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Investigations into the effect of acid on the spectral and kinetic properties of purple membrane from *Halobacterium halobium*

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The formation and reversal of the acid species of purple membrane generated below pH 4.00 (22°C) is studied together with the photochemical cycle over the pH range 6.40–3.20. The buffering capacity of the membrane reaches a peak at pH 4.30, indicating the possibility of a conformational change taking place. The generation of the new spectral species can take place in the dark and is unaffected by the addition of reducing agents. Kinetic parameters measured indicate that the group being titrated below pH 4.00 could be the same as that protonated in the formation of intermediate *O*. The temporal placement of intermediate *O* after *M* in the photochemical cycle is shown to be incompatible with the data presented here. Reneutralization of acidified purple membrane shows that the spectral changes in acid are reversible but the phototransient properties are altered.

The purple membrane of the extreme halophile *Halobacterium halobium* acts as a light-driven proton pump which, when illuminated, transports protons across the cell membrane in a vectorial manner (Oesterhelt & Stoeckenius, 1973; Racker & Stoeckenius, 1974). The purple membrane contains a single species of protein containing a retinal moiety bound via a Schiff-base linkage to a lysine residue (Oesterhelt & Stoeckenius, 1971; Bridgen & Walker, 1976). The absorption maximum of the retinylidene protein, termed bacteriorhodopsin, is shifted from 370nm to a broad band centered at 558nm in the dark and 568nm in the light, indicative of further protein–retinal interactions (Oesterhelt & Stoeckenius, 1971). Bacteriorhodopsin makes up 75% of the dry weight of the purple membrane; the remainder is lipid, mainly the diether analogues of phosphatidylglycerolphosphate, phosphatidylglycerolsulphate, and glycosulpholipids (Kushwaha *et al.*, 1975, 1976; Vaver, 1978).

When illuminated, the bacteriorhodopsin undergoes a photochemical cycle that is accompanied by a reversible deprotonation of the Schiff-base linkage (Lozier *et al.*, 1975, 1976; Kaufman *et al.*, 1976; Sherman *et al.*, 1976*a*). The phototransients observed have been labelled *K*, *L*, *M*, *N*, *O*, and *bR* by Lozier *et al.* (1975), named alphabetically in the order in which they are believed to appear after the absorption of light. Their spectral characteristics have been discussed by Lozier *et al.* (1975) and Kung *et al.* (1975). At low-level illumination (i.e. room light) only intermediate *bR* exists at high enough steady-state concentrations to be observed, and is identified as light-adapted purple membrane. Proton release from the membrane has been shown to follow the formation of *M*, whereas proton uptake is concurrent with the regeneration of *bR* (Stoeckenius *et al.*, 1979).

Both the spectroscopic and kinetic properties of purple membrane have been shown to be dependent upon pH (Lozier & Niederberger, 1977; Moore *et al.*, 1978; Tsuji & Rosenheck, 1979). A blue spectral species of the membrane exists at low pH values that is sensitive to the presence of salts (Edgerton *et al.*, 1978). Mowery *et al.* (1979) have named this species bR^{acid}_{605}. In the present paper the properties of the acid species are further characterized: in particular its formation, the effects on the photochemical cycle, and the reversibility of the changes induced by acid.

Materials and methods

Halobacterium halobium, strain R1, was grown and the purple membrane was extracted and purified according to Oesterhelt & Stoeckenius (1974). It was stored in the presence of NaN_3 (approx. 0.01%) which was removed either by pelleting and resuspending in distilled water or by passage down a Sephadex G-25 column (15cm × 2cm) equilibrated

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with distilled water. The concentration and purity of the bacteriorhodopsin present were measured by amino-acid analysis (Moore *et al.*, 1978). The protein concentration was calculated using the molecular composition values of lysine (7), arginine (7), aspartic acid (15), glutamic acid (16), valine (20), and phenylalanine (13) determined by Bridgen & Walker (1976) for an M_r of 25000.

