# EFFECT OF HIGH PRESSURE ON THE ABSORPTION SPECTRUM AND ISOMERIC COMPOSITION OF BACTERIORHODOPSIN

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ABSTRACT The effects of high pressure upon the absorption spectra and isomeric composition of the dark ($bR^D$) and light adapted ($bR^L$) forms of bacteriorhodopsin were examined. Pressure favors the 13-*cis* form of bacteriorhodopsin ($bR^{13\text{-}cis}$). The equilibrium isomeric composition and absorption spectra of bacteriorhodopsin samples at a given pressure were the same starting from either light or dark adapted bacteriorhodopsin. From the effect of pressure on the equilibrium constant between $bR^{all\text{-}trans} \rightleftharpoons bR^{13\text{-}cis}$ in the dark, the molar volume change between $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$ was found to be $-7.8 \pm 3.2$ ml/mol. This volume change suggests a difference in conformation between dark- and light-adapted bacteriorhodopsin, but the magnitude of the change is small, probably involving only a small number of the protein residues.

## INTRODUCTION

The purple membrane of *Halobacterium halobium* contains a single protein species (bacteriorhodopsin), with retinal as its chromophore. Bacteriorhodopsin exists in two relatively stable states—the dark-adapted form ($bR^D$) absorbing maximally at ~560 nm and the light-adapted form ($bR^L$) absorbing maximally at ~570 nm (Oesterhelt and Stoeckenius, 1973). Irradiation of $bR^D$ with light yields $bR^L$. After irradiation, $bR^L$ converts back to the original $bR^D$. A distinct difference between $bR^L$ and $bR^D$ is the isomeric composition of the retinal chromophore; the former contains 100% all-*trans* retinal ($bR^{all\text{-}trans}$) and the latter is a mixture of 13-*cis* retinal pigment ($bR^{13\text{-}cis}$) and all-*trans* retinal pigment ($bR^{all\text{-}trans}$) with a ratio of 1:1 (Oesterhelt et al., 1973; Pettei et al., 1977; Maeda et al., 1977). As the photochemical cycle and proton pumping ability of purple membrane samples which contain $bR^{13\text{-}cis}$ or $bR^{all\text{-}trans}$ are quite different (Lozier *et al.*, 1978), the conformation of the apoprotein (bacterioopsin) in $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$ is expected to be different. However, spectroscopic studies (UV, ORD, CD) (Becher and Cassim, 1976) and enthalpy studies (Ohno et al., 1977; Dencher et al., 1977) have not found any definite difference.

It occurred to us that molar free volume change ($\Delta V$) might be a useful parameter to consider in this regard because movement or ionization of as little as one residue of a protein during a conformational change possesses a measureable $\Delta V$ (Kauzmann, 1959). In fact, pressure effects on the photochemical reactions of retinal pigments have been successfully studied and have given valuable information (Lamola et al., 1974; Tsuda et al., 1977; Tsuda, 1979).

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In the present study, pressure effects on absorption spectra and the isomeric composition of the retinal chromophores of $bR^D$ and $bR^L$ were studied and $\Delta V$ obtained. The origin of $\Delta V$ will be discussed in terms of the conformational change between $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$.

## MATERIALS AND METHODS

The purple membrane was prepared from *H. halobium* by the method of Becher and Cassim (1975). All samples were buffered at pH 7.2 with 0.01 M imidazole HCl buffer, because for this buffer the pH decreases by no more than 0.1 on increasing the pressure from 1 atm to 6 kbars (Tsuda et al., 1976).

The apparatus used for pressure generation and the optical bomb will be described in detail elsewhere (Tsuda, manuscript in preparation). The high-pressure optical bomb was constructed from stainless steel and rated to 3 kbars. The optical bomb has four windows—three of them are of synthetic sapphire and the other is for thermocouple electrodes. The bacteriorhodopsin solutions were placed in a cylindrical internal cell of quartz (0.6-cm i.d., 300 $\mu$l) and were isolated from the pressure fluid by means of a quartz cylinder. The bacteriorhodopsin solution could also be placed directly in the cylinder holding the quartz cell (4 ml) if necessary. A Cary 118c spectrophotometer was used to record all absorption spectra. The spectrophotometer was optically connected to the high pressure optical bomb by UV grade optical fibers. Actinic light from a projector (400 W) was led to the sample by an optical fiber. Constant temperature was maintained by circulating a thermostatted fluid in the optical bomb.

