Biophys. Struct. Mechanism 1, 259–271 (1975)

Flash Photometric Experiments on the Photochemical Cycle of Bacteriorhodopsin*

N. Dencher and M. Wilms

Institut für Neurobiologie, Kernforschungsanlage Jülich
D-517 Jülich, Fed. Rep. of Germany

Received February 18, 1975 / Accepted March 18, 1975

Abstract. The photochemical reaction cycle of bacteriorhodopsin was investigated by means of flash photometric methods. Three different intermediates with absorption maxima at about 630 nm, 411 nm, and 646 nm could be detected. Kinetic data of the occurrence of these intermediates were obtained from isolated purple membrane in different mediums and from intact halobacteria. An activation energy of 14.1 ± 0.4 kcal · mol^{-1} and of about 19 kcal · mol^{-1} for the formation of bacteriorhodopsin 411 and of bacteriorhodopsin 565, resp., was calculated. pH-changes in the medium caused by the reaction cycle of bacteriorhodopsin were detected by use of the pH-indicator bromocresol green.

Key words: Bacteriorhodopsin — Intermediate Products — Reaction Kinetic — pH-Changes.

Introduction

Bacteriorhodopsin, located in distinct patches (purple membrane) within the surface membrane of the extreme halophile *Halobacterium halobium*, shows a number of similarities to the visual pigment rhodopsin (Oesterhelt and Stoeckenius, 1971). The chromophore 13-*cis* or all-*trans* retinal is bound to a lysine residue of the protein as a Schiff base (Oesterhelt *et al.*, 1973). Bacteriorhodopsin has a molecular weight of about 20,000 determined by gel electrophoresis (Dencher and Kühn, unpublished). The absorption spectrum of the isolated purple membrane, *i.e.* of bacteriorhodopsin, which besides lipids (25%) is the only protein species (75%) in this membrane (Oesterhelt and Stoeckenius, 1971), shows two main bands at about 565 nm and 280 nm (Fig. 1). In preparations of the purple membrane in a salt/ether solution the absorption maximum shifts from 568 nm[1] to 412 nm[1] upon illumination (Oesterhelt and Hess, 1973). This bacteriorhodopsin 412 spontaneously regenerates to the original bacteriorhodopsin 568 in the dark (Oesterhelt and Hess, 1973). The first absorption shift is accompanied by a proton release, the second one by a proton uptake from the purple membrane (Oesterhelt and Hess, 1973).

Several different experiments indicate that upon illumination bacteriorhodopsin in *Halobacterium halobium* acts as an energy transforming system by generating

* Presented in part at the Annual Meeting of Deutsche Gesellschaft für Biophysik, Freiburg, October 1974.

[1] Abbreviation and equivalent names: BR = bacteriorhodopsin; BR 570 (Stoeckenius and Lozier) ≡ BR 568 (Oesterhelt and Hess, 1973) ≡ BR 560 (Kayushin *et al.*, 1974) ≡ BR 565 and BR 412 (Oesterhelt and Hess, 1973) ≡ BR 414 (Kayushin *et al.*, 1974) ≡ BR 415 (Stoeckenius and Lozier) ≡ BR 411, because of slightly different absorption maxima published.

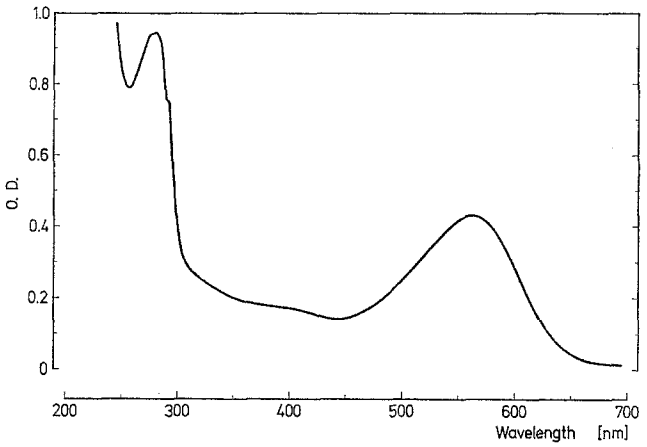

Fig. 1. Absorption spectrum of the dark adapted purple membrane (containing bacteriorhodopsin as the only protein species) in distilled water after purification on a linear sucrose density gradient

a proton gradient across the cell membrane, which is used for ATP synthesis (Oesterhelt and Hess, 1973; Oesterhelt and Stoeckenius, 1973; Danon and Stoeckenius, 1974). Moreover, light-controlled behavioral responses indicate a sensory function of bacteriorhodopsin (Dencher, 1974; Hildebrand and Dencher, 1974).

To come closer to an understanding of the mechanism of these functions, more informations about the photochemical cycle of bacteriorhodopsin under physiological conditions are required. Most of the results previously reported have been obtained with salt/ether preparations (Oesterhelt and Hess, 1973). Some kinetic data on the BR 560→ BR 414 conversion measured on purple membrane of *Bacillus salinarium* by means of flash photometric technique are published without mentioning experimental conditions (Kayushin *et al.*, 1974). Our investigations are concerned with intermediate products of the photochemical cycle, their kinetics and temperature dependence and accompanying pH-changes in the medium. Experiments using the method of flash photometry were performed with intact halobacteria and isolated purple membrane.

