation based on a model with intact membrane vesicle attached to a planar bilayer (Drachev et al., 1976) is therefore called in question. That the concept of chemical capacitance is quantitatively applicable to the bacteriorhodopsin membrane system is the subject of the present article.

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The existence of an ERP-like photosignal in the bacteriorhodopsin model membrane was first pointed out by Hong (1977) in his attempt to apply the concept of chemical capacitance to data reported by Drachev et al. (1974). The first unequivocal demonstration of such a displacement photocurrent was made by Trissl and Montal (1977). Subsequently, Hong and Montal (1979) demonstrated the existence of a still faster component that they named B1 because of its persistence at low temperature, similar to the ERP R1 component. Likewise, the slower component, which can be reversibly inhibited by low temperature, was named B2, in analogy to the ERP R2 component.

Here, we report a new procedure of constructing model membranes that allows us to obtain photosignals that appear to be composed of an almost pure B1 component with a negligible contribution from the B2 component. We found that, over a wide range of experimental conditions, the relaxation of the fast (B1) component is in agreement with the same equivalent circuit that Hong and Mauzerall used to fit their Mg porphyrin data. Furthermore, the B1 relaxation is unaffected by a pH change of the bathing solution from pH 0 to 11, whereas the B2 component exhibits a significant pH-induced kinetic change. As will be discussed in detail, this pH-induced behavior is diametrically different from conclusions based on conventional open circuit measurements. We also report evidence of a small but reproducible temperature dependence for the B1 component and its corresponding activation energy (2.54 ± 0.24 kcal/mol). In addition, the B1 component is insensitive to deuterium-hydrogen exchange, whereas the B2 component is highly sensitive. Our kinetic data, therefore, are consistent with the interpretation that the B1 component is due to the oriented dipole mechanism, whereas the B2 component is due to the interfacial proton transfer mechanism, a suggestion previously made by Hong (1980). In contrast, other investigators ascribe all displacement photocurrents to oriented dipole mechanisms (reviewed by Hong, 1978). The significance of our new findings on the molecular interpretation of light-induced charge movements in bacteriorhodopsin membranes is discussed.

MATERIALS AND METHODS FOR FORMING MODEL MEMBRANES

Purple membrane fragments used in this study were from cell cultures of *Halobacterium halobium* strain R1, grown in a medium as described by Lanyi and MacDonald (1979) and extracted as described by Oesterhelt and Stoeckenius (1974). All the purple membrane preparations used in this study were light-adapted. Two different methods of model membrane formation are used: (*a*) the Trissl-Montal method of transferring an oriented interfacial layer of purple membranes to a thin Teflon film (Trissl and Montal, 1977); and (*b*) a multilamellar coating method that was modified from the Trissl-Montal method.

In the original Trissl-Montal method (e.g., Fig. 1 *A* in Hong and Montal, 1979), an interfacial layer of light-adapted purple membrane fragments was formed in one aqueous phase, which was then apposed to a thin Teflon film (6.35 μm thick, unless otherwise indicated) by slowly raising the water level. The other aqueous phase contains only electrolyte solution. In the multilamellar coating method, an aqueous suspension of light-adapted purple membrane fragments (5–15 μg in protein dry weight) is deposited on a piece of dry and clean Teflon film (area 0.5 cm^2; same thickness) and allowed to dry in the air. The film is then mounted on the same chamber used in the Trissl-Montal method. In both methods, the interfacial film is washed several times with water to remove any loosely attached fragments. The Trissl-Montal method gives rise to photosignals with both B1 and B2 components, whereas the multilamellar coating method gives rise to an almost pure B1 component. Evidence will be presented to support the claim that the photosignal so obtained has a negligible B2 contribution provided that the current amplifier has a sufficient bandwidth to resolve the B1 relaxation.

METHOD OF ELECTRICAL MEASUREMENT

Tunable voltage clamp measurements (Hong and Mauzerall, 1974, 1976) were made through a pair of platinum electrodes connected to a negative feedback current amplifier with adjustable access impedance and instrumental time constants. A 100-MHz fast settling operational amplifier (model 46K; Analog Devices, Inc., Norwood, MA) was used to implement the negative feedback control. The highest achievable time resolution is 150 ns. The output of the amplifier was monitored by a transient recorder (Biomation model 8100; shortest time base 10 ns) interfaced to a PDP 11/34 minicomputer for signal averaging and record storage. Stored raw data along with measurement parameters were then retrieved and analyzed off-line on the same minicomputer by means of a family of programs that are optimized for the required analysis protocols. The light source was a pulsed dye laser (model SLL-625A; Candela Corp., Natick, MA) with rhodamine 6G (590-nm output; half-width duration 0.8 μs; peak energy 1.25 J). The laser pulse intensity was measured by a joulmeter. The laser beam size was adjusted to cover at 40% of the membrane area. No effect on the photosignal can be detected by varying the beam size. The laser intensities used in this study (2–3 J/cm^2) were within the range of linear responses and not close to saturation. The laser discharge and the start of the recorder sweep were synchronized by a delay pulse generator. The noise isolation was achieved through the following combination of approaches. The entire measurement system, including the minicomputer, was enclosed in a Faraday cage. The noises through transmission lines are reduced by means of optocouplers. Further noise reduction is achieved through signal averaging. The temperature of the chamber was controlled by a refrigerator circulator (Lauda Div., Brinkmann Instruments Co., Westbury, NY) and monitored with a digital thermometer (Keithley Instruments Inc., Cleveland, OH). In all the data shown in this article, the polarity of the photocurrent is such that a positive current would flow through the purple membrane/Teflon film assembly from the uncoated side to the coated side.

METHOD OF EQUIVALENT CIRCUIT ANALYSIS

The central feature of the equivalent circuit (Fig. 1) is a chemical capacitance (C_p) that is connected in series to the photoemf (E_p). C_p is physically distinct from the ordinary membrane capacitance (C_m) and is characteristic of a membrane that exhibits displacement photocurrents (Hong, 1976). The transmembrane *dc* resistance (R_s), encountered by the photocurrent, and the ionic membrane resistance (R_m) are set to infinity because of the presence of the Teflon film. The resistance (R_p) is the internal resistance encountered by the process of charge recombination. The true photochemical relaxation time (τ_p) is equal to the product of R_p and C_p. Notice that C_m is connected in parallel to E_p, whereas C_p is connected in series to E_p. In an idealized voltage-clamp measurement when the access temperature R_s is zero (i.e., when R_s is considerably smaller than the source impedance), the photocurrent is described by the following equation:

$$I(t) = \frac{E_p(t)}{R_p} - \left(\frac{1}{\tau_p} - \frac{1}{R_sC_p}\right)\int_o^t \frac{E_p(u)}{R_p}\exp\left(\frac{u-t}{\tau_p}\right)du.$$

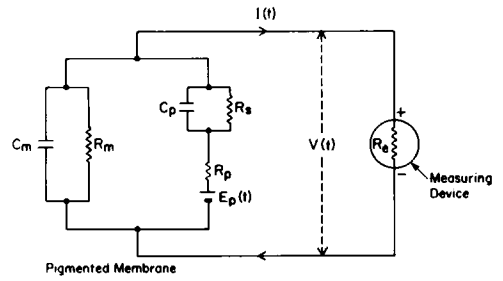

FIGURE 1 An equivalent circuit that incorporates the concept of chemical capacitance. Two such circuits with different sets of parameters are required to fit the B1 and the B2 components separately. Notice that the Teflon film support in the reconstituted membranes used in the present study is an integral part of the membrane dielectric and must be included in the analysis. See text for further explanation. (Reproduced from Hong, 1980.)