For the spectral titrations, purple membrane was suspended in glass distilled water at a protein concentration of 2–3 μmol·dm^{-3} to minimize buffering effects. HCl was added from 0.1 mol·dm^{-3} stock solutions by using a Hamilton syringe. The acid solutions were made up from volumetric HCl purchased from BDH, Poole, Dorset, U.K. Spectra were recorded by using a 1 cm pathlength cell in a Cary 118c spectrophotometer. Initial pH values were measured by using a Russell CMJ microelectrode with a Radiometer pH Meter 51; all other values were calculated from known activity coefficients. Samples were allowed at least 30 min to reach equilibrium before measurements were made, unless otherwise reported.

Flash photolysis experiments were carried out by using the instrumentation described by Moore *et al.* (1978) and Gibson & Greenwood (1965) with a 4 cm pathlength cell, or an Applied Photophysics flash photolysis system with a 5 cm pathlength cell. The sample was excited with a white light pulse of less than 300 μs duration. The energy delivered was 250 J for the former system and less than 200 J for the Applied Photophysics system. The reaction traces were either photographed and analysed using semi-logarithmic plots, or input via a Datalab 905 transient recorder to a PDP 8/A computer. A semi-logarithmic linear regression program was used to analyse traces having a single exponential decay and an iterative procedure adapted from Wilkins *et al.* (1974) was used for analyses involving more than one decay term. The protein concentrations in these experiments were chosen to optimize conditions for the particular apparatus involved. These were typically 7–10 μmol·dm^{-3} for the former instrument and 2–3 μmol·dm^{-3} for the Applied Photophysics system.

Membrane titration experiments were carried out with purple membrane at a protein concentration of 0.33 mmol·dm^{-3} in a water-jacketed cell at 22°C. HCl was added in portions from a 0.1 or 1.0 mol·dm^{-3}-HCl stock solution. The pH values in these experiments were measured by using a Radiometer GK 2321C combination electrode in conjunction with a PHM 64 digital pH meter, and were recorded after the readings had stabilized.

Portions of 0.1 mol·dm^{-3}-NaOH were used to neutralize acidic purple membrane suspensions and samples were allowed at least 30 min to complete spectroscopic changes unless otherwise stated.

Trimethylamine borane and sodium cyanoborohydride were kindly donated by Dr. H. B. Dixon. All other chemicals were AnalaR grade; analytical reagent water (BDH) or glass-distilled water were used throughout. At the start of each experiment, the purple membrane was fully light-adapted. All experiments were performed at room temperature (20–22°C) unless otherwise stated.

Results

Flash photolysis experiments at acid pH values

Absorption changes on a 100 ms timescale were recorded at 412, 640, and 570 nm over the pH range 6.40–3.20. Under these conditions, the wavelengths represent the decay of the *M* species, formation and decay of *O*, and the regeneration of *bR* respectively.

In a previous publication, Moore *et al.* (1978) investigated the effect of acid addition on the static spectrum of purple membrane and the formation of phototransient *O* after flash photolysis at 15°C. Initially, addition of acid was shown both to bleach the 568 nm peak in the static spectrum and to decrease the rate of formation of phototransient *O*. Further addition of acid resulted in the formation of a peak at 610 nm and an increase in the rate of formation of *O* in a manner indicative of a protonation reaction. The rate constant for the decay of *O* decreased over the pH range studied in a manner consistent with a deprotonation reaction. The amplitudes for the changes at 640 nm after flash photolysis first increased then decreased over the pH range studied, the inflection point showing a possible relationship to the transition pH for the spectral titration.

We have repeated these experiments at 22°C in order to compare the kinetic parameters measured at 412, 570 and 640 nm under the same conditions. The pH dependencies of the kinetics observed at 640 nm were qualitatively the same as those recorded at 15°C. At 22°C the rate constants measured were greater than those at 15°C, as would be expected. The inflection point for the rate of formation of phototransient *O* was shifted to a slightly higher pH value at the higher temperature, as is the pH at which the 610 nm peak first appears. Fig. 1(*b*) shows the pH dependence of the rate constant for the formation of intermediate *O* and Fig. 1(*c*) shows its decay rate (cf. Moore *et al.*, 1978). A second-order rate constant for the protonation reaction observed below pH 4.00 is estimated to be 4.4×10^3 dm^3·s^{-1}·mol^{-1} from this data.

