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Photochemistry and Dark Equilibrium of Retinal Isomers and Bacteriorhodopsin Isomers*

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Retinal isomers play an important role in photoreceptors: 11-cis and all-trans retinal in visual pigments (rhodopsins), 13-cis and all-trans retinal in bacteriorhodopsin, the pigment of the purple membrane of *Halobacterium halobium.* The proteins of these pigments bind selectively particular retinal isomers. Bound to the protein, retinal isomers photoisomerize and dark-isomerize only to specific other isomers, a behavior unlike that of retinal in solution.

Three different topics concerning the isomerization and photochemistry of free retinal and of protein-bound retinal will be dealt with in this discussion paper.

I. Thermodynamic Equilibrium of Retinal Isomers

The thermodynamic equilibrium between retinal isomers is of considerable interest. For instance, knowing the equilibrium between 11-cis and all-trans retinal (alternatively also called "trans retinal") is important for an understanding of the visual process and especially of the reaction from trans to 11-cis retinal during regeneration. In the case of bacteriorhodopsin, the equilibrium between 13-cis and all-trans retinal is the basis for the equilibrium between 13-cis and trans bacteriorhodopsin (see part III of this paper).

The equilibrium between the isomers trans, 9-cis, 13-cis, and 9,13-dicis retinal has been measured by NMR techniques [1] (compare also [2]). We determined the

$$trans \rightleftharpoons 13\text{-}cis \rightleftharpoons 11\text{-}cis \rightleftharpoons 9\text{-}cis \rightleftharpoons 9,13\text{-}dicis$$

Fig. 1. Thermodynamic equilibrium between retinal isomers. This equation indicates only the equilibrium state; isomerization is assumed to occur from any isomer to any other one

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thermodynamic equilibrium between trans, 9-cis, 11-cis, 13-cis, and 9,13-dicis retinal in solution by using high-pressure liquid chromatography (Fig. 1).

Equilibration is attained with a catalyzer, since without catalyzer practically no isomerization occurs at room temperature in the dark.

The equilibrium constants

$$K_R = \frac{[\text{trans}]}{[x\text{-cis}]}$$

[trans] = concentration of all-trans retinal

[x-cis] = concentration of x-cis retinal (x = 9; 11; 13; or 9,13), of 11-cis,12s-trans, or of 11-cis,12s-cis retinal

for a temperature of 20° C in chloroform are listed in Table 1, which also includes the changes of free enthalpy for the reaction x-cis → trans according to $\Delta G^0 = -RT \ln K_R$. The table gives also the percentages of these isomers in the thermodynamic equilibrium mixture. Other isomers appear only in negligible quantities and are not listed.

Table 1 shows, in agreement with theoretical expectations, that trans retinal is by far the most predominant of the isomers. 13-cis retinal, whose conjugated system resembles that of trans retinal more than the other isomers, is the second most frequent, and 9-cis the third one. 4.1% of 9,13-dicis retinal were found in the mixture. It is worthwhile to note that calculating the amount of 9,13-dicis retinal in the equilibrium mixture by applying the equilibrium constant $K_{9\text{-cis}} = 3.8$ to the 9-double bond of 13-cis retinal, and the constant $K_{13\text{-cis}} = 3.0$ to the 13-double bond of 9-cis retinal, and treating these two double bonds as independent of each other in the 9,13-dicis retinal, we get the value of 5.2% for this dicis compound. The difference between the theoretical value of 5.2% and the experimental value of 4.1% is a measure for the further loss of resonance energy of the dicis compound.

A very small percentage of 11-cis retinal is found in the thermodynamic equilibrium mixture (Table 1). This is to be expected because steric hindrance between the methyl group at C 13 and the proton at C 10 makes the formation of the planar conjugated side chain more difficult [3], i.e. lowers the resonance energy of the molecule.

For 11-cis retinal in solution a temperature-dependent equilibrium between different conformations has been proposed, with a 12s-trans and a 12s-cis conformation as the two main components [4–6] (Fig. 2). The reason for the existence of these two conformers (atropisomers) is again the steric hindrance. The 11-cis,12s-trans conformer has the lower energy (low temperature form) and has probably a planar or nearly planar conjugated side chain 6–16 [4, 5]. The 11-cis,12s-cis conformer possesses the higher energy and the conjugated side chain is partially dis-

Table 1. Thermodynamic data for the equilibrium mixture of retinal isomers in chloroform. Temperature 20° C

Retinal isomer	trans	13-cis	11-cis	9-cis	9,13-dicis	11-cis, 12s-trans	11-cis, 12s-cis
% isomer	60.2	20.0	0.048	15.7	4.1	0.034	0.014
K_R = [trans]/[x-cis]		3.0	1254	3.8	14.7	1800	4200
$-\Delta G^0_{293}$ [cal mol^{-1}]		640	4150	780	1560	4360	4850

