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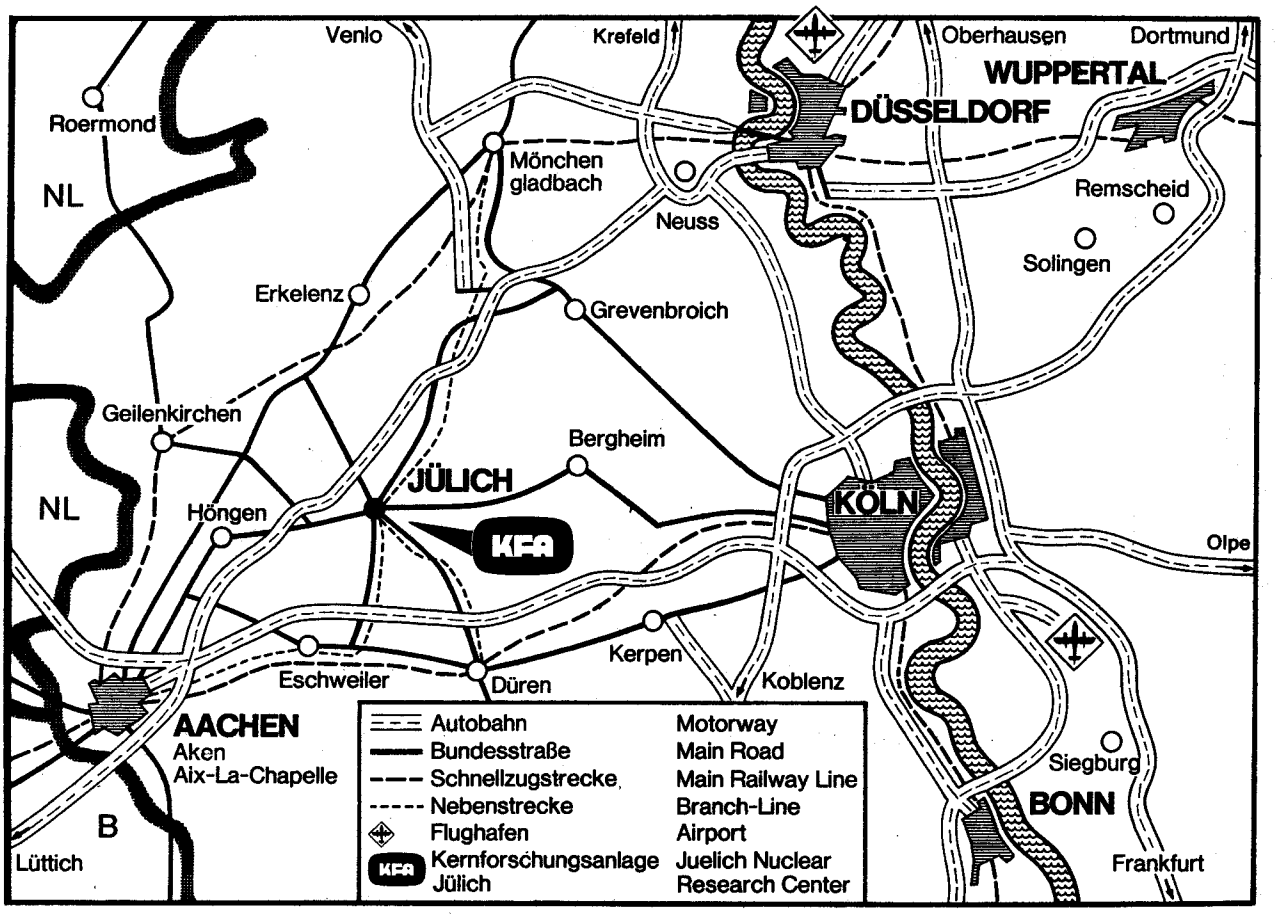
Jül - 1374
Dezember 1976

Als Manuskript gedruckt
ISSN 0366-0885



Berichte der Kernforschungsanlage Jülich - Nr. 1374

Institut für Neurobiologie Jül - 1374

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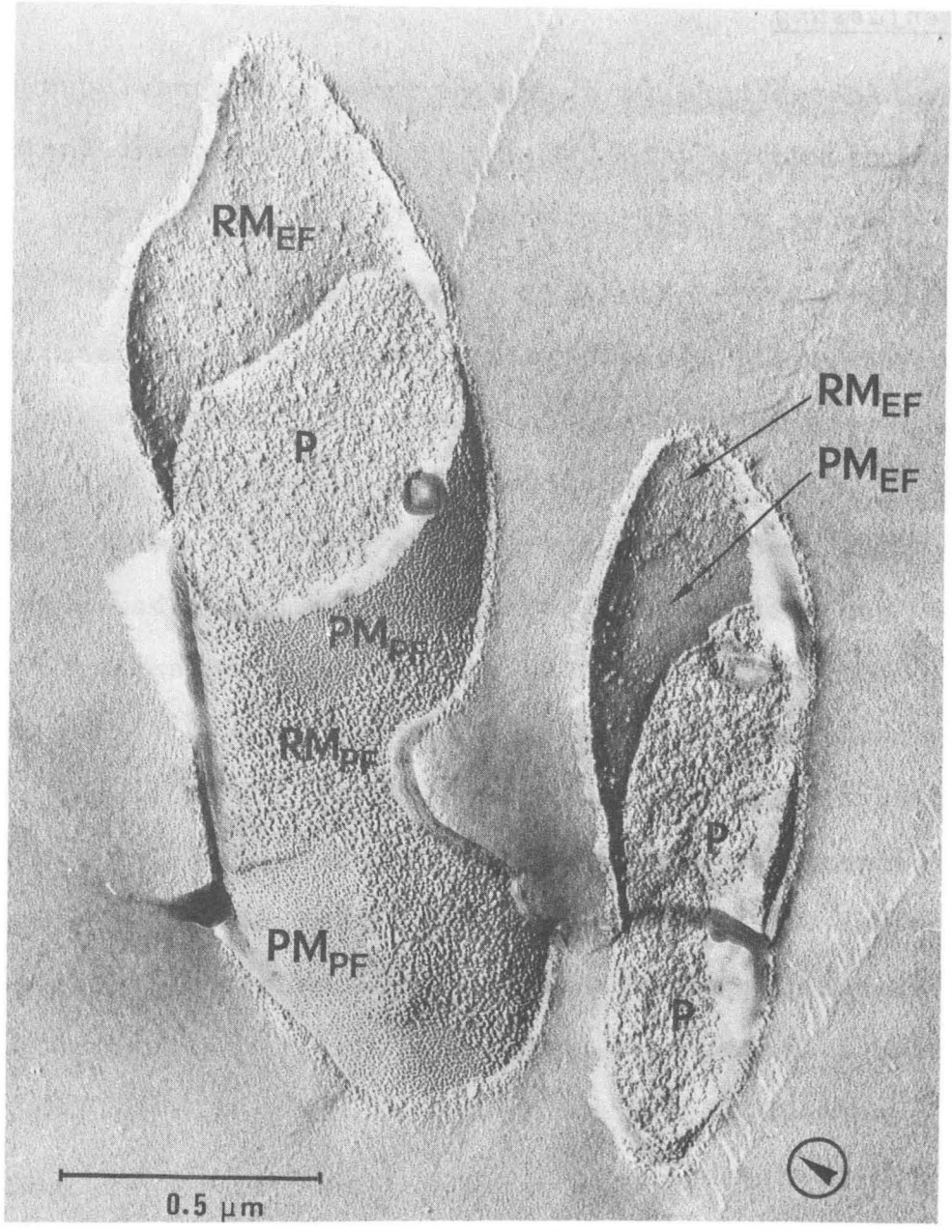

Electron micrograph from freeze-etched *Halobacterium halobium* cells in culture medium. Patches of the purple membrane are surrounded by the red membrane.

PM	purple membrane
RM	red membrane
P	plasma
PF	plasmatic fracture face
EF	extraplasmatic fracture face

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Zusammenfassung

Die Lichtenergie in chemische Energie umwandelnden Bakterien Halobacterium halobium enthalten in ihrem Photopigment Bacteriorhodopsin 13-cis- und all-trans-Retinal als Chromophor.

13-cis- ($BR^{548}_{13\text{-}cis}$) und trans-Bacteriorhodopsin (BR^{568}_{trans}) wurden einzeln hergestellt und ihre Photochemie und gegenseitige Umwandlung untersucht. Nach Lichtanregung findet man für $BR^{548}_{13\text{-}cis}$ und BR^{568}_{trans} jeweils verschiedene Photo- und Folgeprodukte. BR^{568}_{trans} geht durch einen bereits für lichtadaptierte Purpurmembranen beschriebenen Reaktionszyklus. Demgegenüber ergibt $BR^{548}_{13\text{-}cis}$ ein Photoprodukt ^{x}C, das bei -90^{o} in weniger als 50 ns entsteht und bei dieser Temperatur mit einer Halbwertszeit von 1,5 µs in ein weiteres Zwischenprodukt, ^{610}C, übergeht. ^{610}C zerfällt seinerseits mit einer Halbwertszeit von 37 ms ($20^{o}C$) in $BR^{548}_{13\text{-}cis}$ und BR^{568}_{trans}. Die Reaktion $^{610}C \rightarrow BR^{548}_{13\text{-}cis}$ verläuft nicht nur thermisch, sondern kann auch photochemisch erfolgen. ^{x}C und ^{610}C sind Zwischenprodukte bei der Photoisomerisation von $BR^{548}_{13\text{-}cis}$ nach BR^{568}_{trans}.

Auf der Grundlage der verschiedenen Photoreaktionen von BR^{568}_{trans} und $BR^{548}_{13\text{-}cis}$ wurde eine analytische Methode zur Bestimmung ihres Mengenverhältnisses entwickelt. Lebende Bakterien, isolierte Purpurmembranen und regeneriertes Bacteriorhodopsin enthalten im dunkeladaptierten Zustand BR^{568}_{trans} und $BR^{548}_{13\text{-}cis}$ im Verhältnis von jeweils 1 zu 1. Das Gleichgewicht $BR^{568}_{trans}/BR^{548}_{13\text{-}cis}$ ist zwischen 1^{o} und $60^{o}C$ nur wenig temperaturabhängig, so daß sich für die Reaktion $BR^{548}_{13\text{-}cis} \rightarrow BR^{568}_{trans}$ ein ΔH^{o} von etwa 350 cal Mol^{-1} ergibt. In lichtadaptierten Proben wurde nur BR^{568}_{trans} gefunden.