The experiments at 412 nm showed that the decay rates of *M* were best fitted as a single exponential term and were independent of pH over the range studied (see Fig. 1*a*). The average value for the rate constant was 158 s^{-1} and the amount of *M* produced by the flash decreased with pH (see Fig. 2*a*).

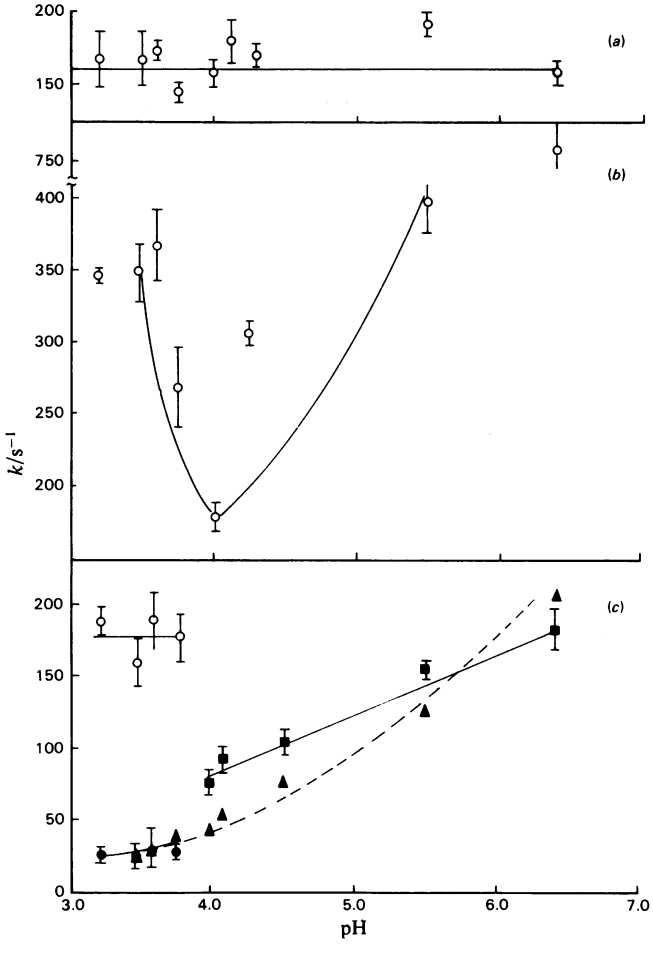

Fig. 1. *Rate constants (k) measured in the flash photolysis experiments over the pH range 6.40–3.20*
The rate constant values are shown for (*a*) the decay of intermediate *M*, (*b*) the formation of intermediate *O*, and (*c*) the decay of intermediate *O* (▲) and the regeneration of *bR*. The regeneration of *bR* has a single rate constant above pH 4.00 (■) and a fast (○) and slow (●) rate constant below that value. Lines have been drawn to provide a guide to the pH dependences of the reactions. The solid line in (*a*) shows the average value for *M* decay over the whole pH range. The solid lines in (*c*) shows the pH dependence of *bR* regeneration and the dashed line shows the same for *O* decay. The error bars for the *O* decay rate (▲) are within the space of the symbol.

If the static absorption at 568 nm represents the amount of bacteriorhodopsin capable of cycling, then the amplitude measured at 412 nm could be expected to decrease with pH even if the absorbance coefficient of *M* does not change.

The kinetics for the regeneration of *bR* over the pH range examined vary. Above pH 5.00, they appear as a single exponential term with a rate constant approximating that of *M* decay. Below pH 4.00, the reaction exhibits two exponential terms, one with a rate constant still approximating that of *M* decay, and the other with a rate constant close to that of *O* decay (see Fig. 1*c*). In the pH range 4.0–5.0, only one exponential term is evident, the rate constant of which decreases with decreasing pH. Fig. 2(*b*) shows the total amplitude of the observed change at 570 nm over the full pH range studied, along with the amplitudes of each of the two terms observed below pH 4.00. As the 568 nm peak is bleached in the static titration, the amplitude of the regeneration of *bR* is decreased.