The procedures used in the measurement of absorption spectra and extraction of retinal from the pressed or unpressed samples were generally as follows. Dark adapted bacteriorhodopsin ($bR^D$) was prepared by keeping bacteriorhodopsin in the dark at room temperature for at least 15 h before use. Light adapted bacteriorhodopsin ($bR^L$) was prepared by irradiation of bR with 500-nm light for 1 h at 0°C using an interference filter and a 400-W projector lamp. The bR sample was poured into the thermostatted high pressure cells at 30°C, kept ~5 min, and then gradually pressed to a given pressure. The absorption spectra were recorded under high pressure. After the high pressure experiment, the pressure was released gradually to 1 atm within 2 min and then the spectral change with time was measured.

When the chromophore was extracted from a pressed bR sample for analysis by high performance liquid chromatography (HPLC), the high pressure optical bomb was cooled to 0°C and the pressure released to 1 atm within 1 min 2 ml of the 4 ml sample was used for extraction. The other 2 ml was kept in the dark at room temperature for several days and then extracted (see below). To have duplicates, the samples were divided into two parts. Each 1-ml sample was vigorously mixed using a transfer pipette with 2 ml cold dichloromethane (0°C), which caused the color of bR sample to bleach. Each solution was then repeatedly sonicated (at 4°C) for 2 min and then rested for 2 min to allow for recooling. This procedure was then repeated. The emulsion was then centrifuged (10 min 4,000 rpm, 4°C; Beckman centrifuge J21 rotor, Beckman Instruments, Inc., Spinco Div. Palo Alto, Calif.) to separate the phases; the lower organic layer was then drawn off with a syringe from the aqueous layer of denatured protein. An additional 2 ml of cooled dichloromethane was added to the aqueous solution of denatured protein and the procedure repeated. The resultant dichloromethane layers were dried over sodium sulfate, filtered, and evaporated under a gentle stream of nitrogen gas. To estimate the yield of extraction, the retinal was dissolved with 2 ml of hexane and its absorption spectrum measured. The moles of retinal extracted were compared to the number of moles of the pigment. In general, the yields were ~75% (see Table I). After measuring the spectrum, the solution was evaporated again under nitrogen gas. The residue was dissolved in 100 $\mu$l of ethanol. All these operations were carried out at 4°C under dim red light.

A 10-$\mu$l aliquot of this sample was then injected into a Waters Associates 6000A HPLC system equipped with $\mu$-Porasil column (Waters Associates, Inc., Milford, Mass.). The sample was eluted at a constant flow rate (2 ml/min) with 2% ether-hexane (vol/vol). Two well resolved peaks were detected by a spectrophotometer ($\lambda$ = 365 nm) (Schoeffel Model SF770); they were identified as the 13-*cis* and all-*trans* isomers of retinal by a comparison of their retention times with those of authentic samples. The percentage of each isomer was obtained by dividing the peak area by their respective extinction

TABLE I
COMPOSITION OF RETINALS OF $bR^L$ AND $bR^D$, BEFORE AND AFTER PRESSING

| Species | Percent composition | | Yield* |
|---|---|---|---|
| | 13-*cis* | all-*trans* | |
| $bR^L$ | 3 ± 2 | 97 ± 2 | 0.75 ± 0.07 |
| $bR^L_{P\sim 2.5\,kb}$‡ | 75 ± 2 | 25 ± 2 | 0.82 ± 0.05 |
| $bR^L_{P\rightarrow 1b}$§ | 51 ± 2 | 49 ± 2 | 0.72 ± 0.04 |
| $bR^D$ | 50 ± 2 | 50 ± 2 | 0.68 ± 0.12 |
| $bR^D_{P\sim 2.5\,kb}$‡ | 73 ± 2 | 27 ± 2 | 0.70 ± 0.08 |
| $bR^D_{P\rightarrow 1b}$§ | 52 ± 3 | 48 ± 3 | 0.78 ± 0.06 |

*Averages of four independent extractions. Four HPLC spectra were taken for each extraction.
‡30°C, 4.5 h in the dark, extracted immediately after releasing the pressure to 1 atm at 0°C.
§Room temperature for 4 d after releasing the pressure to 1 atm in the dark.

coefficients at 365 nm: $4.58 \times 10^4$ for the all-*trans* and $3.84 \times 10^4$ for the 13-*cis* isomer (Hubbard, 1956).

