ENTHALPY CHANGES DURING THE PHOTOCHEMICAL CYCLE OF BACTERIORHODOPSIN

DONALD R. ORT AND WILLIAM W. PARSON, *Department of Biochemistry, University of Washington, Seattle, Washington 98195 U.S.A.*

ABSTRACT We have used a capacitor microphone calorimeter to measure rapid enthalpy changes that occur when bacteriorhodopsin-containing membrane fragments are excited with short flashes of light. We resolved the enthalpy changes into three phases. At about 100 μs after the flash, the bacteriorhodopsins converted into metastable states have an enthalpy about 15-20 kcal mol^{-1} greater than the enthalpy before excitation. Some of this energy ($\sim$10 kcal) is then released to the surroundings as the membrane fragments release protons to the solution. After proton release and before proton rebinding, a large amount of heat is released to the surroundings, equivalent to about 40–45 kcal/mol of bacteriorhodopsin reacting. At this point the energy of the system is about 35 kcal/mol less than it was before the flash; i.e., the system has released all of the energy of the photon (49 kcal/E) plus an additional 35 kcal/mol. Nevertheless, the free energy of the system must still be greater than it was originally, because relaxation to the original state occurs spontaneously. An entropy decrease of at least 125 cal/mol per deg is required to compensate for the heat release. An entropy decrease of this magnitude implies a major increase in molecular order in the purple membrane.

INTRODUCTION

When the purple membranes of *Halobacterium halobium* are illuminated, they can transport protons against an electrochemical potential gradient (1). Bacteriorhodopsin, the photochemically active protein in the purple membrane, is evidently capable of storing some of the free energy of the photons it absorbs. However, no details have been available about the underlying enthalpy and entropy changes during bacteriorhodopsin's photochemical cycle. Information of this type could provide clues regarding the mechanism of proton movement.

In this paper, we use a capacitor microphone calorimeter to measure enthalpy changes that occur when purple membrane fragments are excited with short flashes of light.

MATERIALS AND METHODS

Volume changes resulting from excitation of purple membrane suspensions were measured with the capacitor microphone apparatus described previously (2). The accompanying paper (3) provides information on the isolation of the purple membranes, the procedure for light-adaptation of the samples, the data analysis, and the calibration of the magnitude of the volume changes. The excitation flashes were 588-nm, 0.5-μs pulses from a rhodamine-6-G dye laser, except as noted. In experiments that required varying the temperature, the pH of the suspension was measured and adjusted at each temperature.

RESULTS AND DISCUSSION

The volume change (ΔV) that occurs when a suspension of purple membranes is excited with a flash of light is a function of the temperature (T) and of the buffer (B) present in the solution (2, 3):

$$\Delta V\{T, \mathrm{B}\} = [n_e E_e - n_r \Delta H_r\{\mathrm{B}\}]\alpha\{T\}/\rho C + n_r \Delta V_r\{\mathrm{B}\}. \qquad (1)$$

Here α is the thermal coefficient of expansion of the solvent, which is a function of the temperature. C is the heat capacity of the solution at constant pressure; ρ, the density of the solution; n_e, the number of einsteins of energy E_e absorbed; and n_r, the number of moles of metastable product generated. $\Delta H_r\{\mathrm{B}\}$ is the enthalpy change associated with the conversion of 1 mol of bacteriorhodopsin into the metastable state; the reference point is the enthalpy of the system before the excitation. Because the photochemical cycle involves the transfer of protons to and from a buffer in the solution, ΔH_r will be a function of the nature of the buffer. $[n_e E_e - n_r \Delta H_r\{\mathrm{B}\}]$ is the amount of energy converted to heat, assuming that the system does no work except pressure-volume work. ΔV_r is the difference in molal volume between the reactants and products and is also a function of the buffer.

