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Photochemical and Functional Properties of Bacteriorhodopsins Formed from 5,6-Dihydro- and 5,6-Dihydrodesmethylretinals†

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ABSTRACT: 5,6-Dihydroretinal and 5,6-dihydro-1,1,5,9,13-desmethylretinal are synthesized, and their all-trans isomers are shown to form pigment analogues (λ_{max} at 475 and 460 nm, respectively) of bacteriorhodopsin (purple membrane protein). The shift of the absorption maximum of the pigment from that of the protonated Schiff base of the chromophore for 5,6-dihydrobacteriorhodopsin is small compared to that of the native pigment, suggesting that negative charges similar to those controlling the λ_{max} of visual pigment rhodopsin exist near the cyclohexyl ring. Both pigment analogues undergo reversible light-induced spectral shifts reflecting cyclic photoreactions of the pigments. These results indicate that the absence of the C-5–C-6 double bond and of the five methyl groups of retinal does not abolish the photochemistry of these pigment analogues and strongly suggest that these structural features are not directly required for the photoreactions of native bacteriorhodopsin. The apparent rates of the photochemical transformations of these artificial pigments are quite different from those of bacteriorhodopsin. A *working hypothesis* is proposed for the photocycle of the pigment analogues, which includes a slower light-induced cycling rate (for the light-adapted pigments) than that of native bacteriorhodopsin and an increased rate of dark adaptation. When incorporated into egg lecithin vesicles both pigment analogues show proton pumping ability, again indicating that the missing double bond and the methyl groups are not structurally required for the function of the pigments.

The light-transducing pigment bacteriorhodopsin (bR) is the only protein in the purple membrane of the halophilic bacteria, *Halobacterium halobium*. Its chromophore, retinal, is linked to the apomembrane through a protonated Schiff base bond and can be extracted from the light-adapted form of the pigment as the all-trans isomer. Various synthetic bacteriorhodopsins have been prepared from retinal analogues in order to study the binding of retinal to the apoprotein, as well as the role of chromophore structure on the photochemistry and the function of the pigment (Oesterhelt & Christoffel, 1976; Tokunaga et al., 1977; Marcus et al., 1977; Tokunaga & Ebrey, 1978; Crouch et al., 1979). Chemical modifications of the retinal polyene side chain and/or the β-ionone ring are possible. We report here results of studies of pigments regenerated from bleached bacteriorhodopsin and two retinal

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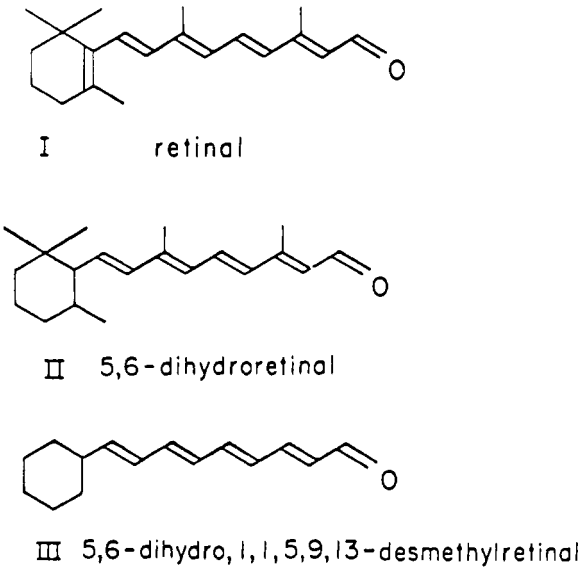

FIGURE 1: Structure of retinal and analogues: (I) retinal; (II) 5,6-dihydroretinal; (III) 5,6-dihydro-1,1,5,9,13-desmethylretinal.

analogues shown in Figure 1: 5,6-dihydroretinal and 5,6-dihydro-1,1,5,9,13-desmethylretinal (5,6-dihydrodesmethylretinal).

Isomers of 5,6-dihydroretinal have been synthesized previously and used to form pigment analogues of bovine rhodopsin (Blatz et al., 1970; Arnaboldi et al., 1979). The synthesis of a novel analogue, 5,6-dihydrodesmethylretinal, is described in the present work. We find that the all-trans isomers of both retinal analogues bind to bacterioopsin to give 5,6-dihydrobacteriorhodopsin and 5,6-dihydro-1,1,5,9,13-desmethylbacteriorhodopsin. (The two bacteriorhodopsin analogues will be abbreviated to [5,6-dihydro]bR and [5,6-dihydrodesmethyl]bR, respectively, where the square brackets are used to indicate specifically that the modifications are made in the chromophores.) The double bond between C-5 and C-6 in the cyclohexyl ring of retinal is saturated in both of the retinal analogues. The conjugated double bond system is thus shortened and resides only in the polyene side chain, leading to blue-shifted absorption maxima. Since the cyclohexyl ring and the polyene side chain are no longer in conjugation in these two molecules, there might be more freedom of motion of the side chain relative to the ring. In addition to the saturation of the 5–6 double bond, all of the five methyl groups are removed in 5,6-dihydrodesmethylretinal so that when complexed with bacterioopsin, the side chain presumably has even more freedom in its binding site.

Both [5,6-dihydro]bR and [5,6-dihydrodesmethyl]bR show light-induced absorption changes. The simplest model consistent with the spectroscopic data to be reported consists of the light/dark adaptation and the photocycling similar to those of bR. The relative rates of these reactions are, however, apparently different from those of bacteriorhodopsin. For bR, irradiation of the dark-adapted form (bR^{DA}, λ_{max} at 558 nm) at room temperature with moderately bright lights (for example, from a 400-W projector) leads to the formation of the light-adapted bR(bR^{LA}, λ_{max} at 568 nm), which in neutral pH reverts to bR^{DA} after ~50 min in the dark at room temperature. Since this irradiation also causes the light-induced cycling of bR^{LA}, the observation of only bR^{LA} immediately after the irradiation is the result of the much slower rate of dark adaptation compared to the rate of decay of the photoproducts. For both [5,6-dihydro]bR and [5,6-dihydrodesmethyl]bR, the light/dark adaptation is not apparent at room temperature but could be observed at low temperatures (e.g., at ~210 K), suggesting that the rate of dark adaptation at room temperature is not significantly slower than that of photocycling at the given light intensity.