## RESULTS

### *Effects of High Pressure upon Absorption Spectra of Bacteriorhodopsin*

Fig. 1 *a* shows the spectral changes of $bR^D$ after being pressed to 2.5 kbar at 30°C. The absorption spectrum of $bR^D$, immediately after raising the pressure to 2.5 kbar, increases in intensity (1.1 times) and shifts slightly (3 nm) to longer wavelengths (compare with Fig. 1 *b*, curve 5). The increase in intensity and also the red-shift probably are due to solvent contraction with pressure. A similar intensity increase and red-shift are seen in rhodopsin (Yoshizawa, 1972) and bacteriorhodopsin (Tokunaga et al., 1976) upon cooling; the spectral shifts upon either cooling or pressing may reflect changes in the dielectric constant of the protein. If the pressure is maintained at 2.5 kbar (Fig. 1 *b*, curves 2–5), the absorption band decreases in intensity and slowly shifts to shorter wavelengths. This change saturates within 2 h. Fig. 1 *b* shows the spectral changes of a pressed bR sample after the pressure is released to 1 atm at 30°C. The absorption band increases in intensity and shifts to longer wavelengths with time, eventually returning to the original spectrum.

The pattern of spectral change between $bR^D$ immediately after pressing and the equilibrated pressed $bR^D$ ($bR^P$) is very similar to that between $bR^L$ and $bR^D$. In both cases there are large blue shifts in the absorption maximum and substantial decreases in extinction.

Fig. 2 *a* shows the spectral changes with time seen in $bR^L$ when pressed to 2.5 kbar at 30°C. The spectral change of $bR^L$ under high pressure is larger than that of $bR^D$. The final spectrum of $bR^L$ under high pressure is almost the same as that of $bR^D$ under high pressure. Upon releasing the sample to 1 atm, the absorption band increases in intensity and shifts to longer wavelengths with time (Fig. 2 *b*). These results are similar to those of pressed $bR^D$ after being released to 1 atm (Fig. 1 *b*).

The chromophore of $bR^L$ is all-*trans* retinal, while $bR^D$ has a 1:1 mixture of 13-*cis* and all-*trans* retinal. Moreover, 13-*cis* bacteriorhodopsin ($bR^{13\text{-}cis}$) can be reconstituted from 13-*cis* retinal and bacterioopsin. Its $\lambda_{max}$ is at 550 nm and its extinction is 0.81 times that of $bR^L$ (Dencher et al., 1977; Aton et al., 1979). Like the all-*trans* pigment, this 13-*cis* pigment