However, when R_e is neither zero nor infinite, the photocurrent $I(t)$ becomes a function of the access impedance and was shown to be described by the following equations instead:

$$I(t) = \frac{1}{R_e C_m \left(\frac{1}{\tau_s} - \frac{1}{\tau_l}\right)} \left[\left(\frac{1}{\tau_s} - \frac{1}{R_s C_p}\right) \int_o^t \frac{E_p(u)}{R_p} \exp\left(\frac{u-t}{\tau_s}\right) du - \left(\frac{1}{\tau_l} - \frac{1}{R_s C_p}\right) \int_o^t \frac{E_p(u)}{R_p} \exp\left(\frac{u-t}{\tau_l}\right) du \right],$$

where

$$\frac{1}{\tau_s} = \frac{1}{2}\left[\frac{1}{R_p C_m} + \frac{1}{\tau_p} + \frac{1}{\tau_m} + \sqrt{\left(\frac{1}{R_p C_m} + \frac{1}{\tau_p} + \frac{1}{\tau_m}\right)^2 - 4\left(\frac{1}{R_p C_m R_s C_p} + \frac{1}{\tau_p \tau_m}\right)}\right]$$

$$\frac{1}{\tau_l} = \frac{1}{2}\left[\frac{1}{R_p C_m} + \frac{1}{\tau_p} + \frac{1}{\tau_m} - \sqrt{\left(\frac{1}{R_p C_m} + \frac{1}{\tau_p} + \frac{1}{\tau_m}\right)^2 - 4\left(\frac{1}{R_p C_m R_s C_p} + \frac{1}{\tau_p \tau_m}\right)}\right]$$

$$\frac{1}{\tau_p} = \frac{1}{R_p C_p} + \frac{1}{R_s C_p}$$

$$\frac{1}{\tau_m} = \frac{1}{R_e C_m} + \frac{1}{R_m C_m}.$$

That is, if R_e is not equal to zero, a single first-order relaxation process will be detected as a biphasic current with two exponential time constants, τ_s and τ_l. The true relaxation time constant τ_p can be computed from the above equations. The agreement of the model with the experiment is checked by comparison of the computed theoretical curve and the experimental curve. In the computation of the theoretical curve, the following experimental parameters are entered as inputs to the computer program that incorporates the equivalent circuit in its algorithm: τ_s and τ_l, the two exponential time constants used to fit the biphasic waveform of relaxation, the access impedance (R_e), and the membrane RC relaxation time (τ_m), the low-pass filter time constant (τ_f) of the amplifier, and the waveform of the laser pulse (recorded by a Lite-Mike, model 560B; EG&G, Salem, MA). All these input parameters to the computer program were measured independently from the same membrane. The photoemf is assumed to have the same waveform as the exciting laser pulse, with its peak amplitude determined by normalization of the theoretical curve against the experimental curve.

While it is understood that the photoelectric signals under true voltage-clamp conditions must be reported as currents and those under the true open circuit condition must be reported as voltages, the intermediate case where R_e is neither zero nor effectively infinity can be reported either as currents or voltages (as the IR drop generated by the follow of current through the access impedance). Since our experimental conditions are closer to voltage-clamp than open-circuit conditions, we routinely report the photosignals as currents. In this regard, some attempted photocurrent measurements appearing in the literature were actually carried out under conditions closer to open circuit than voltage-clamp conditions (reviewed and discussed in Hong, 1980).

In principle, two separate sets of parameters are required: one to fit the B1 and another to fit the B2 components. We report here only the fit to the B1 component.

RESULTS

pH Dependence of the Photosignals in Membranes Formed by the Trissl-Montal Method and by the Multilamellar Coating Method

The pH effect on the photosignal relaxation differs somewhat in membranes formed by the two slightly different methods. In the membranes formed by the Trissl-Montal method, the negative peak (corresponding to the B2 component) diminishes and the positive peak (B1) increases as pH varies from 7 to 2 (Fig. 2 *A*). In contrast, with membranes formed by means of the multilamellar coating method, there is very little change in the waveform from pH 0 to 9, although a mild pH effect on the amplitude is still evident (Fig. 2 *B*; pH 0 not shown). However, if a long instrumental time constant is used so that the bandwidth of the amplifier is no longer sufficient to resolve the decay of the positive peak, then the pH dependence of the photoresponses is evident in membranes formed by either methods (Figs. 2, *C* and *D*). From pH 1 to 9 the change is mostly monotonic and is almost always reversible. At higher pH (above 11), the signal is usually not reversible when the pH is restored to neutral. Comparison of Figs. 2, *C* and *D* shows that the positive peaks are less prominent and the negative peaks are more prominent in membranes formed by the Trissl-Montal method. The simplest interpretation is that photoresponses from membranes formed by the multilamellar coating method contains an almost pure B1 component that is virtually pH independent from pH 0 to 7, provided that the bandwidth of the amplifier is sufficient to resolve the relaxation of B1. In contrast, the photoresponses from membranes formed by the Montal-Trissl method contain a significant contribution of B2 component in addition to the B1 component. However, the B2 component is not completely absent in membranes formed by the multilamellar coating method. By applying a low-pass filter time constant that is longer than the B1 relaxation (τ_f = 35 μs), the faster B1 component is