Membrane titration with acid

The buffering capacity was defined by Perlman *et al.* (1967) as:

$$\beta = \left(\frac{\partial h}{\partial \mathrm{pH}}\right)_{T,P}$$

where *h* is the number of acid equivalents added per mol of protein, *T* is the temperature, and *P* is the

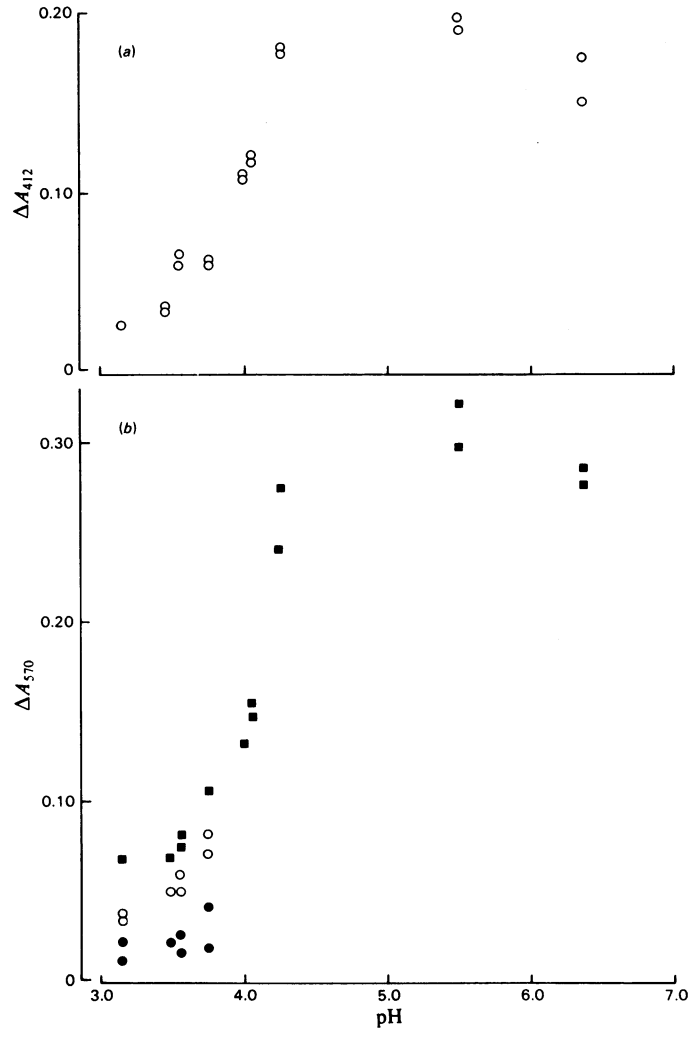

Fig. 2. *Total absorbance changes (△A) recorded at 412 and 570 nm after flash photolysis experiments over the pH range 6.40–3.20*

(*a*) Shows the total absorbance change at 412 nm; (*b*) shows the total absorbance change at 570 nm over the whole pH range (■), and the separate contributions of the fast (○) and slow (●) components observed below pH 4.00.

pressure. The buffering capacity at a particular pH value may be determined as the slope of the titration curve at that point and the number of titratable groups calculated from the area under the curve. Fig. 3(*a*) shows the titration curve for purple membrane at 22°C and atmospheric pressure. Over the pH range 7.00–3.00, approx. 12 titratable groups are protonated. Fig. 3(*b*) shows the shape of the buffering curve, which has a sharp peak at pH 4.30. This value is close to the pH at which the 'acid' spectrum starts to appear in an acid titration at 22°C. Perlman *et al.* (1967) have suggested that a maximum value for β reflects a conformational change taking place. It is difficult to define such a change in a multi-component system such as the purple membrane but the sharp peak in the shape of the buffering curve does indicate that a change in the nature of the titrating processes has occurred at that pH value. This transition is apparently reflected in the spectral titration, and may relate to the inflection in the pH dependence of the formation rate of *O* at the same point (see Fig. 1*b*).

Acid titration of dark-adapted purple membrane

Purple membrane was left to equilibrate in the dark for 18 h, after which acid was added in the dark and the resulting spectrum recorded at low light levels. Control experiments were carried out with

light-adapted membrane to which an equivalent amount of acid was added and the spectral changes were allowed to come to completion in the light. There was no difference between the spectra of the samples titrated in the light or dark.