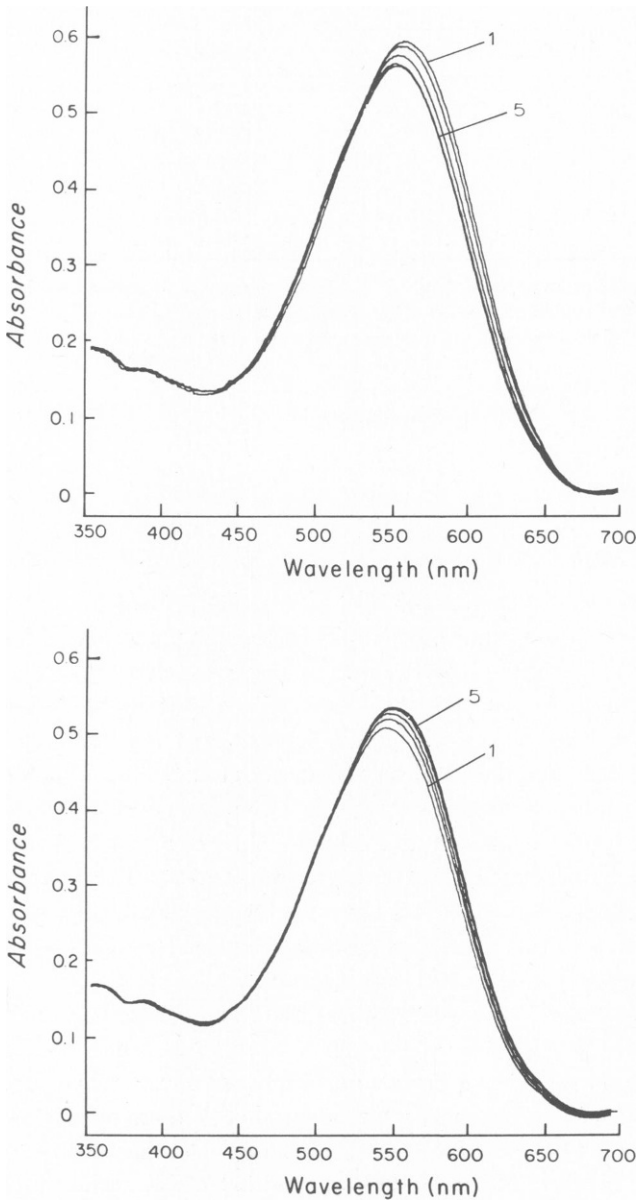


FIGURE 1 (*A*) Spectral changes of $bR^D$ in 10 mM imidazole buffer, pH 7.2, under high pressure. All measurements were done at 2.5 kbar and 30°C in the dark. Curves 1–5 were recorded at t = 0, 10, 30, 60 and 120 min after pressing. (*B*) Spectral changes of $bR^D$ in 10 mM imidazole buffer, pH 7.2, at 1 atm and 30°C after releasing pressure from 2.5 kbar. Curves 1–5 were recorded at t = 0, 10, 30, 60, and 120 min after releasing pressure.

