THE PHOTOCHEMICAL CYCLE OF HALORHODOPSIN: ABSOLUTE SPECTRA OF INTERMEDIATES OBTAINED BY FLASH PHOTOLYSIS AND FAST DIFFERENCE SPECTRA MEASUREMENTS

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ABSTRACT Results of experiments using flash photolysis and fast difference spectroscopy suggest an extended version of the earlier published scheme of the photochemical cycle of halorhodopsin. Detailed experimental verification of the suggested photocycle is given. Due to the high resolution of the time-resolved difference spectra, absolute spectra of the intermediates in the photocycle were derived, allowing the interpretation of complex kinetic absorbance changes.

INTRODUCTION

The retinal protein halorhodopsin (HR) in the cytoplasmic membrane of *Halobacterium halobium* acts as a light-driven chloride pump (1, 2; for a recent review see reference 3). Like the well-known bacteriorhodopsin, HR undergoes a photocycle in the millisecond time range upon illumination. In a previous report we suggested a reaction scheme that explained satisfactorily the experimental results obtained in 1 M sodium chloride (4). Briefly, after formation of the photoproduct HR_{600} (species of HR with maximal absorbance at 600-nm) in 5 ps (5, 6) two main intermediates, HR_{520} and HR_{640}, occur in the millisecond time range. These intermediates are connected via a chloride-dependent equilibrium. Only HR_{640} can revert back to the initial state HR_{578}, which is the educt of the photocycle in 1 M sodium chloride. This initial state splits into two states, HR_{565} and HR_{578}, which also equilibrate Cl^- dependently. Both initial states can deprotonate in the dark and form HR_{410} intermediates. A detailed analysis of these dark equilibria is given by Schobert et al. (7).

HR_{565} can also be excited by light to produce HR_{640} with a much faster rise time than in the presence of Cl^- without being preceded by any blue-shifted intermediate (8).

Here we report on experiments using flash photolysis and fast difference spectroscopy, which allowed us to verify the suggested sequence of reactions and to quantify rise and decay times of the intermediates.

It is noteworthy to point out that we regard in this report the photocycle as the set of reactions occurring up to the millisecond time range, connected linearly and cyclically. Slower reactions or side reactions, such as the formation of HR_{410} or light/dark adaptation, are not considered in this report, because they do not appear during the cycle itself and therefore play no significant role in the understanding of the photocycle. Nevertheless it has been shown (9, 10) that they can regulate the pump activity and therefore play a crucial role in the action of HR as a photochromic pigment.

MATERIALS AND METHODS

HR was isolated from OD2 cells (bacteriorhodopsin negative strain) by the method described previously (11), with some modifications described elsewhere (9). For exchange of the buffer solutions HR was dialyzed twice for 2 h against a 1,000-fold excess of volumes at 4°C in the dark just before the measurements. After dialysis octylglucoside (OG) was added to give a final concentration between 1 and 1.5%. To substitute Cl^- in the buffer solution NO_3^- was chosen, which binds to one of the two anion binding sites of HR and therefore stabilizes the protein. Electrical experiments have demonstrated that transport activity is absent in 1 M NO_3^- (12). Determination of Cl^- concentration was done by titration with silver nitrate in the presence of potassium bichromate as an indicator, according to (reference 13).

Nevertheless it should be stated that in the kinetic analysis of the data the actual Cl^- concentration was taken to be the only variable affected by replacement of chloride. Secondary effects of the replacement of Cl^- by NO_3^-, like the action of KNO_3 as a chaotropic reagent and putative alterations of time constants, were considered to be small and therefore were neglected. In fact occasionally constants in the photocycle were shown to be identical in 1 M Cl^- and 1 M NO_3^-, supporting this assumption.

Isolation of Halobacterial Lipids

Halobacterial cells of strain *H. halobium* JW5 (retinal negative, bacterioruberine negative strain) were grown under standard conditions (14) and harvested from a 30 liter culture. After resuspension in 200 ml basal salt and addition of 10 mg DNase, lipids were extracted with 750 ml chloroform/methanol (1:2 vol/vol) followed by the addition of 250 ml chloroform and 250 ml water. Thorough mixing and short centrifugation at 1,500 *g* for 10 min separated a lower phase, which was dried with anhydrous Na_2SO_4. The solvent was evaporated and the lipids were stored in chloroform (20 mg/ml) at −20°C.

Preparation of HR_L

Halobacterial lipids were freed from solvent and a twofold excess (wt/wt) of OG dissolved in water (10% wt/wt) was added. The lipids were dissolved at 60°C in a sonication bath and mixed with HR_{OG} at a molar ratio of 20:1 on the basis of an average molecular mass of 1,000 D for the lipids. After dialysis of the sample against 1 M NaCl and 10 mM 3(*N*-morpholino)-propane sulfonic acid (MOPS) pH 7 for 6 d to remove OG, the sample was concentrated by centrifugation at 10°C and 100,000 *g* for 16 h.