At a sufficiently long time after the flash, the photochemical system will have relaxed completely, so that $n_r = 0$ and all of the energy of the flash will have been converted to heat. At this time, the volume change is

$$\Delta V_{h_r}\{T\} = n_e E_e \alpha\{T\}/\rho C. \qquad (2)$$

At long times after a flash, the volume changes obtained with purple membrane suspensions thus relax to equal the volume changes given by photochemically inactive solutions of ink (2, 3). Fig. 1 illustrates this at two different temperatures: 2°C, where $\alpha = 0$, and 14.5°C, where α is positive. At both temperatures, the excitation flash causes the suspensions of membranes to expand. The expansion then decays, until the residual volume change is the
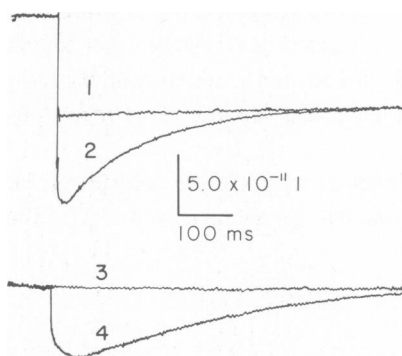

FIGURE 1 The effect of temperature on the magnitude of the flash-induced volume change with ink or purple membrane suspensions. Excitation was from a dye laser flash (588 nm) with an intensity of 10 nE. A downward deflection is a volume increase. In traces 1 (14.5°C) and 3 (2°C), the black ink was dissolved in 200 mM KCl and 5 mM pyrophosphate (pH 8.5). In traces 2 (14.5°C) and 4 (2°C), the ink was replaced by purple membranes. The bacteriorhodopsin (20 μM) and the ink had the same absorbance at 588 nm. Traces 1 and 2 are the average of 9 flashes spaced 20 s apart; traces 3 and 4, the average of 12 flashes.

same as that seen with a solution of ink. If one subtracts the volume change measured at long times (or measured with ink) from the total volume change measured at earlier times, one gets

$$\Delta V'\{T, B\} = \Delta V\{T, B\} - \Delta V_{hv}\{T\} = -n_r \Delta H_r\{B\}\alpha\{T\}/\rho C + n_r \Delta V_r\{B\}. \quad (3)$$

In the remaining discussion we shall be concerned only with the net volume change after this subtraction, $\Delta V'$. We did the subtraction with a computer, in a program that also extrapolated the volume changes back to the time of the flash to eliminate any effects of differences in the decay rates.

One can separate the terms on the right in Eq. 3 experimentally, because α is generally a much stronger function of the temperature than n_r and ΔV_r (2–4). Over the temperature range where this is true, the difference between the values of $\Delta V'$ measured at two temperatures (T_j and T_k) is

$$\Delta V'\{T_j, B\} - \Delta V'\{T_k, B\} = -n_r \Delta H_r\{B\}[\alpha\{T_j\} - \alpha\{T_k\}]/\rho C. \quad (4)$$

The total enthalpy change $\Delta H_r\{B\}$ includes the heat of protonation of the buffer, $\Delta H_p\{B\}$. Values of ΔH_p are known for many different buffers. $\Delta H_r\{B\}$ also includes the enthalpy change associated with the photochemical transformations of bacteriorhodopsin itself, ΔH_{BR}, which should be independent of the nature of the buffer. If u protons are released for each bacteriorhodopsin that the flash converts into a metastable state, then

$$\Delta H_r\{B\} = \Delta H_{BR} + u\Delta H_p\{B\}. \quad (5)$$

We wish to find ΔH_{BR}.

If we measure the temperature dependence of $\Delta V'$ (Eq. 4) with purple membrane suspensions in two different buffers (B_1 and B_2), and subtract, ΔH_{BR} drops out, giving

$$[\Delta V'\{T_j, B_1\} - \Delta V'\{T_k, B_1\}] - [\Delta V'\{T_j, B_2\} - \Delta V'\{T_j, B_2\}]$$
$$= -un_r[\Delta H_p\{B_1\} - \Delta H_p\{B_2\}][\alpha\{T_j\} - \alpha\{T_k\}]/\rho C. \quad (6)$$