Materials and Methods

Halobacterium halobium strain R_1[2] was used for all experiments. Most of the experiments were carried out with isolated purple membrane, prepared by dialysis against distilled water and differential centrifugation (Oesterhelt and Stoeckenius, 1971). Preparation of the purple membrane was performed in dim white light. Samples were dark adapted for about 15 min before use. Since only slight differences (Table 2) in the bacteriorhodopsin reaction kinetics of isolated purple membrane and intact halobacteria were observed and because of less light scattering membrane preparations were preferred. For measurements the purple membranes were suspended either in distilled water (pH ≈ 6.6) or at temperatures below 0° C in a water/glycerol solution (20/80, v/v; pH ≈ 7.1).

[2] We thank Dr. D. Oesterhelt for providing a culture of *Halobacterium halobium* R_1. This mutant strain is lacking gas vacuoles.

The flash photometric apparatus is described elsewhere in detail (Witt, 1967; Sengbusch, 1970; Nöll, 1974). The monochromatic measuring light causes a photocurrent in a photomultiplier after having passed through a temperature controlled cuvette (containing the sample) and an interference filter adjusted to the wavelength of the measuring light. The exciting light (argon flash, half-time ≈ 20 μsec) is filtered through cut-off filters (GG 475 and GG 495, Schott & Gen.). Therefore, if the wavelength of the measuring light and of the adjusted interference filter is shorter than approximately 460 nm, light of the exciting flash ($\lambda > \approx 460$ nm) cannot reach the photomultiplier. The time resolution of the apparatus depends on the wavelength of the measuring light and varies from 230 μsec for wavelengths longer than 460 nm to 5 μsec for those shorter than 460 nm. To improve the signal-to-noise ratio, twenty single signals, obtained at intervals of 1.2 sec from each sample, were averaged (Biomac 1000, Datalab). The time course of absorption changes was measured between 350 nm and 700 nm at intervals of 5 to 10 nm and at different temperatures ($-45°$ C to $+40°$ C). From the averaged signals half-times of the formation of products (*e.g.* Fig. 2) and their difference spectra (maximum absorption change versus wavelength) were calculated.

pH-changes in the medium during the reaction cycle of bacteriorhodopsin were detected by means of the pH-indicator bromocresol green (visual transition interval pH 3.6 to 5.4; absorption peaks at 430 nm [acid] and 618 nm [alkaline]). Two different samples were compared. Both contained equal quantities of isolated purple membranes in distilled water (1 ml, O.D. $_{565\,nm} = 0.7$) and the same amount of aqueous indicator solution (0.6 ml, saturated). A buffer solution (1 ml, 250 mM citric acid/Na_2HPO_4, pH 5.0) was added to one cuvette, the other sample was adjusted to pH 5.0 by addition of 1 ml diluted HCl-solution. (Final concentration of the indicator and of BR 565: 46 μM and 6 μM, respectively.) Control experiments indicated that the reaction kinetics are not significantly changed in the presence of the high buffer concentration (96 mM). All experiments were performed with a measuring light of 620 nm.

Results

The absorption spectrum of the dark adapted (≈ 15 min) purple membrane containing bacteriorhodopsin 565 (BR 565) is shown in Fig. 1. In contrast to rhodopsin of vertebrates there is no visible colour change (bleaching) of bacteriorhodopsin, even at high light intensities. During illumination only a small shift (≈ 5 to 10 nm) of the absorption maximum at 565 nm towards longer wavelengths and an increase of absorption can be measured by means of a spectralphotometer. According to Oesterhelt *et al.* (1973) these in the dark reversible absorption changes are due to the isomerization of 13-*cis* to all-*trans* retinal (see discussion). Using a flash photometric equipment, absorption changes of bacteriorhodopsin at different wavelengths can be detected, which occur in the time scale of microseconds and milliseconds. In this paper we present kinetic data of three intermediate products occurring within the time range between decay and spontaneous regeneration of BR 565. The absorption changes after the flash in Fig. 6, *i.e.* the absorption decrease, increase, and decay to the original value, indicate formation and decay of BR 411 and BR 646, resp., in the photochemical cycle of bacteriorhodopsin. Absorption changes caused by the first appearing intermediate BR 630 (Fig. 5) before formation of BR 411 are not resolved in this recording.