Experimental Setup

Flash photolysis experiments were carried out with the apparatus described earlier (15), except that a dye laser (model FL 2000; Lambda Physics, Göttingen, FRG) was used for excitation. In control experiments with various light intensities and qualities, it was ensured that the light used did not affect the absorbance changes. Actinic light was used in the linear range of the dose effect curve. Additionally, spectra before and immediately after the measurement were taken and no significant changes were observed. Intensity of the measuring light was 20 $\mu W/cm^2$ for the flash photolysis experiments.

The transient difference spectra were recorded using the polychromatic flash apparatus described recently (16). Evaluation of the time constants of the measured traces was done using a least squares fitting routine. The quality of the fit was checked by residual plots.

RESULTS

Rise and Decay of HR_{520}

Excitation of halorhodopsin with green light in the presence of molar concentrations of sodium chloride leads to a dominant photointermediate, HR_{520}. Fig. 1 shows its rise (*A*) and decay (*B*) at room temperature measured at 510 nm. Both rise and decay traces can be fitted with single exponentials corresponding to relaxation times of 0.85 μs (rise) and 14 ms (decay). The residual plots between measured and fitted curves are given in Fig. 1, *C* and *D*.

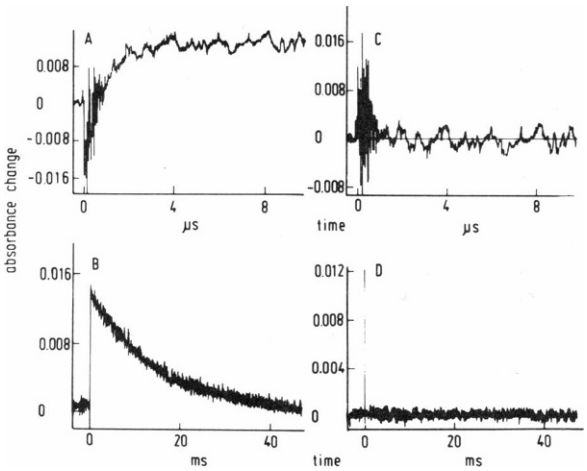

FIGURE 1 Absorbance changes at 510 nm and after flash excitation at 580 nm of a 12.5 μM HR solution in 1 M NaCl, 10 mM MOPS pH 7.0, and 1% OG. (*A*) Rise of the absorbance change at 510 in 0.85 μs, deviations in the beginning of the traces between measured and fitted curve are due to the laser flash. (*B*) Relaxation of the absorbance at 510 nm to the initial value in 14 ms. In *C* like *D* the residual plot between measured and fitted curve is shown.

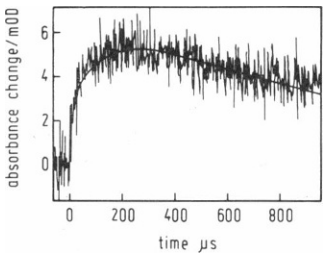

FIGURE 2 Absorbance change at 470 nm after flash excitation at 580 nm of a 20 μM HR solution in 1 M NO_3^-, 10 mM MOPS pH 6.0, and 1% OG. The solid line represents the fitted curve, revealing the time constants mentioned in the text.

Removal of chloride by dialysis against sodium nitrate reduces the intermediate concentration of HR_{520} upon flash excitation and also changes the kinetics of its formation and decay (Fig. 2). However, dialysis does not eliminate chloride completely and we found a residual concentration between 2 and 3 mM in our samples. The experimental curve of absorption changes at 470 nm under these conditions can be fitted in a first approximation by two time constants with 100 μs for the rise and 300 μs for the decay. A refined fit reveals a second decay time constant of 5 ms with a small amplitude. The rise time has a value of 100 μs in nitrate and increases upon chloride addition as shown in Table I, reaching ~1 μs at molar concentrations. This result confirms the general scheme obtained for the HR photocycle by previous experiments (4; Fig. 10), and further quantitates the rise of HR_{520} as a chloride-dependent step in the microsecond range. The chloride concentration does not increase the velocity of HR_{520} formation proportionally but saturates at higher concentration, indicating a more complex kinetic relationship (see Discussion).

The decay of HR_{520} in the virtual absence of chloride leads to HR_{640} with a half-time of 300 μs (see Fig. 10 and below). The residual 2–3 mM chloride will not influence this rate markedly because equilibration of HR_{640} and HR_{520} occurs at ~100 mM (see Fig. 4). Therefore the rise time of 300 μs corresponds to the rate constant $HR_{520} \rightarrow HR_{640}$. The occurrence of a small amplitude of 5 ms

TABLE I
Cl^--DEPENDENCE OF THE RISE TIME OF HR_{520}

Cl^- addition	Fitted time constant
μmol	*μs*
No addition	16/100*
4	13
8	8
16	3
128	3.6
256	0.7
2,000	1.1

Chloride was added from a 1 or 4 M NaCl stock solution to a measuring volume of 2 ml and flash photolysis carried out as described in Materials and Methods.

*100 μs is the time constant determined in an earlier set of experiments (i.e., Figs. 2 and 6). The sensitivity of the rise time to Cl^- in the millimolar concentration range and the impossibility to control perfectly chloride concentration explain larger deviations in different experiments.

half-time is expected after flash excitation due to the build-up of a stationary state involving HR_{520} and HR_{640}. This equilibrium couples the decay of HR_{520} to that of HR_{640} and causes the 5-ms component.

