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Thermodynamic studies of purple membrane

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Differential dilatometric and differential scanning calorimetric measurements have been made of purple membrane with an emphasis upon the temperature range 5°C < T < 45°C. The coefficient of thermal expansion α is about $7 \cdot 10^{-4}$/Cdeg up to 30°C and decreases at higher temperatures. The specific heat increases rapidly with temperature with absolute values in the range 0.30–0.45 cal/Cdeg per g. A nearly constant α juxtaposed with a rapidly increasing specific heat is similar to the properties of lipid bilayers in the gel phase and alkanes in the solid phase. This behavior is explained by the concept of hindered vibrations which would now appear to apply to at least one integral membrane protein. There may also be a small broad transition centered near 20–25°C that would correspond to the melting of less than 25 degrees of freedom per bacteriorhodopsin molecule and associated lipids. Using our measured apparent specific volume the average thickness of purple membrane is calculated to be 43.5 Å. The specific volume of interaction of lipids and proteins is estimated, using the amino acid sequence of bacteriorhodopsin and average amino acid volumes from structural studies of other proteins, to be about 11% of the specific volume of the purple membrane lipids or 4% of the volume of the bacteriorhodopsin protein. A positive volume of interaction is consistent with lipid-protein interactions being an important determinant of the thermodynamic properties of purple membrane.

Introduction

Purple membrane is the specialized patch of the cell membrane of *Halobacterium halobium* [1] that contains the retinylidene protein bacteriorhodopsin [2] arranged in trimers [3] within a two-dimensional crystalline lattice [4] containing 77% protein (w/w) [5]. Upon illumination of the all-*trans* chromophore, protons are pumped across the purple membrane [6] during a cycle of spectral intermediates [7].

There have been a number of studies of non-thermodynamic properties of purple membrane that suggest some sort of break or thermal transition between 25 and 30°C as well as a number of studies that find no evidence for such a thermal anomaly at any physiological temperature. Several investigations of the rates of decay of the longer-lived photocycle intermediates, M and O, show nonlinear Arrhenius plots, with breaks between 25 and 30°C [8–10]. However, three studies report linear Arrhenius behavior [11–13], and another study shows that any quantitative differences in the spectra of the kinetic intermediates between 20 and 35°C are similar in magnitude to those between 5 and 20°C [14]. ^{31}P-NMR data have been interpreted to indicate lineshape narrowing of the phospholipid headgroups from 8 to 30°C and no changes between 30 and 67°C [15], and ESR experiments exhibit a change in slope in the Arrhenius plot of the hyperfine splitting of a stearic acid spin label between 25 and 30°C [16].

Turning to thermodynamic studies, the differential scanning calorimetric data of Jackson and Sturtevant [17] show no thermotropic transitions of purple membrane in the temperature region between 0 and 60°C. In that study [17] the concentrations of purple membrane were rather low, 3.5 mg/ml. Similar concentrations of pure lipid bilayers are generally too small to allow one to obtain specific heats in the single phase regions far away from sharp first-order transitions. However, when higher concentrations are used, single-phase specific heats are obtained for lipids [18,19]. One focus for the present study was to use higher concentrations of purple membrane in order to investigate by calorimetry and dilatometry whether there is a thermodynamic anomaly in the physiological temperature range that is much weaker than the strong first-order transitions excluded by the previous calorimetry study. Another focus was to measure absolute specific heats and coefficients of expansion as functions of temperature.

Materials and Methods

Sample preparation

Purified purple membrane from strain JW-3 (ET 1001) was a gift of Dr. W. Stoeckenius. It was stored at −80°C in the sucrose of the density gradient that is the final step of the purification procedure [20]. For all experiments the frozen purple membrane was allowed to thaw slowly at room temperature and then was washed with double-distilled water by dilution and centrifugation at 5°C at 100 000 × g for 1 h, repeated twice. For dilatometric studies and some calorimetric studies the final pellet was resuspended in buffer consisting of 0.1 M sodium azide/0.03 M sodium phosphate (pH 7.0); the high concentration of sodium azide was employed in order to prevent bacterial growth of any kind during the long times at or above room temperature required for equilibrium dilatometry. For the remaining calorimetric studies, the final pellet was resuspended in double-distilled water.

In order to judge the integrity of the purple membrane after long-duration dilatometer experiments, light adaptation of a dilute aliquot of the dilatometer sample was measured using a Beckman DK spectrophotometer; after intense illumination the maximum red shift and absorbance increase were monitored. In addition, the kinetics of dark adaptation were determined by following the λ_{max} shift from 568 to 558 nm of the dilute sample in the spectrophotometer. After each dilatometer experiment the characteristics of light and dark adaptation were unchanged from those of a freshly thawed sample.