Bacteriorhodopsin 411 and Bacteriorhodopsin 565

Upon a light flash fast changes in absorption of the purple membrane containing BR 565 occur. Fig. 2 shows an example of the raw data observed, *i.e.* the transient change in absorption at 411 nm resulting from the absorption of photons. The decay of the absorption increase to the original value is slowed

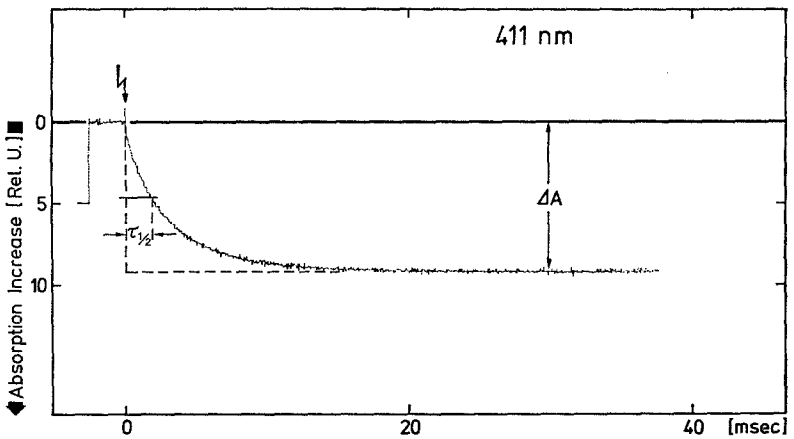

Fig. 2. Time course of the absorption changes at 411 nm measured on purple membrane suspension in aqueous glycerol (80%, v/v; pH ≈ 7.1). Temperature −12.5° C. The arrow depicts the light flash. Half-time ($\tau_{1/2}$) and maximum absorption increase (ΔA) are shown

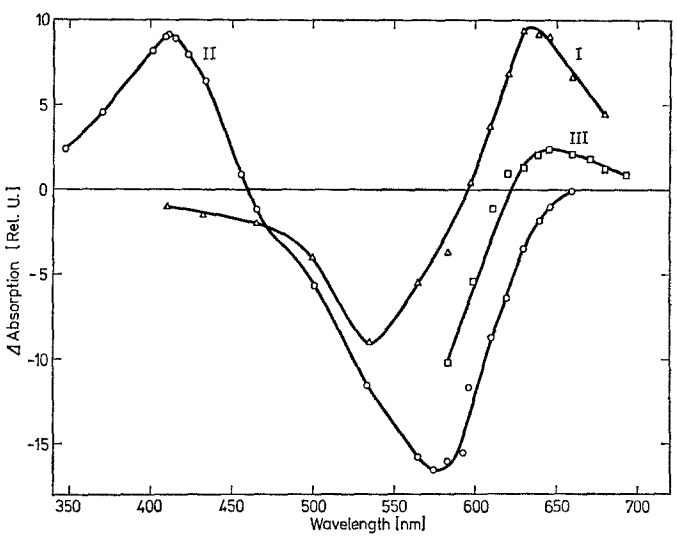

Fig. 3. Separated difference spectra measured on purple membrane suspensions. I. BR 565 → BR 630 transition (water/glycerol, −45° C); II. BR 565→ BR 411 transition (water, 10° C); III. BR 565→ BR 646 transition (water, 10° C). Ordinate: Absorption change in relative units of voltage change caused by the photomultiplier; positive values indicate absorption increase

down at the low temperature (− 12.5° C), thus the decay is not recorded in the measured time range. Calculating the maximum absorption change at a certain time (ΔA in Fig. 2) at different wavelengths, a difference spectrum with a maximum of absorption increase at 411 nm was obtained (Fig. 3, curve II). This difference spectrum indicates a transition from BR 565 to a product with an ab-

Table 1. Half-times of formation of BR 411 at different temperatures, measured on purple membrane suspensions in aqueous glycerol (80%, v/v; pH ≈ 7.1) and in water (pH ≈ 6.6). Mean (n = 3 to 6) and standard deviation are shown

Temperature (° C)	−25	−20	−12.5	−10	0	10	20 (extra-polated)
Half-time (msec) in aqueous glycerol	4.95	2.33 ± 0.07	1.36 ± 0.20	0.97 ± 0.04	0.37 ± 0.04	0.14 ± 0.01	≈ 0.05
Half-time (msec) in water	—	—	—	—	0.32 ± 0.03	0.12 ± 0.02	

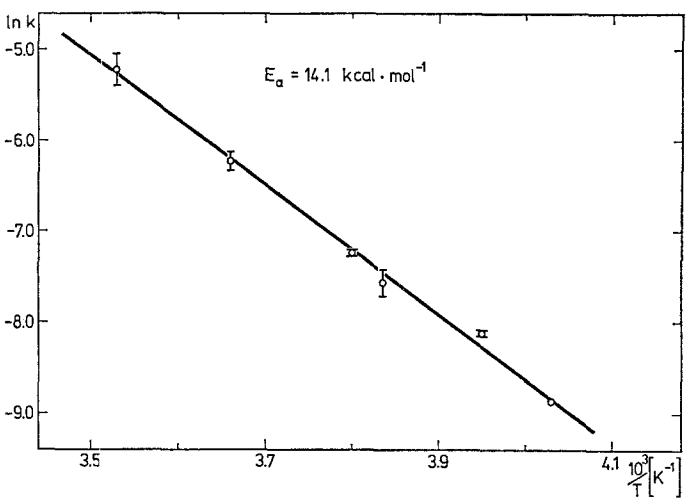

Fig. 4. Arrhenius plot of kinetic data for the formation of BR 411. The kinetics are plotted as rate constants (μsec^{-1}) of the absorption increase at 411 nm (calculated from the half-times in Table 1). Measurements were performed on purple membrane in water and in aqueous glycerol. Vertical bars represent the standard deviation of the mean (n = 3 to 9)

sorption band at 411 nm (BR 411). The reaction was measured at different temperatures between −25° C and 10° C (Table 1). The half-time ($\tau_{1/2}$) of formation of BR 411 was calculated as illustrated in Fig. 2. At 10° C a half-time of 0.14 ± 0.01 msec (s.d., n = 4) was obtained with purple membrane suspensions in water/glycerol (20/80%, v/v). From the measured temperature dependence of formation of BR 411 the activation energy of 14.1 ± 0.4 kcal · mol^{-1} (standard deviation of the slope) and a $Q_{10} \approx 2.7$ was calculated (Fig. 4).