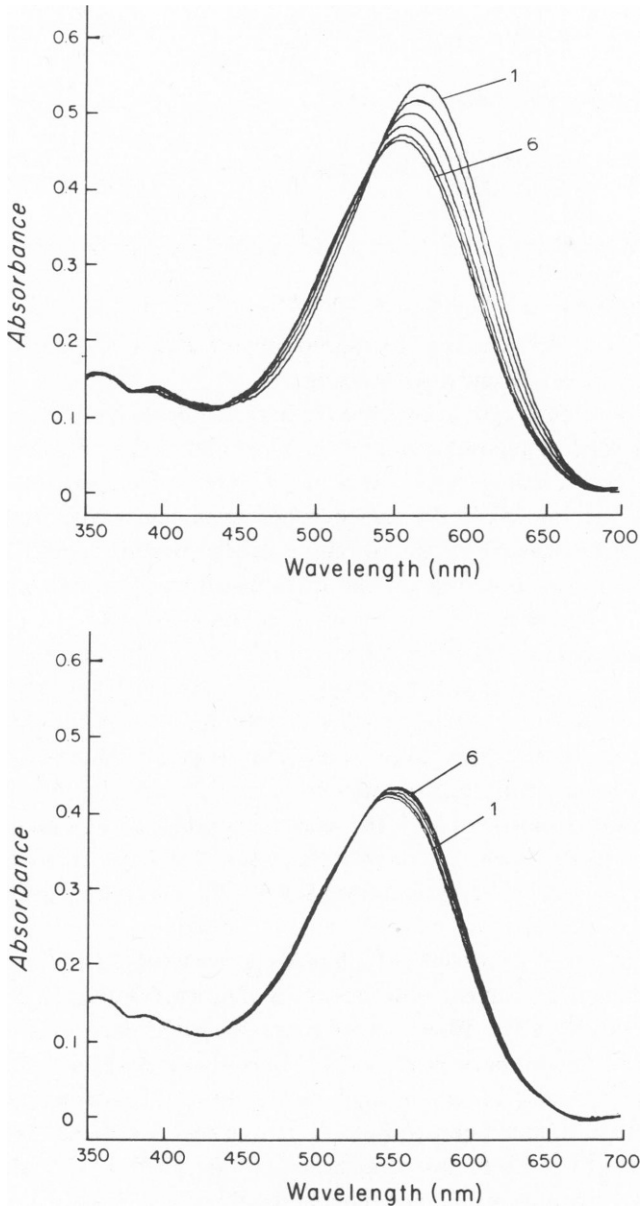


FIGURE 2 (*A*) Spectral changes of $bR^L$ in 10 mM imidazole buffer, pH 7.2, under high pressure. All measurements were done at 2.5 kbar and 30°C in the dark. Curve 1–6 were t = 0, 5, 10, 20, 40, and 60 min after pressing. (*B*) Spectral changes of $bR^L$ in 10 mM imidazole buffer, pH 7.2, at 1 atm and 30°C after releasing pressure from 2.5 kbar. Curve 1–6 were t = 0, 10, 30, 60, 120, and 360 min after releasing pressure.

will also "dark adapt" to the 1:1 mixture of 13-*cis* and all-*trans* pigments. All these observations strongly suggest those two pigments are on equilibrium in $bR^D$.

That the absorption bands of both $bR^D$ and $bR^L$ under high pressure shift to the blue and decrease in intensity suggests that pressure favors $bR^{13\text{-}cis}$ in the equilibrium between $bR^{13\text{-}cis}$ and $bR^{all\text{-}trans}$.

Our proposed scheme is as follows:

$$bR^P \begin{pmatrix} \text{all-}trans\ 0\% \\ 13\text{-}cis\ 100\% \end{pmatrix} \underset{+P}{\overset{\Delta \text{ or } -P}{\rightleftharpoons}} bR^D \begin{pmatrix} 50\% \\ 50\% \end{pmatrix} \underset{\Delta \text{ or } +P}{\overset{h\nu}{\rightleftharpoons}} bR^L \begin{pmatrix} 100\% \\ 0\% \end{pmatrix}, \quad (1)$$

where $+P$ and $-P$ represent an increase and decrease of pressure

*Extraction of the Chromophore from Pressed bR*

To test the hypothesis derived from the spectrophotometry study, we tried to extract the chromophore from bR before and after pressing.

Though it is impossible to extract the chromophore of a pressed sample at high pressure, we believe the chromophore composition of pressed bR at high pressure is the same as that of bR immediately after pressure is released 1 atm at 0°C. Our reasons are as follows: The rate of transformation of $bR^P \rightarrow bR^D$ at 1 atm depends upon temperature; at 0°C, there is no apparent change in the spectra within 10 min after the pressure is released. This relatively slow reversion rate is also observed for the transformation of reconstituted $bR^{13\text{-}cis}$ to $bR^D$ (Maeda et al., 1977; Becher and Ebrey, unpublished observations).

Table I shows the composition of the retinals of $bR^L$ and $bR^D$ before and after pressing. The extraction of retinal from dark adapted pigment at 1 atm yields a ratio of ~1:1 13-*cis* to all-*trans* retinal. When dark adapted bacteriorhodopsin is pressed at 2.5 kbar and 30°C for 4.5 h, cooled to 0°C, the pressure released to 1 atm, and the chromophore extracted, the fraction of 13-*cis* retinal in the pressed sample increases to 73 ± 2% and that of all-*trans* decreases to 27 ± 2%. This result is consistent with the absorption spectral changes. If an aliquot of the above sample is kept at 1 atm and room temperature for 4 d, and then the chromophore extracted, we find 52 ± 3% 13-*cis* retinal and 48 ± 3% all-*trans* retinal, essentially the same as that of $bR^D$.