Abstract

Flash photometric experiments were performed which help to clarify the photochemistry and interconversion of 13-cis bacteriorhodopsin ($BR^{548}_{13\text{-}cis}$) and trans bacteriorhodopsin (BR^{568}_{trans}). The two bacteriorhodopsin isomers were seperately prepared by incubating the retinal-free protein with 13-cis and trans retinal, respectively.

Our results reveal that $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans} form different intermediates upon light excitation:

BR^{568}_{trans} undergoes a reaction cycle identical to that previously described for light adapted purple membrane. The intermediate ^{411}T of this cycle was found to photoreact to BR^{568}_{trans}.

$BR^{548}_{13\text{-}cis}$, in contrast, yields a photoproduct ^{x}C, which is completely present in less than 50 nsec at -90°C and decays with a half-lifetime of 1.5 µsec to a further intermediate, ^{610}C. ^{610}C decays with a half-lifetime of 37 msec (20°C) to both $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans}. In addition to the thermal pathway, ^{610}C is photoconvertible to $BR^{548}_{13\text{-}cis}$. ^{x}C and ^{610}C are intermediates in the photoisomerization pathway of $BR^{548}_{13\text{-}cis}$ to BR^{568}_{trans}.

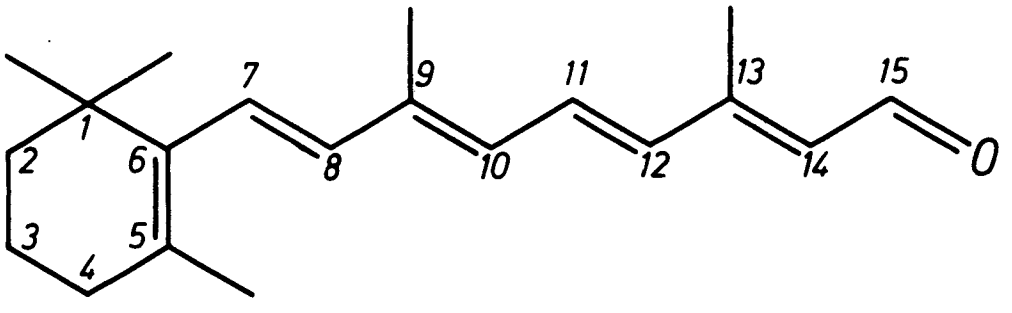
An analytic method based on the distinct photoreactions of $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans} was developed to determine the ratio in a given sample. It was found that in the dark adapted state the purple membrane of living bacteria, isolated purple membrane and regenerated bacteriorhodopsin all contain a mixture of $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans} in a ratio of about 1:1. The equilibrium constant K is only slightly dependent on the temperature between

$0^{o}C$ and $60^{o}C$ (50% BR^{568}_{trans} at $0^{o}C$, 53% BR^{568}_{trans} at $60^{o}C$). ΔH^{o} for the reaction $BR^{548}_{13\text{-}cis} \rightarrow BR^{568}_{trans}$ was calculated to be about 350 cal $mole^{-1}$. Under conditions of light adaptation, only BR^{568}_{trans} is present.

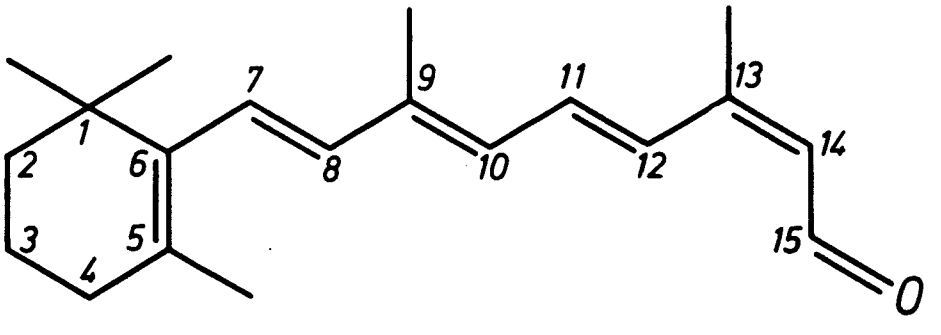
Glossary

1) Abbreviations

PM	purple membrane
BR	bacteriorhodopsin
$BR^{548}_{13\text{-cis}}$	13-cis bacteriorhodopsin
BR^{568}_{trans}	trans bacteriorhodopsin
BO	bacterioopsin
trans	all-trans retinal, alternatively also called trans retinal, because of the s-cis bond in position 6, so it is only "all"-trans in regard to the double bonds.



13-cis 13-cis retinal



Our notation of bacteriorhodopsin and its photoproducts deviates from that used in the literature. There should be no problem to find out which of our intermediates corresponds to any one described in the literature.

2) I/I_o

I_o is the intensity of the measuring light incident on the photomultiplier at the time before the flash

I is the intensity of the measuring light incident on the photomultiplier at the time given on the abscissa

3) Dark adaptation

We define dark adaptation as an equilibrium state where no further absorption changes of the sample can be detected. At lower temperatures (< 5°C) special care has to be taken to be sure the equilibrium between $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} has been reached.

Irreversible spectroscopic changes occurring over a longer time span and due to chemical decomposition are not considered in this definition. Fortunately, all bacteriorhodopsin samples are chemically extremely stable.

4) Light adaptation

A light adapted state is defined as a state, where under continuous and constant illumination with visible light no further absorption changes can be detected. The light intensity used for the samples described in this paper was in the order of 10 mW cm^{-2}. Under these conditions the photoequilibrium is practically completely on the side of BR^{568}_{trans}.

We would like to mention that the light of a normal, not even sunny, mid-European day is sufficient to push the equilibrium nearly totally to BR^{568}_{trans}.

Bacteriorhodopsin (BR) is a chromoprotein and the photopigment of the purple membrane (PM) of *Halobacterium halobium* (1). The purple membrane converts light energy into a proton gradient (2), which in turn is transformed into chemical energy (3), and also directly used for membrane transport processes (4). BR is also a photopigment for sensory functions of the bacterium (5). It is described as the only protein of the PM (1) and consists of a protein moiety and either a 13-cis or an all-trans retinal moiety (6). Protein and chromophore are covalently bound to each other via a Schiff base in a 1:1 ratio (1).

Both isomers, 13-cis bacteriorhodopsin ($BR^{548}_{13\text{-cis}}$) and trans bacteriorhodopsin (BR^{568}_{trans}), are expected to give different and specific photoproducts. Hence mixtures containing both bacteriorhodopsin isomers should be more difficult to study than samples containing only one single isomer. Consequently the investigation of the two separate bacteriorhodopsin isomers is desirable. Therefore we prepared both $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} by incubating the retinal-free protein, bacterioopsin (BO), with the respective isomers, 13-cis and trans retinal. With these two bacteriorhodopsin isomers we have performed flash photometric experiments which clarify their photochemistry and interconversion. An analytic method based on the clearly distinct photoreactions of $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} was developed to determine their ratio in a given sample.

The results of this study and literature (6,7,8,9,10), lead us to the reaction scheme shown in Fig. 1. It summarizes

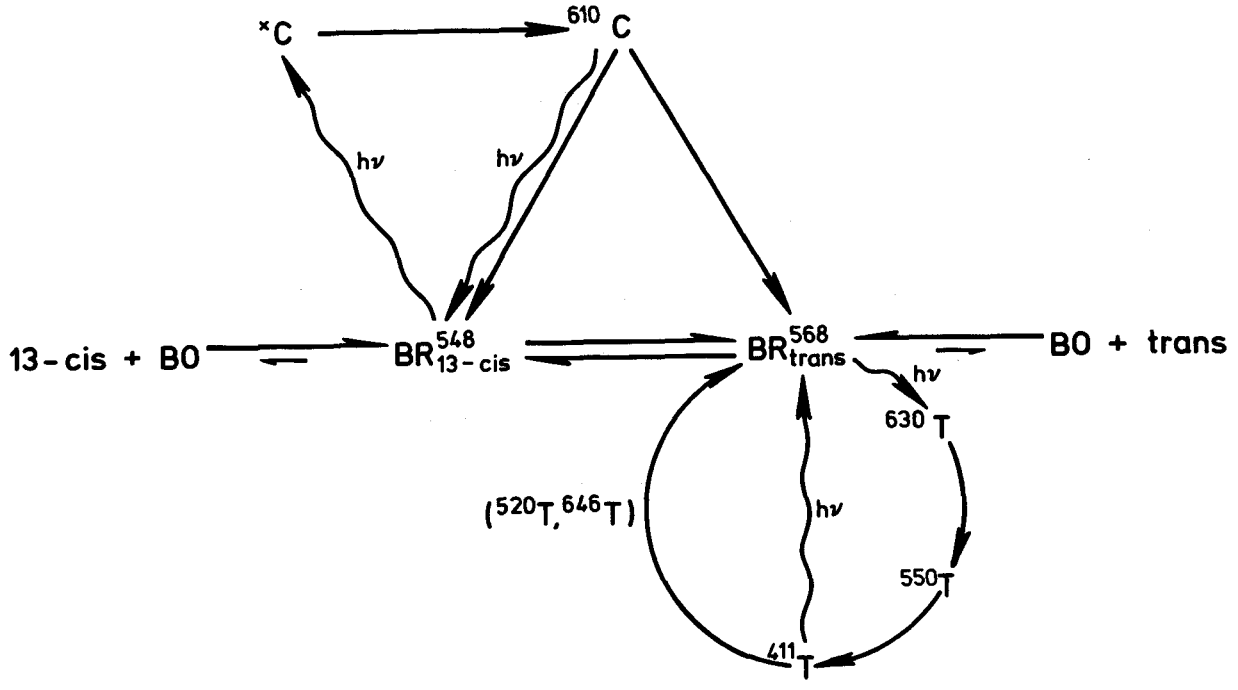


Fig. 1:

Simplified reaction scheme of the photochemistry and of the dark reactions of 13-cis and trans bacteriorhodopsin. The two bacteriorhodopsin isomers are called $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans}. Products originating from $BR^{548}_{13\text{-cis}}$ after light absorption are designated as C, products originating from BR^{568}_{trans} are called T. A superscript on the right indicates the wavelength maximum of the absorption spectrum, a superscript on the left indicates the wavelength maximum of the difference absorption spectrum (spectrum of C product minus spectrum of $BR^{548}_{13\text{-cis}}$; spectrum of T product minus spectrum of BR^{568}_{trans}). Superscript x denotes that the exact maximum of the difference spectrum is presently unknown. The location of ^{520}T and ^{646}T in the cycle is still uncertain.

what is presently known about the photochemistry and thermochemistry of BR.