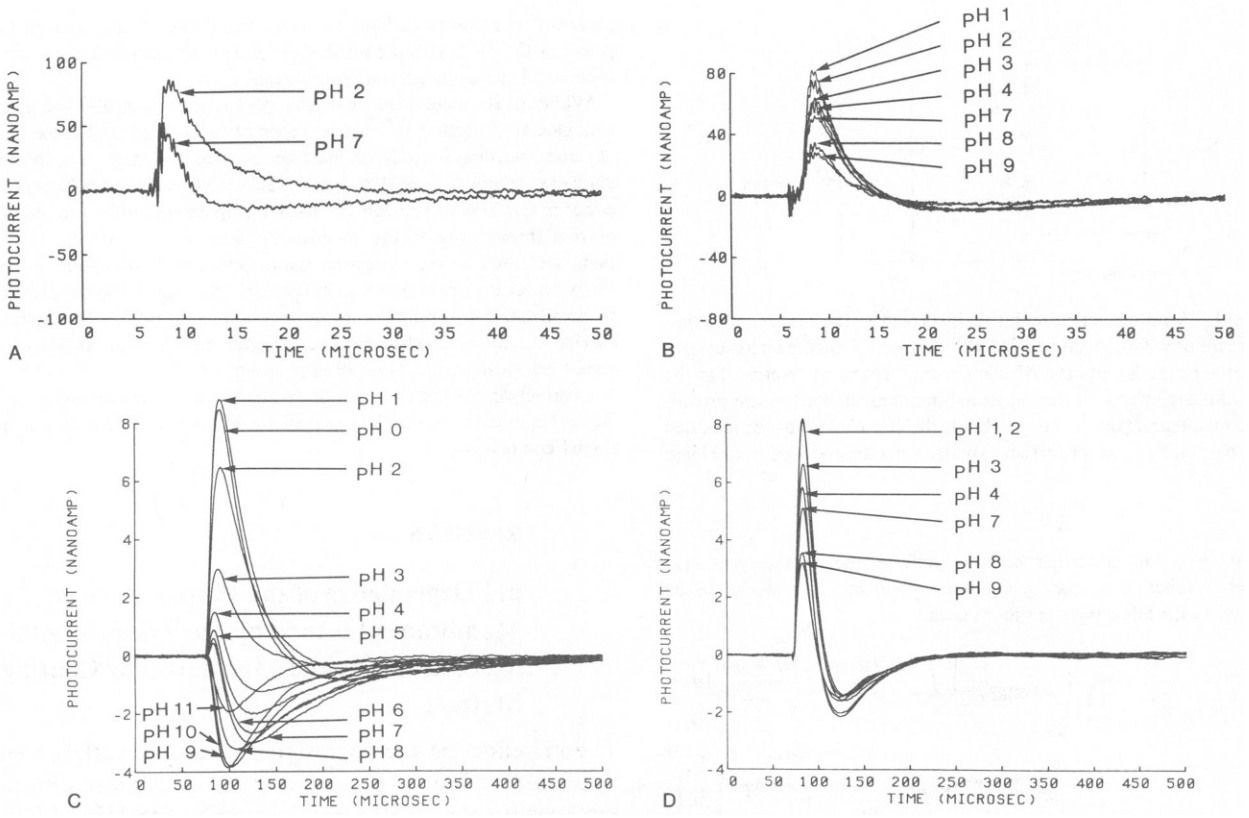

FIGURE 2 pH dependence of photosignal in bacteriorhodopsin-containing membranes. The temperature was 25°C. The bathing solution contained 3 M KCl and 0.05 M L-histidine titrated to various pH values as shown. The access impedance was 40 kΩ. Data in A (τ_f = 0.355 μs) and in C (τ_f = 33 μs) were from a membrane made by the Trissl-Montal method, whereas data in B (τ_f = 0.457 μs), and in D (τ_f = 35 μs) were from a membrane made by the multilamellar coating method. The relative contribution of B1 amplitudes in C and in D is diminished by severe low-pass filtering. Conversely, the relative contribution of B2 amplitudes in A and in B is diminished by the absence of filtering of the B1 signals.

selectively suppressed and the presence of a smaller B2 component is then revealed. Still photoresponses from membranes formed by the Trissl-Montal method have a higher content of B2 component. Additional evidence in support of this interpretation can be found in the D_2O substitution experiment to be described in the next section. The decline of photosignal amplitudes at higher pH in Fig. 2 *B* will be examined later.

Effect of Deuterium Oxide Substitution in the Aqueous Phases

The effect of pH on the B2 component suggests that interfacial proton transfer may be responsible for its generation. If so, substitution of D_2O for water in the aqueous phases may slow down the relaxation and reduce the amplitude of the B2 component, provided that the formation/breaking of the bond in protonation/deprotonation is rate limiting. This expected effect is seen clearly when the photosignal is measured at pH 7 and 25°C in a Trissl-Montal membrane, the conditions under which the B2 component is prominent. As shown in Fig. 3 *A*, the B2 component diminishes to about half the amplitude of the control, and the relaxation is also slowed down. In contrast, if the photosignal is measured at low pH (Fig. 3 *B*), a smaller difference is detected. However, Fig. 3 *B* also indicates that the photosignal measured at pH 2 is not completely free from the B2 contribution. In support of the hypothesis that the multilamellar deposition method gives rise to pure B1 signals, we found no kinetic isotope effect of D_2O substitution at the 5% level of significance in membranes made by this latter method (the next two sections).

Equivalent Circuit Analysis of the B1 Component

A typical photosignal obtained from membranes by means of the multilamellar coating method at 25°C and pH 2 (in water solution of salts) and measured with an instrumental time constant of 0.355 μs is shown in Fig. 4 *A* as the noisy curve. This photosignal is supposed to contain a nearly pure B1 component. A theoretical curve computed by inputing the consistent set of experimental parameters is shown as the smooth curve. Normalization yields a peak photoemf of 155 mV. The corresponding first order relaxation time obtained from the equivalent circuit analysis is 12.7 μs (R_p = 60.3 kΩ, C_p = 211 pF). The error incurred in computing τ_p is <6%, although the errors for R_p and C_p

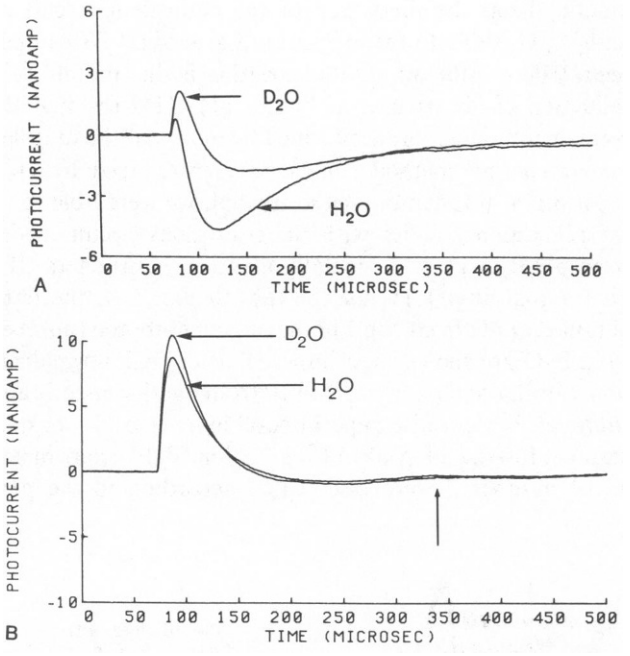

FIGURE 3 Effect of D_2O substitution in the aqueous phases. The membrane was made by the Trissl-Montal method. The temperature was 25°C. The instrumental time constant was 33 μs. The pH was 7 in *A* and 2 in *B*, respectively. The aqueous solution contained 3 M KCl and 0.05 M L-histidine. The access impedance was 40 kΩ. The instrumental time constant was 33 μs. A slight kink indicated by the arrow was due to a reproducible distortion of the baseline. The kink could have been eliminated by baseline subtraction.