The extraction of retinal from the light-adapted species yields 97 ± 2% all-*trans* retinal and 3 ± 2% 13-*cis* retinal, in agreement with the results of others (Oesterhelt et al., 1973; Pettei et al., 1977; Maeda et al., 1977). When $bR^L$ was pressed at 2.5 kbar at 30°C for 4.5 h in the dark, the isomeric composition was 75 ± 2% 13-*cis* retinal and 25 ± 2% all-*trans* retinal, essentially identical to that of the pressed $bR^D$ sample. The isomeric compositions of a pressure released light-adapted preparation which was kept at 1 atm at room temperature in the dark for 4 d was 51 ± 2% for 13-*cis* and 49 ± 2% for all-*trans* retinal. The above results show that isomeric composition of the chromophores of bacteriorhodopsin is the same after 4.5 hours at 2.5 kbar starting from either the light-adapted species or the dark-adapted one. If either of these pressed samples is kept in the dark at 1 atm at 30°C for ~4 d, the isomeric composition of the retinals is the same as that of $bR^D$. These results support mechanism 1 which was derived from the studies of absorption spectra of the purple membrane under high pressure. The hypothesis was that increased pressure shifts the equilibrium between the all-*trans* and 13-*cis* pigments toward the 13-*cis* pigment. That the retinal composition of

dark-adapted bacteriorhodopsin before and after being pressed is the same also suggests that bacteriorhodopsin is insensitive to pressure denaturation up to 2.5 kbar.

*Pressure Dependency of the Dark Equilibrium between $bR^{13\text{-}cis}$ and $bR^{all\text{-}trans}$*

It has been reported that the dark equilibrium between $bR^{13\text{-}cis}$ and $bR^{all\text{-}trans}$ with a ratio

$$K = \frac{[bR^{all\text{-}trans}]}{[bR^{13\text{-}cis}]}$$

is insensitive to temperature (Ohno et al., 1977; Dencher et al., 1977). However, the present results show that the equilibrium constant, K, is dependent on the pressure. This shows that there is a molar volume change between $bR^{13\text{-}cis}$ and $bR^{all\text{-}trans}$. The effect of pressure, $P$, upon the equilibrium constant at constant temperature, $T$, is related to the molar free volume change ($\Delta V$) for the reaction by

$$\left(\frac{\partial \ln K}{\partial P}\right)_T = \frac{\Delta V}{RT},$$

where $R$ is the gas constant and $T$ the absolute temperature.

The equilibrium constant, $K$, at each pressure was determined by the retinal isomeric composition of the pressed bacteriorhodopsin using HPLC. Fig. 3 shows the results. The logarithm of K is plotted versus pressure P. The right ordinate gives the equilibrium constant, K, directly; the left ordinate gives the percentage of 13-*cis* retinal in pressed purple

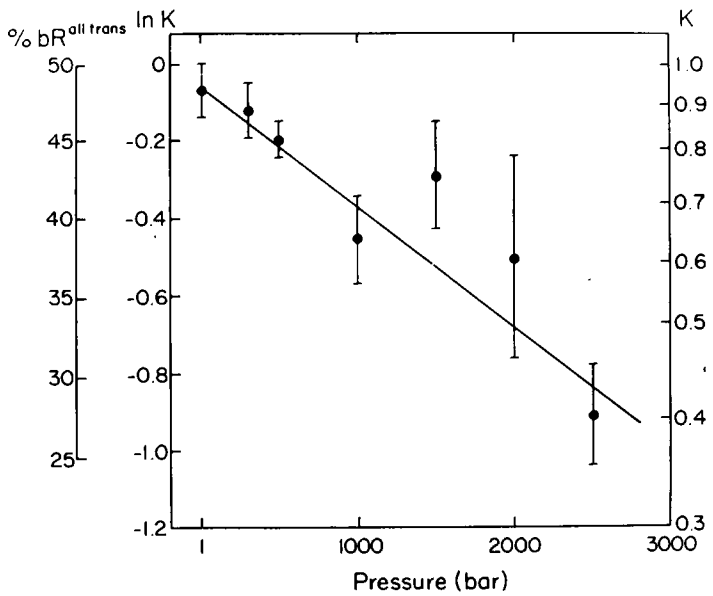


FIGURE 3 Pressure dependence of the equilibrium constant, K. $bR^D$ solution, pH 7.2, was kept at given pressure at 30°C for an appropriate duration (5 h at 2.5 kbar, ~ 63 h at 0.3 kbar). K was determined from the isomeric composition of the pigments at each pressure as measured by the HPLC method described in the text.

membrane. From the Figure, the volume change of the reaction $bR^{all\text{-}trans} \rightarrow bR^{13\text{-}cis}$ was calculated to be $-7.8 \pm 3.2$ ml/mol ($bR^{13\text{-}cis}$ has a smaller volume).