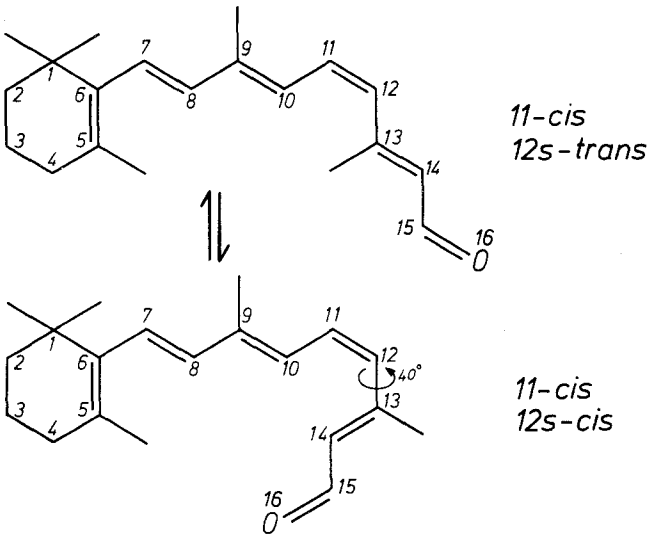

Fig. 2

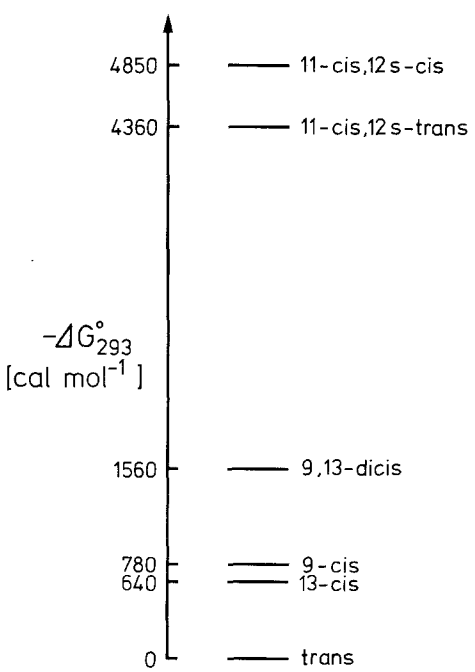

Fig. 3. Free enthalpy differences of retinal isomers at 293 K

rupted at the 12s bond. The angle between the two arising planar systems (6—13 and 12—16) of the 12s-cis form is expected to be close to 39°, the angle found in the crystal state [5].

The energy difference between the two conformers is relatively small, in the order of kT (for T about room temperature). Both conformers lose a considerable

amount of resonance energy compared to unhindered retinal isomers with a planar conjugated side chain, i.e. both are in a higher energetic state. The planar or nearly planar 12s-trans conformer loses resonance energy to overcome the steric hindrance between the methyl group at C 13 and the proton at C 10. The 12s-cis conformer loses resonance energy because of the disruption of the planarity of the conjugated side chain at the 12s bond.

It was estimated that a solution of 11-cis retinal at room temperature contains about 30–35% of the 12s-cis conformation [4, 6]. With the value of 30% for the 12s-cis form we calculate K_R = [trans]/[11-cis,12s-cis] = 4200 and [trans]/[11-cis, 12s-trans] = 1800 (Table 1).

The ΔG^0-values for the reactions 11-cis,12s-cis → trans and 11-cis,12s-trans → trans at room temperature are also listed in Table 1, and illustrated together with those of the other isomers in Figure 3.

The conformation of 11-cis retinal in rhodopsin is still unknown. We would expect that the protein of rhodopsin combines preferably with only one conformer of

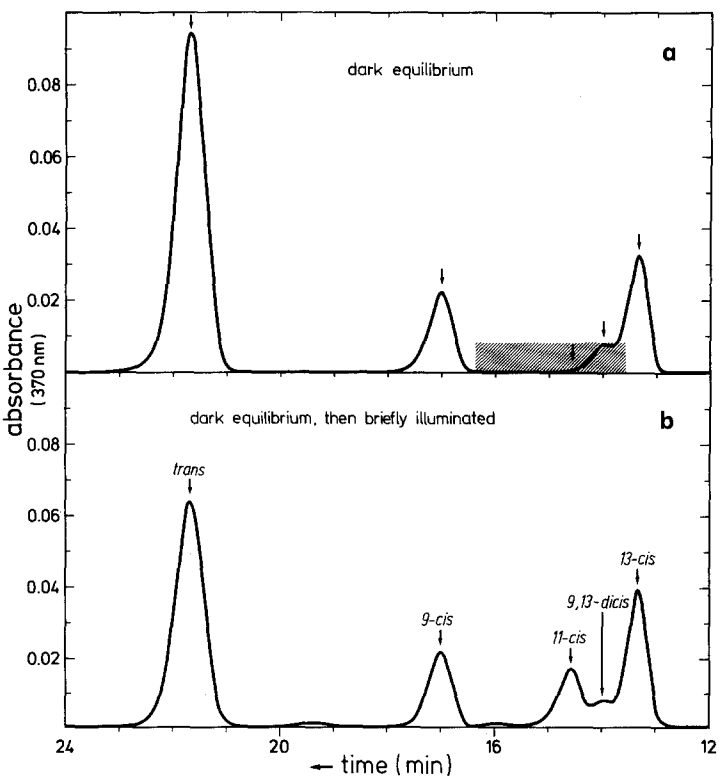

Fig. 4. High-pressure liquid (HPL) chromatograms of retinal isomers. a) Retinal after dark equilibration (at 20° C). b) Retinal after dark equilibration and subsequent short exposure to light. The original sample contained trans retinal. Instrument: Spectra Physics 3500B; column 500 × 3 mm, packed with silica, 5 μm; mobile phase: petrol ether/diethyl ether (90/10, v/v); flow rate: 0.6 ml/min; detector: Schoeffel 770 Spectroflow, measuring light 370 nm; pressure: 1000 psi; column temperature: 23° C