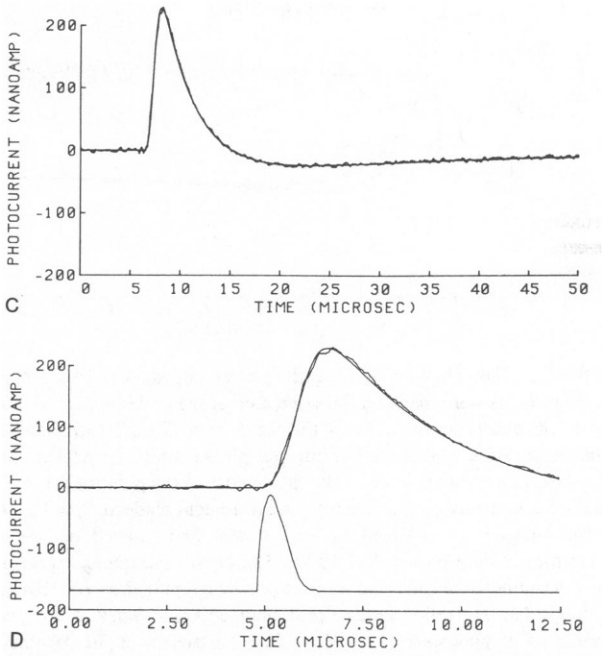

FIGURE 4 (*A*) Equivalent circuit analysis of the B1 component. The membrane was formed by the multilamellar coating technique on a Teflon film. The temperature was 25°C. The bathing electrolyte solution contained 0.1 M KCl and 0.01 M L-histidine titrated to pH 2. The measurement was made at an access impedance of 39.2 kΩ and an instrumental time constant of 0.355 μs (four measurements were signal-averaged). The computed (smooth) curve is superimposed on the measured (noisy) curve. Other experimentally measured parameters used as inputs for computation include: τ_s = 3.4 μs, τ_l = 24.0 μs, τ_m = 6.42 μs. The photoemf is approximated by a triangular waveform with a half-width duration of 0.8 μs, the same as the exciting laser pulse. Normalization yielded a peak photoemf of 155 mV. The root-mean-square value of the noise is 1.6 nA, while the root-mean-square deviation of the computed curve from the experimental curve is 2.2 nA. The derived parameters were: τ_p = 12.7 μs, R_p = 60.3 kΩ, and C_p = 211 pF. Notice that the inclusion of the Teflon film support as an inseparable part of the membrane dielectric increases the effective membrane thickness to 6.35 μm, and, therefore, reduces the membrane capacitance (C_m) to an unusually low value of 164 pF (328 pF/cm^2). However, the true photochemical relaxation time constant (τ_p) remains unaffected by variation of the effective membrane thickness. See text for detail. (*B*) Agreement of the computed rise phase with the measured one. The same data as in Fig. 4 *A* was displayed on an expanded time scale to emphasize the fit of the rise phase. The laser pulse was also shown with an arbitrary vertical scale. Note that the convolution effect due to the finite (nonzero) width of the laser pulse and the instrumental time constant (low-pass filter effect) of 0.355 μs were incorporated in the computation. The photoemf was assumed to have the same time course as the laser pulse. The agreement is an indication of the lack of a detectable latency at a time resolution of 0.355 μs.

may be larger. Notice that the root-mean-square deviation of the theoretical curve from the experimental curve is determined to be 2.2 nA, which is favorably approaching the limit set by the noise level of 1.6 nA (root-mean-square value) in the measured signal. The same set of data is shown on an expanded time scale along with the waveform of the exciting laser pulse in Fig. 4 *B*. Note that the effect of convolution of the nonzero pulse width with the low-pass filter time constant (τ_f) was included in the computation of the rise phase. In view of the assumption that the photoemf has the same time course as the light pulse and the agreement of the computed rise phase and the measured one, we conclude that there is no detectable latency (of the B1 component) at a time resolution of 0.355 μs. Statistical analysis of data collected from separate membranes in either water or deuterium oxide at pH 2 gives the following values of τ_p: 12.3 ± 0.7 μs in H_2O (n = 8); 12.6 ± 1.4 μs in D_2O (n = 6). There is no kinetic isotope effect on the B1 relaxation at the 5% level of significance (t test).

To demonstrate that the agreement of the computed curve and the measured curve is by no means accidental and to demonstrate that the waveform of the photosignal does depend on the access impedance, we made two separate measurements of the photosignal on the same membrane under almost identical conditions except for different values of the access impedance (20 kΩ and 40 kΩ) (Fig. 5). In Fig. 5, the experimental curve obtained at R_e = 20 kΩ is compared with a theoretical curve which was computed in a procedure that is different from what was carried out in Fig. 4: the input parameters used in the computation are the values of circuit parameters (except R_e) obtained from analysis of the experimental curve at R_e = 40 kΩ. Normalization yields a peak photoemf of 120

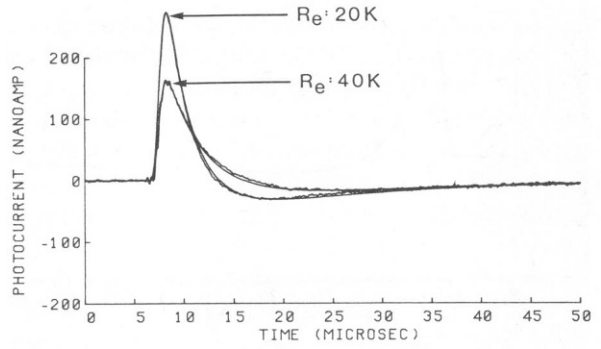

FIGURE 5 The effect of varying the access impedance. Two separate measurements were made on the same membrane under almost identical conditions except for the values of the access impedance: 20 kΩ and 40 kΩ. The temperature was 25°C. The aqueous phases were 3 M KCl and 0.05 M L-histidine titrated to pH 2. The time course of the photosignal at R_e = 20 kΩ is completely predictable from measurement made at R_e = 40 kΩ. A circuit analysis as outlined in Fig. 4 was first carried out on the experimental data obtained at 40 kΩ. The circuit parameters were then used to compute the time course of the photocurrent if R_e = 20 kΩ instead of 40 kΩ. Normalization yields a peak photoemf of 120 mV. A computed prediction of photocurrent at R_e = 40 kΩ based on input parameters determined from the measured data at R_e = 20 kΩ was also shown (determined peak E_p = 122 mV). Separate analyses of the data at the two access impedances resulted in the following parameters: R_p = 61.0 kΩ, C_p = 207 pF, τ_p = 12.6 μs at R_e = 20 kΩ; R_p = 65.3 kΩ, C_p = 192 pF, τ_p = 12.5 μs at R_e = 40 kΩ.

