Effect of Acid pH on the Absorption Spectra and Photoreactions of Bacteriorhodopsin†

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ABSTRACT: Purple membranes (PM) from *Halobacterium halobium* were incorporated into 7.5% polyacrylamide gels to prevent aggregation which occurs in suspensions at low pH. At pH 7.0, the circular dichroism (CD) spectra and visible absorption spectra of light- and dark-adapted bacteriorhodopsin (bR_{568} and bR_{558}, respectively) and the flash photolysis cycle of bR_{568} in gels were essentially the same as those in PM suspensions. Titration of the gels with hydrochloric acid showed the transition to a form absorbing at 605 nm (bR_{605}^{acid}) with $pK = 2.9$ and to a second form absorbing at 565 nm (bR_{565}^{acid}) with $pK = 0.5$. Isosbestic points were seen for each transition in both light- and dark-adapted gels. In addition, a third isosbestic point was evident between pH 3.5 and 7. Visible CD spectra of bR_{568}, bR_{605}^{acid}, and bR_{565}^{acid} all showed the bilobed pattern typical of bR_{568} in suspensions of PM. Flash kinetic spectrophotometry (with 40-μs time resolution) of bR_{605}^{acid} and bR_{565}^{acid} showed transient absorbance changes with at least one transiently blue-shifted form for each and an early red-shifted intermediate for bR_{565}^{acid}. Chromophore extraction from membrane suspensions yielded *all-trans*-retinal for bR_{565}^{acid} and a mixture of 13-cis and trans isomers for bR_{605}^{acid}.

The general features of the purple membrane in *Halobacterium halobium* are well established. Like visual pigments, its protein, bacteriorhodopsin (bR), contains an ϵ-retinylidene-L-lysine chromophore (Oesterhelt & Stoeckenius, 1971), which is protonated in its initial state (Lewis et al., 1974) and shows a large red shift of its main absorption band due to its interaction with the protein. bR undergoes a cyclic photoreaction after absorption of light (Lozier et al., 1975) and transports protons across the membrane. It thus functions as a light-energy transducer, generating an electrochemical gradient (Oesterhelt & Stoeckenius, 1973; Racker & Stoeckenius, 1974; Danon & Stoeckenius, 1974).

The purple membrane exists in a stable dark-adapted form which is characterized by a visible spectral transition with λ_{max} at 558 nm (bR_{558}) and is thought to contain both 13-*cis*- and *all-trans*-retinal isomers in equimolar ratios (Oesterhelt et al., 1973; Pettei et al., 1977). Illumination generates a metastable light-adapted form with λ_{max} at 568 nm (bR_{568}) (Oesterhelt & Stoeckenius, 1971) containing *all-trans*-retinal as its chromophore (Oesterhelt et al., 1973). While both 13-*cis*- and *all-trans*-retinal-containing bRs undergo photoreaction cycles, only the cycle of the all-trans component is thought to be accompanied by proton translocation (Ohno et al., 1977a; Lozier et al., 1978). The cycle of bR_{568} contains at least four intermediates, which are designated by their visible spectral transitions as K_{590}, L_{550}, M_{412}, and O_{640} (Lozier et al., 1975). Another intermediate (N_{520}) is less well established. The Schiff base is believed to be unprotonated in the M_{412} intermediate (Lewis et al., 1974).

Oesterhelt & Stoeckenius (1971) reported that the purple membrane changes its color to blue at low pH, due to a shift of the visible transition to 605 nm at a pH of 2.0. We have undertaken a systematic study of this phenomenon, because it may help elucidate the interaction of the chromophore with its environment and may also give clues to the mechanism of color changes and to the mechanism of proton pumping in the reaction cycle. The purple membrane at low pH also potentially provides a well-defined equilibrium system for forms of bR which may be closely related to photocycle intermediates and on which new investigative techniques can be tested before they are used on the experimentally less accessible photoreaction cycle intermediates.

Materials and Methods

H. halobium S_9 was grown, and the purple membrane was isolated as described by Oesterhelt & Stoeckenius (1974). Spectra were taken at room temperature on a Cary 14 fitted with a scattered transmission accessory and an EMI 9659 photomultiplier with a 160-s scan time (750–350 nm). The purple membrane concentration was based on $\epsilon_{568} = 58\,000$ (R. A. Bogomolni, personal communication) for light-adapted membrane.

Retinals were extracted from purple membrane and analyzed as described by Pettei et al. (1977) on a Waters ALC-GPC 204 high-performance liquid chromatograph fitted with a dual wavelength detector (254 and 365 nm). All solvents were spectroscopic quality or redistilled prior to use. Buffers used in PM suspensions for the extraction were 50 mM Hepes–HCl or 50 mM sodium phosphate–HCl (pH 7.0), 0.1 M potassium phthalate–HCl ($4.0 \geq pH \geq 2$), 0.1 M KCl–HCl ($2.0 > pH > 0$), and HCl ($pH < 0$). Samples ($A_{568} = 0.64$ for light-adapted membranes) were light-adapted, if indicated, by illumination in a 1-cm cuvette (3 mL) with a slide projector through a Schott 0G2 filter for 3 min with a light intensity of 20×10^5 erg cm^{-2} s^{-1}. They were then aspirated through a 20 gauge needle into a hypodermic syringe containing methylene chloride (6 mL), at which time the light was extinguished. Otherwise, all operations were conducted under dim red light.

The methylene chloride–water samples were forced through the 20 gauge needle several times and then were allowed to stand for 20 min at 0 °C. When membrane in 1 M HCl was

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extracted, only a small fraction of the retinal was recovered. After the first extraction, the mixture was sometimes neutralized and then further aspirated through the syringe to try to increase the yield. However, this had little effect. The samples were then centrifuged at 12000*g* for 10 min, the methylene chloride layer containing retinals and other lipids was removed, benzene was added, and the solvents were removed on a rotary evaporator. The residue was transferred to a small vial with methylene chloride, and the solvent was driven off with a stream of dry nitrogen. The lipid fraction was then dissolved in 20 μL of methylene chloride and processed by liquid chromatography on a μPorasil column using 2% ether in hexane at 2.0 mL/min. To calculate yields of retinal, measured volumes of samples were injected, retinals were collected, and UV spectra were taken. The molar extinction coefficients used were 42900 at 380 nm for *all-trans*-retinal and 35000 at 375 nm for the 13-cis isomer in 100% ethanol (Planta et al., 1962).