For dilatometry it is necessary to degas the sample exhaustively. In order to prevent sudden bursting of the highly concentrated purple membrane sample, the degassing was carried out with constant stirring under partial vacuum in two steps: (1) purple membrane in the 10 ml dilatometer flask was placed in a beaker of ice water and degassed until no further bubbles appeared, then (2) purple membrane in the 10 ml dilatometer flask was placed in a beaker of water at 50–60°C and degassed until no further bubbles appeared. Step 2 was repeated two or three times. Care was taken not to heat the sample from below. The degassing was interrupted numerous times in order to place two to three drops of degassed buffer on the surface of the purple membrane dispersion to allow collected air bubbles to be released. This procedure normally took 4 h. For the calorimetry experiments, 1 h of degassing was sufficient.

Determination of α_{PM}

The coefficient of thermal expansion, defined as $\alpha = (\delta V/\delta T)_P/V$, was determined for purple membrane (PM) by differential dilatometry [21] using the following relation:

$$\alpha_{PM}(T) = \alpha_{Buffer}(T) + (\Delta n/\Delta T)(B/V(T)) \quad (1)$$

The term involving α_{Buffer} accounts for the expansion of the buffer reference; $\alpha_{Buffer}(T)$ was determined in separate dilatometry measurements of buffer vs. water. The last term in Eqn. 1 is essentially the measured differential coefficient of expansion of purple membrane compared to buffer. It involves the primary data $\Delta n/\Delta T$, which has been corrected for a small mismatch in the volumes of the sample and reference sides, and the calibration factor B which converts the change in counts of interference fringes, Δn, into change in volumes. B is calibrated using mercury vs. water [21] and has the value 10^5 $B = 1.60 \pm 0.02$ ml/count for

the studies reported in this paper. To convert the volume change associated with the last term in Eqn. 1 into a coefficient of expansion requires dividing by $V(T) = v(T)m_{PM}$ where $v(T)$ is the apparent specific volume per gram of purple membrane and m_{PM} is the mass of purple membrane in the sample. The absolute apparent specific volume per gram of purple membrane at 3.5°C was determined following the procedure of Nagle and Wilkinson [22]. Very dilute dispersions of purple membrane (0.015% w/w) in sucrose/water solutions, from 33 to 45.8% (w/w), were centrifuged at $55\,000 \times g$ for 15 h. By varying the density of the sucrose solution and observing whether the membrane pelleted on the bottom of the centrifuge tube or rose to the top it was possible to obtain an absolute apparent specific volume at 3.5°C to within ± 0.003 cm^3/g. The value of $v(T')$ at all higher temperatures, T', was found using

$$v(T') = v(3.5°C) + \int_{3.5°C}^{T'} (\alpha_{PM}(T)v(T))dT$$

where α_{PM} was determined from Eqn. 1. The mass of purple membrane in these experiments was determined after each experiment by drying three aliquots of 1–3 ml of purple membrane in buffer solution in a vacuum oven at 60°C until no further change in weight was observed. It was essential to subtract out the weight of buffer, since it was a considerable percentage (30–50%) of the total dry weight.

Two experimental protocols were employed to measure $\Delta n/\Delta T$. The first involved typical scanning dilatometry measurements, both heating and cooling, performed at scanning rates of 5 Cdeg/h. The second protocol utilized equilibrium dilatometry. The water bath temperature was held constant (± 0.005 Cdeg) for 2 h; the counts of fringes, $n(T)$, were measured for 30 min–1 h; the temperature was changed by ΔT and allowed to equilibrate for two or more hours; and then another measurement of $n(T)$ was taken for 30 min–1 h: from this $\Delta n/\Delta T$ was determined. Most of the experiments were of this second type, carried out over 1 month for the first purple membrane sample, and one week for the second sample.

Differential scanning calorimetry

DSC scans were performed with a Microcal MC-1 calorimeter (Microcal Inc., Amherst, MA 01002) at scanning rates between 12 Cdeg/h and 60 Cdeg/h. Data was acquired every 0.01 Cdeg using an Apple II Plus computer. Calculation of the specific heats was as in Ref. 18, using the equation.