In Fig. 3 experimental evidence for the conversion of HR_{600} to HR_{520} is presented and best explained with the help of the absolute spectra of intermediates in Fig. 9. The primary photoproduct HR_{600} is formed in 5 ps (5, 6) and causes a positive change in absorption at 605 nm, which is the wavelength selected for the experiment of Fig. 3. In the presence or absence of chloride, a photoproduct with the same kinetics and very similar spectral properties is formed (Franz, A., manuscript in preparation). The positive absorption change due to HR_{600} formation is constant for at least 300 ps, indicating a life-time of more than 900 ps. On the other hand, the time resolution of the equipment used in this work was restricted to 100 ns. In the experiment of Figs. 6 and 3 *A* the positive absorption change at 605 nm within the time resolution of the instrument is followed by a decrease below the zero level with a time constant of 1.1 μs. This change in absorption corresponds to the formation of HR_{520} in sign (see Fig. 9) and time. Thus we conclude that HR_{520} is formed directly from HR_{600} but further experiments in the nanosecond time range will be necessary to demonstrate this conclusively.

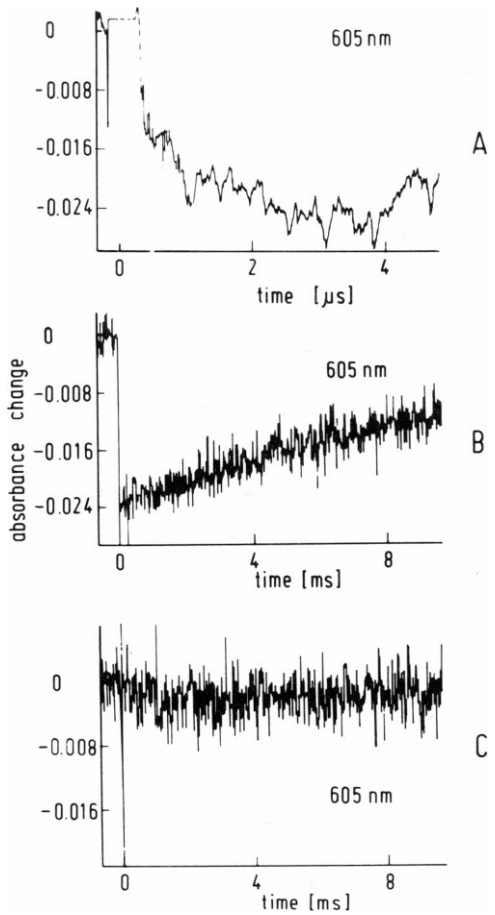

FIGURE 3 Absorbance changes at 605 nm after flash excitation at 620 nm of a 9.5 μM HR solution in 100 mM KSCN, 500 mM Na_2SO_4, 200 mM NaCl, 10 mM MOPS pH 6.0, and 1% OG. (*A*) Depletion with a time constant of 1.0 μs, measured with a time resolution of 10 ns per point. (*B*) Return to the initial value in 12 ms and (*C*) no absorbance changes detectable before addition of Cl^-.

The negative absorption change reverts with a time constant of 12 ms as expected for the re-formation of the initial state HR_{578} from HR_{520} (Fig. 3 *B* and Fig. 9). In Fig. 3 *C* a control experiment is shown where Cl^- was omitted from the sample solution. Under these conditions HR_{640} instead of HR_{520} is the dominant intermediate. The apparent lack of a change in absorption at 605 nm demonstrates that this wavelength is close to the isosbestic point of HR_{578}/HR_{565} and HR_{640} (see Fig. 9) and that the change seen in Fig. 3 *B* must be mainly due to the decay of HR_{520}.

Chloride Dependence of the HR_{520}/HR_{640} Equilibrium

The chloride concentration in flash photolytic experiments determines the main intermediate observed. At 510 nm the difference of the molar extinction between HR_{578} and HR_{520} is 13,000, whereas at 660 nm the difference between HR_{565} and HR_{640} is approximately 24,000 (Fig. 9 and Table II). Thus the cross-over point at ~170 mM chloride in Fig. 4 is not the concentration at which the amounts of the intermediates HR_{520} and HR_{640} are equal. Correction with the differential molar extinction (see Fig. 9) yields the molar ratio of 1:1 at 100 mM Cl^-. From this number the real equilibrium constant between HR_{520} and HR_{640} can be calculated if all kinetic constants are known (manuscript in preparation). The experiment confirms that in contrast to the initial state HR_{565} (10 mM), the intermediate HR_{640} has a lower affinity for chloride (100 mM; see also references 7 and 8). The set of experimental data was then extended to 31 different wavelengths, and representative intermediate spectra after a 25-μs flash are shown in Fig. 5, *A* and *B*. As already seen in Fig. 4 the main intermediate in NO_3^- is HR_{640} and in Cl^- HR_{520}, but both intermediates are present at the optimal conditions for the other species.