Preparation of BO, $BR^{548}_{13\text{-}cis}$, and BR^{568}_{trans}

Halobacteria (*Halobacterium halobium*, strain R_1L_3) were grown and the purple membrane isolated as previously described (11). The chromophore-free protein, bacterioopsin, was prepared according to (10,11): aqueous suspensions of purple membranes (BR concentration 1.3 - 1.7 · 10^{-5} mole l^{-1}) were illuminated (halogen tungsten lamp, only $\lambda > 515$ nm used, 10 mW cm^{-2}) for about 14 hours at 10°C in the presence of hydroxylamine (1.0 - 1.3 mole l^{-1}, pH 6.9). It is likely that one of the transients of the BR^{568}_{trans} photocycle reacts with the hydroxylamine to form retinaloxime and BO (10). The excess hydroxylamine was removed by washing the sample with water. Most of the retinaloxime, however, was not removed, but remained attached to the membranes.

Photoreactions arising from these retinaloximes can be ignored in most of our flash experiments since the oximes absorb at lower wavelengths than we use for our measurements. If excitation and measuring light are within the absorption range of retinaloxime, its photoreactions have to be taken into consideration.

The purity of BO obtained by this method and described by the ratio of BO/BR was about 98/2 (Fig. 6).

Regeneration of $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans} was achieved by incubating a buffered aqueous BO suspension (pH 6.88) with the respective ethanolic retinal isomer solutions: 0.5 ml of BO suspension was mixed in the dark with 2 µl retinal solution. Retinal was added in a 3-6 fold molar excess. The final absorbance of the main visible absorption band of the regenerated BR was about 0.5 in a 1 cm pathlength cuvette. The purity of the retinal isomers was better than 99%, as indicated by high pressure liquid chromatography. Again the photoreactions of the surplus

retinal do not interfere with most of our measurements because of the different wavelength range.

According to Fig. 1, $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} equilibrate with each other

$$BR^{548}_{13\text{-cis}} \rightleftharpoons BR^{568}_{trans}.$$

Equilibration between $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} is mainly an isomerization of the chromophore and a first order reaction in both directions. Regeneration of BR is a second order reaction. Special care has to be taken that on the one hand not too much isomerization occurs during regeneration, but that on the other hand most BR is regenerated at the time of the flash. Because first and second order reactions are involved, this goal can be easily attained. We found that for most purposes a 3-6 fold excess of retinal to BO and a reaction time of 2-5 minutes are sufficient to obtain optimal conditions at room temperature.

Samples

For the flash experiments regenerated $BR^{548}_{13\text{-cis}}$, regenerated BR^{568}_{trans}, isolated purple membranes, and living bacteria were used. The experiments were performed with light adapted as well as with dark adapted samples. Measurements above $0^{o}C$ were carried out in aqueous solutions (phosphate buffer, 0.025 M, pH 6.88), measurements below $0^{o}C$ in a buffered (pH 6.9) glycerol/water mixture (2/1; v/v). The living bacteria were excited directly in their culture medium (pH 7.3 - 7.5), a nearly saturated NaCl solution (4.3 M), containing other ions and a peptone as nutrient.

Spectroscopic Methods

Two flash photometers designed for different time ranges were used in this investigation. One is equipped with a frequency doubled ruby laser as excitation light source (347 nm) and has a resolution time of 50 nsec (12). The other flash photometer has a resolution time of 10 μsec and a xenon flash lamp (stroboscope lamp Strobotac 1538-A, General Radio Co.) serves as a light source. For this lamp cut-off filters were used to limit the wavelength range of the originally white excitation light. The wavelength of the measuring beam of both photometers can be set with a monochromator and/or interference filters.

Only a single flash was applied to each sample.

A Cary 17 and a Cary 118 spectrophotometer were used for measuring room temperature and low temperature spectra.

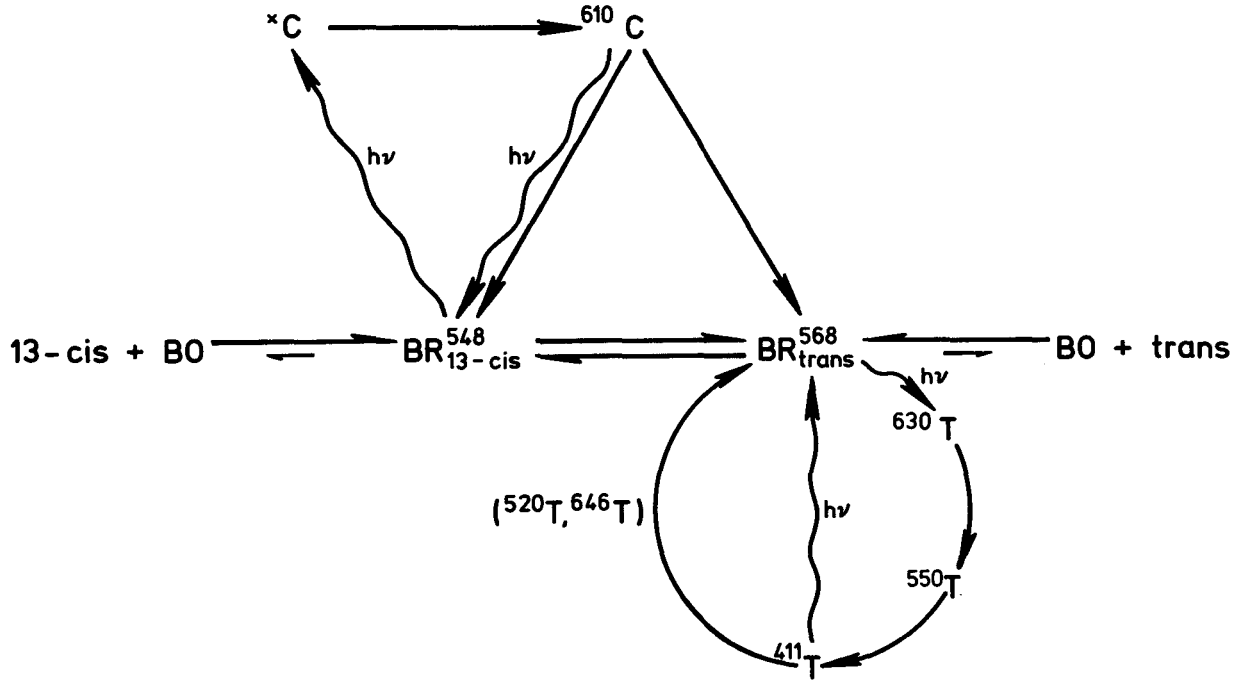


Fig. 1

Photochemistry of $BR^{548}_{13\text{-}cis}$, BR^{568}_{trans}, isolated purple membranes, and living bacteria

Our measurements show that the two bacteriorhodopsin isomers, $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans}, undergo clearly distinct photoexcitations. The results are described in detail as follows.

Photoreaction of $BR^{548}_{13\text{-}cis}$

Upon illumination $BR^{548}_{13\text{-}cis}$ yields a photoproduct, ^{x}C, which is completely present in less than 50 nsec at -90°C, and decays at that temperature with a half lifetime of about 1.5 µsec to the next product, ^{610}C.

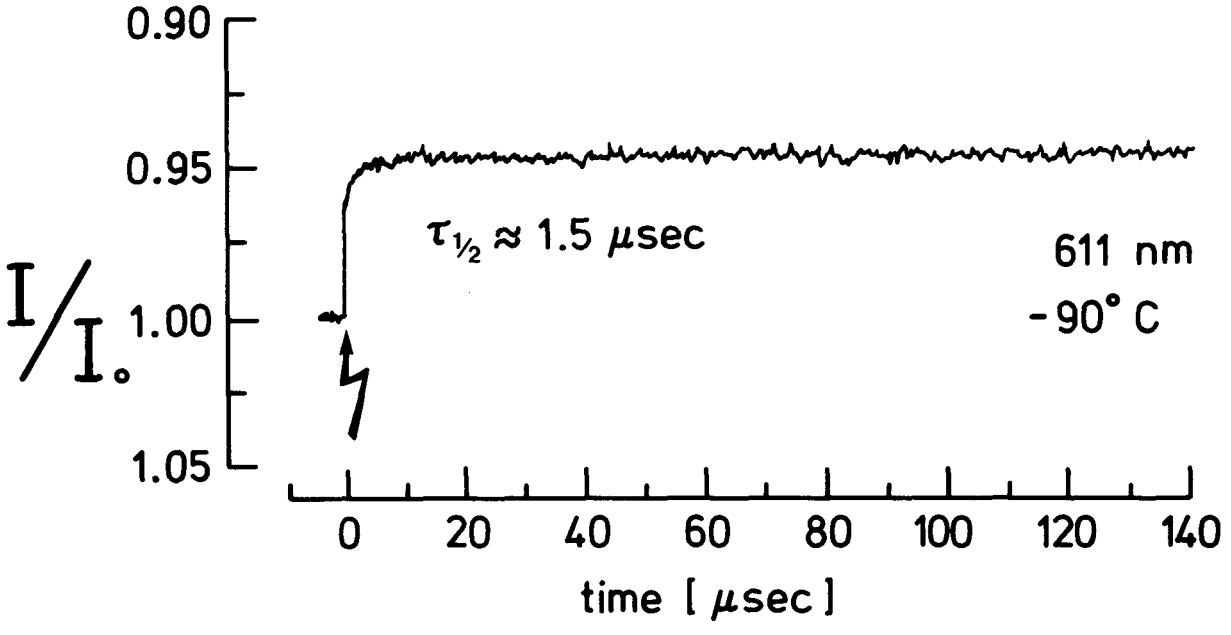


Fig. 2:

Transmission changes at 611 nm after exposure of $BR^{548}_{13\text{-cis}}$ to a laser flash (λ = 347 nm). The arrow depicts the exciting flash. $BR^{548}_{13\text{-cis}}$ was prepared by addition of ethanolic 13-cis retinal solution to chromophore-free membranes suspended in a buffered glycerol/water mixture (2/1, v/v; pH 6.9). After 2 min at 20°C, the sample was cooled to -90°C. The fast unresolved decrease in transmission (increase in absorption) indicates the formation of ^{x}C, the slower further absorption increase the formation of ^{610}C. $\tau_{1/2}$ is the half time of the reaction $^{x}C \rightarrow {}^{610}C$.