When incorporated into egg lecithin vesicles, both pigment analogues give rise to light-induced pH changes in the same fashion as bR, indicating that the artificial pigments can pump protons across the cell membrane. It appears therefore that neither the double bond between C-5 and C-6 nor any of the five methyl groups are required for the photochemistry or the functioning of bacteriorhodopsin.

Materials and Methods

H. halobium is cultured and the purple membrane prepared according to Becher & Cassim (1975). The preparation of bleached purple membrane and the regeneration of the pigment analogues from bleached membrane and all-trans isomers of the retinal analogues have been described in detail (Tokunaga & Ebrey, 1978). Only freshly prepared purple membrane is used, and the pigments are regenerated soon after the bleaching. Residual hydroxylamine in the bleached membrane is removed by repetitive washings with distilled water followed by dialysis against distilled water. Various double-bond isomers of 5,6-dihydroretinal are synthesized, separated by high-performance liquid chromatography (LC), and identified by ^1H NMR and other spectroscopic methods as described previously (Blatz et al., 1970; Arnaboldi et al., 1979).

For the synthesis of 5,6-dihydrodesmethylretinal, the reaction of cyclohexanecarboxaldehyde (Aldrich, 0.02 mol) with triethyl 4-phosphonocrotonate (Aldrich, 0.02 mol) in the presence of sodium amide (0.02 mol) in dry tetrahydrofuran (100 mL) for 12 h at 22 °C under N_2 yields the intermediate "C-15" ester (57%). The ester is reduced to the alcohol with an excess of $LiAlH_4$ in ether and then oxidized with excess MnO_2 in ether to the aldehyde. The above reaction sequence is repeated on the C-15 aldehyde to yield 5,6-dihydrodesmethylretinal with an overall yield of 16%. The final product is purified by preparative thin-layer chromatography on silica gel GF (Analtech) with 10% ethyl acetate in hexane (R_f = 0.62) and then further purified by high-performance LC (μ Porasil, 2% ether in hexane). The product is characterized by mass spectrum [(70 eV) m/e 216 (M^+)], absorption data (λ_{max} at 340 nm for the all-trans isomer in ethanol), infrared ($CHCl_3$, C=O at 1675 cm^{-1}), and NMR [($CDCl_3$) absence of methyl protons; vinyl protons, m at 5.0–6.0–6.3 ppm, CHO, d at 10.0 ppm].

The purity of the retinal analogues is checked by high-performance LC prior to regeneration. The regeneration of pigment analogues is carried out by adding 1-μL aliquots of concentrated ethanolic solutions of the retinal analogues to aqueous suspensions (2–3 mL) of bleached membrane, followed by thorough mixing. The optical density of the final sample was usually ~1.0 in 1.0 cm path length cells.

A Cary Model 118C spectrophotometer was used for recording absorption spectra, and a Jasco-40 spectropolarimeter was used for circular dichroic spectra. Absorption spectra of samples at temperatures as low as that of liquid nitrogen (77 K) were measured by using a glass Dewar that was continuously evacuated. Extraction and isomer identification of membrane-bound chromophore was carried out according to the methylene chloride method (CH_2Cl_2 method) as described by Crouch et al. (1975) and Pilkiewicz et al. (1977) and modified by Tsuda & Ebrey (1980).

A mixture of egg phosphatidylcholine and purple membrane or membrane containing each synthetic pigment analogue (15 mg of lipid/mg of protein) in 150 mM KCl was sonicated under nitrogen for 10 min (in 1-min bursts separated by 2 min

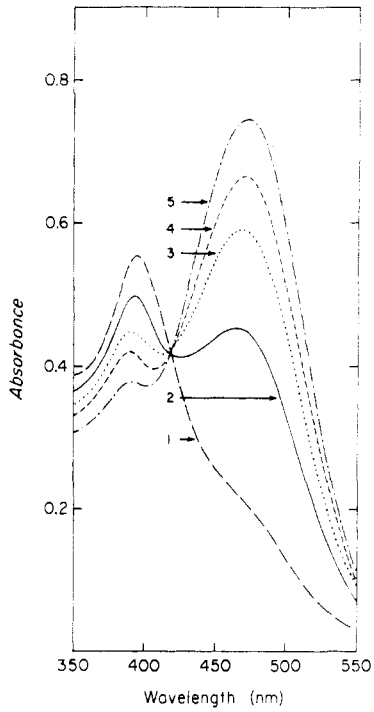

FIGURE 2: Regeneration of [5,6-dihydro]bR in distilled water at room temperature (pH 6.8). The absorption spectra are recorded with a sample of bleached membrane in the reference beam of the spectrophotometer. After the addition of 4 μL of *all-trans*-5,6-dihydroretinal ($OD_{370} \simeq 360$), the spectra (curves 1–5) are recorded at 1, 5, 10, 15, and 30 min, respectively. The final curve represents the dark-adapted pigment and has an absorption maximum at 475 nm. The minor peak of curve 5 at ~380 nm is due to unreacted chromophore and disappears upon further incubation (~2 h more).

of cooling). The vesicle preparation was centrifuged at 20000 rpm for 1 h, and the supernatant was used for the proton-pumping experiments. The pH change was measured, at 18 °C, by a ceramic junction combination pH electrode (Beckman Type 39030).

Results

(*A*) *5,6-Dihydrobacteriorhodopsin*

Regeneration. Figure 2 shows the regeneration of [5,6-dihydro]bR from the bacterioopsin and the retinal analogue. The absorption maximum of the dark-adapted pigment is at 475 nm, red-shifted from 368 nm of the free chromophore in ethanol. An intermediate species preceding the final formation of the 475-nm pigment is indicated by the maximum at 395 nm which appears as soon as the chromophore is mixed with the bleached bR; a regeneration intermediate has also been observed in the regeneration of bR (Schreckenbach & Oesterhelt, 1977).