The primary photoproduct HR_{600} does not contribute to the intermediate difference spectra due to its short life-time. The difference spectra in Fig. 5, *A* and *B* decay with time after the flash, and the expected shifts of the isosbestic points were observed. In Fig. 5, *C* and *D* the time course

TABLE II
SPECTRAL CHARACTERISTICS OF THE DIFFERENT SPECIES OF THE PHOTOCYCLE

Species	λ_{max}	Half-width	Molar absorbance
	nm	*nm*	$M^{-1}cm^{-1}$
HR_{578}	578	104	50,000
HR_{565}	565	112	45,000
HR_{600}	598	82	53,000
HR_{640}	642	75	39,000
HR_{520}	527	96	39,000

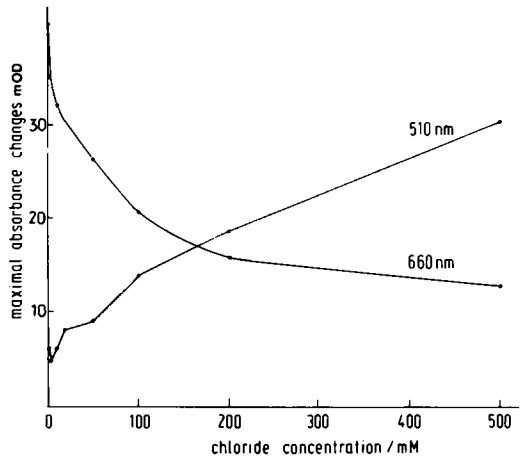

FIGURE 4 Amplitudes of the maximal absorbance changes at 640 and 510 nm after flash excitation at 580 nm under various Cl^- concentrations. To a solution of 11.7 μM HR in 100 mM KSCN, 500 mM Na_2SO_4, 10 mM MOPS pH 6.0, and 1% OG NaCl were added from a 4 M stock solution to give the final concentration as indicated.

of absorption changes at selected wavelengths in the same experiment are shown. The trace measured at 490/500 nm represents mainly concentration changes of HR_{520}, at 570 nm the initial states are monitored, and HR_{640} is the only species absorbing at 680 nm (see Fig. 9). The measured traces in chloride as well as in nitrate can be fitted by a set of two exponentials. In nitrate (Fig. 5 *D*) HR_{520} decays and HR_{640} rises with 300 μs, whereas HR_{640} decays and HR_{565} reappears with 3 ms. In chloride, HR_{520} decays in a biphasic manner with relaxation times of 1 and 14 ms, and HR_{640} rises with 1 ms and decays with 14 ms. Concomitantly, HR_{578} reverts with 14 ms. The biphasic change in HR_{520} concentration is explained by the intermediate establishment of a stationary state mixture of both species after the flash.

HR_{640} is Formed by a Second Route in the Absence of Chloride

In 1 M nitrate our sample still contains ~ 2–3 nM chloride. Nevertheless HR_{565} contributes dominantly to the initial state mixture and therefore a complex pattern of kinetics is expected upon flash excitation. This is demonstrated by the experiment shown in Fig. 6. Absorbance changes in NO_3^- at various wavelengths on the microsecond scale are presented. Whereas the experiments of Figs. 2 and 5 demonstrated the rise of a small HR_{520} amplitude (due to the residual chloride) with a relaxation time of 100 μs and its conversion to HR_{640} with 300 μs, an additional fast and direct formation of HR_{640} from HR_{600} was observed. To make this fact obvious again the absolute spectra in Fig. 9 are helpful. At 600 nm a monotonic decrease in absorption from a positive value to a negative value occurs, indicating