Fig. 2 traces the absorption changes at 611 nm after exposure of $BR^{548}_{13\text{-cis}}$ to a 347 nm laser flash. The steep increase of absorption could not be resolved. We attribute it to the formation of the product ^{x}C, which has a higher absorption at 611 nm than the initial pigment $BR^{548}_{13\text{-cis}}$. As indicated by the further slower absorption increase, ^{x}C, at -90°C, decays with a half lifetime of about 1.5 µsec to the product ^{610}C, which is thermostable at that temperature. At 611 nm, ^{610}C has a higher absorption than either ^{x}C or $BR^{548}_{13\text{-cis}}$ (Fig. 2). At room temperature, ^{610}C itself is present in less than 50 nsec.

Fig. 3 shows the absorption changes recorded with a lower time resolution at 610 nm after flashing ($\lambda > 455$ nm) $BR^{548}_{13\text{-cis}}$ at 20°C. A fast absorption increase during the flash is followed by an absorption decrease with a half lifetime of 37 msec. The absorption does not completely return to the original value, indicating the formation of another product. Analysis of the reaction mixture after the flash (analytic method described below) confirmed our assumption that this product is BR^{568}_{trans}. At 611 nm, BR^{568}_{trans} has a higher absorbance than $BR^{548}_{13\text{-cis}}$, as can be seen from Fig. 4, where their relative absorption spectra are drawn. This explains that the absorption shown in Fig. 3 does not completely return to the original value. The results of analogous recordings, measured in the wavelength range between 400 and 750 nm, are summarized in the difference spectra of Fig. 5. The difference spectrum plotted from I/I_o-values taken 1 msec after the flash (●, corresponding to a in Fig. 3) represents the absorption difference between ^{610}C and the original $BR^{548}_{13\text{-cis}}$. The maximum at 610 nm was taken to designate ^{610}C. The absorption changes 300 msec after the flash (o, corresponding

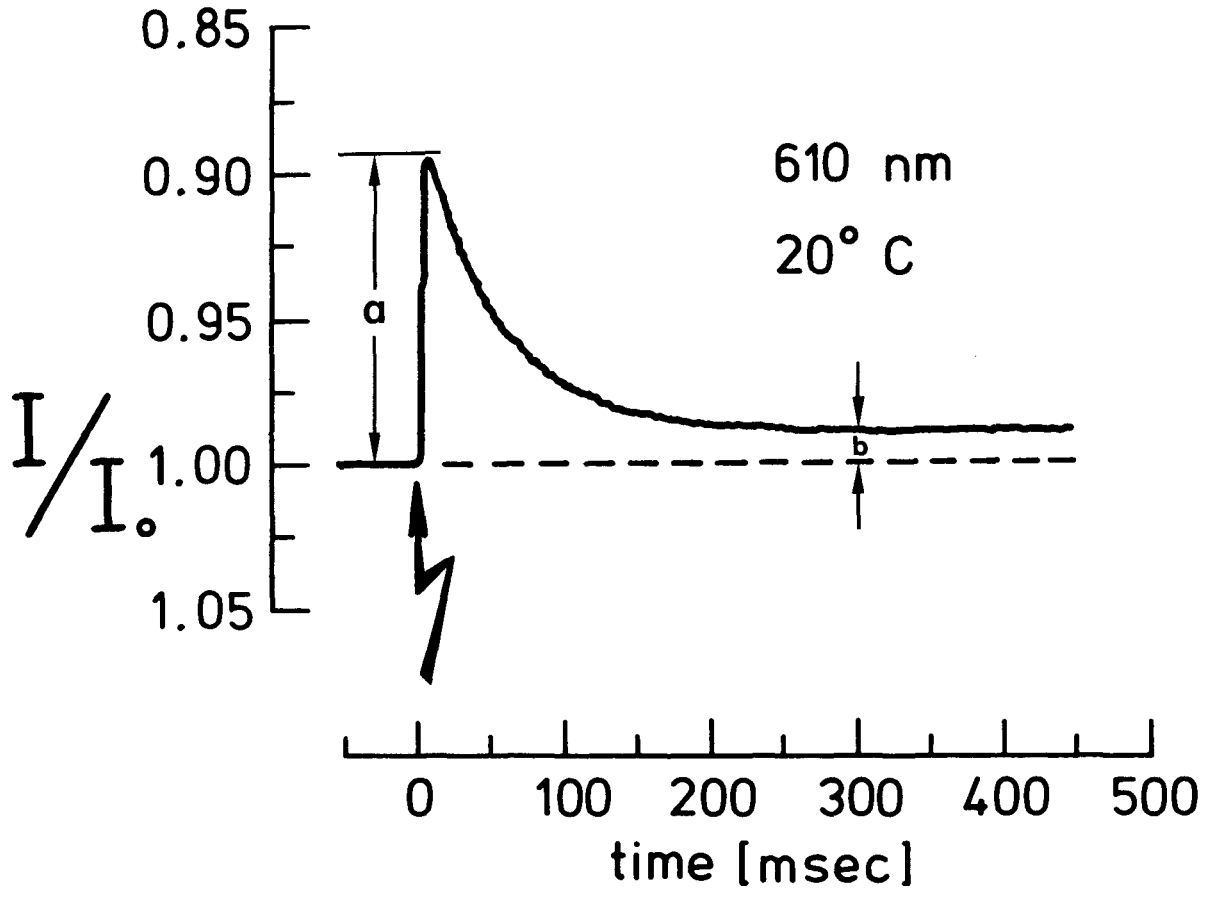


Fig. 3:

Transmission changes at 610 nm after exposure of $BR^{548}_{13\text{-cis}}$ to a flash (λ > 455 nm, Schott GG 455 cut-off filter).

Temperature 20°C.

a represents I/I_o before the flash minus
I/I_o 1 msec after the flash

b represents I/I_o before the flash minus
I/I_o 300 msec after the flash

The kink in the fast unresolved absorption increase is an artifact due to a datum point of the transient recorder.

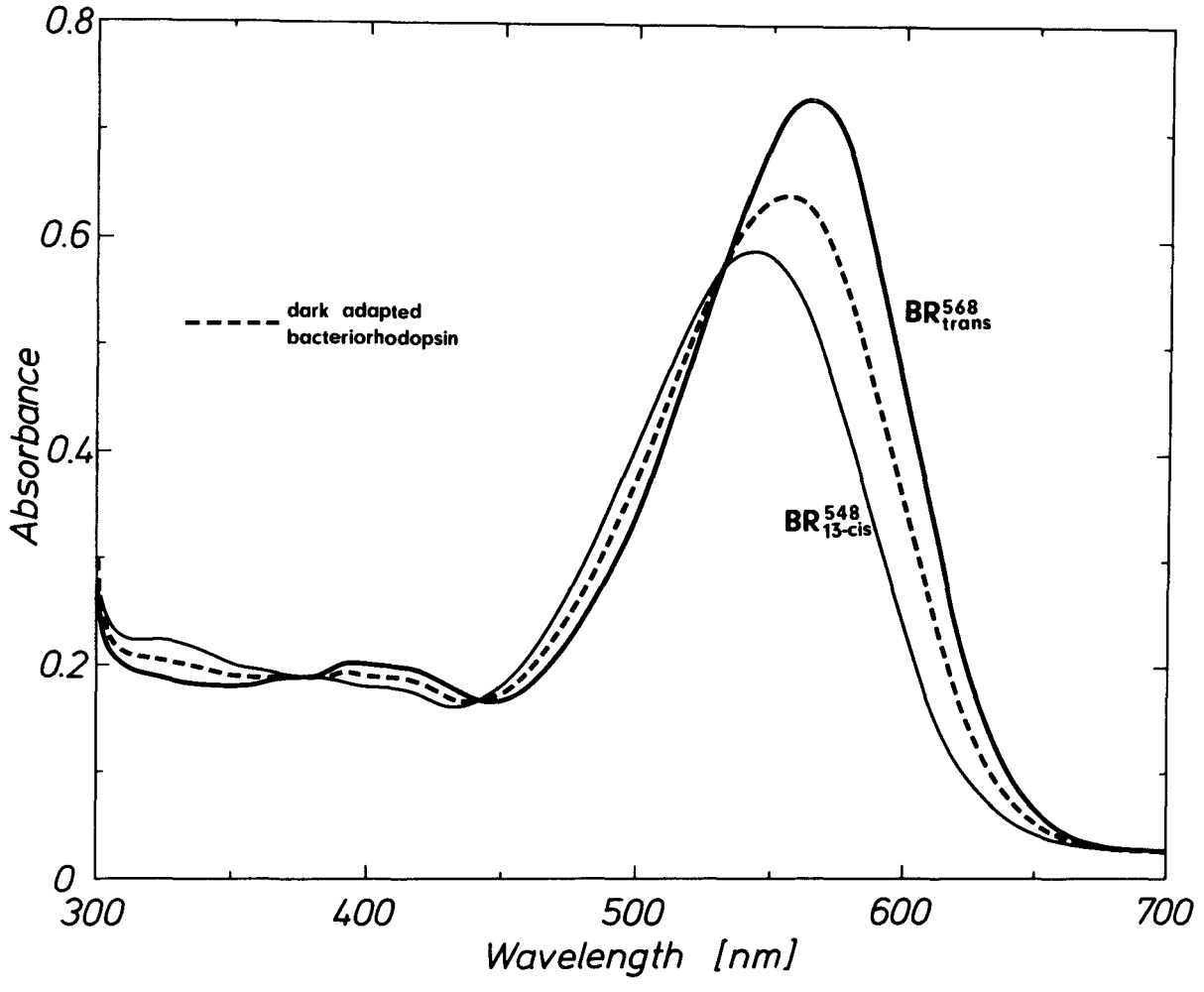


Fig. 4:

Absorption spectra of $BR^{548}_{13\text{-cis}}$, BR^{568}_{trans}, and the mixture of both obtained after dark adaptation, measured at 20°C.

Aqueous suspension of purple membranes measured after light adaptation (——), the same sample after dark adaptation at 20°C (------). Spectrum of $BR^{548}_{13\text{-cis}}$ calculated on the basis of

$$K = \frac{[BR^{568}_{trans}]}{[BR^{548}_{13\text{-cis}}]} = 1 \quad (\text{——}).$$

Optical pathlength 1.00 cm. The photomultiplier was placed near the cell to minimize light scattering. 0.025 M phosphate buffer, pH 6.88. The dark adapted PM has an absorption maximum at 558 nm.