Binding of Retinal Analogue. When *all-trans*-5,6-dihydroretinal is added to native purple membrane, no absorption increase is observed at 475 nm, indicating that the retinal analogue does not bind to the membrane at sites other than the normal retinal binding site of bR. However, when a stoichiometric amount of *all-trans*-retinal is added to a fully regenerated [5,6-dihydro]bR sample, the absorption at 475 nm is slowly displaced by the 558-nm absorption of the dark-adapted form of purple membrane (Figure 3), indicating that retinal and the retinal analogue compete for a common binding site and that the binding of retinal to the apomembrane is stronger than 5,6-dihydroretinal. From this experiment, we infer that the ratio of the maximum extinction of [5,6-dihydro]bR to that of bR is ~0.85.

The circular dichroic spectrum of [5,6-dihydro]bR is similar in shape to that of the native purple membrane. It is biphasic,

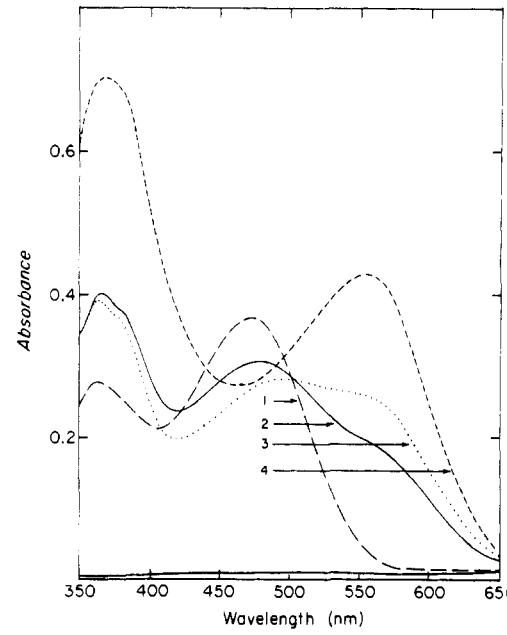

FIGURE 3: Displacement of absorption of [5,6-dihydro]bR (curve 1) by that of bR at room temperature. The apomembrane has been completely regenerated with excess *all-trans*-5,6-dihydroretinal, as indicated by the peak at 370 nm in curve 1. Curve 2 is recorded ~4 h after an addition of *all-trans*-retinal (in 2 μL of ethanol, $OD_{380} \simeq 30.0$; total sample volume is ~2.0 mL). The sample equilibrates after ~10 h (curve 3). Curve 4 is recorded after a second addition of free retinal (6.0 μL, $OD_{380} \simeq 30.0$) and overnight incubation.

and for a sample of OD_{475} equal to 1.0 the ellipticity at 485 nm (the λ_{max} of the negative CD band) is 3.7 m°. This is to be compared to 6.2 m° for a bR sample with OD = 1.0 at 560 nm (Ebrey et al., 1977). The difference in the ellipticity could arise from either different dipolar strengths and/or different geometrical arrangements of the chromophores in trimeric clusters in the membrane (Ebrey et al., 1977).

Photochemistry. The results on the study of the photochemistry of [5,6-dihydro]bR to be presented below indicate that the pigment analogue undergoes light-induced cyclic absorption changes due to the formation of species similar to the M and the K intermediates of bR. Although not all intermediates of the bR photocycle can be correspondingly identified spectrally for this analogue (partially because of the spectral overlap of the intermediates, the relative rates of their interconversions, and possible branching pathways), a bR-like photocycle is consistent with the observations described below and is a good working hypothesis for presenting the data. The novel features of the [5,6-dihydro]bR photocycle in this model are a slower rate of thermal reversion of the short-wavelength "M" intermediate and a relatively faster rate of dark adaptation of the pigment.

Light/Dark Adaptation. There is an *apparent* lack of light/dark adaptation in [5,6-dihydro]bR at room temperature. With a 400-W projector, a brief irradiation (5 min) of [5,6-dihydro]bR^{DA} (i.e., kept in the dark overnight) does not produce a red shift in λ_{max} as in the case of bR. (With prolonged irradiation, a decrease in the main absorption at 475 nm is observed. This decrease is reversible in the dark without shifts in λ_{max} and is associated with the formation of the M-type intermediate discussed below.) The apparent absence of the light-adapted form of [5,6-dihydro]bR, however, could result if the rate of dark adaptation is comparable to or faster than the rate of photocycling at room temperature. Absorption changes observed at ca. 210 K (−65 °C) indeed indicate the existence of a red-shifted species at this temperature which can be identified as [5,6-dihydro]bR^{LA}. As shown in Figure