Accessibility of the Schiff base in acid

Purple membrane at a concentration of 2.7 μmol·dm^{-3} was titrated with acid until the 'acid' spectrum was 95% complete and small amounts of solid trimethylamine borane were added up to a maximum of 0.3 mg/ml. These produced only small changes in the spectrum, which were attributed to a small increase in the pH of the suspension effected by the reducing agent. These changes could be reversed with a small amount of acid. Similar results were obtained with sodium cyanoborohydride.

Properties of purple membrane after reversal of the acid state

NaOH was used to reneutralize samples of purple membrane at low pH values and their spectroscopic and kinetic properties were examined. These experiments were carried out with suspensions in either the first or the second spectroscopic stage of an acid titration (Moore *et al.*, 1978). Upon alkali addition, the spectra in both cases initially resembled that of dark-adapted pigment, having an absorbance peak at 558 nm with a lowered absorbance relative to the original 568 nm peak. Fig. 4 shows the spectrum of purple membrane after reneutralization.

The kinetics of the reversal were complex, but it was possible to measure a very slow rate constant of 3×10^{-3} s^{-1} in the spectrophotometer for the absorbance loss at 640 nm and the gain at 565 nm. This rate constant was independent of the pH of the acidified purple membrane before alkali addition. After 30 min, when the slowest reaction was 99.5% complete, the absorbance peak for the reneutralized sample exhibited a red-shift to 568 nm and an increased absorbance. When the reneutralized sam-

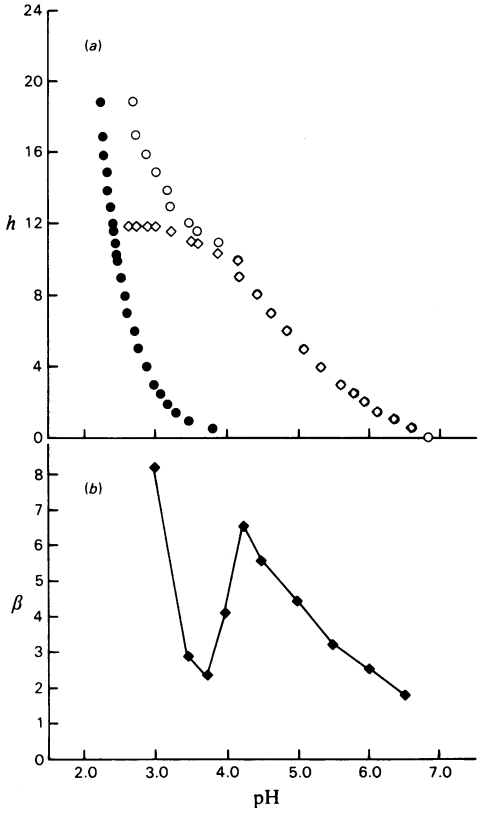

Fig. 3. *Results of the titration of purple membrane with HCl at 22°C over the pH range 7.00–3.00*
The data here show (*a*) equivalents of acid added/mol of protein present (*h*) as a function of the calculated pH values (●), the measured pH values (○), and the difference between the two (◊); and (*b*) the pH dependence of the buffering capacity measured as the slope of the titration curve for purple membrane represented by the open circles in (*a*). The concentration of purple membrane used in this experiment was 0.33 mmol·dm^{-3}.