BR 565 reappears in the dark with an overall half-time of 22 msec at 10° C, monitored at 565 nm (aqueous purple membrane suspension, Table 2). By varying

Table 2. Half-times of formation of BR 630, BR 411, BR 646, and BR 565 in different solvents, measured on isolated purple membrane and intact bacteria

Solvent	Half-time of formation (msec)				Sample
	BR 630	BR 411	BR 646	BR 565	
salt/ether pH ≈ 4.9, 3° C		0.20		40,000	purple membrane
water/glycerol pH ≈ 7.1, 10° C	< 0.23 (— 45° C)	0.14		320	
water pH ≈ 6.6, 10° C		0.12	9	22	
water pH ≈ 6.6, 30° C			2.0	2.4	
culture solution pH ≈ 7.5, 30° C			2.8	2.8	intact halobacteria

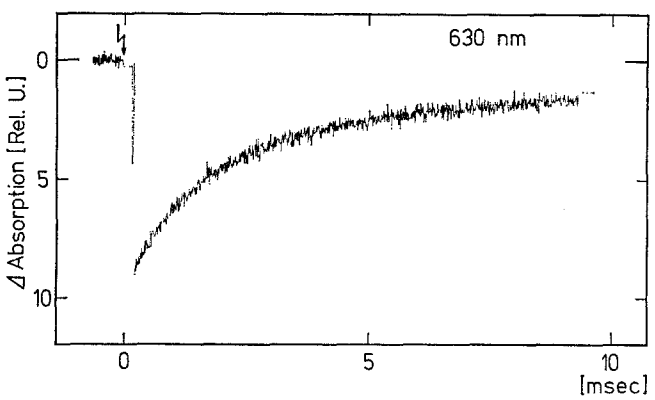

Fig. 5. Time course of the absorption changes at 630 nm measured on purple membrane suspension in aqueous glycerol (80 %, v/v; pH ≈ 7.1). Temperature — 45° C. Ordinate: Absorption change measured as relative voltage change (photomultiplier); positive values indicate absorption increase. The arrow depicts start of light flash; during the first ≈ 230 μsec of the recording the photomultiplier is saturated by the light of the flash

the temperature between 5° C and 35° C, an activation energy of about 19 kcal · mol^{-1} ($Q_{10} \approx 3$) was determined for the regeneration of BR 565.

From the obtained results we could not determine unambiguously the reaction order of the formation of BR 411 and BR 565.

Stoeckenius and Lozier found a product with an absorption maximum at 415 nm appearing with a half-time of 40 μsec at room temperature ($Q_{10} \approx 1.7$). This product decays with a half-time of 10 msec to the original bacteriorhodopsin ($Q_{10} = 2.8$).

An intermediate with an absorption maximum at 412 nm occurring in salt/ether solution (see below) was described first by Oesterhelt and Hess (1973).

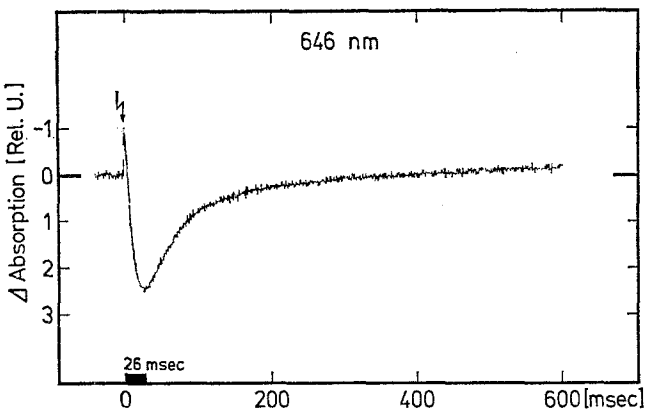

Fig. 6. Time course of the absorption changes at 646 nm measured on aqueous purple membrane suspension (pH ≈ 6.6). Temperature 10° C. Ordinate: Absorption change in relative units of voltage change (photomultiplier); positive values indicate absorption increase. Horizontal bar represents the time between flash (arrow) and maximum absorption increase. The fast decrease of absorption indicates the formation of BR 411

Bacteriorhodopsin 630

Absorption changes recorded at low temperatures indicate that a product occurs before the formation of BR 411 (Fig. 5). The difference spectrum of the formation of this intermediate shows a peak at about 630 nm (Fig. 3, curve I). BR 630 appears in less than 230 μsec at — 45° C (Table 2). Because of the limited time resolution of the measuring equipment mentioned above it was impossible to determine the time course of formation of BR 630. Absorption changes caused by BR 630 are not detectable in Fig. 2 because of the chosen time range, temperature, and monitoring wavelength.