Purple Membrane Containing Gels. Forms for the gels (60 × 50 × 2 mm) were prepared from clamped microscope slides and were sealed with a 1% solution of warm agar. The acrylamide solution of 25 mL total volume contained 7.5% acrylamide, 0.2% bisacrylamide, 0.03% tetramethylethylenediamine, 0.04 M Tris, and 70 absorbance units of purple membrane, pH 7.4 (1 absorbance unit = 1 mL of suspension having an optical density of 1 with a 1-cm light path). The solution was degassed in vacuo, ammonium persulfate was added (final concentration 0.024%), and the solution was poured into the forms and allowed to polymerize for 1 h. The gels were then cut to size (30 × 10 × 2 mm) and stored in water containing 0.01% sodium azide. Blank gels were prepared the same way, omitting purple membrane. Both the blank and purple membrane gels were stirred in 100 mL of the appropriate buffer solutions at the pH of interest for 30 min in the dark at room temperature before spectra were taken. Gels were light-adapted in the same manner as suspensions.

Circular Dichroism Spectra. CD spectra were obtained on an instrument built at the Chemical Biodynamics Laboratory at the University of California, Berkeley (Dratz et al., 1966). A Dumont 2703 photomultiplier with S-20 response was used as a detector, with a cuvette–detector distance of 3.8 cm. The purple membrane gels were placed at the photomultiplier side in a 1-cm cuvette filled with buffer solution at the desired pH.

Flash Kinetic Spectrophotometry. Rapid light-induced transmission changes were measured with the apparatus described previously (Lozier et al., 1976), except that a second monochromator was placed between the sample and detector, in addition to interference filters, to eliminate stray actinic light. A Molectron UV 1000 DL200 nitrogen-pumped dye laser (pulse energy ~1 mJ, pulse duration ~7 ns) or a Phase-R flashlamp-pumped dye laser (pulse energy ~200 mJ, pulse duration ~250 ns) was used as an actinic source. Experiments were performed at room temperature (21 ± 1 °C). At neutral pH, the monitoring light intensity was sufficient to assure complete light adaptation. The stability of the gels was monitored by recording the absorption spectra both before and after the experiment. In addition, the gels were sometimes reequilibrated to pH 7.0 and the absorption spectra and cycle kinetics were checked.

Results

Purple Membrane Gels. Our early studies of purple membrane suspensions at low pH were hampered by aggregation, which caused excessive light scattering. We therefore incorporated purple membranes into 7.5% polyacrylamide gels cross-linked with 0.2% bisacrylamide. No detectable amounts of membrane leaked out while gels were kept in water for 3 weeks. Visible spectra of purple membrane gels were found to be essentially identical with those of the purple membrane suspensions used in preparing the gels. For example, the extinction coefficients are within 3% of each other. Control experiments showed that the spectral characteristics of the gels were stable after 20 min at a given pH. Any changes associated with chromophore destruction are only observable after 4 to 5 h at pH <2. Furthermore, the polyacrylamide matrix apparently does not influence the photoreaction of bR_{568}.

Table I: Spectral Characteristics of the Long-Wavelength Absorption of Bacteriorhodopsin in a Polyacrylamide Gel[a]

expt no.	pH	solutions	light-adapted[b] λ_{max} (nm)	light-adapted[b] A_{max}	dark-adapted λ_{max} (nm)	dark-adapted A_{max}
1	7.0	0.1 M NaH_2PO_4	568	0.575	558	0.493
2	4.0	0.1 M KHPht[c]	566	0.570	560	0.486
	2.0	0.1 M K_2Pht	605	0.462	603	0.460
	0.0	1 M HCl	565	0.513	564	0.498
3	2.0	0.1 M KHPht + 1 M NaCl	575	0.410		
	0.0	1 M HCl + 1 M NaCl	564	0.510		
	0.0	1 M HCl + 2 M NaCl	563	0.523		

[a] Each experiment was done with a different gel. Vertical comparisons for A_{max} for the different experiments are therefore suspect, although λ_{max} values are accurate. [b] Sample was irradiated for 10 min through an orange filter before the spectrum was taken. [c] Pht = phthalate.

The lack of aggregation in the gels allowed us to obtain spectra of two easily distinguishable species which are stabilized by low pH (Table I). The spectrum of purple membrane begins to show a slight red shift and a decrease in absorbance at pH <5.0. At pH <3, the red shift becomes more pronounced. This bathochromic shift appears to be due to the formation of a new complex, bR_{605}^{acid}, which reaches its maximum concentration at pH ~2.0. At pH <1.5, a blue shift begins which apparently is due to the formation of a second acid species with maximal absorbance at 565 nm (bR_{565}^{acid}). The bR_{565}^{acid} formation is reversible. At room temperature bR_{565}^{acid} reverts to bR_{605}^{acid} and bR_{568} over a period of hours in gels at pH 7. Purple membrane is slowly destroyed by pH values below 1.0, and recovery may not be complete. Table I also shows the effect of pH and added sodium chloride on the spectra. High salt concentrations counteract the formation of bR_{605}^{acid} and must be taken into account, particularly if bR_{605}^{acid} is under investigation.