## DISCUSSION

Though $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$ share the same protein (bacterioopsin) and chromophore (retinal), their absorption spectrum, photochemical cycle, and function are different. The only reported difference in structure between $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$ is the isomeric form of their retinal. Little is known about changes in the conformation of the apoprotein between $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$. Becher and Cassim (1976) looked for conformational changes between $bR^{D}$ and $bR^{L}$ by ultraviolet absorption, ORD, and CD techniques. They could not find any definite change in helix content, secondary, or tertiary structure within the errors of their measurements. They concluded that any conformational change involving the secondary and/or tertiary structure of the apoprotein resulting from isomerization must be highly localized at the site of the interaction between the retinal and the apoprotein and the resulting conformation distortion does not spread through the apoprotein. Several groups have tried to measure the enthalpy and entrophy changes between $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$. Ohno et al. (1977) could not find a definite change of the equilibrium constant between $bR^{L}$ and $bR^{D}$ when they varied the temperature in the range between 4°–37°C. They concluded that the enthalpy change of bacteriorhodopsin associated with the isomerization of retinal is close to zero. Dencher et al. (1977) obtained very small values for the change of enthalpy: $374 \pm 170$ cal $mol^{-1}$, and for that of the entropy: 1.3 cal $deg^{-1}$ $mol^{-1}$. This suggests that there is no large change in conformation of bacteriorhodopsin upon the thermal isomerization of the chromophore.

In the present work, we have succeeded in observing a change of equilibrium constant, K, between $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$ in dark adapted bacteriorhodopsin with pressure. From this result, we could calculate the molar volume change of $\Delta V = -7.8 \pm 3.2$ ml/mol. Furthermore, on the basis of these observations we suggest that the ionization of one or two residues or the formation of one or two hydrogen bonds are coupled with the transformation of $bR^{all\text{-}trans}$ to $bR^{13\text{-}cis}$. Arguments in support of this proposition follow.

The volume change $\Delta V$ for a reaction in solution is the sum of the volume change associated with the reactant molecule and the volume change associated with the alterations in solvation which accompany the reaction. The volume changes of either type associated with *cis-trans* isomerization are very small (Hamann, 1963) and thus the volume change is attributed to the apoprotein. While there exists no conclusive interpretation of the observed $\Delta V$ in the reaction of any protein in solution, model studies suggest that volume changes associated with solvation make significant contributions to the total $\Delta V$ and should dominate over other sources in most cases (Hamann, 1963). Some information is available on the volume changes accompanying the formation of hydrogen bonds, hydrophobic bonds, and ionic bonds which might be expected to be associated with solvation of a protein in solution. Suzuki and Taniguchi (1972) summarized the various data and concluded that signs of $\Delta V$ accompanying formations of hydrogen, hydrophobic, and ionic bonds are negative, positive, and positive, respectively. Thus, increasing pressure favors the formation of hydrogen bonds, and the rupture of both hydrophobic and ionic bonds.

The negative sign for $\Delta V$ suggests that either hydrophobic bonds and ionic bonds of the apoprotein are ruptured or new hydrogen bonds are formed upon going from $bR^{all\text{-}trans}$ to $bR^{13\text{-}cis}$. The value of molar volume change we obtain, $\Delta V = -7.8 \pm 3.2$ ml/mol, is small and could correspond to the ionization of no more than two residues or to the formation of about two hydrogen bonds. A change of this many H-bonds probably could not be detected as a change in the secondary or tertiary structure as assayed by the spectroscopic techniques referred to above (Becher and Cassim, 1975; Ohno et al., 1977; Dencher et al., 1977). Thus, our results also suggest that there probably are only small changes in the apoprotein in going from $bR^{13\text{-}cis}$ to $bR^{all\text{-}trans}$ involving only a few and, perhaps, only one or two residues. This small volume change of the apoprotein must change the microenvironment of retinal by isomerizing the retinal from all-trans to 13-cis retinal in the dark.

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