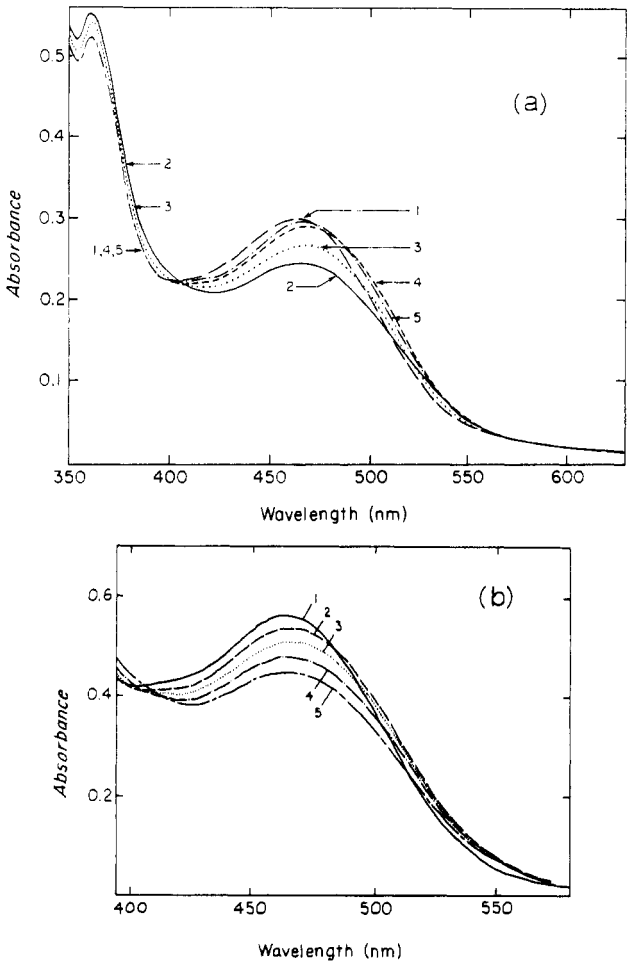

FIGURE 4: Evidence for a "light-adapted" form of [5,6-dihydro]bR. All spectra are recorded at 210 K. The sample contains 67% glycerol, and no reference sample (bleached bR) is used in the spectral recordings. A projector with a 400-W tungsten bulb in combination with interference filters is used for irradiation. (a) (Curve 1) [5,6-Dihydro]bR^{DA}; (curve 2) after 20 min of 480-nm irradiation; (curve 3) warmed to ~240 K (−30 °C) and recooled to 210 K for recording the spectrum; (curve 4) warmed to 250 K (−20 °C) and recooled; (curve 5) warmed to 265 K (−5 °C) and recooled. (b) Irradiation of [5,6-dihydro]bR^{DA} (curve 1) at 210 K with 480-nm light. Curves 2–5 are recorded after 1, 3, 8, and 20 min of irradiation, respectively.

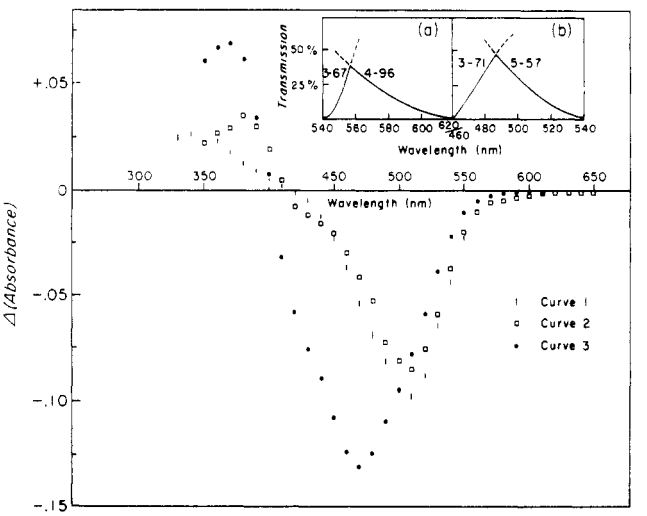

FIGURE 5: Difference spectra between M-[5,6-dihydro]bR and [5,6-dihydro]bR^{DA}. Curve 1 is the difference spectrum obtained at room temperature with "560-nm" irradiation (inset a, where the combined transmission curve of two Corning glass filters, 3-67 and 4-96, is shown). The optical density of the sample before irradiation is 0.9. For this difference spectrum, the absorbance of the sample is recorded every 10 nm from 650 to 330 nm before irradiation and, after an initial irradiation of 30 min (upon which further irradiation produces little change in the absorbance), every 10 nm in the same spectral region with 1 min of irradiation between readings (which requires ~5 s each). The difference between the absorbance (averaged from three differences) is then plotted. Curves 2 and 3 are respectively obtained from "560-nm" and "490-nm" (Inset b) irradiation at 210 K, for a sample containing 67% glycerol. The samples have an optical density of 0.6 at 480 nm and are irradiated for 20 min each. The difference in the shape of the negative bands indicates the presence of two photochemically active species in [5,6-dihydro]bR^{DA}.

4b, curve 2, a stable red-shifted species is produced by irradiating [5,6-dihydro]bR^{DA} with 480-nm light for 1 min. [Prolonged irradiation of the sample produces the "M"-type intermediate discussed below (Figure 4a, curve 2).] [5,6-Dihydro]bR^{DA} is recovered by warming this red-shifted species without the formation of any of the photocycle intermediates discussed below, such as "M". When a sample containing "M" is warmed, however, it goes to a red-shifted species before recovering to [5,6-dihydro]bR^{DA} (Figure 4a). This red-shifted species is probably the same as that shown in Figure 4a, suggesting that it can be tentatively identified as [5,6-dihydro]bR^{LA} and that the fast rate of dark adaptation at room temperature is apparently reduced at 210 K and thus allows the observation of the red shift due to [5,6-dihydro]bR^{LA}.

When the chromophore of [5,6-dihydro]bR^{DA} is extracted from the membrane and identified by high-performance LC, two distinct peaks are observed, indicating a heterogeneous isomeric composition of the chromophore of [5,6-dihydro]bR^{DA} similar to bR^{DA}. The second peak coelutes with authentic *all-trans*-5,6-dihydroretinal (retention time ~13.5 min, μ Porisil column, 2% ether–hexane, 2 mL/min), while the first peak, with a retention time of ~7.7 min, is due to the 13-cis isomer. The ratio of the peak areas measured at 345 nm is approximately 1:1.5 (13-cis/all-trans). Since 13-cis isomers generally have lower extinction coefficients than all-trans isomers, the molar ratio of the two isomers extracted from [5,6-dihydro]bR^{DA} probably lies between 1:1.5 and 1:1.

Formation of a Short-Wavelength, M, Intermediate. Upon prolonged illumination, [5,6-dihydro]bR is converted into a species absorbing near 370 nm (Figure 5a, curve 1). Since this large blue shift is comparable to that seen when bR is photoconverted to M, this short-wavelength intermediate is designated as the M intermediate of [5,6-dihydro]bR. It decays to [5,6-dihydro]bR^{DA} at room temperature with a half-time of ~2 min, indicating a slow rate of the photochemical cycling of the pigment. The decay of M-[5,6-dihydro]bR to the light-adapted pigment and then on to the dark-adapted one is quite different from the pattern seen for bR, where the M to bR^{LA} transition takes place in 10 ms and bR^{LA} to bR^{DA} requires more than 30 min, thus allowing the observation of the dark adaptation. For [5,6-dihydro]bR, the rate of the light-adapted pigment going to the dark-adapted one is faster than that of M-[5,6-dihydro]bR going to the light-adapted pigment.