Spectra were recorded at different pH values between 0 and 7 for a dark-adapted (Figure 1) and a light-adapted (Figure 2) gel. In the dark-adapted gel, apparent isosbestic points are evident for the spectra between pH 7.0 and 3.25 at 598 nm, between pH 3.0 and 2.0 at 578 nm, and between pH 1.5 and 0.9 at 585 nm. The apparent isosbestic points differ slightly for the light-adapted gel, and are at 608, 584, and 584 nm, respectively. The apparent isosbestic points in the light-adapted gel are noteworthy since the rate of dark adaptation increases with decreasing pH (Ohno et al., 1977a). However, the half-time for dark adaptation is 50 min at 25 °C, pH 5.0 (R. Casadio, H. Gutowitz, P. C. Mowery, and W. Stoeckenius, unpublished experiments), and is unimportant here, as witnessed by the presence of the apparent isosbestic points. The isosbestic points are not perfect; this may be attributed in part to the presence of small amounts of additional bR species. Neither bR_{605}^{acid} nor bR_{565}^{acid} shows light-dark adaptation of the

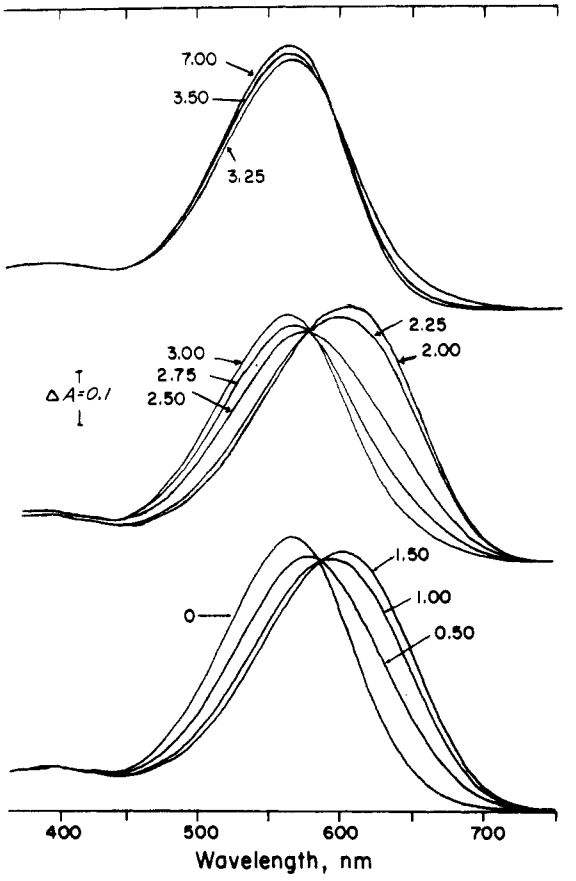

FIGURE 1: Absorption spectra of dark-adapted purple membrane containing polyacrylamide gels at room temperature (24 °C) at various pH values. Sodium phosphate (50 mM) buffer was used for pH <1.

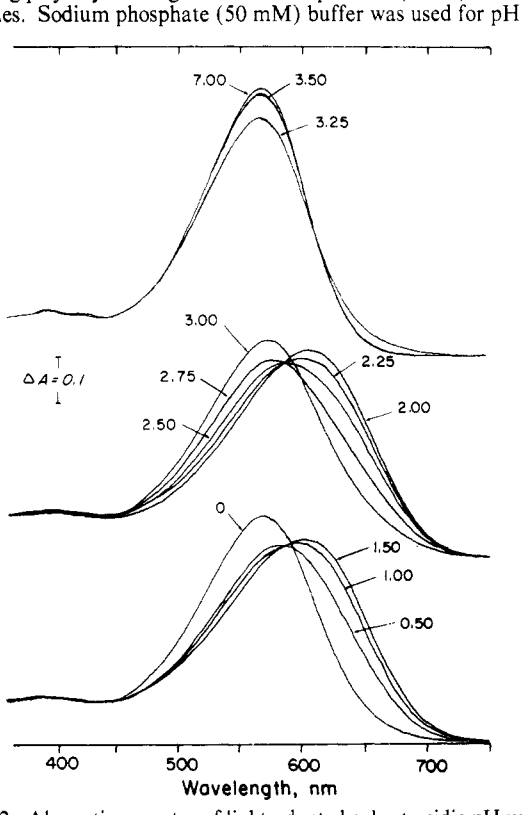

FIGURE 2: Absorption spectra of light-adapted gels at acidic pH values at room temperature (24 °C). The buffers used are listed in Table I.

magnitude found for bR (Table I). Note, though, that data in Figures 1 and 2 cannot be superimposed since they were collected with different gels.

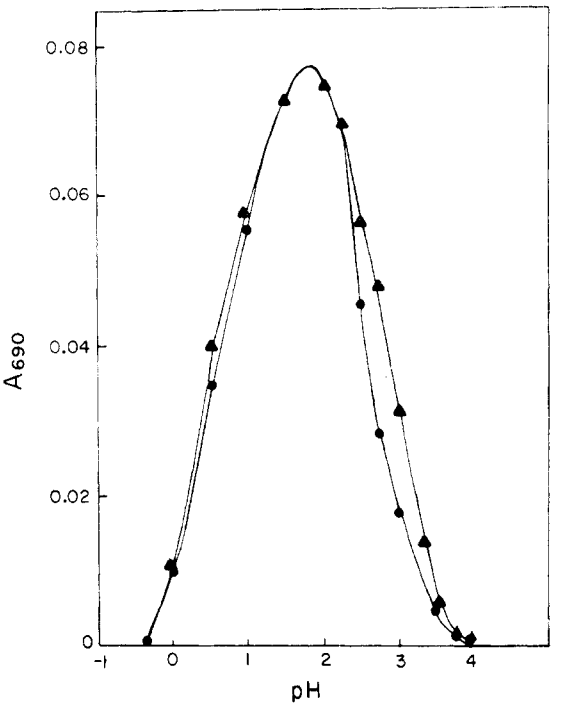

FIGURE 3: Absorbance at 690 nm as a function of pH; from data in Figures 1 and 2.