$$-\Delta_T = C_{PM}m_{PM} - C_s\Delta m_s \quad (2)$$

In Eqn. 2, Δ_T is the measured difference in heat capacity of solvent, and purple membrane in solvent. Operationally, Δ_T was determined by scanning purple membrane in solvent versus solvent, and then scanning solvent versus solvent, taking care that baseline changes were minimized, and finally subtracting the heat capacity of the two different scans. In Eqn. 2, C_{PM} and C_s are the specific heats of purple membrane and solvent, respectively. When the solvent was water, the specific heat C_s was taken from tables [23]. When the solvent was buffer, the specific heat of the buffer was determined in separate calorimetry experiments. m_{PM} is the mass of the purple membrane determined by dry weight after the experiment, and Δm_s is the mass of the solvent displaced by the purple membrane. Since the same volume is always loaded into each calorimeter cell,

$$\Delta m_s = m_{PM}[v_{PM}/v_s] \quad (3)$$

where v_{PM} and v_s are the apparent specific volumes per gram of purple membrane and solvent, respectively, at the loading temperature (25°C). The apparent specific volume of purple membrane at 25°C was calculated to be 0.854 cm^3/g using the result of the apparent specific volume at 3.5°C and the value of α_{PM} (see Results). The apparent specific volume of water at 25°C, 1.002961 cm^3/g, was obtained from tables [24], and the apparent specific volume of the buffer at 25°C was determined to be 0.9953 ± 0.0002 cm^3/g by weighing the same volume of buffer four times.

Results

Dilatometry

The results of equilibrium dilatometry are shown in Fig. 1. There is considerable scatter in the data when compared to pure lipid bilayer data [22]. Better signal to noise can be achieved in lipid

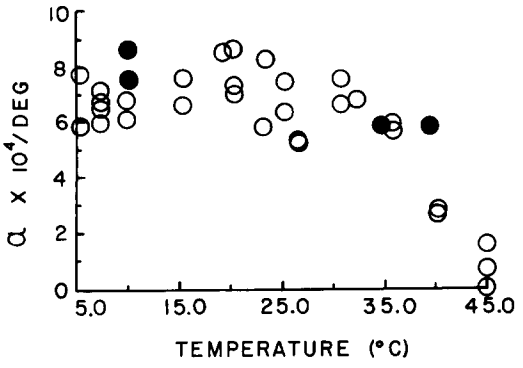

Fig. 1. Equilibrium dilatometry values of α_{PM} versus temperature. Data are for two separate samples, having 132 mg (○) and 106 mg (●) of purple membrane in 10 ml of 0.1 M sodium azide/0.03 M sodium phosphate buffer (pH 7.0). Both purple membrane samples were cycled up to 50°C and down to 5°C in the dilatometer three times before these data were taken.

samples by increasing the concentration; this was not feasible for purple membrane because it was too difficult to degas more concentrated samples than the ones reported on in this paper. The results in Fig. 1 indicate that α_{PM} is nearly constant for $5 < T < 30$°C with perhaps a weak rise in this region to a maximum value between 20 and 25°C. At higher temperatures the data become noisier due to bubble formation; diagnosing bubbles was accomplished by carefully examining the sample visually and by discarding nonreproducible data. It appears that there is a real decrease in α_{PM} over the range $30 < T < 45$°C.

Scanning dilatometry gave more scatter in the data and there was a larger proportion of spurious runs due to bubble formation. However, one cooling scan and two heating scans produced results consistent with the equilibrium measurements, although the maximum values of α_{PM} were about 15% higher than in Fig. 1.

The absolute apparent specific volume of purple membrane measured at 3.5°C by the centrifugation technique was found to be 0.841 ± 0.003 cm^3/g. From this result and Fig. 1 the absolute apparent specific volume can be determined at any temperature; for example, at 20°C it is 0.851 cm^3/g.

Differential scanning calorimetry

Relative specific heats in the high temperature region were obtained in fast scans (60 Cdeg/h) of purple membrane in water and also in buffer. Fig. 2 shows the same two major transitions seen previously by Jackson and Sturtevant [17]. A peak thought to be protein denaturation occurred at 93°C in water ($\Delta H = 87.8 \pm 9$ kcal/mol of bacteriorhodopsin) and 95°C in buffer ($\Delta H = 108 \pm 10$ kcal/mol of bacteriorhodopsin). A smaller peak thought to be the disintegration of the crystalline lattice structure [17,25] occurred at 73°C in water ($\Delta H = 4.7 \pm 0.5$ kcal/mol). In Ref. 17 this peak shifted to 77°C and was much smaller in 0.1 M potassium phosphate buffer; in the buffer scan in Fig. 2B this peak is too small to be clearly seen. In addition, we observe a small broad peak at 63°C in the buffered purple membrane sample which corresponds to a similar third peak observed in Ref. 17.