At 210 K (−65 °C) M-[5,6-dihydro]bR^{LA} is stable; unlike M of bR (Becher & Ebrey, 1977), its stability at this temperature requires neither a high pH nor a high salt concentration. The difference spectra between M-[5,6-dihydro]bR^{LA} and dark-adapted [5,6-dihydro]bR at this temperature obtained with two different irradiations are shown in Figure 5 (curves 2 and 3). The shapes of the negative bands in these two curves are different indicating that two species are present in [5,6-dihydro]bR^{DA}, in accordance with the result of chromophore extraction. In addition, both species are active in the photoconversion to the M intermediate. In conjunction with the observation in Figure 4b, these results suggest that

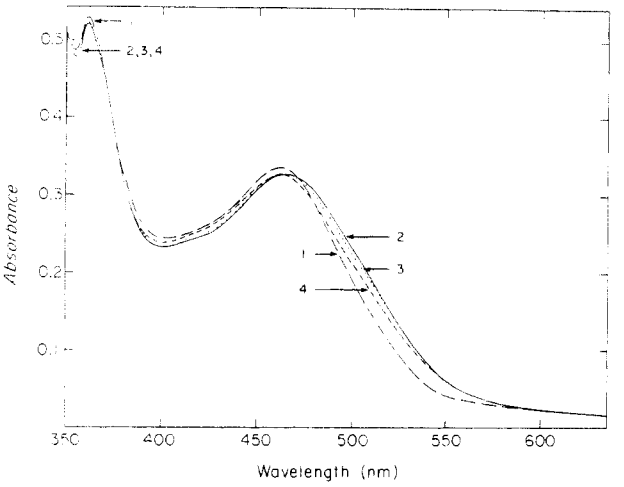

FIGURE 6: Light-induced absorption changes of [5,6-dihydro]bR at 77 K. The sample contains 67% glycerol, and no reference sample is used for the measurements. Curve 1 is for [5,6-dihydro]bR^{DA}. Curves 2–4 are recorded for photostationary mixtures due to 400-, 460-, and 480-nm irradiation, respectively.

540-nm light converts the long-wavelength component of the dark-adapted pigment mixture to the M-type intermediate (curve 2) and 480-nm light converts the [5,6-dihydro]bR species of shorter λ_{max} first to the species of longer wavelength and then to M.

Formation of the K Intermediate. The spectrum of [5,6-dihydro]bR^{DA} at 77 K is shown in Figure 6 (curve 1). Illumination of dark-adapted [5,6-dihydro]bR at 77 K produces a stable red-shifted absorption, indicating a bathochromically shifted species similar to the the K intermediate of bR is formed photochemically at this temperature (curve 2 in Figure 6). K-[5,6-Dihydro]bR decays to M-[5,6-dihydro]bR when warmed to 210 K. Figure 6 also shows the results of irradiating the sample at 77 K with several different wavelengths. The lack of an isosbestic point in the spectral changes indicates the interconversion of more than two species. This observation supports the working hypothesis that [5,6-dihydro]bR^{DA} contains a 13-cis and a trans pigment and each is converted into its own bathoproduct. In the case of native bR, both the ligth-adapted and the dark-adapted forms are photoconverted to bathoproducts at 77 K (Tokunaga et al., 1976).

Light-Induced Proton Translocation by [5,6-Dihydro]bR. One of the physiological functions of bacteriorhodopsin in the halophilic bacterium cell membrane is to use light energy to actively pump the protons across the membrane and set up a proton gradient that is coupled to other energy-requiring metabolic functions of the cell. Purified purple membrane sheets can be incorporated into artificial egg lecithin vesicles which show a light-induced proton-uptake activity (Lozier et al., 1975; Tokunaga et al., 1977; Govindjee et al., 1980). When [5,6-dihydro]bR is incorporated into egg lecithin vesicles, proton uptake is also observed (Figure 7A,B). The slower cycling rate of [5,6-dihydro]bR compared to bR is qualitatively reflected by the slower rate of proton pumping (Figure 7A). Figure 7B shows that light between 400 and 500 nm is more effective than longer wavelength light (>500 nm) in producing the pH changes and the pH change is abolished by FCCP. These results indicate that the pH changes are due to active pumping of protons by the pigment analogue, not by residual bR.

(*B*) *5,6-Dihydrodesmethylbacteriorhodopsin*

Regeneration and Chromophore Binding. The regeneration of [5,6-dihydrodesmethyl]bR is shown in Figure 8. The λ_{max}