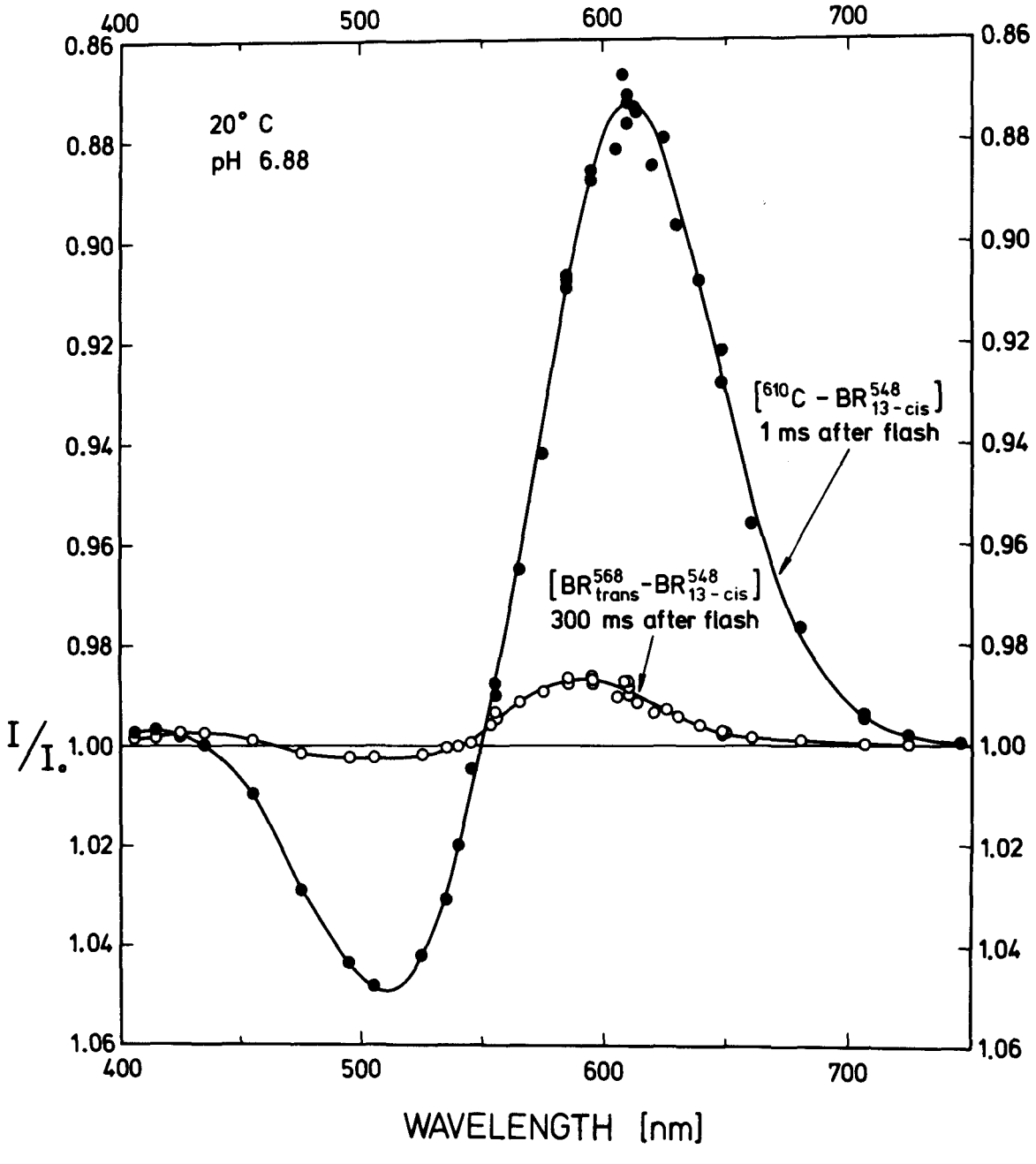


Fig. 5:

Difference spectra obtained after excitation of $BR^{548}_{13\text{-}cis}$ at 20°C. The spectra were plotted from data like those of Fig. 3. The difference spectrum 1 msec after the flash (•) indicates the difference between the ^{610}C and the $BR^{548}_{13\text{-}cis}$ spectrum, the difference spectrum 300 msec after the flash (o) the difference between the BR^{568}_{trans} and the $BR^{548}_{13\text{-}cis}$ spectrum.

to b in Fig. 3) represent the difference of the spectra of $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans}.

Analysis of the experimental data reveals that ^{610}C decays with a half lifetime of 37 ms (20°C) to both $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans}. We will demonstrate that the majority of the ^{610}C molecules decays back to $BR^{548}_{13\text{-cis}}$. The alternative, that $BR^{548}_{13\text{-cis}} \rightarrow {}^{x}C \rightarrow {}^{610}C \rightarrow BR^{568}_{trans}$ is the sole pathway and no back reaction from ^{610}C to $BR^{548}_{13\text{-cis}}$ occurs, can be excluded for the following reason: If all ^{610}C molecules would decay to BR^{568}_{trans}, the molar absorption coefficient (ε) of ^{610}C could be estimated from the difference between the absorption immediately after the flash and the final absorption (a-b in Fig. 3 or $[{}^{610}C - BR^{548}_{13\text{-cis}}] - [BR^{568}_{trans} - BR^{548}_{13\text{-cis}}] = [{}^{610}C - BR^{568}_{trans}]$ in Fig. 5). The ratio of the molar absorption coefficients of BR^{568}_{trans} to $BR^{548}_{13\text{-cis}}$ can be taken from Fig. 4. The absolute absorbance difference in terms of molar absorption coefficients is known within the limits the molar absorption coefficients of BR^{568}_{trans} and $BR^{548}_{13\text{-cis}}$ are known. It is fairly well established that the ε_{max}-value of BR^{568}_{trans} is in the range of 58,000 $\pm$ 3000 l mole^{-1} cm^{-1}. This corresponds to an ε_{max} of 46,000 $\pm$ 3000 l mole^{-1} cm^{-1} for $BR^{548}_{13\text{-cis}}$ (taken from Fig. 4 again). Assuming an ε_{max} of 58,000 l mole^{-1} cm^{-1} for BR^{568}_{trans}, the difference in molar absorbance between BR^{568}_{trans} and $BR^{548}_{13\text{-cis}}$ is 15,200 l mole^{-1} cm^{-1} at 610 nm. This value (15,200 l mole^{-1} cm^{-1}) times $[{}^{610}C - BR^{548}_{13\text{-cis}}]/[BR^{568}_{trans} - BR^{548}_{13\text{-cis}}]$ (see Fig.5), i.e. times 13, plus 11,000 l mole^{-1}cm^{-1}(ε at 610 nm of $BR^{548}_{13\text{-cis}}$), leads to an ε(at 610 nm) of 209,000 l mole^{-1}cm^{-1} for ^{610}C. This is unreasonably high for retinal-protein complexes. If the absorption maximum of ^{610}C is not exactly at 610 nm, ε_{max}

of ^{610}C would be even higher. This evaluation rules out that the reaction $^{610}C \rightarrow BR^{568}_{trans}$ is the sole pathway for the decay of ^{610}C. Because other products were not found, the simplest explanation is a split pathway

$$^{610}C \begin{cases} \nearrow BR^{568}_{trans} \\ \searrow BR^{548}_{13\text{-}cis} \end{cases}$$

From curves like the one shown in Fig. 3 and analytic determination of BR^{568}_{trans} we conclude that $^{610}C \rightarrow BR^{548}_{13\text{-}cis}$ is the main path.

In addition to the thermal pathway to $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans}, ^{610}C is photoconvertible to $BR^{548}_{13\text{-}cis}$. At temperatures below $-80^{o}C$, ^{610}C is, as mentioned before, thermally stable and was accumulated by illuminating $BR^{548}_{13\text{-}cis}$ with green light ($\lambda = 525$ nm). A mixture of $BR^{548}_{13\text{-}cis}$ and ^{610}C results. The absorption band of ^{610}C is noticeably shifted to the red compared with that of $BR^{548}_{13\text{-}cis}$, as revealed by our low temperature measurements with a Cary spectrophotometer (not shown in this paper). Light of a longer wavelength (λ = 610 nm) recovered the original spectrum of $BR^{548}_{13\text{-}cis}$, indicating that ^{610}C photoconverts back to $BR^{548}_{13\text{-}cis}$.

In summary, a photosteady state between $BR^{548}_{13\text{-}cis}$ and ^{610}C is obtained at low temperatures. The ratio of $BR^{548}_{13\text{-}cis}$ to ^{610}C depends on the wavelength of the exciting light. Under conditions of moderate light intensity and at higher temperatures the dark reaction $BR^{548}_{13\text{-}cis} \rightarrow BR^{568}_{trans}$ has to be taken into account and in addition to the photointermediates a mixture of both isomeric forms is obtained.

^{x}C and ^{610}C are intermediates in the photoisomerization pathway of $BR^{548}_{13\text{-}cis}$ to BR^{568}_{trans}.

Photoreaction of BR^{568}_{trans}

The photocycle originating from BR^{568}_{trans} is in principle identical with that previously described for light adapted purple membranes (2,7,9). Its reaction scheme in a simplified form is given in Fig. 1. We confirmed the fast appearance of ^{630}T during the flash, its decay to ^{550}T, and the further reaction to ^{411}T. The pathway of the decay of ^{411}T back to BR^{568}_{trans} was not investigated in detail; however, the formation and decay of ^{646}T was observed.

Photoreactions of transients of the BR^{568}_{trans} cycle, with the exception of ^{411}T, are not shown in Fig. 1. In principal, all retinal containing proteins, including intermediates, are expected to give photoproducts. To our knowledge, this holds for all retinal complexes so far investigated, i.e. retinal protein complexes in the rhodopsin as well as in the bacteriorhodopsin series. The longer the lifetime of a transient the easier it will be to study its photoreactions. We measured directly a photoreaction of ^{411}T (16), which has a relatively long lifetime ($\tau_{1/2}$ = 4.5 msec at 20°C according to Fig. 7). ^{411}T was accumulated at room temperature by illumination of an aqueous suspension of purple membranes with strong white light for about 7 msec. At this time the sample was excited with a 347 nm laser flash (about 20 nsec duration) and the absorption changes recorded. Immediately following the flash, absorption changes characteristic of the formation of BR^{568}_{trans} were detected.

It was reported (2) that the 412 nm complex formed in an aqueous salt/ether solution does not change spectroscopically when illuminated with 415 nm light at 20°C. At 11°C, however, a photoreaction back to the purple complex was reported for the same solvent system (13).