To determine pK values for the formation of bR_{565}^{acid} and bR_{605}^{acid}, we plotted pH vs. absorbance changes at 690 nm (A_{690}), where the purple species of bR absorb negligibly (Figure 3). bR_{568}, bR_{605}^{acid}, and bR_{565}^{acid} ostensibly coexist in sufficiently high concentrations to obviate the determination of exact pK values in a straightforward manner. Furthermore, we cannot independently measure the concentrations of the purple-colored species, nor can we accurately assess the slopes of the titration curves a priori. However, we can estimate both by plotting the raw data. The equilibrium constant may then be defined as in eq 1. Therefore, assuming no destruction

$$K = [H^+]^n[B]/[H_nB] \quad (1)$$

of chromophoric species and that the concentration of bR_{605}^{acid} is proportional to A_{690} while that of bR is proportional to $A_{690(max)} - A_{690}$

$$pK(bR_{605}^{acid}) = n\text{pH} + \log A_{690}/(A_{690(max)} - A_{690}) \quad (2)$$

where bR_{558} = B and bR_{605}^{acid} = H_nB, and similarly

$$pK(bR_{565}^{acid}) = n\text{pH} - \log A_{690}/(A_{690(max)} - A_{690}) \quad (3)$$

where bR_{605}^{acid} = B and bR_{565}^{acid} = H_nB. Analysis of the data in Figure 3 gives n = 1.7 mol of H^+/mol of bR_{558} (pK = 2.9 ± 0.2) and n = 1.5 mol of H^+/mol of bR_{605}^{acid} (pK = 0.58 ± 0.2) for dark-adapted membranes. Analyses of this type at 650 and 450 nm give the same values of pK and n, and those for light- and dark-adapted species are the same within experimental error. These results strongly suggest that the color changes reflect the titration of discrete sites in the purple membrane complex; they also show that bR_{605}^{acid} is never present as a pure species. For example, the contamination of bR_{605}^{acid} by bR_{568}, assuming n = 1 and a pK of 2.7 (2.9 minus the standard deviation), is at most 20% at pH 2.0.

Circular Dichroism Spectra. Spectra of gels can only be obtained in the visible and near-UV region because polyacrylamide begins to absorb appreciably below 270 nm. The CD spectrum of bR_{568} in gels (Figure 4) shows a strong negative band centered at 315 nm and an asymmetric positive and negative band with a maximum at 535 nm, a minimum

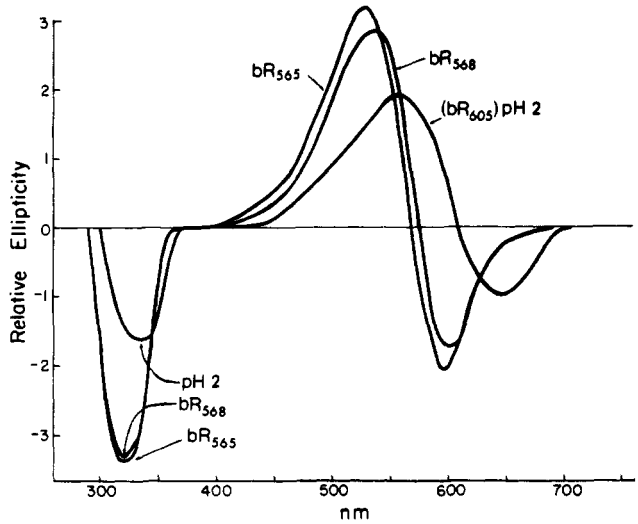

FIGURE 4: CD spectra (single scans) of light-adapted purple membrane gels at pH 7.0 (bR_{568}), pH 2.0 (a mixture containing predominantly bR^{acid}_{605}), and pH −0.3 (bR_{565} at room temperature (25 °C). A spectrum measured at pH 4.0 was indistinguishable from the pH 7 spectrum. For buffers used, see Table I.

Table II: Retinal Isomers Extracted from Bacteriorhodopsin at Various pH Values[a]

pH	light-/dark-adapted[b]	% composition		% extracted[c]
		13-cis	trans	
7.0	LA	7 ± 2	93 ± 2	8
4.0	LA	30 ± 6	70 ± 5	9
2.4	LA	40 ± 3	60 ± 2	2
1.9	LA	37 ± 1	63 ± 1	~1
1.2	LA	28 ± 3	72 ± 4	<1
0.0	LA[d]	5 ± 2	95 ± 2	<1
−0.03	LA[e]	2.8 ± 1	97.2 ± 2	<1
7.0	DA	51 ± 1	49 ± 1	7
4.0	DA	48 ± 4	52 ± 3	5
1.9	DA	43 ± 1	57 ± 1	~1
−0.03	DA[e]	9.5 ± 0.8	90.5 ± 1	<1

[a] Each experiment was performed twice, with a control at pH 7 run simultaneously each time. [b] Dark adaptation (DA) was assured by allowing the solution to stand overnight at room temperature in the dark at pH 7.0. Light adaptation (LA) was accomplished by irradiating with a slide projector through a 570-nm filter for 5 min, followed by immediate extraction. [c] Quantitation was attempted by collecting an aliquot from the liquid chromatographic eluant, taking its UV spectrum, and calculating the total retinal extracted assuming a retinal/protein ratio of 1.0. [d] Disrupted membrane solutions were not neutralized. [e] Disrupted membrane solutions were neutralized with 10 N NaOH.

at 600 nm, and a crossover at 575 nm, which is essentially identical with published CD spectra for purple membrane suspensions (Heyn et al., 1976; Becher & Cassim, 1976). The general features of the low pH spectra are the same as for those near neutral pH, with shifts corresponding to the shifts in the absorption maxima. The spectrum of bR^{acid}_{565} is very similar to that of bR_{568}, with a minimum at 595 nm, a maximum at 530 nm, and a crossover at 570 nm. The pH 2.0 spectrum shows the expected red shift; however, the bands are broader and lower and the crossover point is at 608 nm. Apparently, we are dealing with a mixture of two or more species. Although the 325-nm CD bands are essentially the same for bR_{568} and bR^{acid}_{565}, the pH 2.0 spectrum shows a marked decrease in ellipticity and a red shift.