mV. The role of the two measured curves are then exchanged so that the experimental curve at R_e = 40 kΩ is compared with the computed curve based on parameters obtained at R_e = 20 kΩ. In this latter computation, the photoemf turns out to be 122 mV. The agreement of the time course as well as the determined values of E_p indicate that a cross-prediction can be made entirely from a measurement previously performed at a different value of the access impedance. In other words, the two separate measurements with different R_e values reflect the same kinetic processes though they have different observed time courses. The apparent relaxation time constants, τ_s and τ_l, are the function of the access impedance as well as of other molecular parameters. The fit of model and data is by no means automatic. As a matter of fact, we have never been able to fit a photosignal obtained in the Trissl-Montal method even at pH 2 and low temperature. We tentatively interpret this lack of agreement as an indication that the B2 component is not negligible even at this low temperature and low pH (2) in membranes formed by the Trissl-Montal method. This interpretation is supported by the D_2O substitution experiment described earlier.

However, the agreement of the equivalent circuit and the data shown in Fig. 2 *B* exists only in the range of pH from 0 to 2. At higher pH, the deviation of kinetics from prediction is evident and reproducible, and cannot be explained by experimental errors alone. Furthermore, the amplitude reduction at higher pH is also experimentally reproducible and is not an effect of intensity variation of the laser pulses. We found that the deviation of relaxation kinetics from the prediction of the equivalent circuit at higher pH is due to the existence of a residual B2 component, whereas the amplitude variation is due mainly to a reduction of absorbance at higher pH. This residual B2 component in membranes formed by means of the multilamellar coating method can be further reduced by D_2O substitution. In membranes so treated, we were able to fit the relaxation kinetics with the equivalent circuit model from pH 0 to 11 with virtually identical parameters (the photosignal at pH 11 not shown). In Fig. 6 *A*, the data obtained at pH from 2 to 7 in the same membrane (number 1) in D_2O are shown superimposed, after peak normalization. Similarly, data at pH 7 to 10 from another membrane (number 2) were also superimposable after peak normalization. Instead of peak normalization, data from membrane number 2 were also scaled according to the pH-

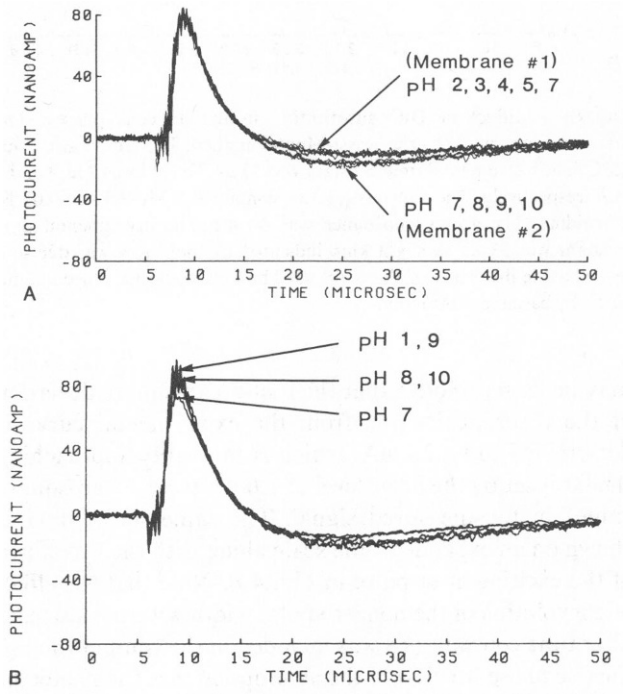

FIGURE 6 pH dependence of the B1 component. The membranes were formed by the multilamellar coating method. The temperature was 25°C. The bathing solution contained 3 M KCl and 0.05 M L-histidine in D_2O titrated with NaOD or DCl to various pH values as shown. (*A*) Data from two membranes with slightly different membrane capacitances were shown (158 pF in membrane number 1 and 205 pF in membrane number 2). The access impedance was 40 kΩ. The instrumental time constants were 0.355 μs in membrane number 1, and 0.457 μs in membrane number 2. The photosignals were normalized to the positive peaks. The slightly different time courses in the two membranes were due to the slight difference in membrane capacitance and hence difference in the charging time constant, τ_m (Hong, 1980). Notice signals at pH 7 appeared in both membranes. Therefore, if the data were all from one signal membrane, all traces would have been superimposable. Thus, pH has no effect on the relaxation of the B1 component from pH 0 to 11 (data at pH 0, 1 and 11 not shown). (*B*) The signals from membrane No. 2 measured at various pH were adjusted in amplitudes for the pH-dependent absorbance changes. The near superposition of all traces indicates that there is no significant pH effect on the B1 amplitudes.

dependent absorbance changes so that the same amount of light was absorbed (Fig. 6 *B*). It is evident that the pH-induced amplitude variation can be largely accounted for by the pH-dependent absorbance changes. We therefore conclude that both the relaxation kinetics and the amplitude of the B1 component is pH dependent in the range from pH 0 to 11.

Temperature Dependence of the B1 Component

Data in the literature indicate that the ERP R1 component is extremely resistant to low temperature, as it is the only component that survives low temperature treatment (Pak and Cone, 1964). On a qualitative level, data about the B1 component of the ERP-like photosignal are also consistent with this notion (Hong and Montal, 1979). But is the B1 (or rather R1) component really temperature independent? Such a question is of importance with regard to the underlying mechanism. If a signal is completely temperature independent, then it may be due to a quantum mechanical tunnelling (of protons or electrons) and is a manifestation of the primary photochemical process. The fact that B1 has a risetime that is faster than one can measure (submicrosecond) is strongly suggestive of such a primary process. After repeated experiments on different membranes made on different dates as well as from different lots of purple membrane preparation, we have concluded that there is a small temperature dependence of the B1 component (Fig. 7). We subjected the photosignals obtained at different temperatures to the equivalent circuit analysis outlined above to obtain the first-order rate constant of the relaxation process ($k_p = 1/\tau_p$). A set of such data are shown in an Arrhenius plot in Fig. 7. The slope yields an activation energy of 2.54 ± 0.24 kcal/mol (n = 20). Similar experiments carried out in deuterated buffer solutions yields an activation energy of 2.45 ± 0.19 kcal/mol (n = 16). Again, there is no difference at the 5% level of significance. That the temperature effect is not due to data scattering is seen in the reversibility of such changes when the temperature is restored as long as a prolonged exposure to extreme temperatures such as 45°C is avoided.