Photoreactions of isolated PM and PM of living bacteria

Isolated PM and regenerated BR are spectroscopically indistinguishable. Dark adapted PM shows flash spectroscopically the features of both $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans}, light adapted PM only those of BR^{568}_{trans}. The same applies to living bacteria. The lifetimes of the two intermediates investigated, ^{411}T and ^{646}T, are, however, considerably longer than those in the isolated purple membrane or in the regenerated BR. As can be seen from Fig. 7, the half lifetime for ^{411}T is about 12 times longer in living bacteria than in isolated purple membrane fragments.

Ratio of BR^{568}_{trans} to $BR^{548}_{13\text{-}cis}$

Assay for $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans}

The fact that upon photoexcitation, $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} yield different photoproducts and different subsequent transients, was exploited to develop an analytical method to determine the ratio of BR^{568}_{trans} to $BR^{548}_{13\text{-cis}}$ in various preparations of BR, under conditions of dark and of light adaptation. The long-lived transient ^{411}T of the BR^{568}_{trans} cycle turned out to be a convenient quantity to measure the amount of BR^{568}_{trans} in a given sample by flash photometry. To use ^{411}T as a measure for BR^{568}_{trans} the following requirements have to be met:

1) The exciting light (analytic flash) should be weak and short enough to avoid secondary photon hits during the photocycle of BR^{568}_{trans}. Under these conditions the concentration of ^{411}T after the exciting flash is proportional to the concentration of BR^{568}_{trans} present before the flash.

2) In samples containing both $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans}, absorption changes of $BR^{548}_{13\text{-cis}}$ and its transients have to be taken into account.

The interpretation of the measurements can be simplified by choosing a wavelength of the measuring beam where the $BR^{548}_{13\text{-cis}}$ path is "spectroscopically decoupled", i.e. no absorption change occurs. Wavelengths where no absorption difference between ^{610}C and $BR^{548}_{13\text{-cis}}$ exists, are their isosbestic points. At the time after the analytic flash when ^{610}C and $BR^{548}_{13\text{-cis}}$ prevail in the $BR^{548}_{13\text{-cis}}$ photoreaction pathway, ^{411}T is predominant

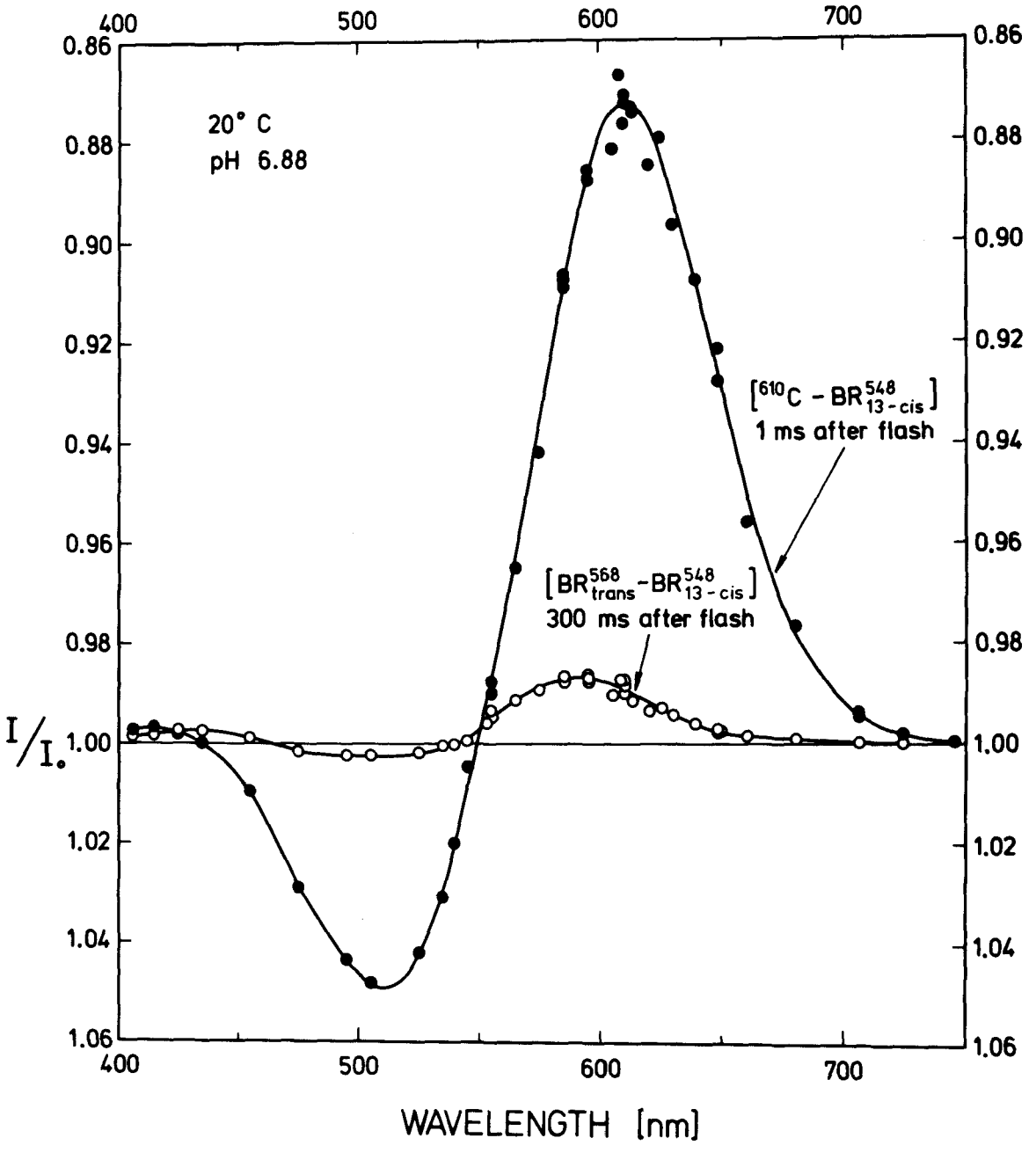

400
500
600
700
0.86
0.88
0.90
0.92
0.94
0.96
0.98
1.00
1.02
1.04
1.06
20° C
pH 6.88
[610C - BR548 13-cis]
1 ms after flash
[BR568 trans - BR548 13-cis]
300 ms after flash
I/I.
WAVELENGTH [nm]

Fig. 5

in the BR^{568}_{trans} cycle. If the appropriate time after the analytic flash (see Figs. 6 and 7) and the right wavelength (isosbestic point) are chosen, absorption changes represent only changes within the BR^{568}_{trans} cycle, i.e. the appearance of ^{411}T (disappearance of BR^{568}_{trans}) and disappearance of ^{411}T (appearance of BR^{568}_{trans}).

The wavelength we chose for the measuring light was 545 nm, which is close to the isosbestic point $^{610}C/BR^{548}_{13\text{-}cis}$ (see Fig. 5). Actually the absorption decrease at 545 nm represents mainly the disappearance of BR^{568}_{trans}, which is, of course, proportional to the appearance of ^{411}T. Test measurements at other wavelengths (e.g. 411 nm) confirmed the consistency of the whole system.

The analytic flashes used were so weak and the turnover of the BR so small that the light of the flash practically did not change the ratio of BR^{568}_{trans} to $BR^{548}_{13\text{-}cis}$.

Summarizing, the ratio $\frac{[BR^{568}_{trans}]}{[BR^{548}_{13\text{-}cis}]}$ was determined as follows:

1) the first analytic flash determined the (relative) amount of BR^{568}_{trans}
2) the sample was light adapted to transfer nearly all BR into the BR^{568}_{trans} form
3) a second analytic flash determined the (relative) amount of BR^{568}_{trans} present after light adaptation. This corresponds to the sum of $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans} present in the original sample. The difference of the BR^{568}_{trans} measured with the second analytic flash and that measured with the first analytic flash is the original amount of $BR^{548}_{13\text{-}cis}$. After transforming the I/I_o-values

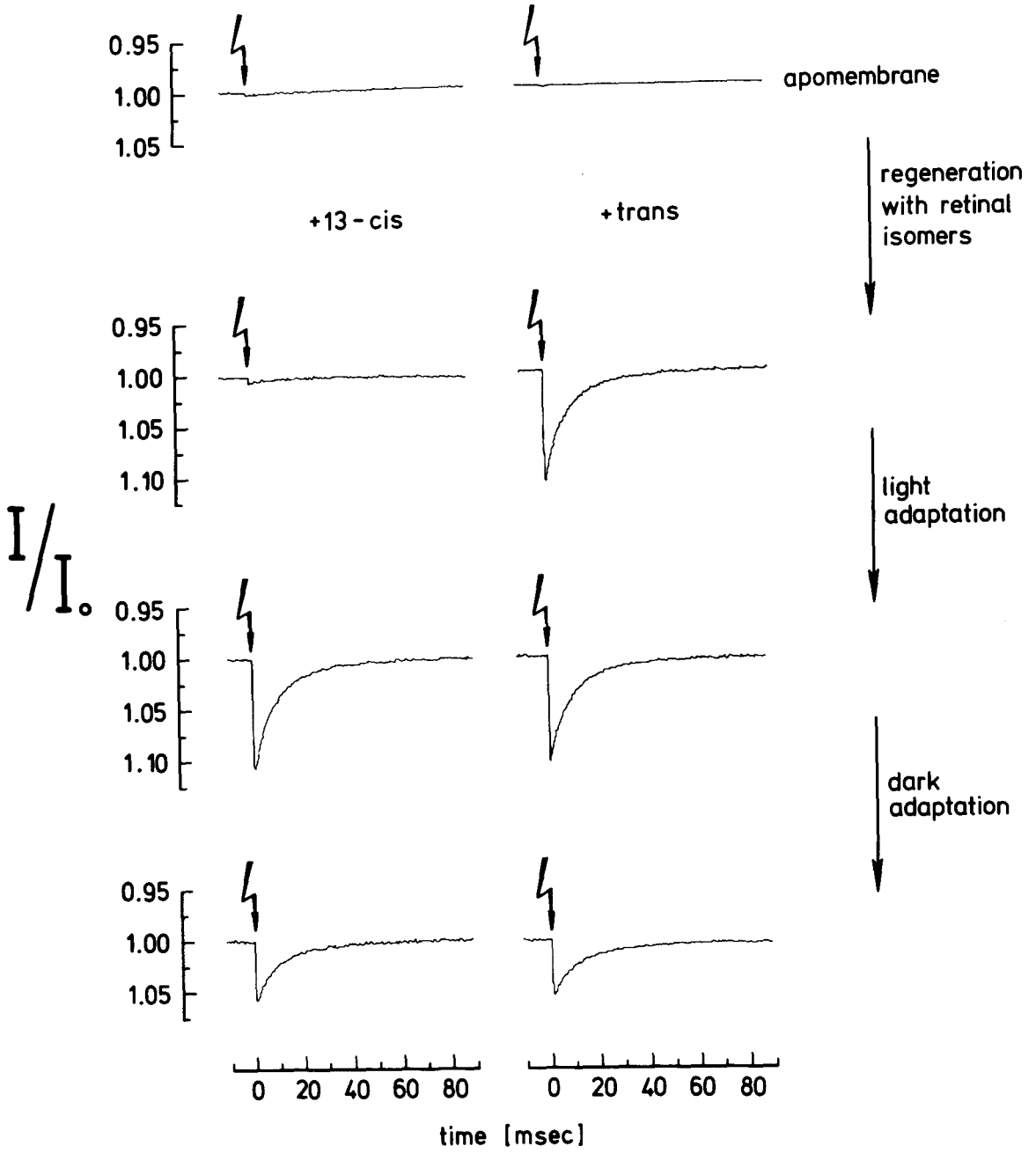


Fig. 6:

Time course of transmission changes at 545 nm of apomembrane and regenerated bacteriorhodopsin. 1., 3., and 4. horizontal line are described in the figure; 2. horizontal line shows transmission changes of regenerated BR. The samples were flashed 3 min ($BR^{548}_{13\text{-cis}}$) and 10 min (BR^{568}_{trans}) after addition of the retinal solution to the apomembrane. $\lambda_{flash} > 570$ nm, Schott filter OG 570; Temperature 20°C; pH 6,88.

into absorbance, which is directly proportional to the concentration, the ratio

$$K = \frac{[BR^{568}_{trans}]}{[BR^{548}_{13\text{-}cis}]}$$

was calculated.

Determination of the ratio BR^{568}_{trans} to $BR^{548}_{13\text{-}cis}$

Fig. 6 states briefly the results obtained with regenerated $BR^{548}_{13\text{-}cis}$ (left side) and with regenerated BR^{568}_{trans} (right side). The first two recordings (first horizontal line) show the absorption changes of the light adapted apomembrane after an analytic flash. The tiny absorption change after the flash indicates that the BO is contaminated with less than 2% BR. The BO was then regenerated with the respective retinal isomers, 13-cis and trans retinal, to the bacteriorhodopsin isomers, $BR^{548}_{13\text{-}cis}$ and BR^{568}_{trans} (second horizontal line).

Regeneration was carried out by incubating 0.5 ml of an aqueous apomembrane suspension with 2 µl ethanolic retinal solution. The final absorbance of the regenerated BR was about 0.5 in the absorption maximum for 1 cm pathlength. About three times the amount of retinal necessary for a complete regeneration was added. For direct comparison of the results $BR^{548}_{13\text{-}cis}$ was flashed 3 minutes after incubation, BR^{568}_{trans}, because of its slower rate of regeneration, after about 10 minutes.

An analytic flash was applied to the regenerated samples (second horizontal line), which were then light adapted and once more flashed (third horizontal line). Following dark adaptation the same samples were again subjected to an analytic flash (fourth horizontal line). All experiments described in Fig. 6 were performed at 20°C.

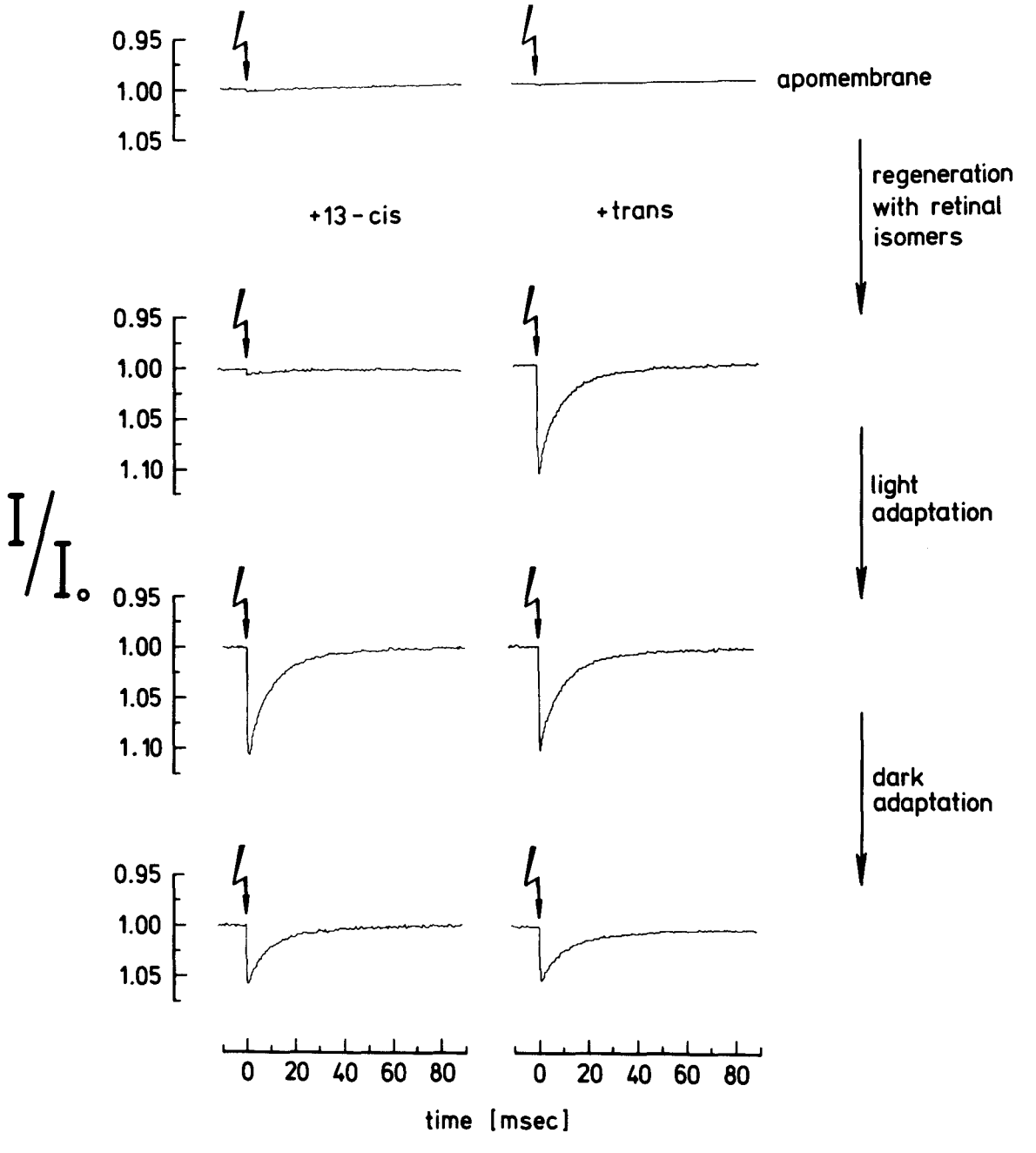

0.95
1.00
1.05
apomembrane
regeneration with retinal isomers
+13-cis
+trans
0.95
1.00
1.05
1.10
light adaptation
I/I₀
0.95
1.00
1.05
1.10
dark adaptation
0.95
1.00
1.05
0 20 40 60 80
0 20 40 60 80
time [msec]

Fig. 6

The following conclusions were drawn from the results summarized in Fig. 6:

1) The second horizontal line demonstrates clearly that $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} form different photoproducts and different subsequent transients. The small absorption change in the case of $BR^{548}_{13\text{-cis}}$ is due to the remaining BR in the apomembrane preparation (see first horizontal line), to a small amount of BR^{568}_{trans} isomerized from $BR^{548}_{13\text{-cis}}$ (see Fig. 1), and possibly a trace of regenerated BR^{568}_{trans} originating from a small contamination of trans retinal in the 13-cis retinal solution used for regeneration.

2) Light adaptation (third horizontal line) yields identical recordings for both samples. This indicates that during light adaptation $BR^{548}_{13\text{-cis}}$ was transformed to BR^{568}_{trans}. The sample with the regenerated BR^{568}_{trans} did not change during light adaptation.

3) Following dark adaptation,again identical tracings were obtained for both samples (fourth horizontal line). Compared with the light adapted samples the I/I_o-values have only about half the size. This indicates the establishment of a dark equilibrium between $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} with a ratio

$$K = \frac{[BR^{568}_{trans}]}{[BR^{548}_{13\text{-cis}}]}$$

of about unity.

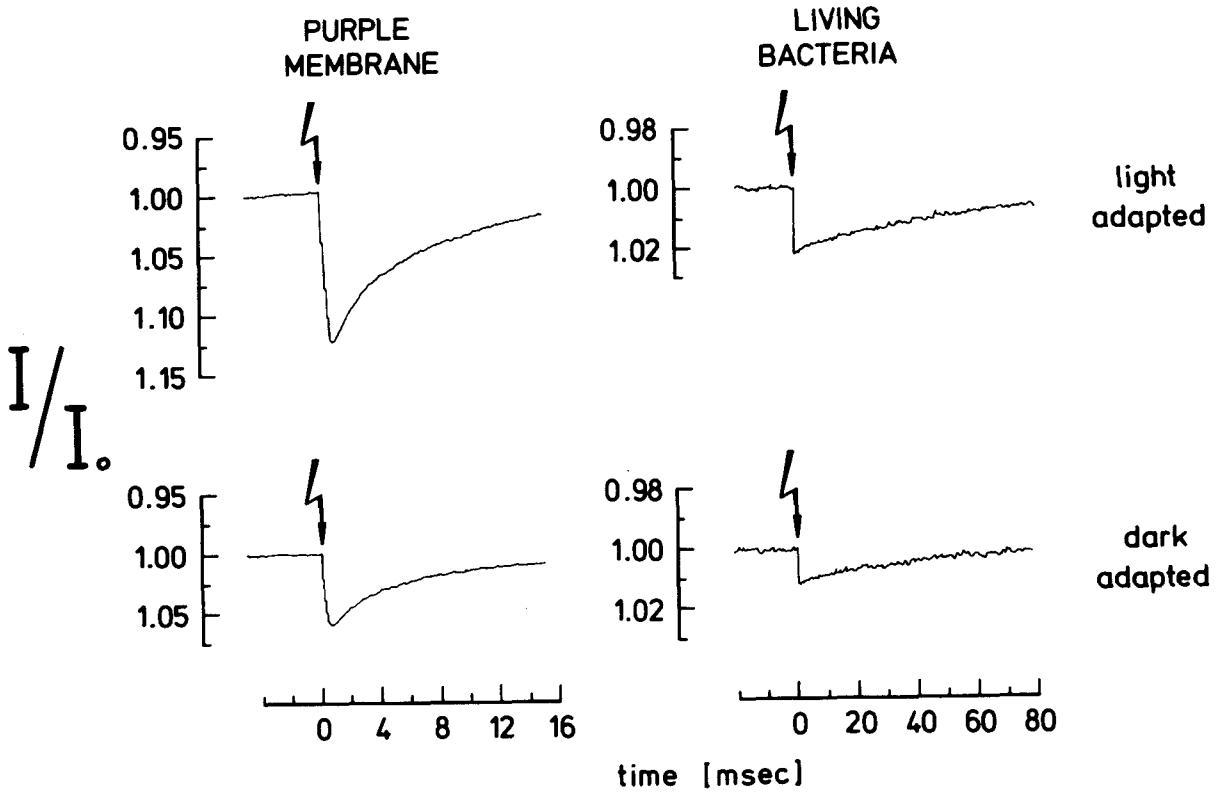


Fig. 7:

Time course of transmission changes at 545 nm of purple membrane fragments and living bacteria. The light of the flash was filtered through a Schott OG 570 cut-off filter. The purple membranes were suspended in aqueous phosphate buffer (0.025 M, pH 6.88), whereas the living bacteria were measured in their culture medium (pH ≈ 7.3). Temperature 20°C.