By rearranging this equation and substituting into Eqs. 4 and 5, we obtain

$$\frac{\Delta H_{BR}}{u} = \frac{[\Delta V'\{T_j, B_1\} - \Delta V'\{T_k, B_1\}][\Delta H_p\{B_1\} - \Delta H_p\{B_2\}]}{[\Delta V'\{T_j, B_1\} - \Delta V'\{T_k, B_1\}] - [\Delta V'\{T_j, B_2\} - \Delta V'\{T_k, B_2\}]} - \Delta H_p\{B_1\}, \quad (7)$$

or

$$\frac{\Delta H_{BR}}{u} = \frac{[\Delta V'\{T_j, B_2\} - \Delta V'\{T_k, B_2\}][\Delta H_p\{B_1\} - \Delta H_p\{B_2\}]}{[\Delta V'\{T_j, B_1\} - \Delta V'\{T_k, B_1\}] - [\Delta V'\{T_j, B_2\} - \Delta V'\{T_k, B_2\}]} - \Delta H_p\{B_2\}. \quad (7')$$

$\Delta H_{BR}/u$ is the enthalpy change associated with the conversion of bacteriorhodopsin into a metastable state, expressed per mole of protons released to the solution, rather than per mole of bacteriorhodopsin. It can be put in the latter form if u is known.

Table I contains data from several typical measurements of $\Delta H_{BR}/u$. These measurements were made by analyzing the relaxation of $\Delta V'$ and extrapolating back to the time of the flash. They therefore pertain to the relatively long-lived metastable state formed in

TABLE I

DETERMINATION OF THE RAPID ENTHALPY CHANGE (CALCULATED ACCORDING TO EQ. 7) ASSOCIATED WITH THE FLASH-INDUCED BACTERIORHODOPSIN REACTION

Buffer B	pH	$\Delta H_p\{B\}$	$\Delta V'\{T_j, B\}$	$\Delta V'\{T_k, B\}$	$\Delta H_r\{B\}$	$\Delta H_{BR}/u$
		kcal/mol	μV	μV	kcal/mol	kcal/mol of H+ released
Phosphate*	6.5	−1.8	972	834	−26.6	
Aces*		−7.2	488	322	−32.0	−24.8
Pyrophosphate*	8.5	−0.5	713	641	−19.4	
Tris*		−11.3	450	338	−30.2	−18.9
Pyrophosphate‡	8.75	−0.5	307	276	−25.8	
Glycl glycine‡		−10.5	222	179	−35.8	−25.3
Pyrophosphate§	8.75	−0.5	433	364	−21.5	
Glycl glycine§		−10.5	281	180	−31.5	−21.0

*5 mM buffer, 200 mM KCl, weak laser flash (<5 nE), average of 16 flashes, $T_k = 2.0°C$ and $T_j = 10.5°C$. Aces is N-(2-acetamido)-2-aminoethanesulfonic acid.

‡20 mM buffer, 200 mM KCl, xenon flash (1.7 nE), average of 150 flashes, $T_k = 2.0°C$ and $T_j = 10.5°C$.

§5 mM buffer, 200 mM KCl, weak laser flash (7 nE), average of 12 flashes, $T_k = 20.1°C$ and $T_j = 29.3°C$.

5–30 ms (depending on pH) at 2°C and lasting for several hundred milliseconds (2). (We shall consider earlier metastable states below.) The table includes measurements made with three different pairs of buffers and two pairs of temperatures. All of the experiments show a large negative enthalpy change of about the same value, i.e., a net release of heat from the membranes to their surroundings. In 10 determinations, the mean value of $\Delta H_{BR}/u$ was -20.9 ± 2.9 kcal/mol of protons released. This average includes measurements made
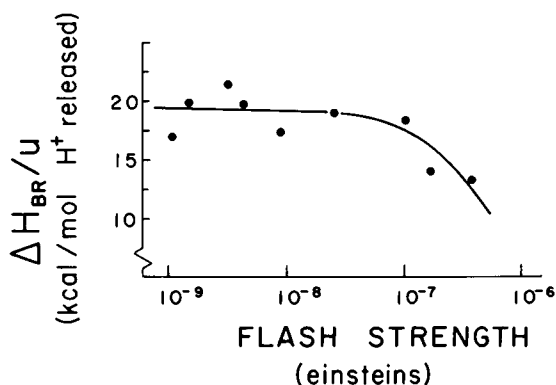