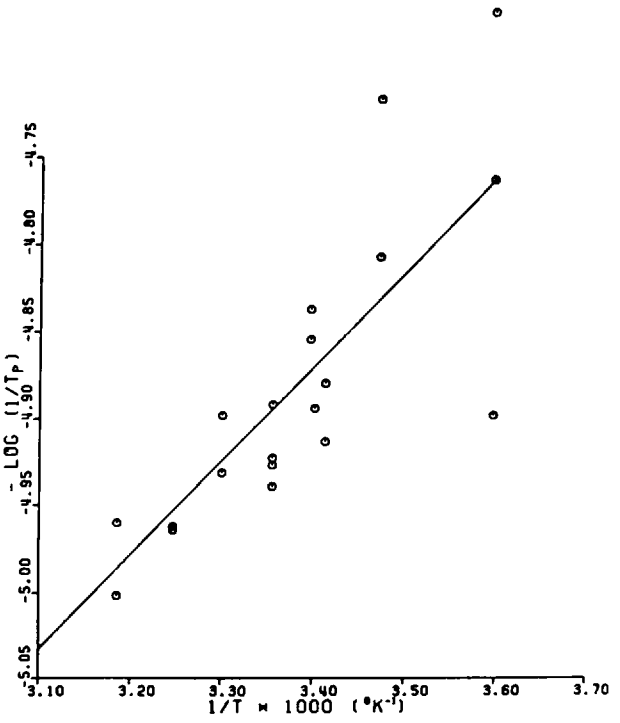

FIGURE 7 Temperature dependence of the B1 component. The relaxation rate constants ($k_p = 1/\tau_p$) of the B1 component were determined as a function of temperature from 5° to 40°C. The Arrhenius' plot is shown. The slope yields an activation energy of 2.54 ± 0.24 kcal/mol.

Dependence on the Fraction of Membrane Area Illuminated

There has been a question as to whether chemical capacitance might be a mathematical artifact generated by partial illumination of the membrane, which partitions the membrane capacitance (C_m) into two parameters, one for the illuminated region and the other for the nonilluminated region (Trissl, 1981). To ascertain that partial illumination of the membrane (40%) has not introduced distortion of the time course of relaxation, we varied the beam sizes of the laser pulse from a 6% coverage to a 100% coverage (full illumination). The measured photoresponses are superimposed in Fig. 8 without normalization. The almost perfect superposition of these responses indicates that there is no detectable dependence of the relaxation time course on the fraction of membrane area illuminated, and that the chemical capacitance is not the ordinary membrane capacitance in disguise. The amplitudes of the photosignals are

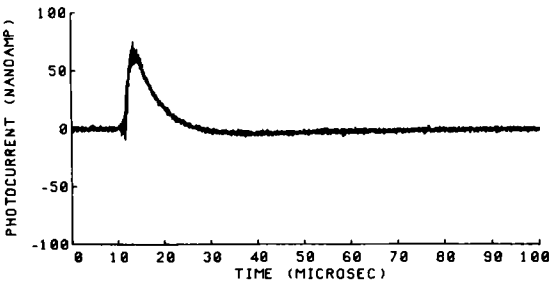

FIGURE 8 Effect of partial illumination on the time course of the photoresponse. The membrane was formed by the multilamellar coating method on a Teflon film. The temperature was 23.2°C. The pH was 2. The aqueous solution contained 0.1 M KCl and 0.01 M L-histidine. The access impedance was 40 kΩ and the instrumental time constant was 0.355 μs. The membrane area was 0.5 cm² (diameter 8 mm). Photocurrent responses from the same membrane with various laser beam sizes were superimposed. The total energy in the laser pulse was kept constant at 0.1 J, but the diameters of the laser beam were 2, 3, 3.5, 4, 5.5, 7, and 8 mm, corresponding to a coverage of 6%, 14%, 19%, 25%, 47%, 77%, and 100% of the total membrane area, respectively. There is no detectable effect on the relaxation time constants. The photoresponses were in the linear range in all measurements.

independent of beam sizes because the photoresponses were all within the linear light dependence range.

Dependence on the Thickness of Teflon Film Used in the Reconstitution

In the equivalent circuit analysis presented above, the Teflon film is treated as a part of the membrane dielectric. Clearly, the thickness of the Teflon film mainly determines the values of C_m and has a major influence on the apparent relaxation time through its effect on the value of the charging time constant τ_m. To make sure that the intrinsic time constant (τ_p) is not also a function of the thickness of the Teflon film being used, we determine τ_p values in model membranes made from Teflon films that are 12.7 μm in thickness with otherwise identical conditions (pH 2, 25°C). The value of τ_p was found to be 12.9 ± 0.6 μs (n = 6). In comparison with the corresponding values obtained from 6.35 μm Teflon films (12.3 ± 0.7 μs [n = 8]), there is no difference between the two at the 5% level of significance.

DISCUSSIONS AND CONCLUSIONS

In this study, we employed two slightly different methods of membrane formation to study the fast photoelectric effect of bacteriorhodopsin-containing membranes. The original Trissl-Montal method gives rise to photosignals that have a rapid B1 component (which is both temperature resistant and acid resistant), and a slower B2 component of opposite polarity (which is inhibited by low temperature as well as by low pH) (Hong and Montal 1979). We also used a modified method, in which the purple membranes are deposited on the Teflon film by direct sedimentation. We believe the second method generates a photosignal that contains a negligible contribution to the B2 component at low pH. First, we would like to set aside the question whether it is really a pure B1 signal, since an independent proof is not yet available. We argue that this signal agrees with our equivalent circuit model. Our theoretical computation uses only experimentally measurable parameters as inputs. The only arbitrary parameter is the amplitude of the photoemf E_p, which is necessary in a linear system. Therefore, there is no built-in arbitrariness. The validity of the model is demonstrated by the fact that the agreement is achieved at almost all temperatures in which the protein pigment bacteriorhodopsin remains stable. Attempts to arbitrarily change certain input parameters such as R_e and τ_m frequently end up with conspicuous disagreement between the model and the measured signal. Furthermore, the time course of the photosignal as a function of the access impedance is correctly predicted by the equivalent circuit model. In view of the fact the equivalent circuit model can be derived from a reasonable molecular model using the Gouy-Chapman theory (e.g., Hong, 1976, 1978) and in view of the inability of the equivalent circuit model to predict the time course of the photosignal when a second component is obviously present, we tentatively take the agreement as an indication of the existence of the B1 signal as a pure entity. That this tentative conclusion is reasonable is further supported by the Arrhenius style behavior of the temperature dependence of the relaxation rate constant as well as the fact the relaxation process can be described as a first order (or pseudo first order) process through a wide range of temperatures. This tentative conclusion is also consistent with the D_2O substitution experiment.