Stoeckenius and Lozier described a fast occurring photoproduct with an absorption maximum at about 610 nm appearing in less than 10 nsec at room temperature.

Bacteriorhodopsin 646

In addition to the fast increase of absorption around 630 nm (BR 630) a slowly occurring increase could be measured in the same wavelength range (Fig. 6). The difference spectrum obtained from these absorption changes has a maximum at about 646 nm (BR 646) (Fig. 3, curve III). At 10° C the maximum of absorption is reached 26 msec after the flash (overall half-time for the BR 565 → BR 646 transition approximately 9 msec, Table 2). The decay of BR 646 is not a single first-order process, but may be described as at least two first-order rate processes with half-times of 21 msec and 73 msec, respectively. The fast decrease of absorption in Fig. 6 before the slowly occurring increase indicates the formation of BR 411.

At different wavelengths not only the absorption changes but also the kinetic data are different. From 600 nm to 646 nm a systematic decrease in rise time occurs. Above 646 nm the rise time remains constant. After the flash the maximum of absorption at 10° C is reached in 88 msec when measured at 620 nm, in 40 msec when measured at 630 nm and in 26 msec

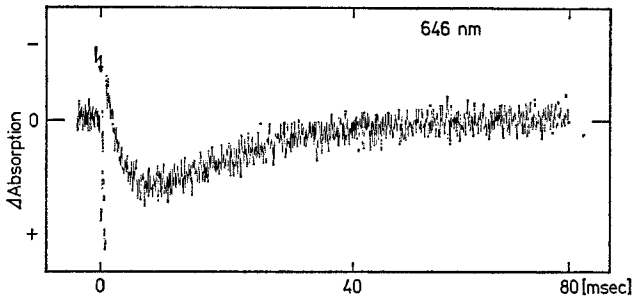

Fig. 7. Time course of the absorption changes at 646 nm measured on intact halobacteria in the culture solution. Temperature 30° C. pH ≈ 7.5. The fast first absorption increase below the arrow is due to the flash. The decrease of absorption indicates the formation of BR 411

when measured at 646 nm and 660 nm. One possible explanation is that the change in the kinetic parameters at different wavelengths is due to interference of the absorption increase caused by BR 646 with absorption changes of the previous intermediates. The difference spectrum in Fig. 3 (curve III) was obtained from the absorption change at 26 msec after flash, *i.e.* the time after which the maximum of absorption change is reached at 646 nm (10° C).

Effect of Different Solvents

The kinetics of the pigment conversion in glycerol, detergents and salt/ether solution were compared with those of purple membrane in aqueous suspension.

The half-time of the BR 565→ BR 411 transition was not altered in the presence of glycerol (80%, v/v), *i.e.* the difference in the half-times measured on purple membrane in water or aqueous glycerol (Table 1) is not statistically significant ($p > 10\%$, *t*-test). The rise time of BR 565, however, was increased by a factor of about 15 ($\tau_{1/2\,\text{(aqueous glycerol)}} \approx 320$ msec, $\tau_{1/2\,\text{(water)}} \approx 22$ msec at 10° C, Table 2). BR 646 could not be detected in aqueous glycerol solution.

In concentrated salt solution saturated with diethylether, a half-time of about 200 μsec at 3° C for the BR 565→ BR 411 transition and a half-time of approximately 40 sec for the decay of BR 411 were measured (Table 2). Preliminary results indicate that the reactions are temperature dependent.

Contrary to our results, Oesterhelt and Hess (1973) reported a not temperature dependent half-time of about 2 sec (23° C) for the BR 568→ BR 412 transition in salt/ether solution. They found a temperature dependence of the decay of BR 412 with an activation energy of 11.4 kcal · mol^{-1}.

In the presence of 2% digitonin (w/w) or 10 mM CTAB[3] (pH < 8) the decay time of BR 411 was increased by a factor 6 and 30, resp., as compared to aqueous purple membrane suspension.

In addition measurements were carried out with intact bacteria in culture solution. As shown in Fig. 7, upon flash a fast absorption decrease and a slow absorption increase appear, finally the absorption decays to the original value. These absorption changes are due to formation and decay of BR 411 and BR 646, respectively. As compared to Fig. 6 (aqueous purple membrane suspension) only

[3] N-Cetyl-N,N,N-trimethyl-ammoniumbromide.