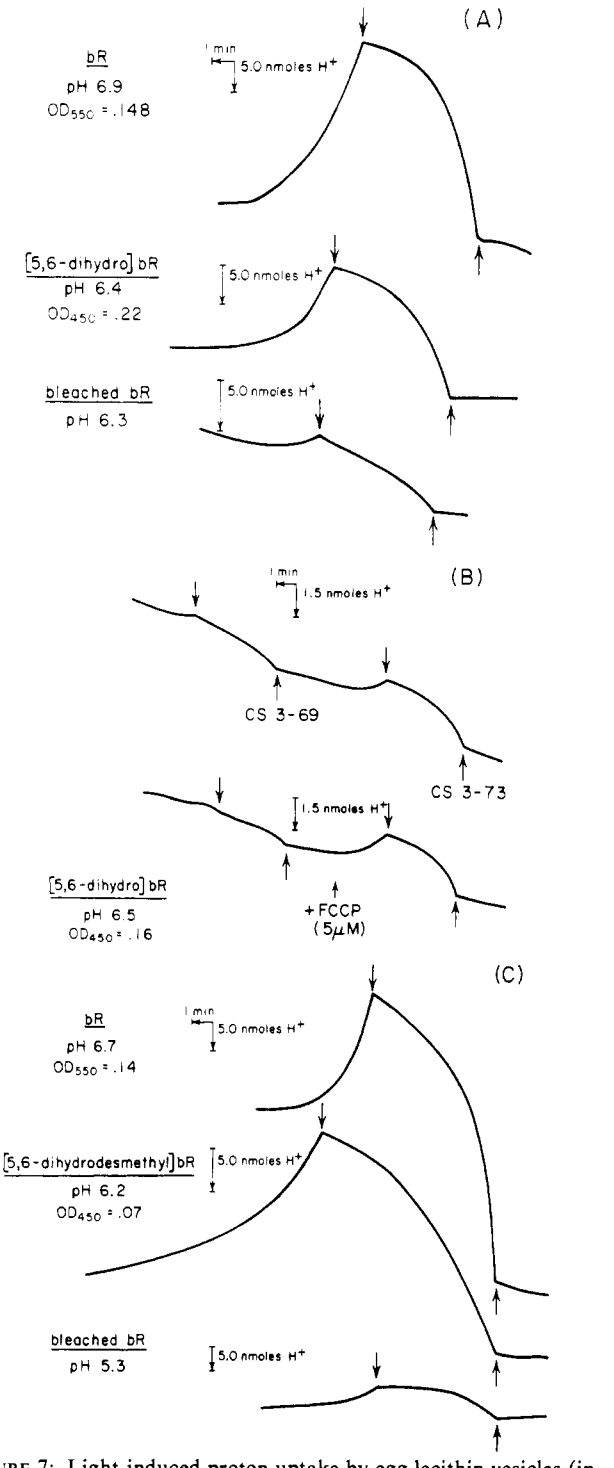

FIGURE 7: Light-induced proton uptake by egg lecithin vesicles (in 150 nM KCl) containing [5,6-dihydro]bR (A and B) and [5,6-dihydrodesmethyl]bR (C). The uptake is measured by the pH increase (upward deflection of the traces) in the suspending (aqueous) medium with a ceramic junction combination electrode (Beckman Type 39030). The traces are recorded from right to left (horizontal scale = 1 min) with the up-going arrows indicating the onset of irradiation and the downward ones the end of irradiation. The reaction volume is ~5.0 mL. The vertical scale next to the traces is the ΔpH produced by the addition of the given number of nanomoles of H^+. pH values and OD of the sample (when applicable) are also shown. The bottom traces in (A) and (C) are recorded for vesicles containing bleached bR, with the residual pH activity observed as before (Tokunaga et al., 1977). The middle traces are recorded for vesicles containing the pigment analogues, and the top traces are recorded for vesicles containing bR. Corning glass filter CS3-73 is used for irradiation. In (B), the top trace shows the light between 400 and 500 nm is more effective than light of longer wavelengths (Corning glass filter CS3-69, $\lambda > 500$ nm) in the proton pumping. The bottom trace shows the effect of FCCP on the light-induced pH changes due to [5,6-dihydro]bR.

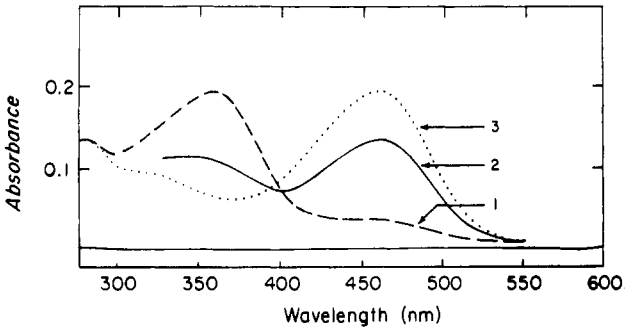

FIGURE 8: Regeneration of [5,6-dihydrodesmethyl]bR at room temperature. Curve 1 is recorded ~5 min after chromophore addition and curves 2 and 3 are recorded 30 min and 3 h, respectively, after the addition. The final spectrum represents [5,6-dihydrodesmethyl]bR^{DA}, λ_{max} = 460 nm.

of the fully regenerated pigment is at 460 nm in the dark, red shifted from 340 nm, the λ_{max} of the *all-trans*-retinal analogue in ethanol. Curve 1 in Figure 8 (λ_{max} at 370 nm) indicates that this is a regeneration intermediate for [5,6-dihydrodesmethyl]bR. As in the case of [5,6-dihydro]bR, the absorption maximum of [5,6-dihydrodesmethyl]bR at 460 nm is replaced by the 560-nm absorption of bR when a stoichiometric amount of *all-trans*-retinal is added, and the addition of 5,6-dihydrodesmethylretinal to native bR does not result in the 460-nm absorption of the pigment analogue. From these observations, it seems that this retinal analogue also binds to the apomembrane at the same binding site as retinal in bacteriorhodopsin and from the long-wavelength maximum of the pigment that the linkage is likely to be a protonated Schiff base as in bR.

Photochemistry. In spite of rather extensive modifications of the chromophore, [5,6-dihydrodesmethyl]bR also undergoes photochemical transformations. The absorption changes associated with these reactions are relatively small, although they are consistent with a photocycle similar to that of bR.

Dark/Light Adaptation. There is no *apparent* red-shifted light-adapted form of [5,6-dihydrodesmethyl]bR after illumination at room temperature. However, a blue-shifted species (blue shifted by ~10 nm) is observed under several conditions. First, irradiation of a dark-adapted sample of [5,6-dihydrodesmethyl]bR with 480-nm light at room temperature produces a transient blue-shifted absorption (Figure 9) which becomes stable at 210 K. Irradiation of the M-type intermediate (discussed below) at 210 K also produces a species near 450 nm which is photointerconvertible with M (Figure 10) and likely to be the same species noted above. Warming up of the 450-nm species leads to the return of the initial 460-nm absorption without the short-wavelength M being formed first. These results indicate that the 450-nm species is not a *thermal* intermediate before the M intermediate, but rather the parent species which, upon irradiation, leads to M. In view of this, the new species, albeit blue shifted by 10 nm from the dark-adapted species, is tentatively considered to be the light-adapted form of [5,6-dihydrodesmethyl]bR.

The M intermediate, formed by prolonged irradiation of [5,6-dihydrodesmethyl]bR^{DA} at room temperature, decays to [5,6-dihydrodesmethyl]bR^{DA} without the appearance of the 450-nm species, suggesting that the rate of dark adaptation (from the 450-nm species to the 460-nm species) is comparable to or faster than that of M returning to the 450-nm pigment.