11-cis retinal (compare also [7]). We think that this is one of the two conformers shown in Figure 2, or a very similar one. The ΔG^0-values for the single-bond cis-trans isomers of 11-cis retinal determine the thermodynamics of the rhodopsin formation.

Experimental Procedure

30 mg of a retinal isomer were dissolved in 0.5 ml chloroform and 40 mg trifluoroacetic acid were added as catalyzer. About one hour after mixing, and standing at 20° C in the dark, the samples were analyzed with a high-pressure liquid chromatograph [8]. At this time equilibrium between the isomers had been reached. Figure 4a shows a chromatogram of an equilibrated retinal sample, Figure 4b the chromatogram of an identical sample, but this time analyzed after a short exposure to light.

To identify the respective isomers, the retention times were used. The quantitative amounts of the isomers are given by the band areas. Both, retention times and band areas, were calibrated with known solutions of either trans, 9-cis, 11-cis, or 13-cis retinal, and known mixtures of them. 9,13-dicis retinal is expected to be the most frequent of the dicis retinals in the equilibrated mixture and was identified as such.

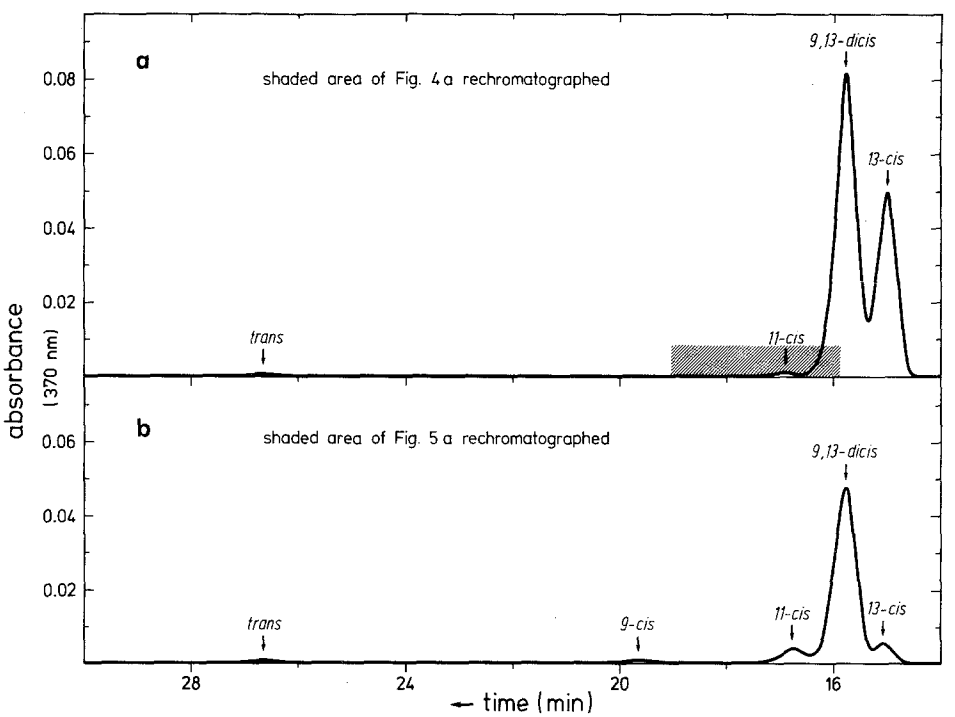

Fig. 5. HPL chromatograms of retinal isomers. a) The eluent of the shaded area of Figure 4a rechromatographed. b) The eluent of the shaded area of 5a rechromatographed. Technical data see Figure 4, except pressure: 1400 psi; column temperature: 0° C

No attempt was made to identify the other isomers in the illuminated sample (Fig. 4b).

Identical chromatograms were obtained by isomerizing samples with either one of the four isomers, trans, 9-cis, 11-cis, or 13-cis retinal. Similar results were also obtained with heptane as solvent and CF_3COOH as catalyst, and with hexane/heptane or chloroform as solvent and iodine as catalyst.

No peak of 11-cis retinal is detectable in Figure 4a. To get the small amount of 11-cis retinal present in the mixture, the fraction of the area where the peaks of 11-cis and of 9,13-dicis retinal are located (shaded area) was collected and rechromatographed (Fig. 5a). The same procedure was repeated (Fig. 5b). Whenever necessary, corresponding fractions from different runs were combined. As the shaded area in Figure 5a indicates, only part of the 9,13-dicis was rechromatographed (Fig. 5b).