FIGURE 2

FIGURE 3

FIGURE 2 The dependence of $\Delta H_{BR}/u$ on the flash strength. Purple membranes were suspended in 200 mM KCl and either 5 mM Tris or 5 mM phosphate at pH 7.8. Measurements were made at 2.0°C (T_k) and 11.4°C (T_j). The laser flash was attenuated with neutral density filters calibrated at the excitation wavelength.
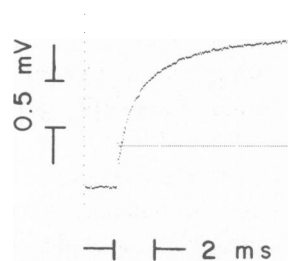
FIGURE 3 Flash-induced volume change at 6.1°C. Purple membranes were suspended in 2 mM pyrophosphate at pH 8.5. The flash strength was approximately 9 nE and this trace is the average of eight flashes. In this trace the volume increases upward. Every other data point is plotted for the first 0.75 ms after the flash, and every fifth point elsewhere. The horizontal, dotted line is the value of ΔV_{hp} determined at a slower sweep rate.

at pH values ranging from 6.5 to 8.75; $\Delta H_{BR}/u$ was not significantly dependent on the pH over this range.

The average value of $\Delta H_{BR}/u$ given above includes measurements made with flash strengths ranging from 1.5 to 10 nE. $\Delta H_{BR}/u$ should not be strongly dependent on the flash intensity, because it expresses the enthalpy change per mole of protons released by the bacteriorhodopsin, rather than per einstein of photons absorbed. Fig. 2 shows that $\Delta H_{BR}/u$ is indeed independent of flash strength, up to about 10^{-7} E. In contrast, the quantum yield of proton release falls off sharply when the flash strength exceeds about 8×10^{-9} E (3). By 10^{-7} E, each bacteriorhodopsin molecule in the front of the microphone cell would receive about 15 photons per flash.

To calculate ΔH_{BR} from $\Delta H_{BR}/u$, we must know the number of protons released for each bacteriorhodopsin converted into the metastable state. The accompanying paper (3) discusses this number in detail. The value of u appears to be 1.0 at low ionic strengths, but to increase to approximately 1.7 at the ionic strengths near 0.2 M used for the measurements described above. This means that ΔH_{BR} for the conversion of bacteriorhodopsin into the metastable state is approximately -36 kcal mol^{-1}.

Note that ΔH_{BR} expresses the enthalpy change relative to the initial state of the bacteriorhodopsin, before the absorption of light. After the excitation, the purple membranes must release 36 kcal of heat to the surroundings for each mole of bacteriorhodopsin converted into the metastable state, in addition to all of the energy of the photon (49 kcal E^{-1}). The total amount of heat released greatly exceeds the energy of the photon. The free energy of the system must still be higher than it was before the excitation, because relaxation back to the initial state occurs spontaneously, but the free energy stored must take the form of a decreased entropy, rather than an increased energy. The entropy of the system must decrease by at least 125 cal mol^{-1} deg^{-1} (35 kcal mol^{-1}/275 deg) in the formation of the metastable state for the net free energy change to be positive.

We have shown previously (2) that at the temperature where $\alpha = 0$ (T_0), the light-induced expansion of purple membrane suspensions can be resolved into two steps, with first-order rate constants that differ by a factor of 15–40, depending on the pH. The faster step is associated with the release of protons into the solution. The slower step may be associated with the transfer of protons between two dissociable groups on the membranes. At temperatures above T_0, the volume changes also include an "immediate" expansion due to a release of heat before the "fast" phase of the expansion. Some of this initial heat comes from bacteriorhodopsin molecules that are excited by the flash but decay back to the ground state; this happens in about 70% of the excitations (5–7). The remainder of the initial heat comes from molecules converted into metastable states with energies lower than the energy of the excited singlet state. Let us now consider how the overall enthalpy change measured above is divided among the immediate, fast, and slow phases.