Formation of a Short Wavelength, M, Intermediate. As shown in Figure 9, continuous irradiation of [5,6-dihydrodesmethyl]bR at room temperature produces an absorption blue shifted to near 350 nm that reverts in the dark to the

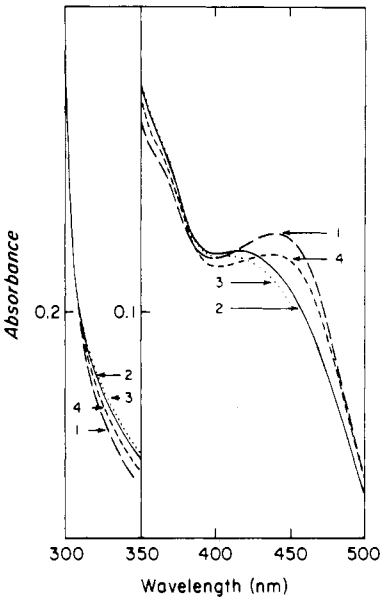

FIGURE 9: Light-induced absorption changes of [5,6-dihydrodesmethyl]bR^{DA} at room temperature. An aqueous suspension (22 mM phosphate buffer, pH 7.0) is used for spectral recording without a reference sample. Curve 1 represents [5,6-dihydrodesmethyl]bR^{DA}. Curve 2 is recorded after 460-nm irradiation (~15 min), curve 3 after 10 min of 420-nm irradiation, and curve 4 after ~25 min in dark.

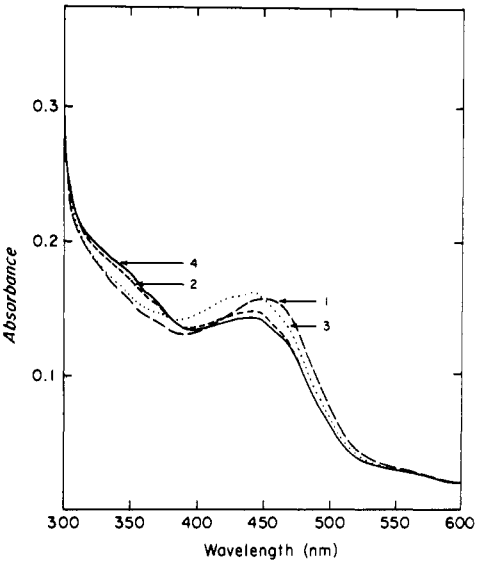

FIGURE 10: Light-induced absorption changes of [5,6-dihydrodesmethyl]bR at 210 K. The sample is in 67% glycerol and 22 mM phosphate buffer (pH 7.0); no reference sample is used. Curve 1 represents [5,6-dihydrodesmethyl]bR^{DA}. Curve 2 is recorded after 460-nm irradiation (20 min), curve 3 after 15 min of near-UV irradiation (Corning glass filter 7-60, transmitting light between 330 and 380 nm and maximally at 350 nm), and curve 4 after irradiation with 420-nm light for 20 min. Note that curves 2 and 4 are blue shifted from curve 1.

initial species. This large blue shift is similar to the shift seen in the formation of the M intermediate of bacteriorhodopsin, and an M-type intermediate absorbing near 350 nm is assumed to exist for the pigment analogue. M-[5,6-Dihydrodesmethyl]bR becomes stable at 210 K (Figure 10).

Absorption Changes at 77 K. The light-induced absorption changes of [5,6-dihydrodesmethyl]bR at 77 K shown in Figure 11 are quite small compared to those of bR. The lack of an isosbestic point in these changes indicates that there is more than one photoproduct. In addition, at least one of the photoproducts is not bathochromically shifted from the parent pigment, but rather is slightly blue shifted giving rise to the

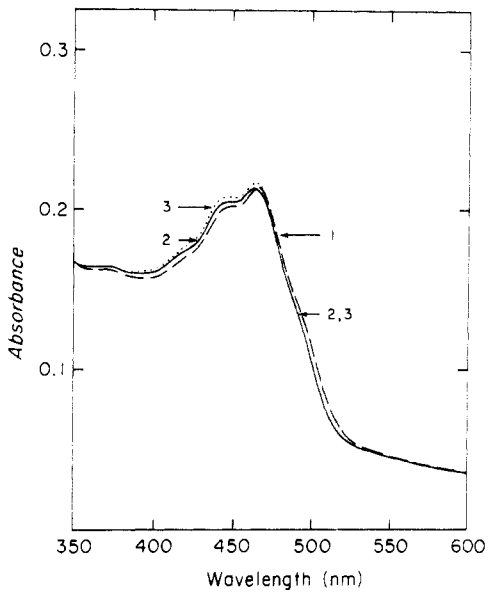

FIGURE 11: Light-induced absorption changes of [5,6-dihydrodesmethyl]bR at 77 K. The sample is identical with that used in Figure 10. Curve 1 represents [5,6-dihydrodesmethyl]bR^{DA}. Curves 2 and 3 are recorded for 440- and 520-nm photostationary state mixtures, respectively.

blue shift in absorption. The small absorption changes could be the results of small differences between the absorption spectra of [5,6-dihydrodesmethyl]bR and its photoproducts and/or the low quantum yields for the formation of the photoproducts.

Light-Induced Proton Translocation by [5,6-Dihydrodesmethyl]bR. Egg lecithin vesicles containing [5,6-dihydrodesmethyl]bR show light-induced proton uptake (Figure 7C). This result indicates that the proton-pumping ability of bacteriorhodopsin can be maintained in the absence of the five methyl groups of the chromophore.

Discussion

Two retinal analogues, 5,6-dihydroretinal and 5,6-dihydro-1,1,5,9,13-desmethylretinal, were synthesized, and their trans isomers were shown to regenerate pigments from bacterioopsin. The existence of photocycle intermediates somewhat analogous to the M and the K intermediates of bacteriorhodopsin and the ability of the pigment analogues to pump protons suggest that the changed features of the chromophores are not essential to the functioning of pigment.