9,13-dicis was collected together with 11-cis retinal and serves as a standard. The absorbance of 9,13-dicis retinal relative to that of 13-cis retinal was taken from [9].

Exact evaluation of the band areas led to the values listed in Table 1.

II. Experiments on the Photoisomerization of Retinal Isomers

Several different pathways for the photoisomerization of retinal isomers are conceivable and more than one are probably realized, depending on the experimental conditions. Figure 6 describes different possible pathways for the photoisomerization of a cis retinal to trans retinal and vice versa. The scheme is simplified and omits, for example, n, π^* transitions, other electronically excited states, vibrational energy levels, etc. The main two pathways discussed in the literature [10] for the

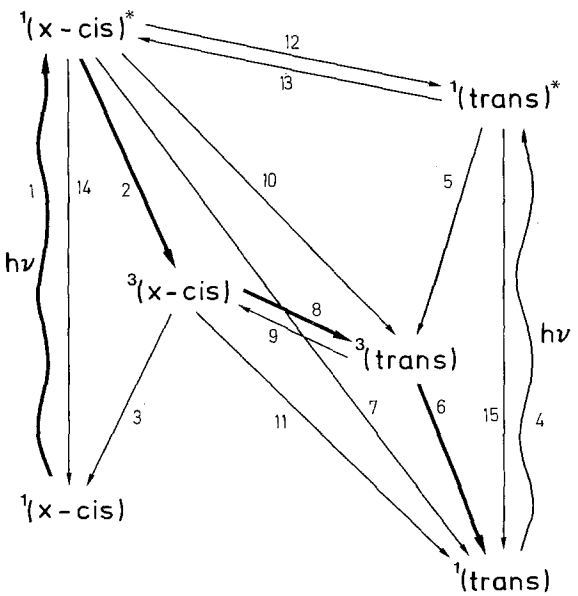

Fig. 6. Radiationless paths in retinal. (*x*-cis) stands for 9-cis, 11-cis, and 13-cis retinal, as well as dicis and tricis isomers. The scheme is simplified and restricted to two retinal isomers, albeit in reality all possible retinal isomers are involved. Isomers containing 7-cis bonds do not participate in the triplet pool

photoisomerization of cis to trans retinal are the direct isomerization in the excited singlet state of the cis retinal and subsequent decay to the ground state of trans retinal, and isomerization via the triplet state. To decide between these two pathways we used a method already briefly described in [11]. There the path via the triplet state was favored. This has been substantiated in the meantime.

In this discussion paper we cannot include all the experimental details, instead we give only the methodology and the results. Figure 7 shows the singlet absorption spectra of 11-cis and of trans retinal, as well as the triplet spectrum of trans retinal. The triplet spectrum of 11-cis retinal is not drawn in Figure 7. It differs distinctively from that of trans retinal. Figure 8 demonstrates with two examples which absorption changes could be expected for different assumed pathways after photoexcitation of trans retinal. At first we consider the simple case of a retinal isomer being excited to its first excited singlet state, dropping to its triplet state, and then falling back to its original ground state (4 + 5 + 6 in Figure 6). The absorption changes drawn in Figure 8a–c should occur. At the wavelength of the isosbestic point λ_i between the absorption spectra of the retinal isomer and its own triplet, no absorption change should be seen (Fig. 8a). On the short wavelength side of λ_i ($\lambda < \lambda_i$) one measures curves like the one shown in Figure 8b, on the long wavelength side of λ_i ($\lambda > \lambda_i$) such like the one shown in Figure 8c. All curves return to the baseline.

Now let us consider the case, where isomerization occurs on the triplet level. We assume that the triplet lifetime is long enough to allow thermal equilibration of the

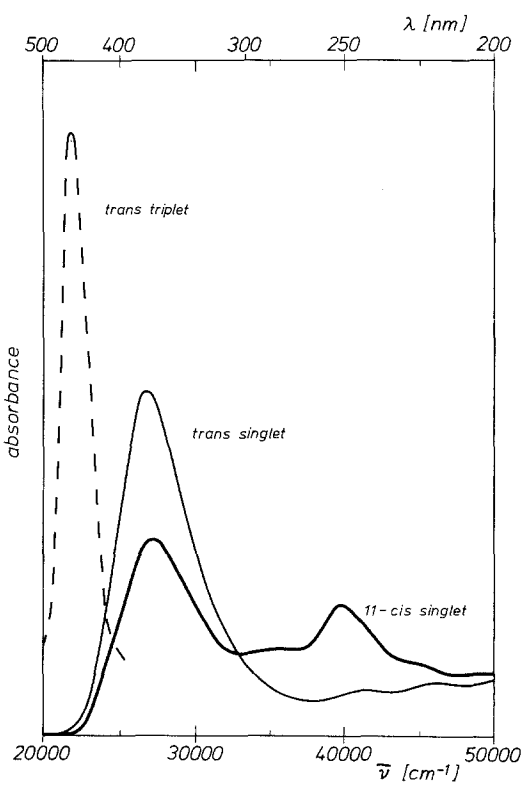

Fig. 7. Absorption spectra of all-trans retinal, 11-cis retinal, and the triplet of all-trans retinal, measured in methylcyclohexane at room temperature