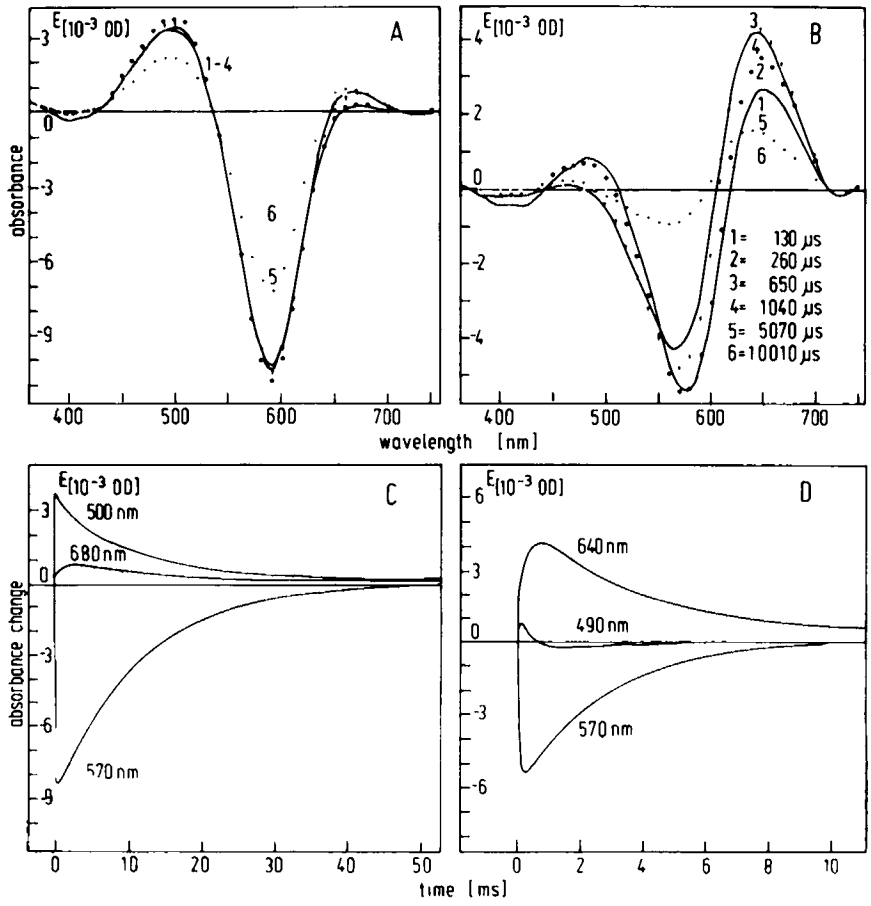

FIGURE 5 Difference spectra recorded at marked time intervals after flash excitation in 1 M Cl^- (*A*) and 1 M NO_3^- (*B*). (*B*) Curve *1*, 130 μs; *2*, 260 μs; *3*, 650 μs; *4*, 1,040 μs; *5*, 5,070 μs; *6*, 10,010 μs. The absorbance changes at selected wavelengths as a function of time is shown in *C* (Cl^-) and *D* (NO_3^-).

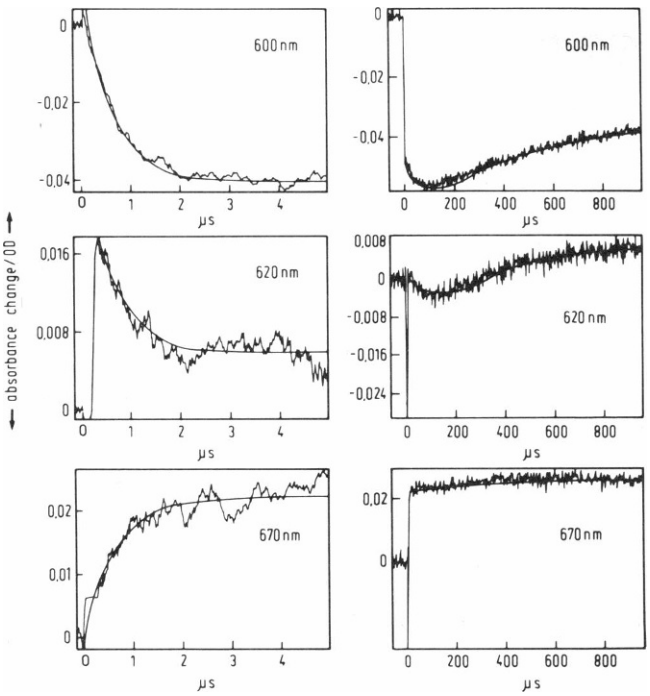

FIGURE 6 Absorbance changes at selected wavelengths measured with a time resolution of 20 ns per point and 1 μs per point. The experiments were done with a 16 μM HR solution in 1 M KNO_3, 10 mM MOPS pH 6, and 1% OG. Excitation wavelength was 580 nm; the traces shown are the average of 128 flash responses.

that the formation of HR_{600} (positive absorption change) is followed by conversion to HR_{640}. The solid line represents a fit with a relaxation time of 0.6 μs. At 620 nm the same time constant fits the experimental curve and the signs of the amplitudes are predicted by the fact that at this wavelength HR_{640} has a higher molar extinction than HR_{565} but a lower value than HR_{600}. At 670 nm only a positive absorption change with the same time constant of 0.6 μs occurs (the contribution of HR_{600} is hidden in the initial phase by the laser artifact).

In Fig. 6 (*right*) the absorption changes up to 1 ms in the same experiment are shown and clearly the complex pattern indicates the participation of more than one rate. At 600 nm after the formation of the main portion of HR_{640} directly from HR_{600} with 0.6 μs the minimal absorption is reached with a time constant of 100 μs typical for the HR_{520} formation in nitrate. Then the absorption change decreases with 300 μs halftime due to conversion of HR_{520} to HR_{640}. The traces at 620 and 670 nm can be fitted by the same two relaxation times and confirm that nitrate, which contains small amounts of chloride HR_{640}, is formed by two alternative pathways starting from HR_{565} and HR_{578} with time constants of 0.6 μs ($HR_{600} \rightarrow HR_{640}$) and 300 μs ($HR_{520} \rightarrow HR_{640}$).

It should be stressed that all our experiments are done under experimental conditions that keep HR in the light-adapted state and in the presence of either 1 M or, alternatively, a few millimolar concentration of chloride. HR is not in its natural lipid environment but the sample contains 1% OG and traces of Lubrol PX, a leftover from the purification procedure. To study the detergent effect, if any, two additional samples were prepared: one by addition of halobacterial lipids and removal of OG by dialysis, and the other by additon of 1% Lubrol. Fig. 7 shows a representative experiment with all three samples. If scaled, due to the different amounts of HR in the sample, negligible differences in the time course of the absorption changes are found, indicating that the three conditions do not significantly change the photochemical behavior of HR.