Chromophore Extraction. The extraction data (Table II) for pH 7.0 are essentially consistent with the data in the literature, except that we usually find 5–10% 13-*cis*-retinal in bR_{568}. The ratio of 13-cis/trans is close to unity in bR_{558}

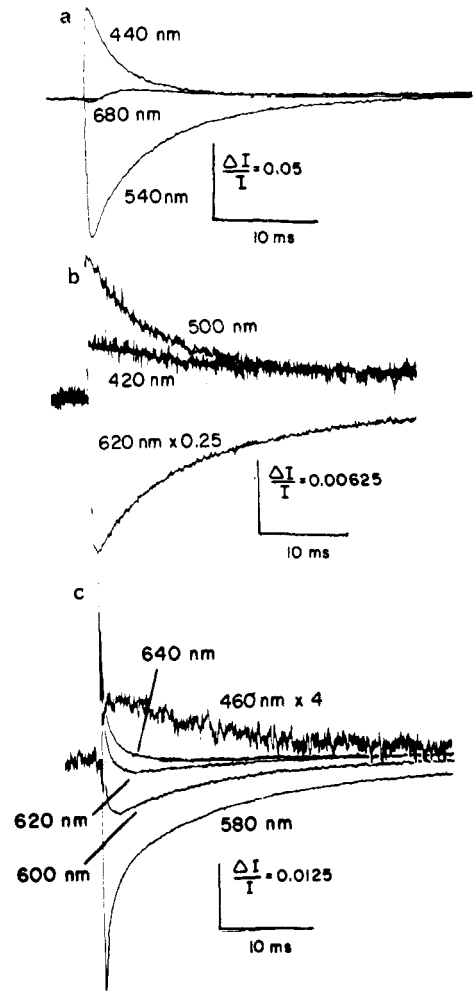

FIGURE 5: Representative flash kinetic data used in (a) Figure 6, (b) Figure 7, and (c) Figure 8.

as found by others (Oesterhelt et al., 1973; Pettei et al., 1977). Extraction of bR^{acid}_{605} yields a mixture of 13-cis and all-trans (40:60) isomers with only a small difference between light- and dark-adapted samples. The 30% 13-*cis*-retinal extracted in the light at pH 4.0 is higher than expected and cannot be explained as an increased rate of dark adaptation or the presence of ~8% bR^{acid}_{605}. The isomer ratio in bR^{acid}_{565} is nearly the same as for bR_{568} and hardly changes with exposure to light.

Photoreaction Kinetics and Spectra. Laser flash induced absorption changes at selected measuring wavelengths for PM gels at pH 7.0, 2.0, and −0.3 are shown in parts a, b, and c of Figure 5, respectively. Difference spectra at selected times are shown in Figures 6–8. At pH 7 (Figure 6) the M and O intermediates (difference spectra maxima at ~420 nm at 0.6 ms and at ~660 nm at 4.6 ms) are found to be similar to those in purple membrane suspensions (Lozier et al., 1975). Note that neither the K_{590} nor L_{550} intermediates are observable on the time scale used in these studies.

At pH 2.0 (Figure 7) when the bR is present predominantly as bR^{acid}_{605}, no red-shifted intermediate is found but at least one, and possibly several, blue-shifted species is observed. A rapid (<80 μs) blue shift is followed by a further blue shift which is complete within ~1 ms; the kinetics do not follow a single exponential and are wavelength dependent. Absorption spectra for these intermediates cannot be calculated because their concentrations are unknown. Interpretation of the spectra is also complicated by the likelihood that a mixture of photoactive species is present in the pH 2.0 sample in the dark.

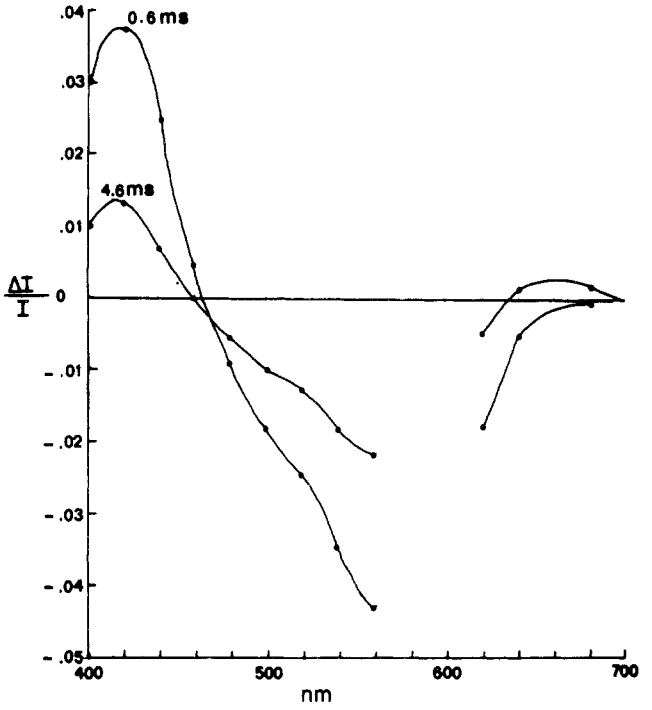

FIGURE 6: Flash-induced difference spectra of purple membrane gels at pH 7.0 in 0.1 M potassium phosphate buffer. Ordinate: difference intensities (in percent transmittance) between times t (indicated) and before the flash, normalized to the preflash intensities. Abscissa: monitoring wavelengths. Actinic wavelength: 588 nm. Phase-R laser, 0.5 pulses/s, 16 flashes of 200 mJ at laser.

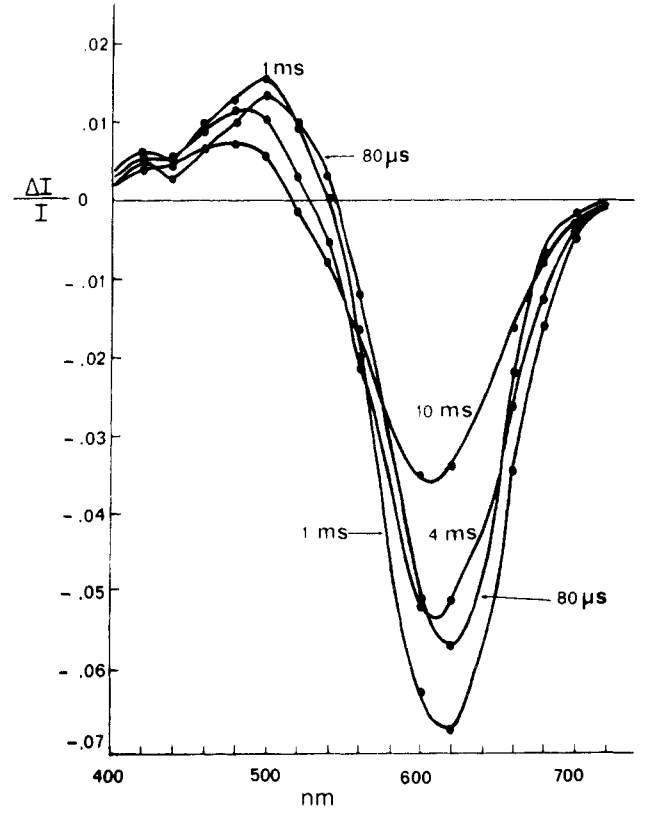

FIGURE 7: Flash-induced difference spectra of purple membrane gels at pH 2.0 in 10 mM potassium phthalate–HCl. Molectron laser, 580 nm, 2 pulses/s, 128 pulses of 8 mJ at laser.