Pigment Formation. Both 5,6-dihydroretinal and 5,6-dihydro-1,1,5,9,13-desmethylretinal form bacteriorhodopsin analogues indicating that the C-5–C-6 double bond and the five methyl groups of retinal are not required for pigment formation. The long-wavelength absorption maxima of the pigments also suggest that the covalent linkage is formed between the retinal analogues and the apoprotein is very likely to be a protonated Schiff base as in bR. This is also indicated by preliminary resonance Raman experiments (R. Schiffmiller and R. Callender, personal communication). Both [5,6-dihydro]bR and [5,6-dihydrodesmethyl]bR are converted to bR when stoichiometric amounts of *all-trans*-retinal are added. It seems most likely that retinal displaces the 5,6-dihydroretinal analogues by virtue of a stronger association equilibrium constant with the apomembrane. In addition, the two pigment analogues we have studied here are rapidly destroyed (within 1 h) by hydroxylamine in the dark (~250 mM, pH 6.8). The increased dark sensitivity of these two pigment analogues to NH_2OH probably is related to the ability of retinal to displace the chromophores.

Absorption Spectra of Pigments. The absorption spectra of these two pigment analogues are red shifted from their corresponding chromophores. However, the spectral red shift of the pigments relative to protonated Schiff bases of the chromophores is much smaller for [5,6-dihydro]bR (2500 cm^{-1}) than for bR (4900 cm^{-1}). It has been proposed that the red shift in the absorption of retinal-containing pigments is due to a counterion next to the protonated Schiff base together with a "second negative charge" located close to the chromophore (Honig et al., 1976). In the case of visual pigments, a remarkable *increase* in the spectral red shift of pigment formation is observed when the C-11–C-12 double bond is saturated (i.e., 5300 cm^{-1} for 11,12-dihydrorhodopsin vs. 2700 cm^{-1} for rhodopsin, Arnaboldi et al., 1979). From this it was deduced that the second negative charge is localized close to both the 13–14 and 11–12 bonds in rhodopsin (Nakanishi et al., 1979; Honig et al., 1979a). The large decrease in the spectral shift for [5,6-dihydro]bR, on the other hand, suggests that in bR and its analogues similar charge(s) may exist near the 5–6 bond, i.e., close to the cyclohexyl ring.

Photochemistry. One of the most prominent intermediates in the photochemical cycle of bR is the blue-shifted M intermediate (M-bR). Blue-shifted species are also clearly observed for both [5,6-dihydro]bR and [5,6-dihydrodesmethyl]bR after irradiation. The assignment of these intermediates corresponding to M-bR is based on their large spectral blue shifts (relative to the parent pigments) similar to the shift of M-bR from bR and on their cycling back to their parent pigments. This observation alone (independent of the detailed scheme of the cycling) proves that the ability of the bacteriorhodopsin analogues to undergo cyclic photochemical reactions is not blocked by either the saturation of the C-5–C-6 bond or the absence of all the five methyl groups of retinal. This, in turn, suggests that in native bacteriorhodopsin both the C-5–C-6 double bond and the methyl groups are not structurally required for the photochemistry. In addition, both pigments show absorption changes at 77 K upon irradiation (although [5,6-dihydrodesmethyl]bR has smaller changes). The direct participation of the C-5–C-6 double bond, in particular, is required in several models of the primary photochemistry of bacteriorhodopsin and rhodopsin (van der Meer et al., 1976; Peters et al., 1977; Applebury et al., 1978). Similar conclusions that the C-5 methyl group and the C-5–C-6 double bond of the cyclohexyl ring of the chromophore are not involved in the primary photochemistry of rhodopsin pigments were reached also by Kropf (1976) and Kawamura et al. (1979), respectively, for 5-desmethylretinal- and retro-γ-retinal-based analogues.

The quantum yield of the photocycling cannot be determined directly from irradiating the dark-adapted pigments since they contain more than one photochemically active species, as shown in Figure 5 for [5,6-dihydro]bR. An estimate can, however, be obtained from irradiating [5,6–dihydro]bR^{LA}. By use of rhodopsin as an actinometer (quantum yield of bleaching equals 0.67), the quantum yield for the initial bleaching of [5,6-dihydro]bR^{LA} (by 480-nm light to form M-[5,6-dihydro]bR) is estimated to be 0.32 ± 0.10, on the same order of magnitude for the quantum yield of bR photocycling. This further supports the conclusion that the photochemistry of the pigments is not drastically affected by the chromophore modifications in these two analogues.

For the photochemical changes of [5,6-dihydro]bR and [5,6-dihydrodesmethyl]bR, a bacteriorhodopsin-like photocycle has been used as a reasonable working model. Although the model is not proven as a unique photocycle for the analogue

pigments, it is consistent with the spectral observations reported above. If the model represents the true photocycle, the chromophore modifications appear to affect the rate-determining processes of the photocycle of the pigments, e.g., the rate of pigment cycling is slower compared to bR. The spectral evidence, on the other hand, suggests the existence of an unstable (short-lived) light-adapted form for both the pigments that dark adapts much more rapidly at room temperature than does native bR. Therefore, while the initiation of the pigment's photochemistry is not greatly affected by the chromophore modifications, some rate-determining dark (thermal) processes apparently are.

Proton Pumping. In addition to the photochemical cycling, both [5,6-dihydro]bR and [5,6-dihydrodesmethyl]bR pump protons as measured by the light-induced pH increases of the suspending medium of pigment-containing vesicles. These results indicate that not only are the double bond between C-5 and C-6 and all of the five methyl groups not required for the photochemistry but also they are not required for the proton pumping action of the artificial pigments. Indeed, the [5,6-dihydrodesmethyl]bR chromophore consists essentially of a simple polyene chain which, judging from its absorption spectrum and resonance Raman data, is attached to the protein via a protonated Schiff base bond. Trans–cis isomerization of the chromophore [as suggested by Rosenfeld et al. (1977), Pettei et al. (1977), and Honig et al. (1979b)] would therefore seem to be the simplest type of model for the primary photochemistry of this pigment, as well as for bacteriorhodopsin itself.

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