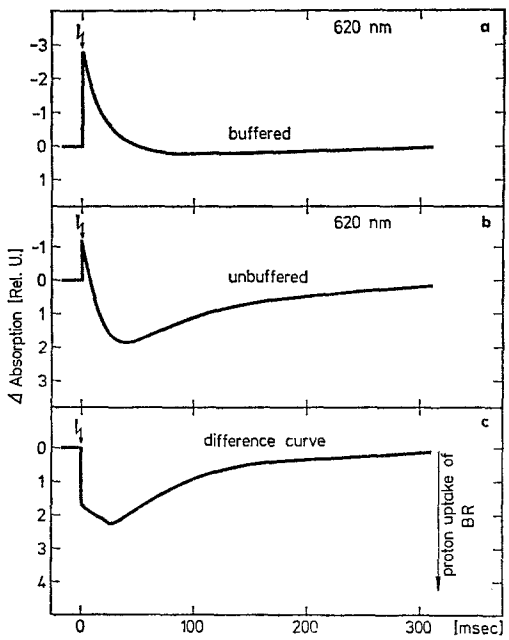

Fig. 8a—c. pH-changes in aqueous suspensions of purple membranes measured by means of the pH-indicator bromocresol green at 620 nm. Temperature 10° C, pH 5.0. Ordinate: Absorption change in relative units of voltage change (photomultiplier). Arrow: flash. (a) buffered solution; (b) unbuffered solution; (c) calculated difference curve (b—a) representing absorption changes of the indicator. Absorption increase (positive values) indicates pH increase in the medium

the noise of the recording is increased because of higher light scattering of the intact bacteria. The kinetic data obtained with intact halobacteria are only slightly different from those obtained with aqueous purple membrane suspensions (Table 2). Half-times of about 2.8 msec (intact bacteria) as compared to about 2.0 msec (purple membrane) for the overall BR 565 → BR 646 transition and 2.8 msec (intact bacteria) as compared to 2.4 msec (purple membrane) for the regeneration of BR 565 were measured at 30° C.

pH-*Changes in the Medium*

In order to detect pH-changes in the medium during the bacteriorhodopsin reaction cycle, experiments were performed with the pH-indicator bromocresol green. In these experiments a buffered and an unbuffered sample were compared, both containing aqueous purple membrane suspension and aqueous indicator solution adjusted to pH 5.

The lacking release of protons from the purple membrane at pH values below 5.4 reported by Oesterhelt and Hess (1973), might be due to the used solvent salt/ether. Under these experimental conditions, the photochemical reaction ceased to be fully reversible at pH values below 4.5 and above 6.5 (Oesterhelt and Hess, 1973).

Our experiments indicate that in aqueous purple membrane suspensions the photochemical reaction cycle of BR 565 is reversible in the measured pH range between 4.2 and 9.0.

According to Racker and Stoeckenius (1974), who measured uptake of protons by reconstituted purple membrane/phospholipid vesicles, the absolute values of H^+ uptake during illumination showed only little variation between pH 5.2 and 7.4.

After a light flash absorption changes at 620 nm can be measured in both cuvettes. In the buffered solution only absorption changes of the bacteriorhodopsin should be expected if buffering is sufficient (Fig. 8a). The absorption changes in

the unbuffered solution are attributed to absorption changes both of the chromoprotein and the indicator (Fig. 8b). The differences between the recordings of the absorption changes in the two cuvettes (determined graphically) should represent the absorption changes of the indicator (Fig. 8c). Curve 8c shows a fast unresolved absorption increase of the indicator after the flash, followed by a slower further increase. Finally the absorption decreases to the original value. The absorption changes indicate that the pH of the environment increases in parallel with the formation of BR 646 and decreases in parallel with its decay to the original value. The absorption increase of the indicator (Fig. 8c) and the absorption increase caused by formation of BR 646 (Fig. 6) both reach their maximum about 26 msec after flash. The fast absorption increase of the calculated difference curve (Fig. 8c) may be an artefact due to the lower time resolution of the measuring equipment at this wavelength (about 230 μsec). This part of the recording will not be considered in the discussion of the pH-changes. (Three experiments with different samples were performed. The results are not significantly different.)

[a] Measurement by means of pH-electrodes with a time resolution of seconds by Oesterhelt and Hess (1973) indicated that the purple membrane releases protons during the BR 565 → BR 411 transition and takes protons up again in the BR 411 → BR 565 reaction.

Discussion

The flash photometric technique used for this investigation permits one to observe the formation of intermediates in the photoreaction cycle of bacteriorhodopsin under physiological conditions (medium and temperature). The kinetic data of aqueous suspensions of purple membranes and of intact halobacteria are similar (Table 2). This suggests a similar mechanism of the reactions *in vitro* and in the living system.

After a short light flash bacteriorhodopsin undergoes a fast cyclic reaction. Because of different retinal isomers, two different forms of bacteriorhodopsin are distinguishable, which are in equilibrium in the dark:

$$\text{13-}cis\text{ BR and all-}trans\text{ BR} \underset{\text{dark. } \tau_{1/2\,(35°C)} = 21\text{ min}}{\overset{\text{light, } \tau_{1/2} \approx 10\text{ sec}}{\rightleftharpoons}} \text{all-}trans\text{ BR}$$

(Oesterhelt *et al.*, 1973; Stoeckenius, personal communication). Usually our experiments were carried out with partly dark adapted purple membrane containing both forms of bacteriorhodopsin. Control experiments using light (100 mW/cm², ≈ 1 min) or dark adapted (≈ 120 min) purple membrane or intact bacteria, resp., showed no discernible differences in the kinetic data of the three intermediates under our conditions. Thus the photochemical cycles of 13-*cis* BR and of all-*trans* BR are probably not different and a *cis-trans* isomerization of retinal is not necessarily involved in the first step of the cycle.