At pH −0.3 (Figure 8) when bR is present predominantly as bR_{565}^{acid}, a photocycle containing at least two intermediates is observed, one of which is red-shifted and has an unresolved rise time. Its decay time ($t_{1/2}$ ~0.5 ms, although a single

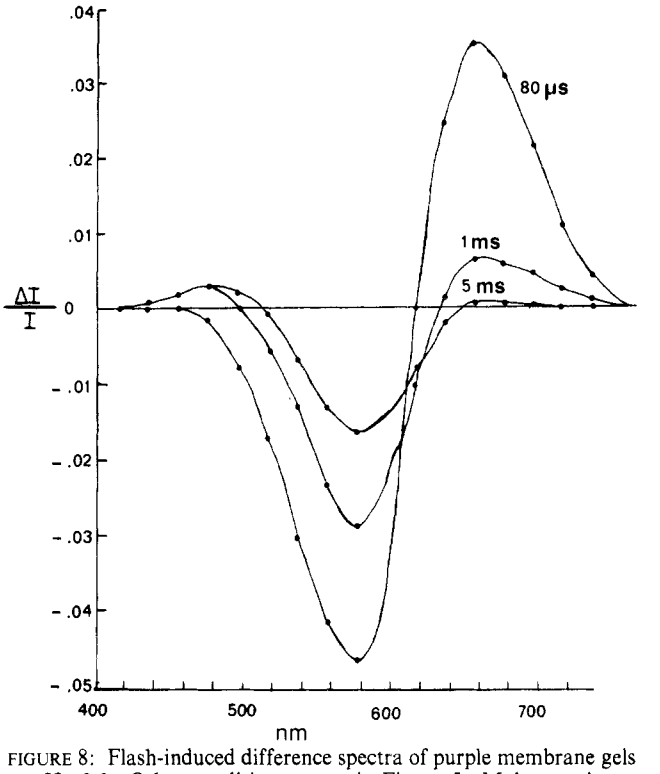

FIGURE 8: Flash-induced difference spectra of purple membrane gels at pH −0.3. Other conditions are as in Figure 5. Molectron laser, 580 nm, 5 pulses/s, 128 flashes of 1.4 mJ at laser.

exponential is not found) roughly matches the rise time of the blue-shifted intermediate, whereas the decay of the latter ($t_{1/2}$ ~21 ms) approximately matches the rise of the bR_{565}^{acid} (slower phase of recovery at 580 nm). Again, the spectra for the red-shifted ("640") and blue-shifted ("580") species cannot be determined.

To understand the flash spectroscopic data, we must assign the phototransients to the different species of bR present. The spectra of bR_{568} and its phototransients are known; that of bR_{565}^{acid} was taken at pH −0.3, ~0.8 pH unit below its pK, so that it was ~86% pure, whereas bR_{605}^{acid} at pH 2.0 comprises only 80% of the species present. We monitored the phototransients of these gels as a function of exciting wavelength to determine whether intermediates might be assigned according to their parent species aborption spectra. The results are summarized in Table III. Note that in the control experiment at pH 7.0 the ratio of maximal transient absorbances at 420 and 500 nm does not vary significantly with actinic wavelength. At pH 2.0, however, this ratio is high only at an actinic wavelength of 550 nm, where bR_{568} and bR_{565} preferentially absorb light. At pH 2.0 the ratio of the maximal amplitudes of the light-induced transients at 620 and 500 nm is essentially invariant with actinic light wavelengths where bR_{605}^{acid} preferentially absorbs. The dramatic effect of actinic light at 550 nm for both the 420/500 nm and 620/500 nm ratios probably represents a preferential depletion of contaminating bR_{565}^{acid} and bR_{568}. Since bR_{565}^{acid} shows no transient absorbance change around 420 nm in its photocycle, we conclude that the 420-nm transient at pH 2.0 is due to residual bR_{568}.

To demonstrate that the two phototransients observed at pH −0.3 are due to bR_{565}^{acid} and not to contaminating bR_{605}^{acid}, we have shown the ratios of the phototransients at 660 and 460 nm (Table III). The high ratio for 630-nm actinic light is in the wrong direction for a contribution from bR_{605}^{acid} and reflects experimental error due to the small intensity of the 460-nm transient.

Table III: Wavelength Dependence of Photointermediates as a Function of Wavelength of Exciting Light and pH

pH	actinic wavelength (nm)	transient absorbance ratios (I_{max}) 420/500 nm	620/500 nm	660/460 nm
7.0	550	−1.7 ± 0.04		
	588[a]	−1.9	1.0	
	630	−1.75 ± 0.04		
2.0	550	21.2 ± 0.1	−50 ± 11	
	580[a]	0.45	−3.5	−3.5
	630	0.28 ± 0.02	−3.4 ± 0.1	
	696	0.0	−3.9 ± 0.1	
−0.3	550			9.4 ± 0.4
	580[a]	−0.34	−1.6	10
	630			19 ± 10

[a] Values from data used in constructing Figures 6, 7, and 8 for pH 7.0, 2.0, and −0.3, respectively. Gels are different from those used for the other actinic wavelengths.

Flash spectroscopy may also help to show whether the blue shift observed in bR_{605}^{acid} when it is exposed to high NaCl concentrations must be attributed to bR_{565}^{acid} or bR_{568} formation (Table I). Flash spectra were taken with the actinic flash at 580 nm and the monitoring beam at 410 nm in 0.01 M HCl and 0.01 M HCl plus 4.0 M NaCl. Visible spectra show that bR_{605}^{acid} blue-shifts to 565 nm in 4 M salt. However, flash spectra show no change in the small 410-nm transient, whereas the 640-nm transient characteristic of bR_{565}^{acid} is present. Hence, the purple complex in 4 M NaCl at pH 2.0 appears to be mostly bR_{565}^{acid}, contaminated by small quantities of bR_{568}, and the formation of bR_{605}^{acid} is suppressed.

