Light-induced currents from oriented purple membrane

II. Proton and cation contributions to the photocurrent

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ABSTRACT The sign of B2, the microsecond component of the photocurrent from oriented purple membrane, is that of positive charge moving away from the purple membrane in the direction of proton release. B2 could be due to internal dipole or proton movement, proton release, or metal cation release. We found that the waveform of B2 is virtually insensitive to changes in the salt concentration as long as it is >40 mM KCl, >5 mM $CaCl_2$, or >0.5 mM $LaCl_3$. However, below these limits, B2's apparent rate of decay increases as the salt concentration decreases without any change in the initial amplitude. This salt dependence suggests that B2 is due to a positive charge, either a metal cation or a proton, moving from the membrane into the solution. That the positive charge is not a metal cation is suggested by the waveform of B2 remaining unchanged upon replacing the cations both in solution and in the binding sites of the purple membrane. Direct evidence that the positive charge movement is due to protons was obtained by examining the correlation of B2 with the proton dependent processes of bacteriorhodopsin in buffers and dyes. Based on these observations, we suggest that most, if not all, of the intrinsic B2 component of the photocurrent at moderate salt concentration is due to proton release.

The photocurrents from purple membranes whose surface potential has been reduced by delipidation or chemical modification of carboxyl groups with methyl esters were found to be only modestly changed. This suggests that the salt effect is not through its modulation of the surface potential. Rather, we propose that in low salt B2 represents the sum of a proton release from the surface of the purple membrane and a second current component, due to cations moving back towards the membrane, which is only important in low salt. The cation counter current is induced by proton release which creates a transient uncompensated negative charge on the membrane.

INTRODUCTION

A wide variety of techniques have been used to study the light-driven movment of protons by bacteriorhodopsin in the purple membrane of *Halobacterium halobium*. Resonance Raman experiments (reviewed in Smith et al., 1985), as well as the large spectral shift accompanying the L-M transition in the bacteriorhodopsin photocycle, indicate that during this transition the Schiff base, which binds the chromophore to the apoprotein, becomes deprotonated. pH indicators and other methods (Lozier et al., 1976; Ort and Parson, 1978; Govindjee et al., 1980: Drachev et al., 1984; Grzesiek and Dencher, 1986), have shown that protons are released from the membrane in the submillisecond time range. These released protons must give rise to a net electrical current from oriented purple membranes (PM). Because B2, the microsecond component of the photocurrent, is the only photoelectric component in that time range and it has the proper sign for a positive charge moving in the direction of proton movement (see Fig. 1 of previous paper [Liu, 1990]), at least part of B2 must be due to a proton current. Indeed, it is often assumed that B2 is due only to protons. However, this has not been rigorously demonstrated because other possible components have not been excluded and the kinetic correlation of B2 with H^+ release has not been established. Firm identification of B2 as a proton current will be very useful in studying the proton pump.

In the preceding paper (part I [Liu, 1990]), it has been shown that the microsecond (B2) photocurrent signal is exactly correlated with the L-M optical transition in both lifeime and amplitude when measured in at least moderate concentrations of salt (e.g., >5 mM $CaCl_2$). Here we will show that B2 is much more sensitive to the surface environment of the purple membrane than the L-M transition suggesting that the main part of B2 is due to interfacial rather than internal charge movement. We call the usual B2 photocurrent signal (lifetime, 85 μs) measured in moderate salt concentration and neutral pH "intrinsic B2", because at low salt or in the presence of most pH buffers some new photocurrent components modify the waveform of B2. By studying the effect of pH buffers on B2, by comparing the B2 photocurrent component with the proton release signal detected by pH indicators and by ion substitution experiments, we demonstrate that most, if not all, of the intrinsic B2 signal

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represents a proton current. In contrast, we propose that the altered B2 waveform seen in low salt has current contributions from both protons and metal cations.

It is known that the surface of purple membrane is negatively charged at neutral pH. The surface charge density on either surface of purple membrane is in the range of -2 to -5 charges/bR (Ehrenberg et al., 1989). This surface charge density gives rise to a surface potential whose value depends on the concentration and type of ions present. Because light induces proton release in the 100-μs time scale and proton uptake is in the 10-ms range, upon deprotonation an additional negatively charged group will be transiently produced, probably near the surface of purple membrane. This negatively charged group (e.g., a COO^-), generated by the proton being released and lasting until bR gets reprotonated, will cause a transient surface potential increase (it becomes more negative). In this paper, we suggest that in high salt, the transient negative surface charge will be easily compensated by the many cations that will be near the new charge. However, in low salt a counter current opposite to the direction of the proton release current is induced by cations, initially far from the new charge, moving toward the membrane to compensate for the charge. This counter current dramatically changes the waveform of the B2 photocurrent in low salt.

MATERIALS AND METHODS

The purple membrane preparation, oriented purple membrane gel, photocurrent measuring set-up, and data processing methods were the same as those in the preceding paper (Liu, 1990).

Lipid depleted purple membrane (LD-PM) gels

Eight gel pieces were incubated in 15 ml of 20 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) containing 5 mM acetate buffer (pH 5.4) for 36 h at room temperature to delipidate the membrane (Szundi and Stoeckenius, 1987). The solution was stirred and changed every 12 h. After delipidation, the CHAPS was removed by washing the gel in distilled water for 2 d during which time the water was changed four times.

Arginine methyl ester and glycine methyl ester modified purple membrane (AME-PM and GME-PM) gels

Six gel pieces were incubated in 30 ml of 100 mM arginine methyl ester or glycine methyl ester (pH 4.9) containing 0.5 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) at room temperature for 4 h (Renthal et al., 1979). The gels were stirred and fresh EDC added after 2 h incubation. After treatment, the gels were washed with distilled water for 24 h; the water was changed every 4 h.

Reconstituted purple membrane gels

Gels were incubated with DOWEX 50W cation exchange resin overnight until the color changed to blue. Then they were put into 5 mM KCl, 5 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, or 0.5 mM $LaCl_3$, respectively. During the incubation, the pH was monitored and continually adjusted to 6.0, until the gels turned the normal purple color again.

pH Titration of B2 with buffers

Because different amounts of buffer will be ionized at different pHs even if the total concentration is constant, the buffers will have different conductivity or ionic strength at different pHs. To control the amplitude of B2 in buffers of different pHs, the conductivity of the buffer was adjusted to be constant within $\pm$ 0.5 kΩ by adding small amounts of KCl. For pH titrations in 5 mM bis-Tris propane, the conductivity of the buffer was adjusted to be equivalent to that of 8 mM KCl. For the pH titration in 5 mM *N*-2-acetamido-2-aminoethanesulfonic acid (ACES), the conductivity of the buffer was adjusted to be equivalent to that of 4 mM KCl.