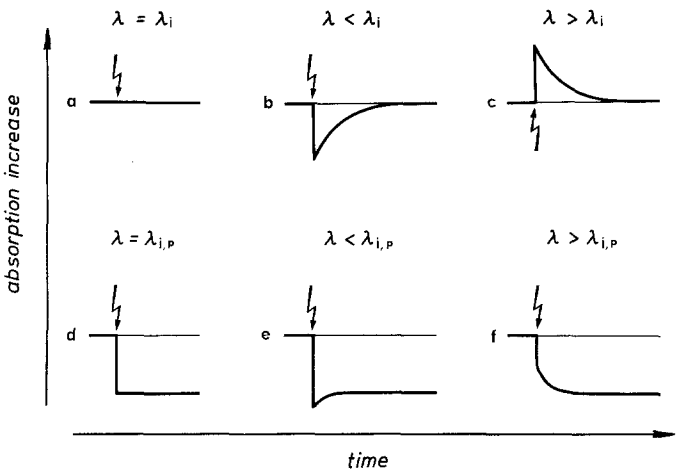

Fig. 8. Schematic drawings of absorption changes after photoexcitation of retinal. **a–c:** Expected changes for retinal going through the cycle 1 + 2 + 3 or 4 + 5 + 6 (Fig. 6). **d–f:** Expected changes for retinal isomerizing from trans to cis retinal (4 + 5 + 9 + 3). Cis retinal isomerizing to trans (1 + 2 + 8 + 6) gives the mirror images about the time axis. λ: Wavelength of the absorption change. λ is the isosbestic point or near to it. λ_i and $\lambda_{i,p}$ represent the long-wavelength isosbestic points. λ_i: Isosbestic point between the absorption spectrum of a retinal isomer and the absorption spectrum of its triplet. $\lambda_{i,p}$: Isosbestic point between the pool of the equilibrated isomers in their triplet state and their ground states

retinal isomers in the triplet state, i.e. a pool of thermally equilibrated isomers is established. The ratio of the isomers formed in the triplet pool should then be independent of the isomers originally flashed. Each of the isomers will decay from its triplet to its own singlet ground state. The pool of the triplets will form an isosbestic point ($\lambda_{i,p}$) with their ground states. $\lambda_{i,p}$ is slightly different from the isosbestic point λ_i, which in contrast to $\lambda_{i,p}$ refers to an individual isomer. If we measure absorption changes at $\lambda_{i,p}$ (not shown in Figure 7) and close to this point on both wavelength sides, we should get curves like those shown in Figure 8d–f. The absorption after the flash does not return to the baseline, because cis isomers are formed, which absorb less than trans retinal in this wavelength range. The two examples given are concerned with photoreactions of trans retinal. The reader should have no difficulties to draw the corresponding curves for the photoreactions of cis retinals or for any other conceivable pathway of Figure 6.

Our experiments were performed with different retinal isomers dissolved in ethanol, EPA [4], or hydrocarbons. To excite the retinal molecules, the light of a frequency-doubled ruby laser was used (excitation wavelength 347 nm) [12]. Absorption changes were recorded in the range between 250 and 800 nm.

We restrict this report to results obtained with solutions of the four retinal isomers 9-cis, 11-cis, 13-cis, or trans in n-hexane or PMh (2-methylbutane: methylcyclohexane = 5 : 1, v/v) [6] in the temperature range from 77–298 K. From a careful analysis of our data we obtained the following results:

1) At low temperatures (77 K) each of the four isomers photoreacts with a relatively high quantum yield to its own triplet state and decays with a half lifetime of about 20 μs to its original ground state. After the flash the absorption returns always to the absorption level present before the flash (as exemplified in Figure 8a–c). Practically no photoisomerization occurs. This was also confirmed by analyzing the sample after the flash (or even after many flashes) by high-pressure liquid chromatography. In terms of Figure 6, the retinal isomer goes only through the cycle 1 + 2 + 3 or 4 + 5 + 6, respectively.

The absorption spectrum of each isomer has its specific isosbestic point with its own triplet spectrum, i.e. the wavelength λ_i is distinctly different for each isomer. This was also confirmed for other isosbestic points further in the ultraviolet.

2) At higher temperatures (room temperature) strong photoisomerization occurs. The ratio of the different isomers formed after the flash is independent of the isomer originally flashed.

In regard to the isosbestic points a striking difference is found between retinal excited at low and at high temperature. Whereas at low temperatures, as just mentioned, each retinal isomer forms its own isosbestic point λ_i with its triplet, at room temperature only one isosbestic point ($\lambda_{i,p}$) is found for all four isomers. We suppose that isomerization occurs on the triplet level, and interpret the common isosbestic point of all isomers as the isosbestic point between a *pool of isomers* in the triplet state and their ground states. Dark equilibration between the isomers is established in the triplet state. The thermal equilibration is faster than the decay of the isomers from the triplet to their corresponding singlet ground states. The different isomers decay from their triplet levels to their respective ground states according to their intrinsic lifetimes. The absorption changes caused by a flash have the characteristic features of the curves typified in Figure 8d–f. In the isosbestic point, for example, we observed a fast (unresolved) absorption change as symbolized in Figure 8d. The difference between the absorption before and after the flash depends on the isomer originally excited.