Discussion

The purple membrane impregnated gels at room temperature differ only slightly, if at all, from bR suspensions in their extinction coefficients, their CD spectra, their light–dark adaptation cycles, and their photocycles at pH 7. The spectral changes in gels between pH 5 and 3.5, mainly small decreases in absorbance, are probably due to secondary effects on the chromophore arising from the titration of the membrane. The apparent isosbestic point for this change at 598 nm for dark-adapted gels is red-shifted to 608 nm in light-adapted gels. The apparent isosbestic points for the bR_{605}^{acid} formation are at 578 and 589 nm, respectively, whereas the isosbestic points for the bR_{565}^{acid} transitions are the same for light- and dark-adapted gels, indicating that light- and dark-adapted membranes produce the same low pH species.

Clearly, bR_{565}^{acid} is formed at the expense of bR_{605}^{acid} at high concentrations of NaCl, and the nature of the anion is important because bR absorbs at 578 nm when 2 N H_2SO_4 is used in lieu of 2 M HCl (H. Niv, unpublished experiments). However, λ_{max} is 575 nm in 1 M NaCl at pH 2.0 whereas it is 565 nm in 1 M HCl (Table I), which shows that a decrease in pH increases the effectiveness of anions. Similar conclusions have been reached by U. Fischer and D. Oesterhelt (personal communication) in more extensive experiments on the anion effect at low pH.

The reliability of the extraction data is difficult to assess. The yield of retinal extracted into methylene chloride is poor, especially at low pH. Imine hydrolysis is both general acid and general base catalyzed (Jencks, 1969). Therefore, the quantity of retinal extracted decreases at high pH because of the low acid concentration and at low pH due to the lack of base. The membrane plates out as a yellow waxy substance at pH <3, whereas it forms a more easily extractable dispersion at pH 7. Neutralizing the membrane after mixing with methylene chloride was of some help. The yields reported here at pH 7.0 are comparable to those recently reported by Maeda et al. (1977). An alternative method of extraction was developed by Bligh & Dyer (1959). Their method extracts 30–40% of the theoretical content of retinal (Kushwaha et al., 1975), but is quantitative if the retinal is removed as the oxime or the thiobarbituric acid product (M. Kates, personal communication). However, the time required results in isomer equilibration (Maeda et al., 1977).

Dark adaptation during which the chromophore is known to isomerize has a half-time of about 1 min at pH 4.0 to 40 min at pH 7 and 30 °C (Ohno et al., 1977a), and little if any dark adaptation appears to occur in the low pH species. Yet, the PM in methylene chloride bleaches almost instantly. Hence, rapid dark adaptation is unlikely as a mechanism for chromophore isomerization before and during extraction, but because of the low yield we cannot exclude the possibility that one isomer may be selectively extracted. However, others (Sperling et al., 1977; Kalisky et al., 1977; Dencher et al., 1976; Ohno et al., 1977a,b) have reported a flash photolytic method for measuring the isomer distribution in the intact membrane at pH 7.0 and have shown that it agrees with the extraction data. In addition, U. Fischer and D. Oesterhelt (personal communication), with different techniques, have obtained data similar to ours for the acid complexes. We shall therefore take the extraction results at their face value despite the low yield with one reservation. Resonance Raman spectra indicate that the conformation of protonated Schiff base in bR is different from the conformation observed in solution of model imines (Marcus & Lewis, 1978). The isomers found in these extracts are therefore presumably the product of a rearrangement occurring during protein denaturation and strictly speaking should be designated as such. For the sake of simplicity, we will, however, continue to use the terms 13-*cis*- and *all-trans*-retinal even when referring to the intact bR chromophores [see also Pettei et al. (1977)].

The small amounts of 13-*cis*-retinal extracted at pH 7.0 from bR_{568} have not been reported by others. Sperling et al. (1978) recently observed "dark adaptation" in PM suspensions illuminated by intense light, but found no 13-*cis*-retinal at the intensities used here for light adaptation, which are only ~30 photons/(molecule of bR s) for 3 min. High light intensities may allow the buildup of intermediates in the normal photocycle to concentrations where they can be optically pumped into intermediates of the 13-cis photocycle found in dark-adapted membranes (Dencher et al., 1976), but this seems hardly possible in our case. While most other observers do not specify the intensities used for light adaptation, they are probably comparable to ours, and the discrepancy in the amount of 13-*cis*-retinal found remains unexplained.

The difference in chromophore distribution between light- and dark-adapted bR_{605}^{acid} at pH 1.9 (Table II) should be corrected for the presence of other species even though this cannot be done rigorously. If we again assume $n = 1$ (eq 1) and pK values of 2.9 and 0.5, there is 10% bR_{568} and 5% bR_{565}^{acid} present at pH 1.9. Furthermore, if we assume that the contaminating bR_{565}^{acid} contains only the trans isomer and if we use the isomer ratio for light-adapted bR at pH 4, i.e., 30:70, in the calculations, we get 13-cis/all-trans = 40:60 for light-adapted bR_{605}^{acid}. Taking the possible errors into account, we may conclude that bR_{605}^{acid} contains slightly less of the 13-cis than the all-trans chromophores and shows little change during light–dark adaptation, whereas bR_{565}^{acid} contains only *all-trans*-retinal in the light-adapted state with a less than 10% conversion to 13-cis upon dark adaptation if light–dark adaptation occurs at all.

Recently, Moore et al. (1978) published spectra of bR in suspension between pH 7 and 3. The spectra and pK values were temperature dependent and also lacked reproducibility (e.g., the apparent isosbestic points varied over 16 nm), which was probably due in part to the aggregation problem. However, their published spectra at 25 °C agree closely with the first two transitions reported here, showing apparent isosbestic points at 604 (pH >4) and 580 nm (pH <4) for presumably partially dark-adapted membranes. In addition, Tokunaga & Ebrey (1978) have reported the acid titration of the chromophore in studies of PM reconstituted with 3,4-dehydroretinal and found apparent isosbestic points corresponding to the formation of two low pH species, but no spectral changes were evident between pH 4 and 7. This discrepancy may be attributed to inherent differences in chromophores. However, they further stated that the absorption spectra of PM were unchanged between pH 3.5 and 10, which is at odds with the data reported here and those reported by Moore et al. (1978).