RESULTS

Effect of varying salt on B2

Fig. 1, *A–C*, shows the B2 component of the photocurrent measured in the mono-, di-, and trivalent cationic salt solutions of different concentrations. As can be seen, the waveform of B2 is very similar in the different salt solutions when the salt concentration is relatively high, the concentrations depending on the valence of the cations. However, they all become faster and apparently smaller upon decreasing the salt concentration. If we compare the traces in KCl with those in $CaCl_2$ and $LaCl_3$ we see that the traces which have the same amplitude do not have exactly the same shape. In low KCl, the photocurrent decays to zero within 100 μs after the flash. In low $CaCl_2$, the photocurrent does not decay quickly to zero; rather there is a long slow tail. The traces in $LaCl_3$ are very similar to those in $CaCl_2$. Computer fitting indicated that the traces in KCl can be well fit with a single exponential plus a baseline, representing the much longer lifetime component, B3. The traces at low concentrations of $CaCl_2$ and $LaCl_3$ need two exponentials plus a baseline to get a satisfactory fit. Fig. 1 *D* shows the decay rate of B2 vs. the KCl concentration. When the KCl is below 10 mM, the rate constant of B2 has a simple linear relationship with the logarithm of the KCl concentration. Fig. 1 *E* is a semilogarithmic plot of the traces shown in

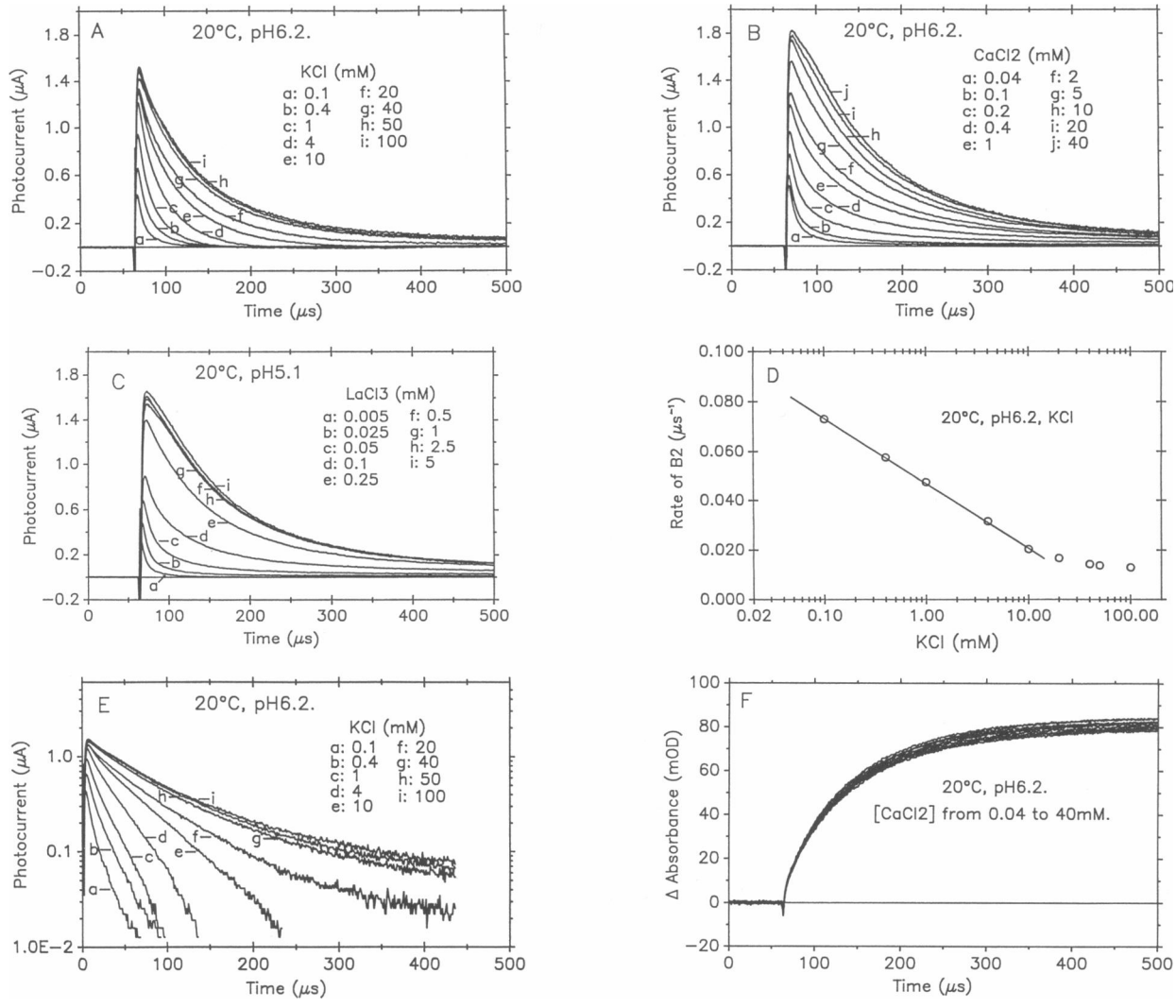

FIGURE 1 Salt dependence of the B2 photocurrent component. (*A*) B2 in different concentrations of KCl. (*B*) B2 in different concentrations of $CaCl_2$. (*C*) B2 in different concentrations of $LaCl_3$. (*D*) Decay rate of B2 in different concentrations of KCl. (*E*) Semilogarithmic plot of data in *A*. (*F*) L-M photocycle transition measured under the same conditions as in *B*.

Fig. 1 *A*. By extending each trace to time zero, the peak amplitudes are essentially the same except for the trace measured in 0.1 mM KCl. This indicates that the apparent decrease in B2's amplitude with decreasing salt concentration is caused by the increasing decay rate of B2. The intrinsic amplitude at time zero is almost constant over the salt range studied. The deviation of the 0.1 mM KCl trace is because the rise and decay lifetime of B2 become close (τ = 1.6 and 13 μs), significantly changing the amplitude at $t = 0$.

We have also measured the B2 component in NaCl and $MgCl_2$ (data not shown). We found no difference between KCl and NaCl. For the same concentration of $CaCl_2$ and $MgCl_2$, B2 decays slightly faster in $MgCl_2$ although the peak amplitude and the slow tail are the same. We have also measured monovalent salts with different anions, NaF and Na_2SO_4, and found that the waveform is not dependent on the type of anion in the solution. The salt concentration dependence of the B2 component in D_2O is very similar to H_2O. It becomes faster when the salt concentration in D_2O is reduced. Because of the slowing down of B2 in D_2O by about five times (see previous paper [Liu, 1990]), the two exponential phases measured in low $CaCl_2$ become clearer (data not shown). Varying the concentration of bR from 6.5 to 44.5 μM (with the concentration of acrylamide fixed at 7.5%), or varying the

concentration of acrylamide from 5.5 to 15% (with the concentration of bR fixed at 41 μM) does not change the salt effect (data not shown). Measuring the temperature dependence of the decay of B2 (from 8 to 35°C) indicates that the activation energy is the same for high and low salt conditions, 13.5 ± 0.2 kcal/mol.