The distribution of the isomers in the triplet pool is still unknown. It was possible, however, to measure the ratio of the isomers after decaying to the ground state. This ratio is different from that found for the dark-equilibrated ground state described in part I (Table 1). Trans retinal is again the predominant isomer, whereas 11-cis retinal now appears in a much higher percentage. With the knowledge of the intrinsic triplet lifetimes of the different isomers, it would be possible to calculate the ratio of the isomers in the triplet state and thus determine the thermodynamic equilibrium in the triplet state.

3) With the knowledge of 1) and 2), predictions for the behavior of retinal isomers in the *intermediate temperature* range between 123 an 223 K can be made. In this temperature range the thermodynamic equilibrium between the isomers in the triplet state should not be reached during the triplet lifetime. The decay of the triplets competes successfully with the isomerization. Accelerating the decay of the triplet (Fig. 6, path 3 or 6), for instance with the triplet quencher oxygen, should change the number of isomerized molecules. The total number of excited molecules going via the triplet, however, should not be altered.

Oxygen is expected to have the following effects on the photoisomerization:

a) At high temperatures (room temperature), where equilibration of the isomers in the triplet state is fast compared to the triplet decay, oxygen should have no effect on the photoisomerization: the gross number of retinal molecules isomerized after the flash as well as the ratio of the formed isomers has to be independent of the oxygen concentration. Our experiments showed that this is exactly the case.

b) Lowering the temperature one would expect that fewer and fewer molecules on the triplet level surmount the activation energy necessary for isomerization, and that the rate of decay of the triplets competes more and more with the rate of isomerization in the triplet state. In this temperature range oxygen should have a strong influence on the isomerization. Again, the experiments confirmed this expectation: in the temperature range of about 123–223 K a much higher number of molecules is isomerized in the absence of oxygen than with oxygen present. The number of triplet molecules formed after the flash, however, is practically independent of the oxygen, but the number of isomerized triplet molecules is strongly oxygen-dependent. At even lower temperatures (e.g. 77 K), where thermal energy is insufficient to overcome the energy barrier between different isomers, practically no isomerization occurs. Oxygen has no influence.

The final proof that isomerization occurs on the triplet level, and not, for example, on the excited singlet level, is given by our oxygen experiments in the intermediate temperature range. (If isomerization would occur on the excited singlet level, then oxygen should have no effect on the number of molecules isomerized.)

In summary, the four retinal isomers 9-cis, 11-cis, 13-cis, and trans photoisomerize under our experimental conditions via their triplet states. Figure 6 shows as an example the path a cis molecule takes when it isomerizes to trans (thick line, 1 + 2 + 8 + 6). Both triplets, that of the cis and that of the trans retinal, are intermediates. The same mechanism is valid for a cis retinal going to any other cis retinal, and for trans retinal going to any other cis retinal.

We think that all other dicis and tricis isomers are also photo-generated and photoisomerized via the triplet state. Isomers containing 7-cis bonds do not have to be considered in this discussion, because both the activation energy and the free enthalpy are probably too high to obtain a noticeable amount of these isomers under our experimental conditions.

III. Photochemistry and Dark Equilibrium of 13-cis and trans Bacteriorhodopsin

Bacteriorhodopsin (BR) is the chromoprotein of the purple membrane (PM) of *Halobacterium halobium*. BR consists of a protein moiety and either a 13-cis or all-trans retinal moiety [13]. Protein and chromophore are covalently bound to each other via a Schiff base in a 1 : 1 ratio [14].

We have performed flash photometric experiments which help to clarify the photochemistry and interconversion of 13-cis bacteriorhodopsin ($BR^{548}_{13\text{-cis}}$) and trans bacteriorhodopsin (BR^{568}_{trans}). $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} were prepared by incubating bacterioopsin (BO), the retinal-free protein, with the respective isomers, 13-cis

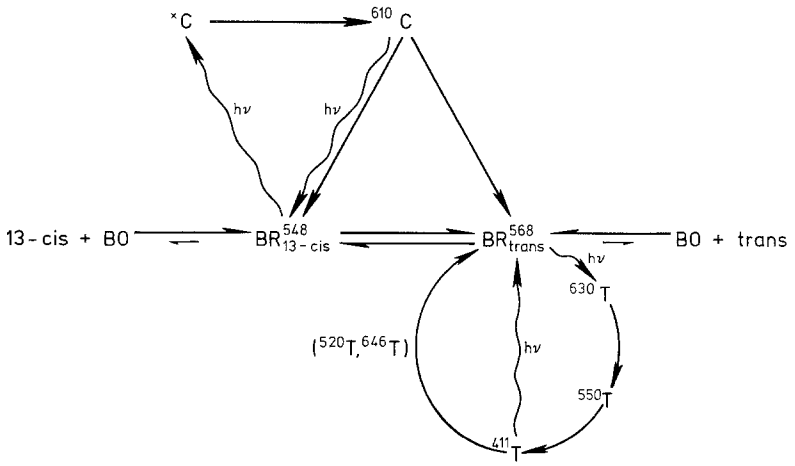

Fig. 9. Simplified reaction scheme of the photochemistry and of the dark reactions of 13-cis and trans bacteriorhodopsin. The two bacteriorhodopsin isomers are called $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans}. Products originating from $BR^{548}_{13\text{-cis}}$ after light absorption are designated 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