Three different intermediates could be identified in the photochemical cycle of bacteriorhodopsin under our experimental conditions.

BR 630: The first measurable reaction is a shift of the absorption maxima from 565 nm to 630 nm (Fig. 1 and Fig. 3, curve I) which appears in less than 230 μsec at −45° C (Fig. 5). Perhaps this BR 630 and the product with an absorption maximum at about 610 nm (BR 610) found by Stoeckenius and Lozier by means of laser flash spectroscopy and low temperature spectroscopy are identi-

cal, but it is also possible that BR 610 and BR 630 are different products appearing successively.

According to Stoeckenius and Lozier the transition BR 570→ BR 610 is photoreversible (*i.e.* by absorbing a photon BR 610 is directly converted to BR 570) and the only step in the reaction cycle requiring photons.

BR 411: In the photochemical cycle of bacteriorhodopsin, BR 411 appears after BR 630. The formation of BR 411 (Table 1) in aqueous purple membrane suspensions was found to be temperature sensitive ($E_a = 14.1 \pm 0.4$ kcal · mol^{-1}, Fig. 4). In contrast to this, Oesterhelt and Hess (1973) did not detect a temperature dependence of this reaction measured in salt/ether solution. Therefore the transition was believed to be a photochemical reaction with a half-time of about 2 sec. These contrary results are not explainable by the different solvents used, since our preliminary results indicate that formation of BR 411 (half-time at 3° C about 200 μsec) is temperature sensitive also in salt/ether solution.

The differences in the measured half-times and in the temperature dependence of this reaction in salt/ether solution are probably due to the lower time resolution ($>$ 2.5 msec; Oesterhelt and Hess, 1973) of the measuring equipment used by Oesterhelt and Hess as compared to our time resolution ($>$ 5 μsec). Therefore, differences in the half-time of formation of BR 411 at different temperatures, which are in the time scale of microseconds, possibly could not be resolved by their method. It should be mentioned, however, that they used constant light for illumination of the sample.

Stoeckenius and Lozier found an additional intermediate with an absorption maximum at 550 nm and a rise time of ≈ 2 μsec at room temperature, which appeared in the cycle between BR 610 and BR 415. We could not detect this intermediate.

BR 646: The absorption maximum at about 646 nm (Fig. 3, curve III) which appears with a overall half-time of about 9 msec upon the flash at 646 nm and 10° C (Fig. 6) might be due to the existence of another intermediate in this cycle. The dependence of the kinetic data on the monitoring wavelength is explainable by interference of the absorption increase caused by BR 646 with absorption changes of the previous intermediates. Like BR 411, BR 646 could be measured in intact bacteria (Fig. 7, Table 2). Temporally BR 646 appears after BR 411, but at the present time it is impossible to decide whether BR 646 is an intermediate between BR 411 and BR 565 or occurs in an alternate parallel pathway. Preliminary results (*e.g.* the temperature dependence of BR 646 and BR 565, and the partial regeneration of BR 565 in the absence of BR 646 in aqueous glycerol solution) favour the latter assumption.

BR 565: After a light flash BR 565 regenerates in the dark (Table 2). The different activation energies for the regeneration of BR 565 reported by Oesterhelt and Hess (1973) ($E_a = 11.4$ kcal · mol^{-1} in salt/ether solution) and us ($E_a \approx 19$ kcal · mol^{-1} in water) might be due to the different solvents used.

In contrast to the formation of BR 411, the following steps are influenced by the solvents used. In the presence of glycerol, salt/ether solution, etc. the reaction rates for the decay of BR 411 and for the formation of BR 565 are decreased, and BR 646 cannot be detected. One possible explanation is that during the observed conformational change of bacteriorhodopsin in the BR 565→ BR 411 transition

(Oesterhelt and Hess, 1973) previously masked chemical groups are exposed and then affected by the solvent.

During illumination intact halobacteria as well as isolated purple membranes generate pH-changes in the medium. Measurements by means of pH-electrodes with a time resolution of seconds indicated that the purple membrane releases protons during the BR 565→ BR 411 transition and takes up protons again in the BR 411→ BR 565 transition (Oesterhelt and Hess, 1973). This result is supported by the fact that the Schiff base retinylidene lysine linkage is protonated in BR 570 but unprotonated in BR 412 (Lewis *et al.*, 1974).

No proton release during the formation of BR 411 and no proton uptake in the BR 411→ BR 565 transition could be measured by means of the pH-indicator bromocresol green, which permits the detection of pH-changes in the medium in the time range of microseconds. However, proton uptake and release by the purple membrane which paralles in time the formation and decay of BR 646 was measured (Fig. 8). Thus it could be possible that BR 646 is a protonated intermediate which may be in equilibrium with another unprotonated intermediate of the cycle, *e.g.* BR 411. The purple membranes in solution represent membrane sheets (Blaurock and Stoeckenius, 1971) and most probably do not reseal to vesicles, thus the detected pH-changes cannot be due to a transmembrane proton transport.