In contrast to the B2 component, there is no detectable change in either the rate or the amplitude of the L-M transition for the concentrations of the KCl and $LaCl_3$ solutions used in Fig. 1, *A* and *C* (data not shown).[1] For $CaCl_2$, the amplitude of M increased slightly as $CaCl_2$ increased (Fig. 1 *F*). However, this very small change is in contrast to the dramatic changes of B2 under the same conditions.

There are several reasons to believe that the salt concentration dependence of B2's decay rate cannot be due to an artifact of the photocurrent measuring system, especially due to change in the system resistance with salt concentration. First, we have shown that the frequency response of our measuring apparatus in the microsecond range is flat over a wide range of salt concentrations (Liu and Ebrey, 1988). Second, from Fig. 1 *A* we can see that increasing the salt concentration, which decreases the sample resistance (R), increases B2's lifetime. If there is a capacitor in the system which caused an RC distortion in the microsecond range, any current component induced by this RC will become slower upon increasing R. Clearly, B2 is not this component because it becomes faster. Third, a comparison of B2 measured in 4 mM KCl with that in 0.5 mM $LaCl_3$ shows that its decay rate is faster in KCl. However, the sample resistance is higher in 0.5 mM $LaCl_3$ (10.6 KΩ) than in 4 mM KCl (6.5 KΩ), indicating that the increase in decay rate is not related to the sample resistance. Fourth, the apparent decrease of B2 amplitude with the decrease in salt concentration is not due to an increase in the sample resistance. Previously, we showed that the signal amplitude is proportional to $R_s/(R_s + 2R_l)$, where R_s is the source resistance formed by sum of the microscopic resistances across each membrane and $2R_l$ is the sample resistance (Liu and Ebrey, 1988). Changing the salt concentration will change both R_s and R_l similarly, so $R_s/(R_s + 2R_l)$ will be kept constant. Finally, from Fig. 1 *C*, we can see that when the concentration of $LaCl_3$ changed from 0.5 to 5 mM, which corresponds to a sample resistance change from 10.8 to 1.2 KΩ; B2 is unchanged. That means an order of magnitude change in the sample resistance has almost no effect on the amplitude of the photocurrent.

Identification of the source of the intrinsic B2 photocurrent component

We have already noted that proton release must make at least a partial contribution to some component of the photocurrent having the same sign as B2. Other possible contributors to the charge displacement are protein dipole movements and movements of metallic cations. Four kinds of evidence suggest that intrinsic B2 is due to the movement of protons from purple membrane into solution. Direct evidence for proton movement comes from studies in pH buffers, pH-sensitive dyes, and D_2O effects, while indirect evidence, eliminating other possible current contributions, comes from studies of the effect of salt on the B2 photocurrent.

Effect of pH buffers on B2

Fig. 2 *A* shows a pH titration of the B2 component in the presence of 5 mM bis-Tris propane buffer (pK_l = 6.8).

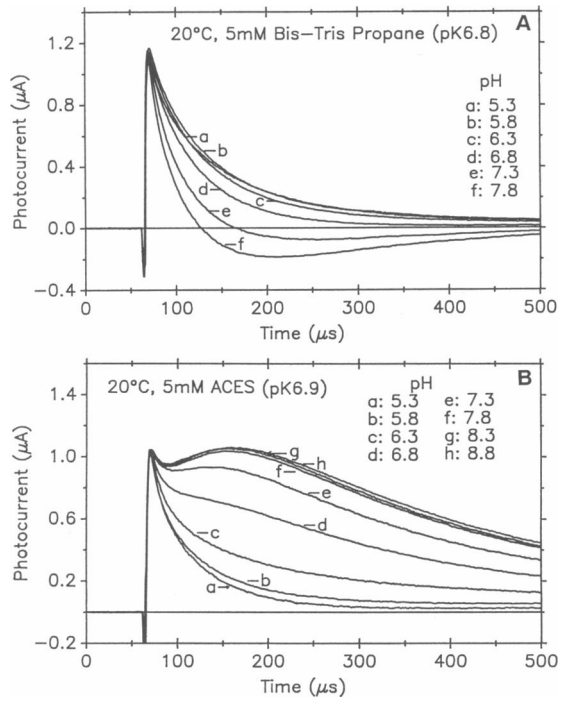

FIGURE 2 pH titration of B2 component of the photocurrent in the presence of (*A*) 5 mM bis-Tris propane pH buffer, and (*B*) 5 mM ACES pH buffer. The conductivities of the buffer were adjusted to be equivalent to that of 8 mM KCl for *A* and 4 mM KCl for *B* by adding small amounts of KCl.

[1]Results in the gel system are almost the same as for purple membrane in suspension (data not shown). In suspension the amplitude of M decreases slightly with increasing KCl concentration. The kinetics of M rise and decay in gel and suspension are almost identical.

With this buffer, a slow negative component is superimposed onto the B2 component causing the apparent shape of the signal to eventually become negative. This figure represents a typical pH titration of B2 with a pH buffer which carries two positive charges in its protonated form. Fig. 2 *B* shows a pH titration of B2 with 5 mM ACES buffer (pK = 6.9). With this buffer, a slow component, a mirror image of the new negative component seen with the bis-Tris propane buffer, is superimposed onto B2. Fig. 2 *B* is typical for a zwitterionic pH buffer that carries only one pair of charges in its protonated form. Other buffers which carry one or two negative charge(s) in their unprotonated form and have a pK >5.5, e.g., phosphate, barbital, and bicarbonate, also cause B2 to have a similar shape. The detailed shape of this new component varies slightly from buffer to buffer. The rate of the rising phase of the "buffer-induced" photocurrent component is proportional to the buffer concentration (data not shown). As can be seen from Fig. 2, the buffer is effective only in the pH range higher than its pK, i.e., when the buffer is able to readily accept a proton. The effect increases with an increase in the unprotonated form of the buffer and becomes saturated when the buffer is 100% in its unprotonated form. Thus this new component must be due to the availability of a proton to protonate the buffer. The time scale of the new current components strongly suggests that B2 is the source of a proton current that protonates the buffer molecules and causes the new buffer-induced photocurrent.