and trans retinal. Separate experiments were performed with $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} in buffered aqueous solution (pH 6.9), and, concerning the low temperature measurements, in a buffered glycerol/water mixture (2/1, v/v; pH 6.9).

The following simplified reaction scheme (Fig. 9) summarizes what is presently known about the photochemistry and thermochemistry of BR, based on the results of this study and literature [13, 15–18]. More details of our work are published in [19].

Experimental results and conclusions of our investigation are: $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} form different photoproducts and subsequent intermediates upon light excitation. 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}. Isolated PM and regenerated BR are spectroscopically indistinguishable.

As outlined in Figure 9, $BR^{548}_{13\text{-cis}}$ yields a photoproduct, ^{x}C, which is completely present at −90° C in less than 50 ns, the minimum time interval which our apparatus could resolve. At −90° C, ^{x}C decays with a half lifetime of 1.5 μs to the next product, ^{610}C, which at room temperature is present in less than 50 ns. ^{610}C decays in the dark with a half lifetime of 37 ms (20° C) to both $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans}, with $^{610}C \rightarrow BR^{548}_{13\text{-cis}}$ being the main path. In addition to the thermal pathway, ^{610}C is photoconvertible to $BR^{548}_{13\text{-cis}}$. At temperatures below −80° C, ^{610}C is thermally stable and was accumulated by illuminating $BR^{548}_{13\text{-cis}}$ with green light (λ = 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 ($\lambda = 610$ nm) recovered (not completely) 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}.

As indicated above, BR^{568}_{trans} undergoes a reaction cycle identical to that previously described for light adapted PM [15, 17, 20, 21]. A photoreaction of the relatively long-lived intermediate, ^{411}T, was directly measured. ^{411}T was accumulated at room temperature by illumination of an aqueous suspension of PM with strong white light. Immediately following excitation at 347 nm with a laser flash, transmission changes characteristic of the formation of BR^{568}_{trans} were detected. More details are discussed in [19]. In principle, each retinal containing protein, if intermediate or not, is expected to give photoproducts when illuminated.

The fact that different sets of photoproducts and subsequent transients are formed on excitation of $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans}, was exploited to determine the ratio of $BR^{548}_{13\text{-cis}}$ to BR^{568}_{trans} in various preparations containing BR, under conditions of dark and light adaptation. It was found that the PM of living bacteria, isolated PM, and regenerated BR all possess a mixture of $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} in the dark adapted state. The ratio

$$K = \frac{[BR^{568}_{trans}]}{[BR^{548}_{13\text{-cis}}]} \quad \text{is near unity for all preparations.}$$

The equilibrium constant, K, is only slightly dependent on the temperature between 0° C and 60° C (50% BR^{568}_{trans} at 0° C; 53% BR^{568}_{trans} at 60° C). ΔH^0, the change of enthalpy for the reaction, $BR^{548}_{13\text{-cis}} \rightarrow BR^{568}_{trans}$, is about 350 cal mol^{-1}. ΔS^0, the change of entropy, was calculated to be 1.3 cal deg^{-1} mol^{-1}. Under conditions of light adaptation (10 mW cm^{-2} white light incident on the sample), only BR^{568}_{trans} was found in PM of living bacteria, isolated PM, and regenerated BR.

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 [22]. 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 ± 2.3% trans retinal, mean and s.d. of seven experiments, PM dark adapted at 1° C).

From curves like those shown in Figure 10 we drew our conclusions concerning the photochemistry and dark equilibrium of BR. The figure states 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 exciting flash. The tiny absorption change after the flash indicates that the BO is contaminated with less than 2% BR. The BO