Absolute Spectra of Parent Species and Intermediates

The concomitant occurrence of HR_{640} and HR_{520} after a flash prevents the derivation of absolute spectra in a simple way. If, however, time windows can be found during which one of the intermediate concentration is stationary, the changing concentration of the other intermediate must correlate in a 1:1 ratio to the repopulation of the parent species. As seen from the time course in Fig. 5 *C*, HR_{640} remains constant in the time range 1.4–4.0 ms and therefore the time-resolved difference spectra reflect the HR_{520} to HR_{578} transition. The same procedure was repeated in 1 M nitrate in the time range 4–5.5 ms (compare Fig. 5 *D*) analyzing the HR_{640} to HR_{565} transition. The result is given in Fig. 8, *C* and *D*. The occurrence of a clear isosbestic point in these two difference spectra validate the assumption of A → B transition.

At the blue and red edges of the difference spectra little contribution of the respective intermediates is to be expected, so this part of the spectra can be used for calculation of the fraction of cycling HR. Additon of this fraction of the spectrum of the respective parent species shown in Fig. 8, *A* and *B* to the difference spectra yields the

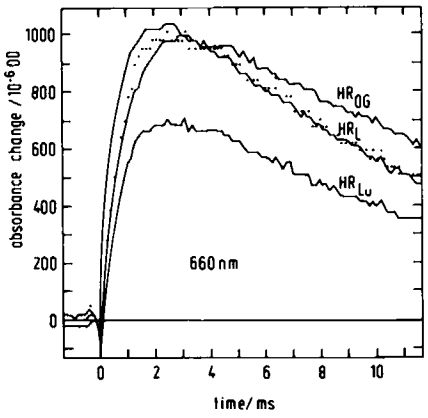

FIGURE 7 Absorbance changes at 660 nm of HR in 1% *n*-octylglucoside (HR_{OG}), in 1% Lubrol (HR_{Lu}), and mixed with halobacterial lipids (HR_L). No significant difference in the kinetic behavior of the three samples could be detected. The trace of HR_{Lu} was scaled up to have the same amplitude as the trace of HR_L (*dots*). The concentration of HR was 14 μM for the traces of HR_{OG} and HR_L and 9 μM for HR_{Lu}. All three measurements were done in 1 M NaCl and 10 mM MOPS, pH 6.0.