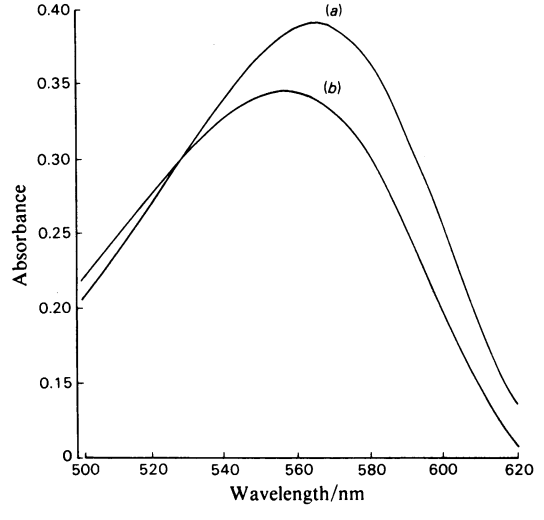

Fig. 4. *Comparison of the absorption spectrum for purple membrane before acid addition and immediately after reneutralization of the acid state*
The absorption spectrum of purple membrane (*a*) in distilled water at pH 6.80 and (*b*) after the pH had been lowered to 3.30 with HCl and then the suspension neutralized with an equivalent amount of NaOH. The spectrum in (*b*) was recorded immediately after the addition of alkali.

Table 1. *Analysis of the spectrokinetic data at 412 nm in water, NaCl (0.8 mmol·dm^{-3}), and after neutralization of the acid species*

Decay rate constants are given as k_1 and k_2 with their relative proportions, A_1 and A_2, expressed as percentages. The pH values shown are those of the acid species before alkali addition. These experiments were carried out at room temperature.

Medium	A_1 (%)	k_1/s^{-1}	A_2 (%)	k_2/s^{-1}
Water	100	150	—	—
0.8 mmol·dm^{-3}-NaCl	100	150	—	—
HCl (pH 3.65)/NaOH	72	122	28	15
HCl (pH 3.08)/NaOH	65	126	35	15

Table 2. *Analysis of the spectrokinetics at 412 nm after neutralization of the acid species in the presence of 10 mmol·dm^{-3}-NaCl*

Decay rate constants are given as k_1 and k_2 with their relative proportions A_1 and A_2 expressed as percentages. The pH values shown are those of the acid species before alkali addition. These experiments were carried out at room temperature.

Medium	A_1 (%)	k_1/s^{-1}	A_2 (%)	k_2/s^{-1}
HCl (pH 3.75)/NaOH	74	170	26	17
HCl (pH 3.00)/NaOH	20	160	80	15

ple was exposed to bright light (750 W) for 15 min immediately after alkali addition, a light-adapted spectrum was recorded indicating that the slowest reaction was dependent upon light intensity. However, the absorbance of the final spectrum was less than that of the original (before acid and base addition) with or without further exposure to light.

It was shown that the acid titration and the 'back-titration' with alkali were not superimposable. However the spectroscopic properties of purple membrane in acid have been shown to be sensitive to the presence of cations (Edgerton *et al.*, 1978), and the possibility existed that NaCl generated in the neutralization was responsible for the discrepancy, so the experiments were repeated in 10 mmol·dm^{-3}-NaCl where the salt generated was at most 10% of the total salt. The forward and back-titrations then become superimposable.

In view of the dramatic changes in the photochemical cycle at low pH values, flash photolysis experiments were carried out with the reneutralized acid species. The kinetics recorded at 640 nm after alkali addition were similar to those before addition of acid and alkali, but the decay rate of *O* was slowed by approx. 45%. The absorption increase at 570 nm had altered considerably and could be resolved into two distinct components with rate constants of 124 s^{-1} and 15 s^{-1}. The rate constant for the same purple membrane in distilled water which had not been treated with acid was 150 s^{-1}. When NaCl equivalent to that generated by the acid and base additions was added to purple membrane the observed change was monophasic with a rate constant of 150 s^{-1}. Table 1 summarizes the results of these experiments together with the kinetics of samples that were neutralized from either the first or the second spectral stage of an acid titration. It can be seen that the rate constants are the same for both, but the relative proportion of the slower reaction is greater for the sample that had been at a lower pH value. The parameters were measured immediately after the alkali addition and showed no change after 17 h.

The kinetic experiments were repeated in 10 nmol·dm^{-3}-NaCl and the reaction at 412 nm was characterized. Table 2 shows the results of these experiments, which are similar to those in Table 1. The extent of the slow reaction was much greater for the sample reneutralized from the second spectral stage (pH 3.00) in 10 mmol·dm^{-3}-NaCl than in the experiments described above.