Fig. 3 shows a signal-averaged trace of the volume change that occurs on excitation of purple membranes at 6.1° ($T > T_0$). One can see the immediate and fast phases of the expansion and part of the slow phase. The horizontal, dotted line superimposed on the trace shows $\Delta V_{hv}\{6.1°\}$, the expansion measured after the bacteriorhodopsin cycle was complete and all of the energy of the flash had been released to the solution as heat (Eq. 2). ΔV_{hv} was extrapolated back to the time of the flash by the computer. The initial expansion at very short times after the flash is clearly smaller than ΔV_{hv}; i.e., $\Delta V'$ initially is nega-
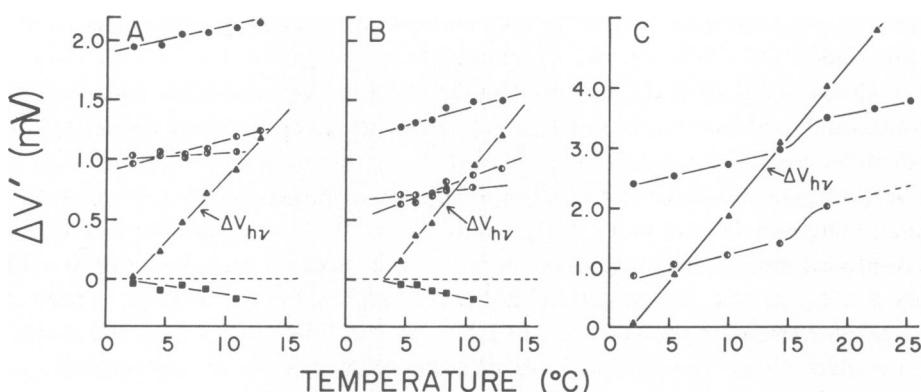
FIGURE 4 The dependence of flash-induced volume changes on temperature. Purple membranes were suspended in 2 mM pyrophosphate (pH 8.5) and 200 mM KCl (Part A), 2 mM pyrophosphate (pH 8.5) alone (part B), or 5 mM pyrophosphate (pH 8.2) and 200 mM KCl (Part C). ▲, $\Delta V_{h\nu}$, is the very slowly decaying component of the volume change due to heating; ●, the total amplitude of $\Delta V'$, determined by the analysis of the relaxation kinetics; ◐, the contribution of the fast formation component to the $\Delta V'$; ○, the slow formation component; and ■, the instantaneous $\Delta V'$, calculated from the difference between the total $\Delta V'$ and the sum of the fast and slow formation components. The lines are linear least-squares fits to the data points. At temperatures above about 18°C, accurate separation of the formation processes was not possible, due to a ringing in the signal with a period of about 150 μs and a damping time constant of 500 μs. The ringing results from a shock wave set up by the sudden heating.

tive. The uncertainty in the extrapolation of $\Delta V_{h\nu}$ from long times is quite small compared to the difference. $\Delta V'$ becomes positive during the subsequent phases of the expansion.

Fig. 4 shows the temperature dependence of $\Delta V'$ immediately after the flash (■), and of the positive contributions to $\Delta V'$ that occur subsequently in the fast (◐) and slow (○) steps. Measurements were made at both high (Figs. 4 A, C) and low (4 B) ionic strengths. The filled circles show the temperature dependence of the final (cumulative) value of $\Delta V'$ after the slow phase, as measured from the relaxation of the total volume change. The values for the immediate $\Delta V'$ were generally calculated by subtracting the sum of the changes in the fast and slow phases from the final values. The buffer in these experiments was pyrophosphate, which has a relatively small heat of protonation (0.5 kcal mol^{-1}). The temperature dependence of $\Delta V'$ should therefore reflect primarily ΔH_{BR}. We were able to resolve the different phases of the volume changes at temperatures up to 16°C. Up to this point, the initial $\Delta V'$ becomes gradually more negative with increasing temperature; the positive contribution to $\Delta V'$ during the fast step becomes gradually larger; and the contribution during the slow step becomes substantially larger.

The enthalpy changes that occur in each of the three phases can be estimated by comparing the temperature dependences of $\Delta V'$ for the different phases with the temperature dependence of $\Delta V_{h\nu}$. Measurements of $\Delta V_{h\nu}$ are included in Fig. 4 (▲). We assume again that all of the temperature dependence of the volume changes in Figs. 4 A and 4 B is due to the temperature dependence of α. From Eqs. 2 and 3, the ratio of the slopes of the curves for $\Delta V'\{B, T\}$ and $\Delta V_{h\nu}\{T\}$ is then

$$\frac{\partial \Delta V'\{B, T\}}{\partial T} \bigg/ \frac{\partial \Delta V_{h\nu}\{T\}}{\partial T} = -\frac{n_r \Delta H_r}{n_e E_e}. \tag{8}$$

The ratio n_r/n_e is the quantum yield of the metastable state that forms in a given step. The comparatively long-lived state (M), studied spectrophotometrically, has a quantum yield of about 0.25–0.30 (6, 7), and we shall assume that the yield is the same for each of the intermediate states of interest here. (To maximize the quantum yield, the experiments in Fig. 4 were done with weak flashes.)