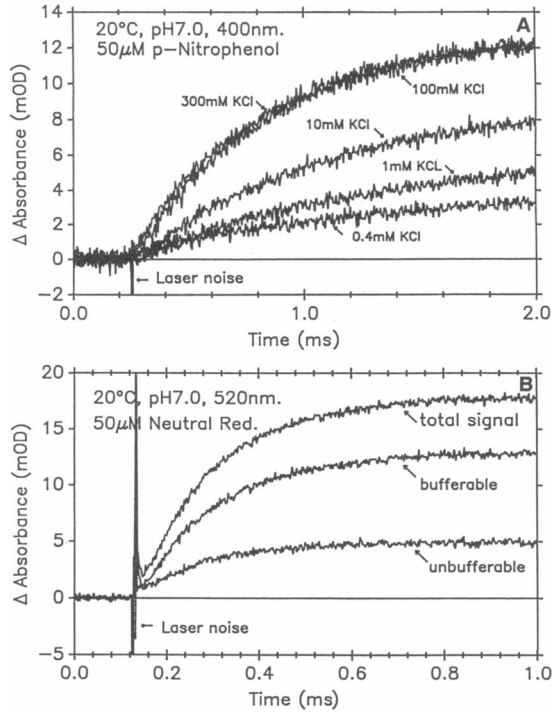

FIGURE 3 Light-induced pH changes of purple membrane sheets in solution (*A*) at different concentrations of KCl by using *p*-nitrophenol as the pH indicator, and (*B*) in 100 mM KCl using neutral red as the pH indicator. PM concentration is 5.5 μM.

Response of pH-sensitive dyes

Fig. 3 *A* shows the light-induced proton release from PM sheets in solution at different concentrations of KCl using the pH indicator *p*-nitrophenol. We can see that upon reducing the salt concentration, the apparent number of protons released is reduced. The salt dependence of proton release we observed is consistent with previous measurements of Ort and Parson (1979) and Govindjee et al. (1980) but differs from the result of Grzesiek and Dencher (1986). As with B2, when the salt is lowered, the apparent size of the *p*-nitrophenol signal is reduced. However, the reduction of proton release detected by the dye is less than the reduction of B2. In going from 100 mM KCl to 0.4 mM, the area of B2 is reduced by 90% while the *p*-nitrophenol signal is only reduced by 74%. This is discussed below.

In some aspects, *p*-nitrophenol is a very good pH indicator for these experiments. Its pK is almost independent of the salt concentration (Kolthoff, 1928) and it does not bind to PM (our unpublished results). We found no spectral or pK changes in the presence of PM over the salt concentrations used. When we added a fixed number of protons to the purple membrane/dye mixture, the spectral change of the dye was always the same for all KCl concentrations. However, one drawback of *p*-nitrophenol is that its response time is somewhat slow. As we can see from Fig. 3 *A*, the rise time of the dye signal is about six times slower than the decay of B2 or L-M transition. Drachev et al. (1984) have shown that the response of the dye becomes faster in the presence of a pH buffer, and when the buffer is increased to 4 mM and the temperature reduced to 5°C, the response is about the same as the L-M transition. However, the presence of buffer makes the measurements very difficult. The reason why the dye response is usually slow and why a buffer increases the response is not clear; it may be related to the dye being negatively charged and thus excluded from the region near the surface of the negatively charged PM. According to this reasoning, the rising phase of the dye signal should become slower at lower salt concentrations where a more negative surface potential will exclude even more the negatively charged *p*-nitrophenol. As predicted, the lifetime of the rising phase of the *p*-nitrophenol signal is slowed from 550 μs to 1.2 ms when the concentration of KCl is changed from 100 to 0.4 mM (Fig. 3 *A*).

Another test of the notion that physical separation caused by membrane/dye charge repulsion strongly

influences pH kinetics is shown by the response of another pH indicator, neutral red (Fig. 3 *B*). Neutral red is uncharged when it is unprotonated; it can get very close and even bind to PM. In this case, the rise time of the pH change is much closer to that of the L-M transition.[2] The kinetics of the bufferable portion of the neutral red signal (τ_{rise} = 170 ± 8 μs) indicate that proton release is much faster than the *p*-nitrophenol response (τ_{rise} = 550 ± 20 μs) and is closer to that of the B2 signal (τ = 85 ± 5 μs). Thus as the dye molecules are allowed to approach the purple membrane surface, the dye response times become much shorter and approach the B2 kinetics. These pH dye and pH buffer experiments indicate that at least part of B2 is due to proton movement.

Effect of salt

Indirect evidence for a dominant contribution of proton movement to intrinsic B2 comes from a consideration of the effect of salt on B2. As discussed earlier in this section, at moderate or high salt the waveform of B2 is independent of the type or concentration of salt; however, at lower salt concentrations B2 is dramatically affected while the L-to-M transition of the photocycle is not. This salt dependence of B2 suggests two things. First that the processes giving rise to both intrinsic and low salt B2 are predominantly due to charge movements from PM to the external solution, because internal proton or dipole movements should not be accessible to influence by the salt containing phase.[3] Moreover, when we change the pH, it is likely that charged groups on the protein, which could make up a moving dipole contribution to the photocurrent, would change their protonation state and thus their dipole moment, while in the previous paper we have shown that in high salt, B2 is essentially independent of pH from 4 to 9. These results indicate that the major contributors to B2 must be proton and/or metallic cation movements at the membrane interface.

To see if any metallic cation made a contribution to intrinsic B2, we measured B2 from a PM gel which was first deionized and then reconstituted with Na^+, K^+, Ca^{++}, Mg^{++}, or La^{+++}. Under these conditions, the gel system should contain only one type of metallic cation (native purple membrane has ca. one Ca^{++} and four Mg^{++} [Chang et al., 1985]). If the cation that is hypothesized to be involved in producing B2 is removed from native PM, B2 should have an altered waveform. For example, if Ca^{++} is the cation that produces or contributes to B2, then the B2 of PM reconstituted with K^+ and measured in a KCl solution should have a very different waveform. We found that B2 from native purple membrane is essentially the same as that from purple membrane reconstituted with K^+, Ca^{++}, Mg^{++}, or La^{+++} when measured at high concentrations of KCl, $CaCl_2$, $MgCl_2$, or $LaCl_3$, respectively. Thus, we conclude that these bound cations make no contribution to the intrinsic B2 normally observed. The salt effect (increase of B2's lifetime as the concentration of salt is lowered) is the same for PM reconstituted with Ca^{++} or Mg^{++} as for native membrane. For PM reconstituted with K^+, a small difference in B2 was observed when measured in 5 mM KCl compared with native PM (Fig. 4). These results indicate that weakly bound monovalent cations have some influence on B2 but only when measured in low salt (discussed below).