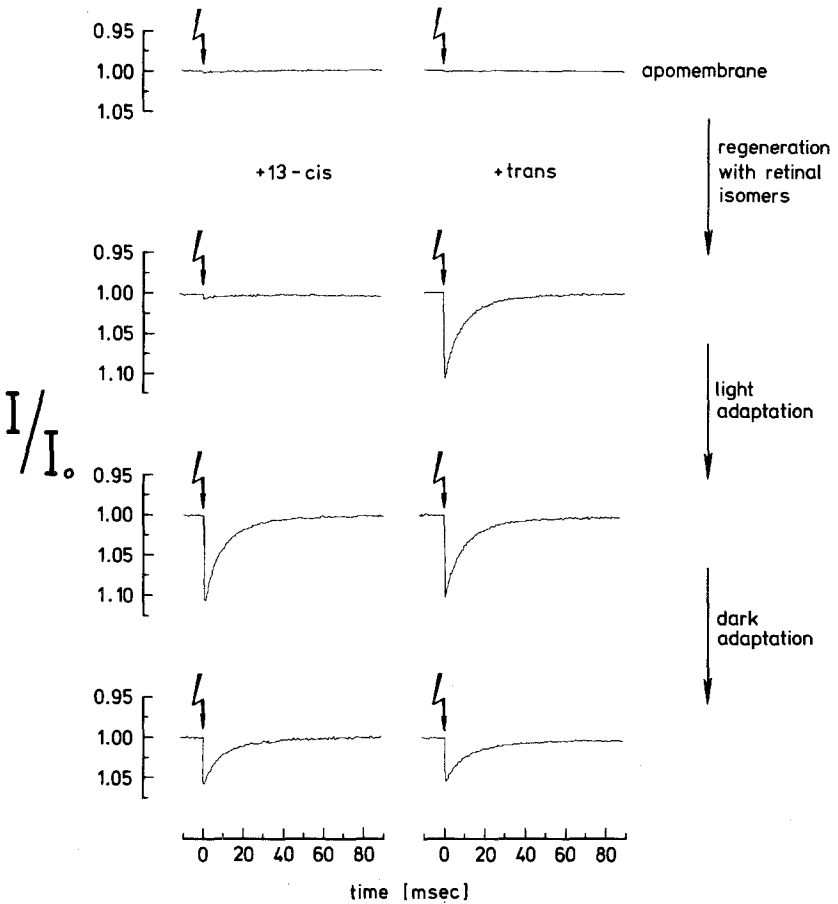

Fig. 10. Time course of transmission changes at 545 nm (which is close to the isosbestic point between the absorption spectra of ^{610}C and $BR^{548}_{13\text{-cis}}$) of apomembrane and regenerated bacteriorhodopsin. 1., 3., and 4. horizontal line are described in the figure; 2. horizontal line shows transmission changes of regenerated BR. 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 min after incubation, BR^{568}_{trans}, because of its slower rate of regeneration, after about 10 min. $\lambda_{flash} > 570$ nm, Schott filter OG 570; temperature 20° C; pH 6.88

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). 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. 9), 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. Light adaptation (third horizontal line) yields identical recordings for both samples. This

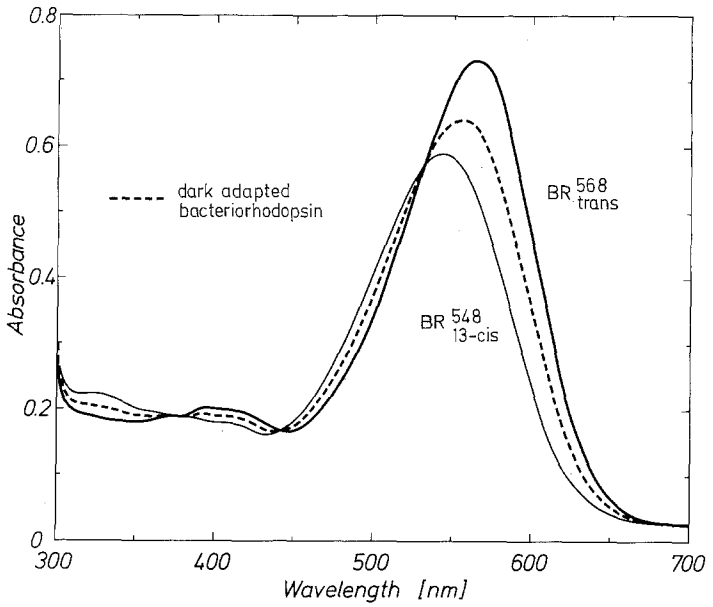

Fig. 11. Absorption spectra of $BR^{548}_{13\text{-cis}}$, BR^{568}_{trans}, and the mixture of both obtained after dark adaptation, measured at 20° C. Aqueous suspension of purple membranes measured after light adaptation (——), the same sample after dark adaptation at 20° C (– – –). Spectrum of $BR^{548}_{13\text{-cis}}$ calculated on the basis of

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

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

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. Following dark adaptation, again identical tracings were obtained for both samples (fourth horizontal line). Compared with the light adapted samples the I/I_0-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.

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 $\rightleftarrows$ trans isomerization. We never found any other retinal isomers besides 13-cis and trans retinal. The ratio $[BR^{568}_{trans}]/[BR^{548}_{13\text{-cis}}] = 1$ requires that 13-cis retinal is stronger bound to the protein than trans retinal, since the thermodynamic

ratio of trans retinal to 13-cis retinal in solution is 3.0 (see part I of this paper).

Knowing the ratio of the two bacteriorhodopsin isomers in the dark and light adapted state it is possible to calculate the absorption spectrum of $BR^{548}_{13\text{-cis}}$ (Fig. 11). Measuring the spectra of $BR^{548}_{13\text{-cis}}$ and BR^{568}_{trans} after regeneration gives the same results, but is more complicated because of scattering and especially dark isomerization during regeneration.

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