With n_r/n_e = 0.3, and E_e = 49 kcal E^{-1}, the slopes of the curves in Fig. 4 A give enthalpy changes of about +18 kcal mol^{-1} for the immediate step, −7 kcal mol^{-1} for the fast step, −40 kcal mol^{-1} for the slow step, and −31 kcal mol^{-1} for the overall enthalpy change at the end of the slow step. (The values for the fast step and the overall change have been corrected for the small contribution of $\Delta H_p\{PPi\}$ to ΔH_r, on the assumption that $u = 1.7$.) Measurements of this type were made three times, with similar results. The value for the final enthalpy change agrees reasonably well with the value of −36 kcal mol^{-1} obtained above for the measurements of $\Delta H_{BF}/u$. (We consider −36 kcal mol^{-1} the more reliable estimate, because it is based on a more extensive series of measurements.) The enthalpy changes measured at low ionic strength (Fig. 4 B) are similar, but the instantaneous enthalpy increase (about +23 kcal mol^{-1}) and the enthalpy decrease that occurs during the fast step (about −12 kcal mol^{-1}) both appear to be somewhat larger.

In addition to its modest effects on the enthalpy changes in the immediate and fast steps, an increase in the ionic strength causes substantial increases in ΔV_r for both the fast and the slow steps (Fig. 4 A, B). Although the differences between the enthalpy changes measured at high and low strengths are only marginally significant, one might expect the enthalpy change for the fast step to depend on the ionic strength. The number of protons released by the bacteriorhodopsin during this step is larger at high ionic strengths (3). If the protons are removed from groups with a positive enthalpy of protonation, the deprotonation would make a negative contribution to the enthalpy change.

If the pyrophosphate buffer used in Fig. 4 is replaced by Tris (ΔH_p = −11.3 kcal mol^{-1}), $\Delta V'$ for the fast expansion has the expected larger dependence on temperature (data not shown). In agreement with Fig. 4, most of the temperature dependence can be accounted for by the heat of protonation of the buffer; ΔH_{BR} is relatively small for this step. Also, as expected, changing the buffer had no significant effect on the temperature dependence of $\Delta V'$ for the slow step.

Between 14° and 17°C, there is a discontinuity in the amplitude of the volume changes, which appears to arise mainly in the slow step (Fig. 4 C). We do not know the cause of this effect. The discontinuity evidently reflects a change in ΔV_r, rather than in ΔH_{BR}, because $(\partial\Delta V'/\partial T)/(\partial\Delta V_{hr}/\partial T)$ is essentially the same above and below this temperature range. Measurements of $\Delta H_{BR}/u$ also give similar results, whether the pair of temperatures used are above or below the discontinuity (Table I).

Hoping to clarify the origin of the discontinuity, we measured the temperature dependence of the decay kinetics of the volume changes. Fig. 5 shows Arrhenius plots of the relaxation kinetics at pH 5.45, 7.6, and 8.6. The plots are nonlinear, with the suggestion of an inflection near 17°C, but we do not feel justified in drawing distinct breaks in their slopes. Sherman et al. (8) have reported Arrhenius plots for the formation and decay of flash-induced absorbance changes at 410 and 660 nm. They observed a break in the Arrhenius plots at 25–30°C and interpreted this as evidence of a phase transition in the membrane. Jackson and Sturtevant (9), however, were unable to detect any endothermic phase transitions in
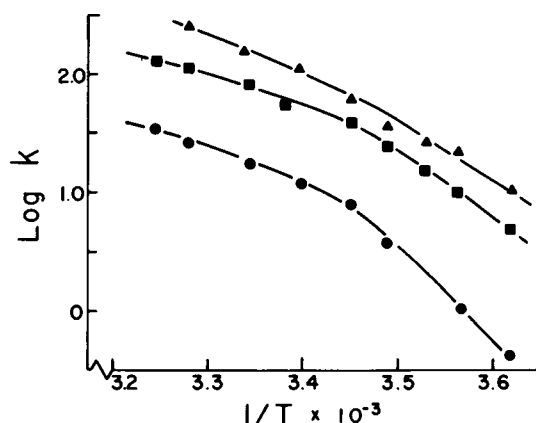
purple membranes between 0° and 70°C by differential scanning calorimetry. They point out that nonlinear Arrhenius plots can have several other possible explanations.