In summary, the pH buffer and pH indicator dye experiments, together with the effect of salt on the B2 photocurrent suggest that B2, the only photocurrent component in the time range of proton release, consists

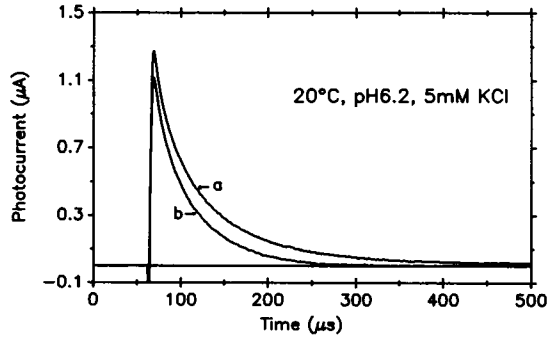

FIGURE 4 B2 component of the photocurrent measured after reconstituting purple membrane with (a) K^+ and (b) Ca^{++} in the cation binding sites (see text).

[2]About 26 ± 2% of the dye signal cannot be buffered with 12 mM bis-Tris propane. We have used both positively and negatively charged buffers such as phosphate, ACES, imidazole and bis-Tris propane from 5 to 20 mM, and the unbufferable portion of the signal is always about the same. This may be because some of the dye is bound to the PM and the buffer cannot easily interact with it. By centrifugation, we found 20 ± 0.5% of dye pellets with PM and thus is probably bound under our conditions. The reason for the 6% difference between the unbufferable signal and the bound dye is not clear; perhaps during the centrifugation of the PM, some loosely bound dye was released to the supernatant. On the other hand, because neutral red changes its spectrum and pK upon binding to the PM due to the metachromatic effect (Dell'Antone et al., 1972), the reasons may be much more complicated. However, no matter what the unbufferable signal may be due to, our interest is in the bufferable portion of the signal which represents the proton concentration change in the solution.

[3]There is one alternative explanation by which salt could influence internal charge motion and that is by altering asymmetrical surface potentials which would lead to a change in the transmembrane potential. We believe this is unlikely because the decay of M is very sensitive to the transmembrane potential (Westerhoff and Dancsházy, 1984) while we have found that M decay changes only slightly (data not shown) over the salt and pH range studied.

almost entirely of a proton current at moderate concentrations of salt.

Effect of modifying the surface charge of purple membrane on B2

The effect of salt on B2 could be due to either direct action or to its modulation of the surface potential of purple membrane which in turn would directly or indirectly affect B2. To see if the salt effect is due to its effect on the surface potential we have compared the B2 photocurrent component of native PM to that of PM in which the surface potential has been modified.

Lipid-depleted purple membrane

Because Szundi and Stoeckenius (1987) have reported that the surface potential of PM which has been delipidated with CHAPS is reduced, we compared the photocurrent of LD-PM with native PM gels. The delipidation procedure removed most of negatively charged lipids from purple membrane, greatly reducing the surface potential. The LD-PM gel is prepared *after* the PM has been oriented, by incubating the gel with CHAPS, thus insuring the same degree of orientation as the control sample. To make sure the same degree of delipidation has been achieved as the original method described by Szundi and Stoeckenius (1987), several characteristic properties of LD-PM were studied and found to be identical. As for LD-PM made by the original method, our LD-PM has its absorption maximum at 561 nm; light and dark adaptation did not change this spectrum; incubating the LD-PM gel with DOWEX 50W does not change it to blue color; it does not change to blue until the pH is below 1.5; the amplitude of the M photo intermediate was about half that of native PM; and the lifetime of M decay is significantly longer than for native PM (Jang et al., 1988). In agreement with this report, we found that the amplitude of M of LD-PM is 53% that of PM. All these properties indicate a similar degree of delipidation of PM in the gel was achieved as for the original procedure.

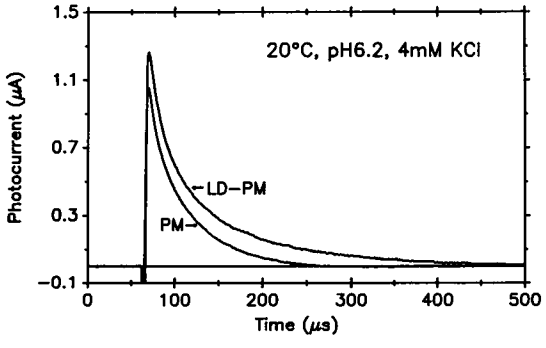

FIGURE 5 Comparison of the B2 component of the photocurrent from native purple membrane (PM) with that of lipid-depleted purple membrane (LD-PM) in 4 mM KCl.

The rising phase of the M intermediate of LD-PM has very similar kinetics as in native PM. Because the amplitude correlation of B2 and the L-M transition has been established (see previous paper), we multiplied the B2 of LD-PM by 1.89 (1/0.53) for comparison with that of native PM both measured in 4 mM KCl (Fig. 5). After this correction, we can see that reducing the surface potential by removing most of the negatively charged lipids does cause B2 to become a little slower. However, the effect is even smaller than the change of B2 of native PM upon going from 4 to 10 mM KCl (Fig. 1 *A*).

Chemical modification of purple membrane with glycine methyl ester and with arginine methyl ester

To further investigate the B2 photocurrent of PM that has an altered surface potential, we modified the carboxyl groups on the surface of the PM with glycine methyl ester (GME) and arginine methyl ester (AME). For GME-PM, the negatively charged carboxyl group becomes neutral upon modification, while for the AME-PM, the negative charge on the carboxyl group is converted to a positive charge by the arginine methyl ester. In the same salt solution, the surface potential will be more negative in the order of AME-PM, GME-PM, and PM. Unlike PM, the color of AME-PM does not change to blue when incubated with Dowex 50W. Also, upon lowering the pH, it does not start to change to blue until the pH is below 2.0. These characteristics of AME-PM are very similar to those of LD-PM reported by Szundi and Stoeckenius (1987). This not only confirms that the carboxyl groups on the surface of PM have been successfully modified with arginine methyl ester but also is consistent with the conclusion that both LD-PM and AME-PM do have reduced surface potentials.

Fig. 6 *A* shows the B2 of PM, AME-PM, and GME-PM in 1 mM KCl. For ease in comparison, the B2 traces of GME-PM and AME-PM in Fig. 6 *A* have been normalized based on the amplitude of their M intermediates. The amplitude of M in the modified gels is 5% smaller than native PM. The B2 photocurrents of the gels with a less negative surface potential are slightly slower. However, these changes are small, smaller than the change in B2 of native PM when the salt concentration is changed from 1 to 5 mM KCl (Fig. 1 *A*). The kinetics of the L-M transition are almost identical for GME-PM, AME-PM, and PM (Fig. 6 *B*). These results are consistent with the absorbance and photovoltage measurements of Ovchinnikov et al. (1982) for AME-PM in 100 mM NaCl.