If after regeneration of $BR^{548}_{13\text{-cis}}$ or BR^{568}_{trans} the samples were first dark adapted and then light adapted, corresponding results were obtained (i.e. the recordings of the fourth line are first obtained, and then those of the third line).

Fig. 7 shows recordings for isolated PM (left side) and living bacteria (right side), both in the light adapted (upper row) and dark adapted (lower row) state. The results concerning the equilibrium constant K agree with those obtained for regenerated $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans}, which are described in Fig. 6 (third and fourth line).

PM of living bacteria (Fig. 7), isolated PM (Fig. 7), and regenerated BR (Fig. 6), all contain only BR^{568}_{trans} in the light adapted state. In the dark adapted state, however, they all possess a mixture of $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans}. The ratio

$$K = \frac{[BR^{568}_{trans}]}{[BR^{548}_{13\text{-cis}}]}$$

is near unity for all preparations.

Temperature dependence of K

A careful analysis of the data revealed that the equilibrium constant, K, is only slightly dependent on the temperature between $0^{o}C$ and $60^{o}C$. Fig. 8 shows the temperature dependence of the dark equilibrium between BR^{568}_{trans} and $BR^{548}_{13\text{-cis}}$. The logarithm of K is plotted versus the reciprocal temperature, T^{-1}.

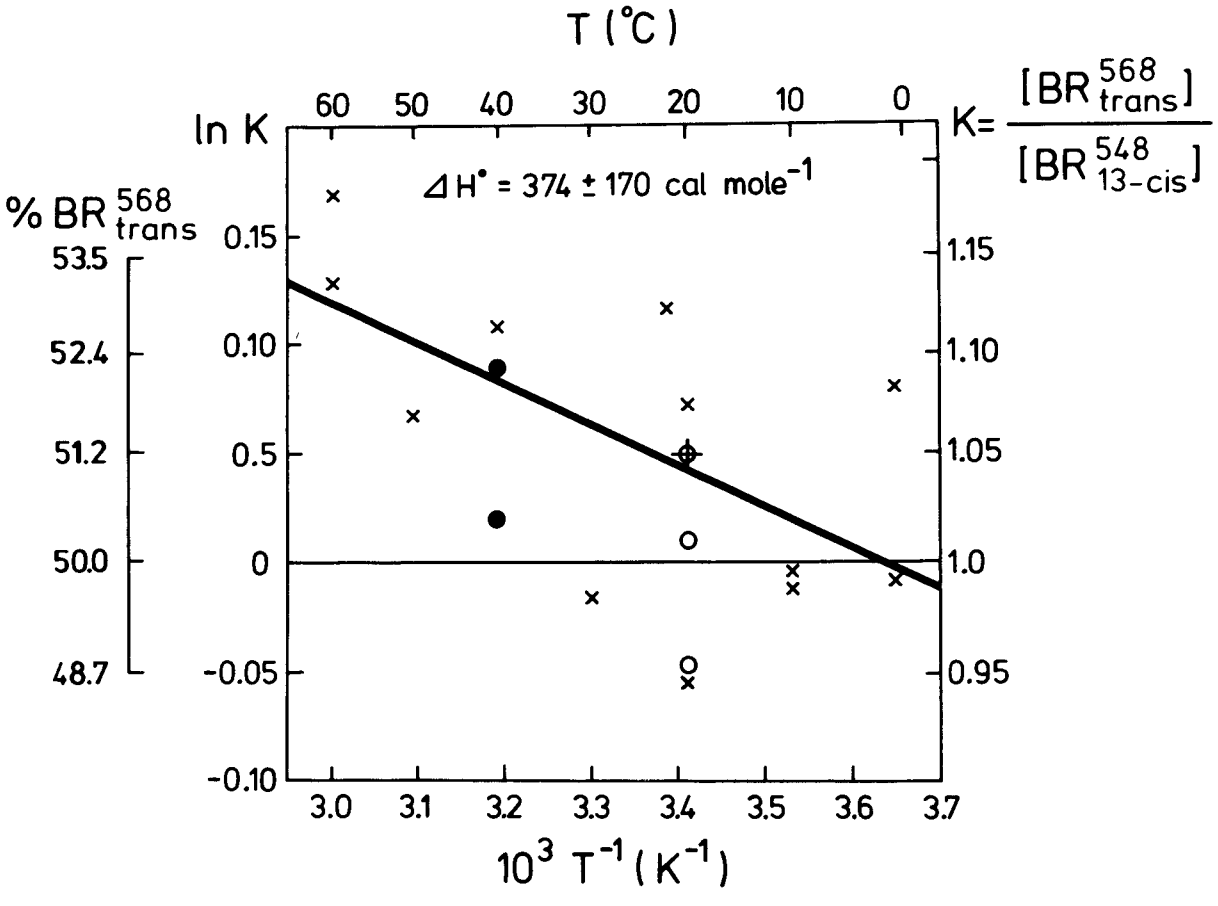


Fig. 8:

Temperature dependence of the equilibrium constant K. Aqueous suspensions (pH 6.88) of isolated purple membranes (x) or bacteriorhodopsin regenerated with 13-cis (o) and trans (⊕) retinal, respectively, as well as living bacteria (•) in culture medium (pH ≈ 7.3), were dark adapted at temperatures between 1°C to 60°C. K was determined by flash photometry as described in the text. The straight line was obtained by a least squares fit. To illustrate the small, but probably significant temperature dependence of the equilibrium, the ordinate was spread and shows only the range between 47.5% and 55.0% BR^{568}_{trans}.

The right ordinate gives the equilibrium constant, K, directly; the left one an additional scale with the percentage of BR^{568}_{trans} present in the sample. According to the procedure already described for Fig.6 and 7, K was determined for isolated PM (x), regenerated $BR^{548}_{13\text{-cis}}$ (o), regenerated BR^{568}_{trans} (⊕), and PM of living bacteria (●). The straight line was obtained with least squares fit for the PM values (x).

At $0^{o}C$ the equilibrium mixture contains 50% BR^{568}_{trans}, at 60^{o} 53% BR^{568}_{trans}. ΔH^{o}, the enthalpy change of the reaction

$$BR^{548}_{13\text{-cis}} \longrightarrow BR^{568}_{trans}$$

is very small and was calculated to be 374 ± 170 cal $mole^{-1}$ (95% confidence interval), the change of entropy, according to $\Delta S = -\frac{\delta \Delta G}{\delta T}$ ($\Delta G = -R\,T \ln K$), is $\Delta S^{o} = 1.3$ cal deg^{-1} $mole^{-1}$.

The K-value is independent of the origin of the sample, be it regenerated $BR^{548}_{13\text{-cis}}$, regenerated BR^{568}_{trans}, isolated PM, or PM of living bacteria.

We would like to emphasize two points of BR concerning the retinal isomers in BR. The thermodynamic equilibrium of trans retinal and 13-cis retinal in solution was determined to be

$$K_R = \frac{[\text{trans}]}{[\text{13-cis}]} = 3.3 \qquad \text{at } 20^{o}C \quad (14,15)$$

To achieve $\frac{[BR^{568}_{trans}]}{[BR^{548}_{13\text{-cis}}]} = 1$, a stronger binding of 13-cis than of trans retinal to the protein is required. Of all cis retinal isomers 13-cis retinal has the lowest K_R-value. From an evolutionary standpoint it may be interesting to point out that a thermodynamic ratio of about 1:1 for protein-bound cis retinal to protein-bound trans retinal is easier to attain with 13-cis

retinal than with any other cis retinal. 11-cis retinal, for example, would be a very unlikely candidate because of its extremely high K_R-value (15).

The second point concerns the fast equilibrating of

$$BR^{548}_{13\text{-cis}} \rightleftharpoons BR^{568}_{trans}$$

In solution the retinal isomers are fairly stable and do not isomerize considerably at room temperature without catalyzer. The fast equilibration of the BR isomers indicates that the protein of BR functions as an isomerase. It is specific to catalyze the 13-cis $\rightleftharpoons$ trans isomerization. We never found any other retinal isomers besides 13-cis and trans retinal.

According to (17), three BR molecules are close neighbors in the purple membrane. Close neighborhood of the retinal sites should mutually influence their absorption spectra. Occupation of these three chromophore sites with different retinal isomers should lead to slightly different absorption spectra. Likewise also partial occupation of these sites, especially happening during regeneration of BR, should have the same effect.

Small absorption changes due to these effects were neglected in this paper.

The analytic data concerning $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans}, described in this paper and obtained by flash photometry, are in quantitative agreement with earlier results (16). In these earlier experiments the retinal isomers were extracted with ethanol and identified by high pressure liquid chromatography. Light adapted PM yielded close to 100% (> 98%) trans retinal, dark adapted PM

about equal amounts of 13-cis and trans retinal (50.1 $\pm$ 2.3% trans retinal, mean and s.d. of seven experiments, PM dark adapted at 1°C).

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Diese Arbeit wurde im Rahmen des SFB 160 von der Deutschen Forschungsgemeinschaft unterstützt.