Fig. 6 gives a schematic summary of the enthalpy changes calculated from the data of Fig. 4 and from the measurements of $\Delta H_{BR}/u$. At the end of the fast step the enthalpy
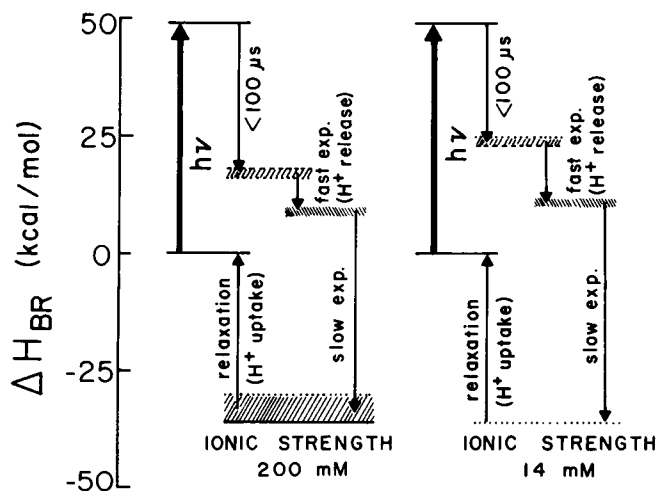

FIGURE 6 Diagrammatic representation of the enthalpy changes in flash-excited purple membranes at high and low ionic strength. Two values are given for the immediate storage of energy. One of these (------) comes from the least-squares slope of the temperature dependence of the immediate $\Delta V'$ calculated at various temperatures (■ in Fig. 4). The other (· · · ·) is calculated from the difference between the least-squares slope of the temperature dependence of the total $\Delta V'$(● in Fig. 4) and the sum of least-squares slopes of the temperature dependence of the fast and slow components (○ and ◑ in Fig. 4). Two values also are given for the enthalpy of the system after the slow expansion. One of these (· · · ·) comes from the least-squares slope of the total $\Delta V'$(● in Fig. 4). The other (———) is calculated from $\Delta H_{BR}/u$ with u taken to be 1.7 protons per cycle (text and Table I).

of the bacteriorhodopsin is still greater than it was before the excitation; it goes strongly negative during the slow step. Because we do not know the magnitude of the free energy changes at various stages of the photochemical cycle, we cannot calculate the entropy changes in the individual steps. During the immediate and fast phases of the cycle, the entropy of the system could either increase or decrease. By the end of the slow step, however, there must be an overall decrease in entropy of at least 125 entropy units (EU). This large entropy decrease implies a substantial increase in molecular order. One can compare it with the entropy increase of 140 EU that accompanies the unfolding of lysozyme at pH 7 and 25°C (10), or with the entropy decrease of 90 EU that accompanies the binding of the S-peptide to the S-protein of ribonuclease (11). Sturtevant (12) has provided a detailed tabulation and discussion of entropy changes in processes involving proteins.

The conclusion that large entropy changes occur during the photochemical cycle agrees with the observation that illumination causes major alterations in the absorption and fluorescence of bacteriorhodopsin's aromatic amino acids (13-15). Spectroscopic effects such as these are frequently attributed to changes in protein conformation. The detailed nature of these structural changes and their relationship to the mechanism of proton pumping remain to be explored. It is perhaps worth noting that the enthalpy decrease accompanying the conversion of bacteriorhodopsin into the metastable state is qualitatively quite different from the enthalpy changes that Cooper and Converse (16) have measured in bovine rhodopsin. The conversion of rhodopsin into metarhodopsin I involves an enthalpy increase of about 17 kcal mol^{-1}. Formation of metarhodopsin II involves a further enthalpy increase of about 10 kcal mol^{-1}.

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