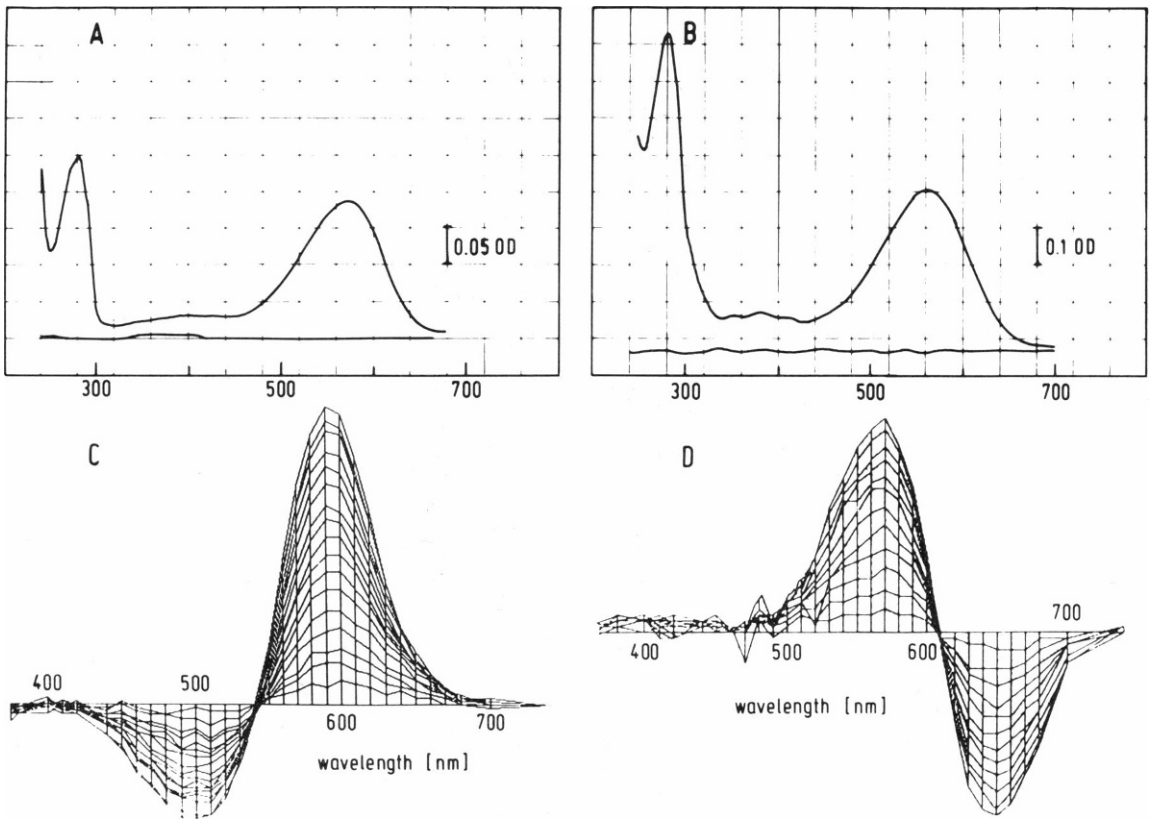

FIGURE 8 Absolute spectra of the two initial states HR_{578} (*A*) and HR_{565} (*C*) and the calculated difference spectra at various times after flash excitation with the two main intermediates HR_{520} in 1 M Cl^- (*B*) as well as HR_{640} in 1 M NO_3^- (*D*). Note the pronounced isosbestic point in the time-resolved difference spectra, justifying the validity of the calculations used. Time difference between the difference spectra is 130 μs.

absolute spectrum of the intermediate. Conveniently, the HR_{640} spectrum is obtained from experiments in nitrate and that of HR_{520} in chloride. As a control, the HR_{640} spectrum was additionally derived from experiments in chloride and the HR_{520} spectrum from nitrate samples. The results obtained were congruent and the spectra of the five known HR species are shown in Fig. 9. Absorption maxima, molar absorbance, and half-width of the bands are summarized in Table II. The molar absorbance of 50,000 $M^{-1}cm^{-1}$ of HR_{578} in OG was taken from reference 11, and the value of HR_{565} was derived from data in references 17 and 18. The absolute values for the molar absorbancies of

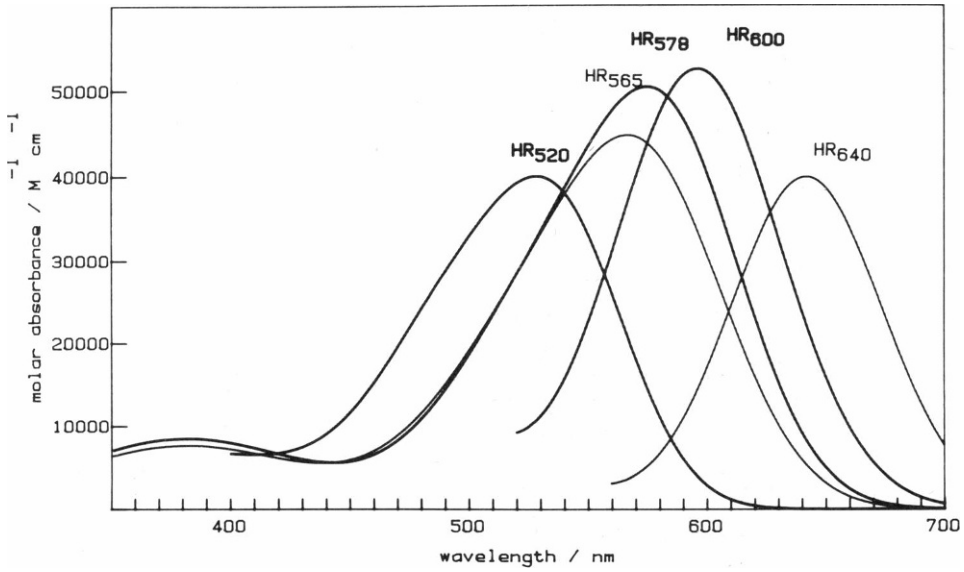

FIGURE 9 Absolute spectra of all known species in the HR photocycle. The spectrum of the first photoproduct HR_{600} was taken from reference 5. The error for the spectra due to the scaling procedure is estimated to be ~5%.

HR_{520} and HR_{640} were directly derived by the calculation procedure described above with the absolute value of HR_{578} as reference. The absolute spectrum of HR_{600} was taken from reference 5.

DISCUSSION

From the kinetic measurements reported here we confirm the model of the photochemical cycle in halorhodopsin as proposed earlier (4). For the first time, however, the rise time of HR_{520} is measured and it is shown that this step is chloride dependent. HR_{520} was thought not to occur in the absence of chloride but could be observed in samples containing 1 M nitrate. We found 2–3 mM chloride in these solutions in contrast to the analyses given by the suppliers.