In summary, both LD-PM and carboxyl-modified PM

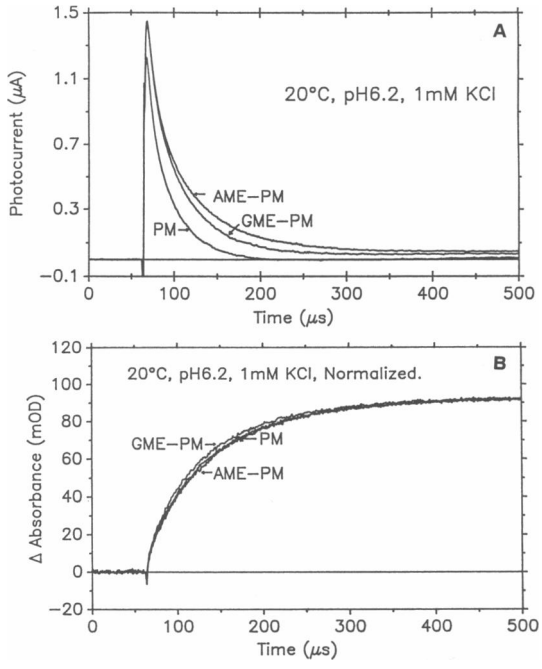

FIGURE 6 (*A*) Effect of carboxyl group modification on the B2 component of the photocurrent. (PM) Native purple membrane; (GME-PM) purple membrane of which the carboxyl groups on the surface of membrane were modified with glycine methyl ester; (AME-PM) purple membrane of which the carboxyl groups on the surface of membrane were modified with arginine methyl ester. (*B*) L-M transitions of three different purple membrane gels used in *A*. The traces are normalized at the maximum.

experiments suggest that changing the surface potential does not significantly change the waveform of B2. Thus, the effect of salt concentration on B2 is probably not through the salt modulating the surface potential.

DISCUSSION

Physical origins of B2

Intrinsic B2 as a proton current

B2 must be due to some type of charge movement. The origin of the charge movement is somewhat controversial. Whereas some authors have stated that B2 is due entirely to proton release to the external medium, others have attributed it to proton movement within the protein, dipole orientation changes of groups within bR, or movements of ions other than protons. The observation that B2 is quite sensitive to the concentration of salt surrounding the membrane, so that in very low salt the area of B2 is <6% of its area in moderate salt (Fig. 1 *B*), suggests that B2 is due almost entirely to charge movements from PM to the bathing solution rather than internal charge movements, such as dipole orientation changes or internal proton movement. That B2 of PM reconstituted with different cations is invariant with salt type at moderate salt concentrations also suggests that under these conditions cations other than protons do not make a contribution to B2. Finally, besides these experiments excluding metallic cation release and internal charge movement contributions to B2, three experiments suggest directly that protons are released into the external medium in a time scale comparable with the time course of the decay of B2. First, in the previous paper we showed that the B2 is slowed by about five times when the H_2O is replaced by D_2O, suggesting a proton is involved in a primary proton dissociation process. Second, we found that when buffer molecules are poised so that they can accept protons, they dramatically alter the waveform of B2 (Fig. 2), strongly suggesting that protons are appearing in solution in the time range of B2. Third, we found that the time course of B2 is approximated by the response of pH-sensitive dyes, and that the response gets closer to the time course of B2 (Fig. 3 *B*) as the indicator dye gets closer to the surface of PM. Thus the most straightforward interpretation of our experiments is that under moderate salt conditions, B2 is composed almost entirely of a current arising from the release of protons into solution. However, there are several unexplained features in the data that suggest that under certain conditions, especially with low salt, B2 may have a more complicated origin.

Effect of salt on B2

We were quite interested in how salt might affect B2. As presented in the results section, the most obvious possibility was that salt was modulating the surface potential which somehow affected proton release. Not only would decreasing the salt increase the surface potential, but the general order in which different salts were effective, trivalents > divalents > monovalents, was consistent with their effectiveness in influencing surface potential. However, the large reduction of the surface potential in going from native PM to the lipid depleted PM (LD-PM) or carboxyl modified PM (GME-PM and AME-PM) preparations cause only modest changes in the waveform of B2. Moreover, the most obvious ways we could propose for how surface potential might affect a proton release process seemed to be inconsistent with the data in Fig. 1. If the surface potential somehow prevented the protons from leaving the surface, the average distance that a proton moved would be shorter during the L-M transition, then we would expect a change only in the amplitude of B2, not its kinetics. However, the opposite was observed. Or if the surface potential affected the apparent pK of the proton releasing group, as suggested by Hess and Kushmitz (1978), then one would again expect only the amplitude, not the kinetics of B2 to change. Finally, it may be that the salt effect cannot be explained by the classical surface

potential theory because the assumption of this theory of uniform surface charge density is not valid for proton movement from the purple membrane. The specific electrostatic forces at small distances between a few charges on the purple membrane, the proton release site, and cations may have to be calculated specifically. Thus, for these reasons, we believe it seems unlikely that the primary effect of salt is through its modulation of the surface potential and we must look elsewhere for its site of influence on B2.

Model for the ionic currents underlying the B2 photocurrent

From the above discussion we can see that all of the alternative hypotheses are troubled by two major points. (*a*) Reducing the salt concentration changes B2's rate of decay without changing its amplitude. As we have mentioned, the amplitude of B2 is proportional to the number of transitions per unit time. If the distance the charge moved during the transition is the same, the amplitude of B2 should increase when the rate becomes faster. (*b*) Reducing the salt concentration increases the rate of decay of B2 without changing that of the L-M transition. In part I, we have shown that the L-M transition is closely correlated with B2 when both are measured at moderate or high salt. Based on these two points, we propose that the part of the waveform of B2 due to proton release, the intrinsic B2, is not changed by reducing the salt because the L-M transition is not changed; the change of B2's waveform upon salt reduction is due to an additional "countercurrent" component of opposite sign to the proton release B2 component and summed to it; this countercurrent component is a consequence of the proton release and has a close relationship with the salt concentration (Fig. 7). Introducing the countercurrent component can explain most of the above results.

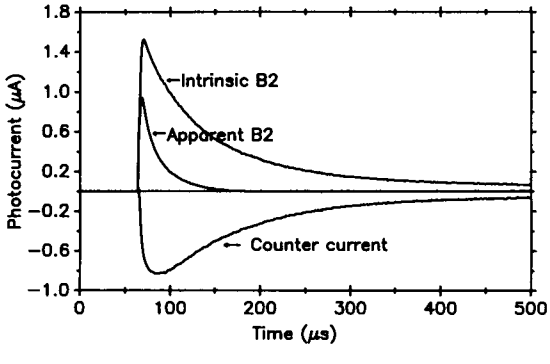

FIGURE 7 The relationship between intrinsic B2 due to the protons leaving the membrane, the "countercurrent" due to cations moving in the opposite direction due to the negative charge created on the membrane, and the apparent B2, the sum of the first two currents.