Conclusion

In a previous publication it was suggested that the low pH species of purple membrane represented an acid-stabilized form of phototransient *O*. Kinetic data indicated that a single protonation of the membrane was involved in the formation of *O* over the same pH range that the second stage of the acid titration took place (Moore *et al.*, 1978). Similar conclusions have been reached by Fischer & Oesterhelt (1979). In the present paper it has been shown that a change in the nature of the buffering by the membrane, suggestive of a conformational change, occurs at the same pH value where the first spectral stage is complete and the second begins.

Tsuji & Rosenheck (1979) have reported changes in the protein conformation at low pH. Using circular dichroism they have measured a 10% loss in the helical content of the protein at pH 3.00 as compared with pH 6.00.

We suggest that the first stage of the acid titration can be attributed to a structural rearrangement in the protein and the second to the titration of a group associated with phototransient *O*. This association is supported by the kinetic data at low pH values. The rate of *O* formation increases whereas the rate of decay of *O* decreases in the pH range where the 610nm peak is seen in the static spectrum. The pH dependencies indicate that phototransient *O* could be stabilized in acid.

The identification of the acid species as phototransient *O* has been questioned (Tsuji & Rosenheck, 1979; Mowery *et al.*, 1979). Mowery *et al.* (1979) have shown that the equilibrium constant for the 13-*cis* and all-*trans*-retinal isomers might be affected by acid. They have detected some 13-*cis*-retinal at low pH values and express doubt that a photochemical intermediate with two different retinal isomers exists. The acid species of purple membrane may not have an identical structure to phototransient *O* as observed at neutral pH values. However, the same group that is protonated to form the kinetic species *O* could be that protonated to form the acid species of purple membrane.

Although this titratable group cannot be identified from the data here, the relatively small second-order rate constant for *O* formation with respect to H^+ concentration suggests that it is not exposed to the solvent. The inaccessibility of the Schiff base to reducing agents at acid pH values indicates that it also is not exposed to the external medium. Schreckenbach *et al.* (1977, 1978) have indicated a pH dependence in the reconstitution reaction of retinol with bacterio-opsin, having an apparent p*K* value of 3.80. They suggested that a group (B_1H) close to the chromophore, interacts with retinol such that a higher pH values the β-ionone ring and side chain are coplanar, whereas at lower pH values they are not.

The similarity of the spectrum of purple membrane to that of dark-adapted pigment after neutralization of the acid species may be explained by a slow relaxation of the 13-*cis*/all-*trans*-retinal equilibrium to its value at neutrality. It is probable that the kinetic irreversibility indicates that at least some of the changes caused by acid cannot be simply reversed by the addition of alkali. These effects must be taken into account in any treatment of the membrane with acid. Korenstein & Hess (1977*a*,*b*) have reported similar kinetics at 412nm for dehydrated purple membrane. They have attributed the change in the kinetics to a conformational change in the membrane.

In the scheme of the photochemical cycle presented by Stoeckenius *et al.* (1979) the formation of phototransient *O* follows *M* decay, sometimes via phototransient *N*. Sherman *et al.* (1976*a*,*b*, 1979) favour a branch in the cycle at *M* formation with one reaction path leading through *O*. The data presented in this study show that the rate of formation of *O* exceeds the decay rate of *M* over the majority of the pH range 6.40–3.20. At pH 6.40 the rate of formation of *O* was determined to be $762\,s^{-1}$ whereas that for *M* decay is $150\,s^{-1}$. Therefore, phototransient *O* cannot follow phototransient *M* under these conditions. In addition the rate of *O* formation exhibits a complex pH dependence whereas the rate of *M* decay is independent of pH over the range studied. If the cycle branches, it apparently does so before *M* formation.

The reactions recorded at 570nm could be explained by considering that *bR* is regenerated from parallel pathways incorporating *M* and *O* separately. At pH 6.40, the rate constants for the decay of *M*, *O* and *bR* are approximately equal. As the pH is lowered, the decay of *O* is slowed, but not enough to allow separation of the *M* and *O* rate constants in the analysis of *bR* regeneration. Below pH 4.00, the decay rate of *O* has decreased such that the regeneration of *bR* can now be resolved into two distinct reactions, one with the same rate constant as the *M* decay reaction, and the other which is attributed to the *O* decay reaction.