It is not known, to what extent these measured pH-changes are connected to the energy converting function of bacteriorhodopsin in *Halobacterium halobium*. Morphological observations indicated an asymmetric distribution of bacteriorhodopsin in the purple membrane (Blaurock and Stoeckenius, 1971). Therefore, it might be possible that *in vivo* during illumination protons are taken up on the cytoplasmic side of the purple membrane during formation of BR 646 and released on the outer surface during decay of BR 646. This vectorial process would generate an electrochemical gradient across the cell membrane which might be used for ATP synthesis by the cells.

It should be mentioned, however, that the reaction rate of the BR 411→ BR 565 transition is insensitive to the pH of the medium (Stoeckenius and Lozier) and that the proton bindung process according to Oesterhelt and Hess (1973) is not directly coupled to this shift of the absorption maximum.

At least four intermediates are engaged in the reaction cycle of BR 565, which is probably responsible for the energy converting function of the purple membrane in *Halobacterium halobium*. Two (possibly three) intermediates appear before and one after the formation of BR 411. Only the first step in the cycle is a photochemical reaction, the following ones are temperature sensitive dark reactions. During formation and decay of BR 646, which may occur in an alternate parallel pathway, protons are taken up and released by the purple membrane. The reaction step which is responsible for the sensory function of bacteriorhodopsin in *Halobacterium halobium* is unknown.

Acknowledgements. We are grateful to Dr. E. Hildebrand and Prof. Dr. H. Stieve for insightful discussions throughout the course of the work and for helpful comments during preparation of the manuscript. We wish to thank Drs. M. Behrens, G. Nöll, and W. Sperling for reading the manuscript.

This work was supported in part by the Deutsche Forschungsgemeinschaft and belongs to the research program SFB 160.

References

Blaurock, E. A., Stoeckenius, W.: Structure of the purple membrane. Nature New Biol. **233**, 152—154 (1971)

Danon, A., Stoeckenius, W.: Photophosphorylation in *Halobacterium halobium*. Proc. Nat. Acad. Sci. USA **71**, 1234—1238 (1974)

Dencher, N.: Functions of bacteriorhodopsin. In: Biochemistry of sensory functions (ed. L. Jaenicke), pp. 161—163. Berlin-Heidelberg-New York: Springer 1974

Hildebrand, E., Dencher, N.: Photophobische Reaktionen von *Halobacterium halobium* und ihre Beziehung zum Retinal-Protein-Komplex der Zellmembran. Ber. dtsch. botan. Ges. **87**, 93—99 (1974)

Kayushin, L. P., Sibeldina, L. A., Lasareva, A. B., Vsevolodov, N. N., Kostikov, A. C., Richireva, G. T., Chekulaeva, L. N.: Membrane protein bacteriorhodopsin from halophilic bacteria. Studia biophys. **42**, 71—74 (1974)

Lewis, A., Spoonhower, J., Bogomolni, R. A., Lozier, R. H., Stoeckenius, W.: Tunable laser resonance Raman spectroscopy of bacteriorhodopsin. Proc. Nat. Acad. Sci. USA **71**, 4462—4466 (1974)

Nöll, G. N.: Reaktionskinetische Untersuchungen an Rinder-Rhodopsin und Stäbchenaußensegmenten sowie 11-cis Retinal. Aachen: Thesis 1974

Oesterhelt, D., Stoeckenius, W.: Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. Nature New Biol. **233**, 149—152 (1971)

Oesterhelt, D., Hess, B.: Reversible photolysis of the purple complex in the purple membrane of *Halobacterium halobium*. Europ. J. Biochem. **37**, 316—326 (1973)

Oesterhelt, D., Meentzen, M., Schuhmann, L.: Reversible dissociation of the purple complex in bacteriorhodopsin and identification of 13-*cis* and all-*trans*-retinal as its chromophores. Europ. J. Biochem. **40**, 453—463 (1973)

Oesterhelt, D., Stoeckenius, W.: Functions of a new photoreceptor membrane. Proc. Nat. Acad. Sci. USA **70**, 2853—2857 (1973)

Racker, E., Stoeckenius, W.: Reconstitution of purple membrane vesicles catalyzing light-driven proton uptake and adenosine triphosphate formation. J. Biol. Chem. **249**, 662—663 (1974)

Sengbusch, G. v.: Reaktionskinetische Untersuchungen an Stäbchen-Suspensionen und Rhodopsin-Lösungen mit der Blitzlicht-Methode. Aachen: Thesis 1970

Stoeckenius, W., Lozier, R. H.: Light energy conversion in *Halobacterium halobium*. J. Supramol. Struct. (in press)

Witt, H. T.: On the analysis of photosynthesis by pulse technique in the 10^{-1} to 10^{-8} second range. In: Nobel Symposium 5: Fast reactions and primary processes in chemical kinetics (ed. S. Claesson). New York: Interscience Publishers 1967

N. Dencher
M. Wilms
Institut für Neurobiologie
Kernforschungsanlage Jülich
D-517 Jülich 1
Postfach 365
Federal Republic of Germany