During the L-M transition, a proton is released into the solution. The release of each proton will leave a newly created negative charge on the membrane. These negative charges will induce a transient increase in the surface potential, causing the free cations in the solution to move towards the membrane (the mobility of the proton is five to seven times greater than a typical cation). When the salt concentration is high, the cations near the surface of membrane only need move a very short distance to compensate for this new negative charge, while when the salt concentration is low, the cations must move a longer distance (see Fig. 8). Because this cation movement is in the opposite direction to proton release, it will produce a current of the opposite sign. The longer the distance that the cations move, the larger this countercurrent will be. The waveform of this countercurrent will be related to the rate of producing protons. In this way, the countercurrent will have a rising phase that is proportional to the salt concentration (when the salt concentration is low) and a decaying phase that is the same as that of intrinsic B2

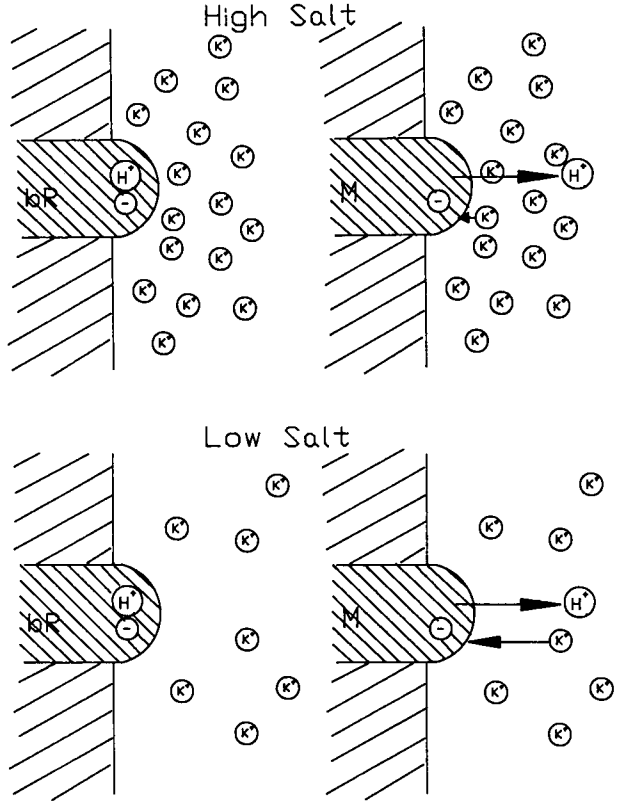

FIGURE 8 Schematic diagram of the "countercurrent" model to explain the waveform of the B2 photocurrent, especially as a function of salt. The backward movement of cations, e.g., K^+ is the source of countercurrent. In low salt, on the average, cations move a longer distance than in high salt. Therefore, the amplitude of counter current is larger in low salt than high salt.

(measured under high salt). Combining the intrinsic B2 current and the countercurrent will result in the apparent B2 as observed in low salt. It should be mentioned that the countercurrent does not have to be composed only of metallic cations such as K^+ or Ca^{++}. Protons near the surface of PM will also contribute depending on their concentration. The different efficiencies of monovalent, divalent, and trivalent ions in modulating the B2 waveform (Fig. 1) may arise because their initial distribution near the membrane will be set by the surface potential (Barber, 1980). This difference in preflash ion distribution may also explain the small differences in the waveform of B2 in the presence of cations of different valencies.

This model can also explain the effects of different kinds of pH buffers on B2 (Liu, S. Y., and T. G. Ebrey, manuscript in preparation). The difference in waveforms between native PM and reconstituted PM with K^+ in the binding site at low salt (Fig. 4) is suggested to be due to a difference in the strength of the binding site. For native PM, the divalent cations in the binding sites are tightly bound. During proton release and cation countermovement to the membrane, they are not involved. In contrast, when the divalent cations are replaced with monovalent cations, these monovalent cations will also be involved in the backward movement because they are more weakly bound than the divalent cations (Chang et al., 1985). Since the monovalent cations in the binding sites are closer to the membrane than the cations in the double layer solution, they will travel a smaller distance; therefore, the amplitude of the countercurrent will become smaller, resulting in a larger B2 (Fig. 4).

With the introduction of the cation countercurrent in response to proton release, it is not surprising that the reduction of the area of "apparent" B2 is inconsistent with the reduction of the *p*-nitrophenol proton signal when the KCl concentration is reduced (Fig. 3 *A*). Because the amplitudes of the L-M transition and B2 at time zero are almost not changed, we propose the number of protons released is same in 100 mM as in 0.4 mM KCl. The reduction of the *p*-nitrophenol signal by 74% when the concentration of KCl is lowered from 100 mM to 0.4 mM could be due to three reasons. (*a*) Some of the protons may also be part of the countercurrent, reducing the number of protons reaching the dye. (*b*) Because unprotonated *p*-nitrophenol is negatively charged, reducing the salt concentration will cause it to stay even farther away from the negatively membrane. (*c*) When the KCl is reduced to 0.4 mM, the response time of the *p*-nitrophenol is slowed to 1.2 ms, while the proton uptake rate is ~5 ms. Therefore, the apparent amplitude of *p*-nitrophenol signal will be reduced because the rates of formation and decay are too close to each other.

It should be noted that such dramatic effect of salt on the B2 component have not been previously reported. Dér et al. (1985) have measured B2 in both salt-free and 100 mM KCl solutions. In contrast to our results, the lifetimes of B2 reported are about the same. We don't understand the exact reason for this apparent discrepancy, but we think it may be due to one of the following possibilities. (*a*) Because they used a high-input impedance preamplifier, it will be very hard for them to determine if the amplitude of signal changed with the salt. The amplitude of the signal will be very sensitive to the sample resistance and it will increase with the increasing of sample resistance (see Liu and Ebrey, 1988). However, this should not affect the determination of lifetime in microsecond range if the amplifier is fast enough. (*b*) The higher concentration of purple membrane used by Dér et al. may make the attainment of the "salt-free" condition difficult. (*c*) The purple membrane they used may contain some unknown trivalent cations that inhibits the salt effect as La^{+++} does. We do find that a gel made with purple membrane from a different lab showed a very small salt effect. However, after deionization of the gel and reconstitution with Ca^{++}, it showed the normal salt effect.

Effect of pH buffers

As noted above, some insight into B2 comes from the effect of pH buffers on it. A similar effect to that seen in Fig. 2 *A* has been reported by Tóth-Boconádi et al. (1986) and Dér et al. (1988) for another diamine buffer. Rather than attributing the effect of their buffer on the kinetics of B2, they proposed that the diamine binds to bR and reverses the proton pump. However, an alternative explanation to this buffer effect was given by Marinetti (1987). He plausibly explained the change in the sign of B2 based on a "counterion collapse" theory. This theory gives a simple explanation for the Ca^{++} dependence of the diamine effect and can also explain our pH titration traces with such buffers as bis-Tris propane, a diamine (Fig. 2 *A*). Marinetti assumes that B2 is a proton release signal. We found piperazine, a diamine buffer that has a very different structure and polarity than the diamine tested by Tóth-Boconádi et al. and from bis-Tris propane has the same effect on B2 (data not shown). We also think that a reversal of proton pumping in the presence of diamine is doubtful because it seems unlikely that all the diamines, which vary greatly in distribution of charges and polarity will all bind to the same "special site." To explain in detail the effects of many of different type of buffers on B2, a modified "counterion collapse" theory is being developed (Liu, S. Y., and T. G. Ebrey, manuscript in preparation). The purpose here in showing the effect of pH (proton) buffers is because they provide strong evidence that B2 is a proton release signal.