The precursor to *O* cannot be identified from the results presented here. Acquisition of data at earlier times in the photochemical cycle at low pH values could clarify the point at which the *M* and the *O* pathways diverge. Further information could also be obtained using studies of the temperature dependences of the photocycle intermediates.

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References

Bridgen, J. & Walker, J. D. (1976) *Biochemistry* **15**, 792–798

Edgerton, M. E., Moore, T. A. & Greenwood, C. (1978) *FEBS Lett.* **95**, 35–39

Fischer, U. & Oesterhelt, D. (1979) *Biophys. J.* **28**, 211–230

Gibson, Q. H. & Greenwood, C. (1965) *J. Biol. Chem.* **240**, 2694–2698

Kaufman, J. L., Rentzepsis, P. M., Stoeckenius, W. & Lewis, A. (1976) *Biochem. Biophys. Res. Commun.* **68**, 1109–1115

Korenstein, R. & Hess, B. (1977*a*) *FEBS Lett.* **82**, 7–11

Korenstein, R. & Hess, B. (1977*b*) *Nature* (*London*) **270**, 184–186

Kung, M. C., Devault, D., Hess, B. & Oesterhelt, D. (1975) *Biophys. J.* **15**, 907–911

Kushwaha, S. C., Kates, M. & Martin, W. G. (1975) *Can. J. Biochem.* **53**, 284–292

Kushwaha, S. C., Kates, M. & Stoeckenius, W. (1976) *Biochim. Biophys. Acta* **426**, 703–710

Lozier, R. H. & Niederberger, W. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 1805–1809

Lozier, R. H., Bogomolni, R. A. & Stoeckenius, W. (1975) *Biophys. J.* **15**, 955–962

Lozier, R. H., Niederberger, W., Bogomolni, R. A., Hwang, S.-B. & Stoeckenius, W. (1976) *Biochim. Biophys. Acta* **440**, 545–556

Moore, T. A., Edgerton, M. E., Parr, G., Greenwood, C. & Perham, R. N. (1978) *Biochem. J.* **171**, 469–476

Mowery, P. C., Lozier, R. H., Chae, Q., Tseng, Y.-W., Taylor, M. & Stoeckenius, W. (1979) *Biochemistry* **18**, 4100–4107

Oesterhelt, D. & Stoeckenius, W. (1971) *Nature* (*London*) *New Biol.* **233**, 149–152

Oesterhelt, D. & Stoeckenius, W. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2853–2857

Oesterhelt, D. & Stoeckenius, W. (1974) *Methods Enzymol.* **31**, 667–678

Perlman, G. E., Oplatka, A. & Katchalsky, A. (1967) *J. Biol. Chem.* **242**, 5163–5168

Racker, E. & Stoeckenius, W. (1974) *J. Biol. Chem.* **249**, 662–663

Schreckenbach, T., Walkhoff, B. & Oesterhelt, D. (1977) *Eur. J. Biochem.* **76**, 499–511

Schreckenbach, T., Walkhoff, B. & Oesterhelt, D. (1978) *Biochemistry* **17**, 5353–5359

Sherman, W. V., Korenstein, R. & Caplan, S. R. (1976*a*) *Biochim. Biophys. Acta* **423**, 238–248

Sherman, W. V., Silfkin, M. A. & Caplan, S. R. (1976*b*) *Biochim. Biophys. Acta* **430**, 454–458

Sherman, W. V., Eicke, R. R., Stafford, S. R. & Wasacz, F. M. (1979) *Photochem. Photobiol.* **30**, 727–729

Stoeckenius, W., Lozier, R. H. & Bogomolni, R. A. (1979) *Biochim. Biophys. Acta* **505**, 215–278

Tsuji, K. & Rosenheck, K. (1979) *FEBS Lett.* **98**, 368–372

Vaver, V. A. (1978) in *Membrane Transport Processes* (Tosteson, D. C., Ovchinnikov, Yu. V. & Latoore, R., eds.), vol. 2, pp. 21–30, Raven Press, New York

Wilkins, C. L., Klopfenstein, C. E., Isenhour, T. L. & Jurs, P. C. (1974) *Introduction to Computer Programming for Chemists: BASIC Version*, Allyn and Bacon, Boston