Early research on halorhodopsin already demonstrated the occurrence of two light reactions in halorhodopsin, one starting from HR_{578}, the other from HR_{565} (8, 19). We include this reaction in our photocycle scheme by connecting HR_{565} to HR_{640} via the primary photointermediate HR_{600} seen in picosecond experiments in 1 M nitrate solutions (Franz, A., W. Zinth, J. Tittor, and D. Oesterhelt, manuscript in preparation). Quantum yield measurements gave approximately the same value for the reaction of HR_{565} as found for HR_{578}. Based on this quantum yield the absolute spectrum of the primary photoproduct in nitrate is found to be identical to that in 1 M chloride and we therefore call the species in both cases HR_{600}. This is somewhat surprising because in the parent species the retinal Schiff base in halorhodopsin encounters different ionic environments. Thus, in HR_{565}, binding site I (7) is occupied by nitrate, but binding site II is empty. In HR_{578} both binding sites are occupied by chloride. This difference in ionic environment causes a λ_{max} difference between the two species of 13 nm and is explained by the general effect of binding anions, which cause a small blue shift upon occupation of site I, but a small red shift upon occupation of site II. The transition from HR_{578} to HR_{600} is connected to the release of a chloride ion. This is required by the chloride uptake- and release balance of the scheme of the photocycle shown in Fig. 10. The alternative assumption of the release of chloride during transition from HR_{640} to HR_{565} is excluded because the second photoreaction starting from HR_{565} is not chloride dependent. As a consequence, the HR_{600} species in nitrate and chloride are different based on the ions occupying sites I and II. HR_{600} in nitrate solution has nitrate at site I and HR_{600} in chloride has chloride either at site I or at site II. Since the absorption maximum of both species is identical within the limits of error we take this as an indication that the primary step of the HR_{600} in chloride is accompanied by release of chloride from site II. An alternative explanation would be that in HR_{600} the occupancy of site II does not influence the chromophore anymore, indicating that the physical distance between site II and the retinal has enlarged to the extent that any interaction is interrupted.

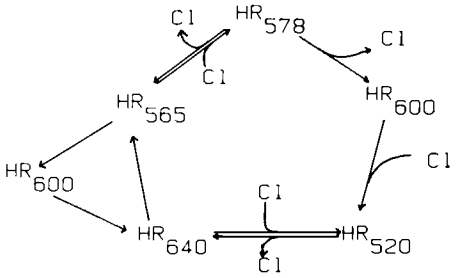

FIGURE 10 Scheme of the photocycle of HR. The indices denote the maxima of absorbance of the species. Side reactions of the cycle such as the formation of HR_{410} are omitted because they are not treated in the context of this report.

The occurrence of two photocycles in halorhodopsin raises the question of its physiological significance. In saturated brines, where chloride is the dominant anion, the photochemistry of halorhodopsin will be governed dominantly by the photocycle starting from HR_{578} and the non-translocating ion cycle starting at HR_{565} bears no significance. It should be mentioned, however, that the known retinal proteins are in general photochemically active in all their conformational states under non-physiological conditions, but only very few of these reactions have physiological significance. In halorhodopsin such activity of physiological significance is found for the reaction of HR^{L}_{410} with blue light regulating the activity of halorhodopsin (9). In addition to the photochemical reaction of HR_{565} there might be more light reactions to be detected for halorhodopsin in light- and dark-adapted states.

The model in Fig. 10 implies a direct conversion of HR_{600} to HR_{520}. HR_{520} rises with 800 ns and, so far, we have no possibility to cover the window from 1 to 100 ns. The absorption changes due to formation of HR_{600} are constant for more than 300 ps (5), indicating that HR_{600} has a half-time of at least several nanoseconds since the accuracy of absorption change measurements in our experiments is within ~5%. If an additional intermediate were present in the cycle it would have to be a red-shifted species compared to HR_{520}, and have a rise time of more than 10 ns and a decay time of 800 ns. No intermediate with these characteristics has been demonstrated in retinal proteins, but further experiments in this time range will be necessary to examine this point.

A very interesting result is obtained by finding the chloride dependence of HR_{520} rise. We interpret this according to our model of chloride translocation (20) with the approach of a chloride ion to the positively charged nitrogen of the Schiff base after the movement of this group in the primary reaction leading to HR_{600}. This is in agreement with the saturation phenomenon observed for the acceleration of the HR_{520} rise by chloride.

Another result that will be important for quantitation of the photochemical cycle is the aboslute spectra presented in Fig. 9. Using the procedure described here we obtain absolute spectra for HR_{520} and HR_{640} under both ionic

conditions applied in 1 M nitrate solution as well as in 1 M chloride solutions. Under both conditions identical absolute spectra were obtained demonstrating the reliability of the procedure applied. This result answers the question of whether HR_{640} and HR_{520} in chloride and nitrate are spectroscopically identical (8, 21). The spectral bands of the intermediates have the usual asymmetry deviating from the typical Gaussian shape. This is known from most absorption bands of retinal proteins, but interestingly not found for HR_{640} (Fig. 9). An interpretation of this fact will be given in the context of more theoretical considerations of the absorption bands in halorhodopsin.

We would like to point out that with the help of the spectra presented the photocycle scheme and the relaxation time of rise and decay of the various intermediates allow the simulation of all different spectra observed experimentally and their kinetics. As a result, within 10% accuracy all experimental results can be simulated with one set of rate constants, and this will be described in a future paper.

We thank Susanne Meeßen for technical assistance and Drs. Peter Hegemann and Joe Farchaus for critical reading of the manuscript. Many hours of very helpful discussions with J. K. Lanyi are gratefully acknowledged.

This work was supported by Deutsche Forschungsgemeinschaft Oe 52-16/1.

Received for publication 28 April 1987 and in final form 3 August 1987.

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