Correlations between photocycle, proton release, and photocurrent

There have been several studies of the possible correlations among the rise of the M intermediate, the appearance of protons in solution, and the microsecond (B2) phase of the photoelectric effect. These different studies have, in our opinion, led to a fairly good consistency between these measures of bR's photochemical processes. However, discrepancies have been continually referred to (e.g., Rayfield, 1985; Trissl, 1985; Okajima and Hong, 1986; Holz et al., 1988). Previously we noted that some of the disagreements probably are due to inaccuracy in recording the photoelectric signals. Other reasons for these discrepancies are due to measurements in low-salt solutions or comparing bR in different environments (i.e., as sheets, incorporated into proteioliposomes or bilayers, etc.). In the preceding paper (part I) it was shown that the L-to-M transition of the photocycle correlated extremely well with the B2 component of the photocurrent under the conditions used, moderate concentrations of salt. In this paper we have shown that, under these conditions, the B2 component of the photocurrent is essentially a pure proton current, and its kinetics approach the kinetics of proton release as conditions are changed to reduce the distance between the membrane and the detecting dye. Thus we suggest that all three processes, the L-to-M transition, the B2 photocurrent, and proton release have identical kinetics at 20°C, in the presence of at least moderate salt concentrations. This conclusion is consistent with that of Drachev and co-workers (1984), although in their work the photoelectric effect did not quite follow the L-to-M transition, probably because the measurements were done on bR in different environments.

Characteristics of proton movement as measured by B2

Assuming that intrinsic B2, from pH 2 to 9, is a pure proton current, some characteristics of the proton pump which are difficult to elucidate by other methods can be studied by photocurrent measurements. For example, because B2 is a direct measure of the proton movement, it does not have the problems of a pH indicator's slow response time, salt-dependent pK, or restricted pH range. Thus the 85-μs lifetime component of B2 seen in moderate salt from pH 2 to 9 (Figs. 2 and 7 of the previous paper [Liu, 1990]) indicates that protons are released with these kinetics at 20°C and the rate is independent of pH. We see no evidence for the suggestion of Ort and Parson (1979) that the kinetics of proton release are either pH-dependent in the range of 4 to 8.5, or that at some pHs proton release is faster than the L-to-M transition. Following Kalisky et al. (1981), the 85-μs rate for proton release would require that the pK of the releasing group must be <6. Indeed, for the amplitude of B2 and thus the number of protons released at moderate salt concentrations to be reduced to half at pH 2.8 (Fig. 4 *B* of the previous paper), the intrinsic pK of the proton releasing group of purple membrane must be $\leq$2.8.

B2 as a direct measure of the proton current allows us to study the effect of La^{+++} on proton release. Fig. 1 *C* shows that proton release is normal in 5 mM $LaCl_3$, pH 5.0. We have also measured B2 at higher $LaCl_3$ concentrations and higher pH (15 mM and pH 7.0) and found no effect on B2. Therefore, the notion that La^{+++} inhibits the proton pump (Seigneuret and Rigaud, 1985) is misleading. More accurately, La^{+++} does not affect the proton release process of the proton pumping. But when the La^{+++} concentration is high and the proton concentration is low (>10 mM $LaCl_3$ and >pH 6.5), La^{+++} will inhibit the proton uptake step in proton pumping, causing a slowing down of the M decay and proton uptake. In steady-state illumination, the net number of released protons in the bulk solution will be low. This is consistent with the early reports by Drachev et al. (1981) and Chang et al. (1985).

When the pH is below 5.0, the pumping ability of PM decreases with the pH, as the blue membrane is formed. However, Fig. 4 *B* of the first paper shows there is no change in the direction or rate (85 μs) of proton pumping upon lowering the pH. Proton release still occurs before uptake. This is consistent with the results of Mitchell and Rayfield (1986) but not of Dencher and Wilms (1975) using pH indicators. The difficulty in studying proton release with a pH indicator at low pH is that the pH change induced by the proton release becomes smaller with decreasing of pH.

At high pH (>9) B2 changes from an 85-μs species to one with faster components (0.3 and 6 μs) which are, in contrast to the 85-μs species, insensitive to the salt concentration (see previous paper). This suggests that this new, faster decaying B2 does not represent proton movement from the surface of purple membrane but rather internal charge movement. The reduction in the total area of these two fast components further confirms this point. Therefore, we propose this new photocycle present at high pH (Hanamoto et al., 1984; Dancsházy et al., 1988) does not pump protons. This pH profile of proton pumping is qualitatively consistent with the results of Lozier et al. (1978), Renard and Delmelle (1980), Li et al. (1986), and quantitatively consistent with Kouyama et al. (1987) in whole cells and cell envelope vesicles. Previously we had noted (Li et al., 1984) that the apparent salt dependence of proton release (Ort and Parson, 1978; Govindjee et al., 1980) but salt independence of M formation

suggests that these processes are not necessarily coupled. However, the interpretation of the salt dependent dye signals presented above suggests that salt does not affect proton release, only its detection. Nevertheless, at high pH it does appear that the fast forming (τ = 6 and 0.3 μs) M is not coupled to proton release.

As reflected in the B2 component, proton release is not significantly affected by replacing the divalent cations in bR's binding site with other cations. Chemical modification of the carboxyl groups on the surface of purple membrane also does not greatly affect proton release. Delipidation of purple membrane decreases its pumping ability probably because the number of bR's photocycling is decreased; and since delipidated PM's light-induced M decay is significantly slowed down (Jang et al., 1988), the steady-state net proton release will probably be much lower. However, the number of protons released per M formed (Fig. 5) is unchanged.

The advantages of using oriented gels rather than other photocurrent measurement techniques were summarized in Liu and Ebrey (1988). However, an important advantage of the method was not apparent until now: the ease of the control experiment. In each group of data we presented, the gels were sliced from one large piece of an oriented membrane gel. So all the gels have the same PM concentration and degree of orientation. Therefore, when measuring the salt concentration dependence of B2, the aggregation induced by La^{+++} and Ca^{++} is not a problem. When we compare the B2 of native and modified PM, we don't need to worry about the extinction change and the orientation change because the modifications were done after the gels were prepared.

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