If we are willing to accept the notion that the multilamellar coating method generates a nearly pure B1 component, then the following conclusion can be drawn from our data. The B1 component is pH independent in the range of 0–11. The effect of pH has been studied in the range of 0–6 previously by Drachev et al. (1981) in a different model collodion membrane system. A direct comparison leads to a conspicuous discrepancy. In the data they reported (Fig. 8 in Drachev et al., 1981), the component I (corresponding to the B1 component) shows little pH dependence in the range from 6 to 2 but its amplitudes double as the pH is varied from 2 to 0. In addition, the component II (corresponding to the B2 component) exhibits little pH dependence from 6 to ~4 and then drops to zero at pH 2, as does their component III. As pH is decreasing, it is evident from Drachev's data that as the component II and III diminish the component I starts the rise significantly. We further point out that these components were taken as the amplitudes of the positive peaks and negative peaks directly without further analysis. This protocol of decomposition of a signal into two components is valid only if the time courses of these components are independent of pH (only the amplitudes vary) and only if the rise times and the decay times of the two components do not overlap significantly. Our experimental data fail to support these conditions. On the other hand, we found no contradiction between the data of Drachev et al. (1981) and our interpretation with regard to the B1 and the B2 component. This can be made clear by referring to the curves shown in Fig. 9, which was originally proposed by Hong and Montal (1979) to explain the effect of temperature and pH on a model membrane formed by the Trissl-Montal method. The lower curve represents the measured photocurrent which contains both B1 and B2 together. The upper and the middle curves represent individual B1 and B2 components. Since these two components have opposite polarity, the inhibition of B2 component by low temperature and/or by low pH results in an increase of the positive peak of the photosignal, thus giving an apparent impression of an increase in the B1 amplitude. We further suggest that the component II and the component III reported by Drachev et al. (1981) are the two apparent exponential components of B2, as stipulated by the equations for the photocurrent response according to our equivalent circuit model. This is clearly illustrated in Fig. 9. As shown in the lower curve of Fig. 9, Hong and Montal (1979) predicted that a second positive phase may exist as a result of mixing of four

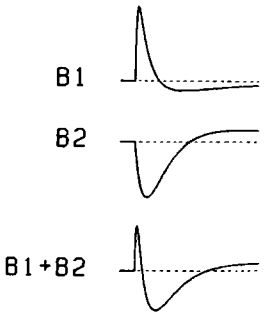

FIGURE 9 Schematic diagram originally proposed by Hong and Montal (1979) to illustrate the decomposition of the photosignal into two components. Note that both B1 and B2 components decay at two exponential time constants. Also notice a second positive phase which may or may not be observable, because of the complexity of the tails of the four exponentials. (Reproduced from Hong and Montal, 1979.)

exponentials. We have observed this speculated slow positive phase in a bacteriorhodopsin-containing lipid bilayer formed according to the method of Dancsházy and Karvaly (1976) (Fig. 10). Our interpretation of the component II and III is further supported by their observation that the component II remains proportional to component III through the entire pH range, indicating that component II and component III have the same pH dependence, a coincidence that requires an explanation. Their alternative explanation supplied is not unique and requires further experimental support.

Implicit in the comparison of our present data and data reported by Drachev et al. (1981) is the assumption that the B1 component points in the same direction as their component I, namely in the direction opposite to that induced by continuous light. Concurrent stationary state and transient measurements using a lipid bilayer membrane incorporated with purple membrane fragments by means of Dancsházy and Karvaly's (1976) method permit us to infer the orientation of purple membranes in the model systems and demonstrate that it is indeed the case. The extracellular surface of the purple membrane sheets is attached to the Teflon support whereas the intracellular surface is facing the aqueous compartment where the pH is being varied. This polarity assignment also agrees with that reported by Fahr et al. (1981). Therefore, the B1 and the B2 peaks are formally equivalent to the negative and the positive peaks, respectively, reported by Fahr et al. (1981), by Keszthelyi and Ormos (1980), and by Drachev et al. (1981). That is, our present sign convention for the photosignals is opposite to theirs. Most investigators reported photoelectric relaxation in four exponential time constants, but the values varied widely in ranges among them (Table I). We believe that the variation originates from differences in access impedances of measurement rather than differences in methods of model-membrane formation. For example, the data of Drachev et al. (1981) were performed under an open circuit condition. They correctly identified τ_4 as the pure membrane RC relaxation, but failed to realize the distortion of the remaining three time constants. The conditions in the data reported by Fahr et al. (1981) were close but not quite equal to the idealized voltage clamp (short circuit) condition. If the condition were truly short circuit, τ_2 would approach the true photochemical relaxation time constant of B1 (12.3 ± 0.7 μs). However, their data can still be reconciled from the reported estimate of their experimental conditions: an access resistance R_e of 500–1000 Ω and a membrane capacitance C_m of ~5 nF, resulting in a τ_m value of 2.5–5 μs. From Eq. 8 in Hong (1976), a crude estimate of the true B1 relaxation time constant can be obtained: $\tau_p = \tau_1 \times \tau_2/\tau_m$, which yields 4–9 μs. Unfortunately, most other investigators did not even report a crude estimate of the access impedance or the membrane capacitance. It is therefore not possible to rescue their data by a similar maneuver. From the foregoing analysis, it is clear that even a qualitative conclusion based on open circuit measurements is not to be trusted unconditionally.

With regard to the generating mechanisms of these displacement photocurrents, Hong (1978) has analyzed two prototype mechanisms: an oriented dipole mechanism, based on light-induced intramolecular charge separation, and an interfacial charge transfer mechanism, based on a second-order interfacial charge transfer reaction involving

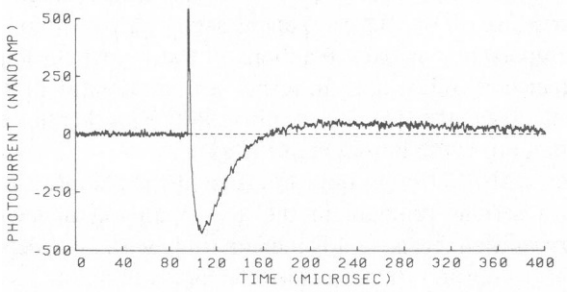

FIGURE 10 Photosignal from a lipid bilayer containing bacteriorhodopsin formed by the method of Dancsházy and Karvaly (1976). The temperature was 25°C. The instrumental time constant was 0.355 μs. The aqueous phase contained 0.1 M NaCl buffered with 0.005 M Trizma at pH 7. The dotted line is the base line. Notice the second positive phase.