The presence of the apparent isosbestic points in Figures 1 and 2 was not expected. Three transitions and therefore four species are evident in each figure. The simplest conclusion is that each transition is dominated by only two species, the absorption spectra of which obey Beer's law. Contaminating pigments can be present in only small quantities or even the apparent isosbestic points would not be observed. Pathways leading to the interconversion of these species are apparently complex, and knowledge of them would undoubtedly contribute to the understanding of reactivity in bR. However, we are only observing thermodynamic relationships here.

The CD spectrum of bR_{568} in gels is virtually indistinguishable from that in purple membrane suspensions, where the maximum in ellipticity is at 535 nm, the crossover, red-shifted from the absorption maximum, is at 574 nm, and the minimum is at 600 nm (Heyn et al., 1976; Becher & Cassim, 1976; Ebrey et al., 1977). The only discernible difference is in the ratio of minimum to maximum ellipticities, which is 0.74 in solution and 0.62 in gels. Since the spectra in gels are superimposable with the base line at 710 and 375 nm, artifacts due to light scattering should be minimal. Interestingly, X-ray diffraction shows no lattice structure in bR_{605}^{acid}, and in bR_{565}^{acid} reflections are diffuse (G. I. King, personal communication) even though the bilobed CD patterns are pronounced as at neutral pH.

It is clear that the bR chromophore undergoes two major transitions between pH 0 and 7. Both acid species are formed by titrating the membranes with 1 to 2 mol of H^+/mol of bR, indicating that they result from the titration of 1 or 2 groups and not from the random titration of many lipid or protein functional groups. At low ionic strength bR_{605}^{acid} is formed, whereas bR_{565}^{acid} is formed in concentrated NaCl solutions or at pH <1. The apparent isosbestic points in the visible spectra, the bilobed pattern in the CD spectra, and the radically differing flash kinetic cycles also imply that the complexes bR_{568}, bR_{565}^{acid}, and bR_{605}^{acid} are discrete entities. The pK for the formation of bR_{605}^{acid} (2.9) is consistent with the titration of protein carboxyl groups (Schuster et al., 1977). However, that for bR_{565}^{acid} (0.5) is not so easily explained, since it is too high for amide (p$K \approx -0.3$) or sulfolipid and too low for phosphate groups. Since the formation of bR_{565}^{acid} is dependent on the ionic strength and the nature of the anion, it is probably not only due to a simple titration of a functional group. Furthermore, pK values depend on the environment and may show large changes when the dissociative groups are embedded in proteins or membranes (Bender & Kezdy, 1975). No firm conclusions can therefore be drawn.

Each of the acid species clearly has its own photocycle, but so far they are poorly characterized. This is particularly true of bR_{605}^{acid}. Its resolvable transient at 520 nm may, for example, contain more than one intermediate due to the cycles of the contaminating pigments. Earlier intermediates may not be observed due to the limited time resolution of the study. Whether the intermediates arising from bR_{565}^{acid} represent parallel pathways or a photocycle cannot be decided. Some of these questions are not fully resolved for bR_{568} either. A provisional photocycle model appears to be justifiable for bR_{565}^{acid}. The rise of the 680-nm transient is unresolvable, and it decays approximately with the rise of bR_{565}^{acid}.

The properties of the two acid-stable complexes emphasize the role of bR_{565}^{acid} in the proton-pumping cycle [see also Lozier et al. (1978)]. The data presented here show that neither bR_{605}^{acid} nor bR_{565}^{acid} at pH 2.0 has an intermediate absorbing at 410 nm, whereas residual bR does, which suggests that neither complex pumps protons. This conclusion is supported by the report of Drachev et al. (1978) that bR-impregnated films do not create a transmembrane photopotential at pH 0.9.

Moore et al. (1978) and also U. Fischer and D. Oesterhelt (personal communication) have suggested that bR_{605}^{acid} is an acid-stabilized form of the "O" intermediate in the photocycle. One could argue as well that it is an equilibrium form of the "K" intermediate. However, a kinetic intermediate probably contains only a single isomer of the chromophore, whereas bR_{605}^{acid} obstensibly contains two.

Work on the acid-stable complexes shows that they have potential value in studies of the mechanism of the bR photochemistry and of the purple membrane proton pump. They are easy to prepare, are very stable, and are easy to handle. A question of particular interest is the cause of their formation. The bR_{605}^{acid} complex differs from bR_{568} by 1 or, at most, 2 mol of H^+/mol of bR. U. Fischer and D. Oesterhelt (personal communication) believe that they differ by only 1 mol of H^+ and that bR_{565}^{acid} formation is the result of binding one anion near the chromophore. In this regard, one should notice that n in eq 1–3 may be regarded as the Hill coefficient used in studies of enzyme cooperativity (Koshland, 1970) instead of as a coefficient of proton molarity. It may, for example, indicate a positive cooperativity in the titration of neighboring bR molecules. Its physical significance is therefore uncertain.

One explanation for the formation of acid-stable complexes would be that one or two groups are titrated, which causes the retinylidene moiety to uncouple from the proton-translocating system. This may well be true for bR_{605}^{acid}. Becher & Cassim (1976) have stated that the negative ellipticity in the 325-nm region in the CD spectra of bR is an indication of the degree of chromophore mobility. If this is true, an uncoupled, relatively mobile chromophore in bR_{605}^{acid} is suggested by the CD data (Figure 4) and by the extraction of both 13-*cis*- and *all-trans*-retinal (Table II). However, bR_{565}^{acid} is as tightly coupled as is bR_{568} by both of these criteria. A plausible explanation for the inhibition of the formation of the M_{412} intermediate in bR_{565}^{acid} is that a protonation from the exterior membrane surface has increased the exterior proton potential sufficiently to inhibit deprotonation of the Schiff base and therefore proton pumping.

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