TABLE I
RELAXATION TIME CONSTANTS OF BACTERIORHODIPSIN PHOTOSIGNALS

Source	τ_1	τ_2	τ_3	τ_4
	μs		ms	
Fahr et al. (1981)	1.3	17	0.06	0.9
Dér et al. (1985)	25	150	2.4	5.8
Keszthelyi and Ormos (1980)	4.4	81	2.5	8
Rayfield (1983)*		57	1.06	13
Trissl (1983)*‡		115	4.5	640
Drachev et al. (1981)	<0.2	200	2	1,000

*τ_1 not reported.
‡τ_4 derived from Fig 1 *d* in Trissl (1983).

reactants in both the membrane phase and the adjacent aqueous phase. The latter mechanism was shown to be involved in the displacement photocurrent elicited by a laser pulse from a Mg porphyrin lipid bilayer membrane separating a redox gradient (interfacial electron transfer). The former mechanism was repeatedly invoked to explain many displacement photocurrents including the ERP and the ERP-like signals in bacteriorhodopsin membranes. Although proton uptake and release exist in both cases, an interfacial proton transfer mechanism has not been seriously considered mainly because of the lack of a significant pH effect on the photosignals (R2 and B2 components; reviewed by Hong, 1978). From the present analysis, the lack of a pH effect is more apparent than real. Thus, our kinetic data indicate the following. The B1 component may be generated by an oriented dipole mechanism. We have not found any experimental evidence that the B1 relaxation varies with an aqueous component in a pseudo first-order manner. Thus, an interfacial charge transfer mechanism for B1 generation is unlikely. The B1 charge displacement can be generated by conformational changes involving a segment of molecule that carries a permanent electric dipole moment or generated by hydrogen-bonded chain mechanisms (Nagle and Tristram-Nagle, 1983). The fact that direction of charge displacements in B1 component happens to be opposite to that of proton translocation should not cause any conceptual difficulty, as the path of least resistance for proton conduction need not be the same as the path of least (geometrical) distance. We failed to observe any effect on B1 after overnight hydrogen-deuterium exchange. The conflicting reports of kinetic isotope effect on τ_1 by Keszthelyi and Ormos (1980) and by Fahr et al. (1981) can be explained on the basis of overlap of B1 decay and B2 rise as elaborated above. The lack of a kinetic isotope effect in B1 relaxation suggests that either the displaced charges are not exchangeable protons or the bond formation or breaking is not rate-limiting. As for the B2 component, its graded pH-dependence over a wide range from 0 to 10 strongly suggests that B2 is generated by an interfacial proton transfer mechanism with an initial uptake of protons followed by a release. Quantitative kinetic evidence of a pH-dependent pseudo first-order relaxation of B2 is not yet available. However, an interfacial proton transfer mechanism for B2 generation appears most likely. The suppression of B2 at low pH can be understood by retardation of proton release by virtue of mass action in a second-order reaction. Since the total number of protons released is presumably conserved, retardation of the release process will reduce the peak amplitude of the photocurrent (see Hong, 1976; Ormos et al., 1980). This is further supported by the D_2O substitution experiment reported in this article, and by AC conductimetric measurements reported by Marinetti and Mauzerall (1983). The latter investigators found evidence of proton uptake and release by purple membranes at pH 4. However, the interfacial proton transfer reaction may not be the whole event of B2. Marinetti and Mauzerall (1983) found the transient conductance change of bacteriorhodopsin suspension to be independent of buffer compositions at pH 8, indicating that ions other than protons are involved.

With regard to correlation of photoelectric signals and the intermediates in the bacteriorhodopsin photocycle, most investigators cited above associated the fast (B1) component with formation and decay of the K intermediate. In view of the lack of a kinetic isotope effect in B1 relaxation as well as in the photocycle preceding the decay of L intermediate (Korenstein et al., 1976), there is no inconsistency in their interpretation. However, the situation may be more complex. Dér et al. (1985) assigned τ_1 and τ_2 (i.e., B1 component) to the L to M transition. Ormos et al. (1983) found two additional relaxation processes at low temperature that they associated with the formation of K intermediate. As for the slow component, most investigators associated it with the formation and/or decay of M intermediate (e.g., see Drachev et al., 1984), despite the lack of agreement among the data reported. This correlation implies that the slow component reflects the process of proton release and subsequent uptake as the reactions proceed in a unidirectional cycle. This interpretation is, however, incompatible with our observation on B2, as the presence of a Teflon support in our present system precludes the observation of proton release at the extracellular surface of purple membranes, which itself is in contact with the insulating Teflon support. At a more fundamental level, the interpretation in terms of a unidirectional photocycle is also incompatible with the notion of displacement photocurrents, as the amount of electric charges moving in one direction initially must equal that moving in the opposite direction subsequently. Thus, the B2 component reflects a proton uptake at the intracellular interface and a subsequent release into the same intracellular aqueous phase, i.e., the B2 relaxation is a back reaction. In the absence of an insulating barrier, the slow component may also reflect the release of protons into the extracellular aqueous phase and subsequent uptake of protons from the same phase. This question can be answered by independent manipulation of the pH in the extracellular and in the intracellular aqueous phases. For the time being, our observation on the B2 component serves as a reminder of the importance of back reactions in the interpretation of photoelectric relaxations in terms of a unidirectional photocycle (see also Ormos et al., 1980; Stoeckenius and Bogomolni, 1982; Parodi et al., 1984).

Our rather different interpretation of the pH effect does pose a serious problem in the accompanying molecular interpretation, because it is incompatible with an accepted spectral interpretation: bacteriorhodopsin in acidic conditions is generally considered to be a different (spectral) species with different photochemical properties (Mowery et al., 1979; Druckman et al., 1982; Kimura et al., 1984). Yet we found absolutely no changes of the B1 signal over a wide pH range (see also Fig. 6 in Hong and Okajima,

1986). Furthermore, we treat the pH dependence of the B2 component as a consequence of the mass action law. This clue leads to the proposal of a new concept of local reaction conditions (Hong, 1986). Because the bacteriorhodopsin molecule is exposed to both aqueous phases, the two interfacial reactions may depend on the pH of the adjacent aqueous phases only, if the two interfacial reactions are sufficiently fast to be decoupled from each other. Thus, despite the fact bacteriorhodopsin is a single molecule, the description of a single set of experimental conditions is no longer adequate: two sets of conditions pertaining to the two aqueous solutions are needed instead. The effect of these local conditions on the heterogeneous reactions in a purple membrane has significant impact on the conventional practice of assigning each decay component of a transient absorption spectrum to a different photointermediate in bacteriorhodopsin. Depending on the pH difference across the membrane, the protonation/deprotonation processes at the two interfaces may follow two distinct decay time courses, giving rise to an additional spectrally identifiable species, while in reality a simple electrochemical explanation is available. This analysis casts doubt on the validity of using spectral characteristics alone as a means of identifying photointermediates, strengthens the relevance of primarily using electrokinetic information to monitor the membrane function, and places a greater emphasis on electrical data in elucidating the molecular mechanism.

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