To obtain absolute specific heats in the physiological range, higher sensitivity specific heat data were taken on more concentrated samples at the slower scanning rate of 12 Cdeg/h. There are differences in the absolute specific heat at any one temperature from one scan to the next; the standard deviation of the average for the purple membrane in water at 30°C is shown in Fig. 3. However, the slopes in C_P versus T are consistent in all scans. As shown in Fig. 3, the specific heat increased between 15 and 45°C in a slightly nonlin-

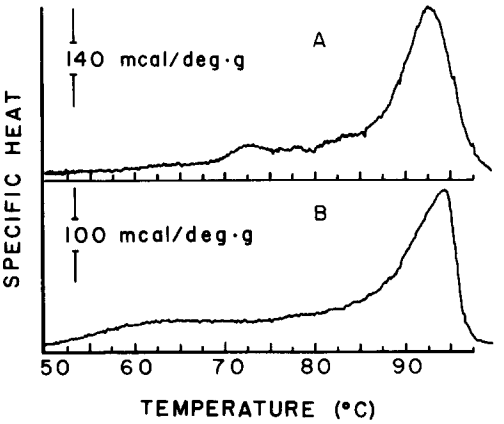

Fig. 2. Differential scanning calorimetry of purple membrane at high temperatures. A. 5.3 mg of purple membrane in 0.9156 ±0.0003 ml water was scanned at 60 Cdeg/h. B. 4.97 mg of purple membrane in 0.9156±0.0003 ml 0.1 M sodium azide/0.03 M sodium phosphate buffer (pH 7.0) was scanned at 60 Cdeg/h. The enthalpy of the transitions near 100°C and 80°C were calculated by straight line interpolation of the baseline under the peaks and integrating the excess heat capacity.

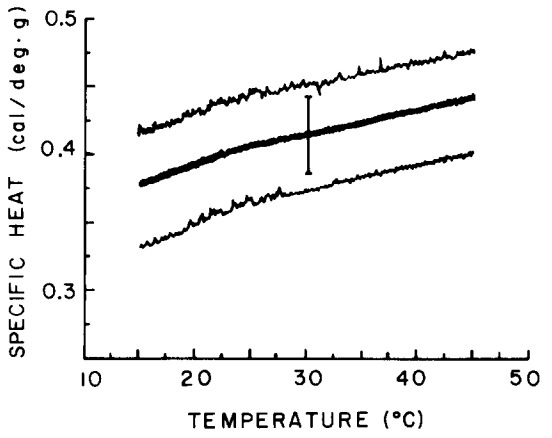

Fig. 3. The central solid curve shows the averaged absolute specific heat data of purple membrane in water vs. temperature. Two samples contained 23.3 ad 24.5 mg/ml purple membrane in 0.92 ml double-distilled water, and were scanned at 12 Cdeg/h. Data were collected in the following order: (1) five scans of water vs. water, (2) three scans of the first purple membrane sample vs. water, (3) three scans of the second purple membrane sample vs. water, and (4) two scans of water vs. water. The upper and lower traces show the extremes of the data and the error bar shows the standard error in the absolute value of all the data. Errors in slope were much lower as illustrated by the three curves in the figure.

ear fashion. The same trend of increasing specific heats as a function of temperature is seen in Fig. 4 for purple membrane in buffer and the nonlinearity is much more marked than in Fig. 3. Ignoring

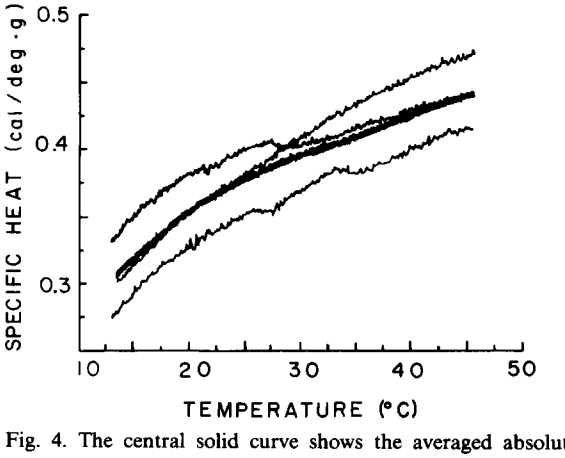

Fig. 4. The central solid curve shows the averaged absolute specific heat of purple membrane in buffer versus temperature. The sample contained 14.8 mg purple membrane in 0.92 ml sodium azide buffer and was scanned at 12 Cdeg/h. These data were obtained using two scans of buffer versus buffer as the reference baseline.

the nonlinearity, the average relative rate of change of the specific heat, defined as

$$\mathrm{RRC} = (\mathrm{d}C_P/\mathrm{d}T)/C_P \qquad (4)$$

is about 0.006/Cdeg for purple membrane in water and about 0.011/Cdeg for purple membrane in buffer.

Discussion

Our calorimetric and dilatometric data agree with previous calorimetric data [17] in showing that there is no obvious sharp first-order transition in the physiological temperature range. However, our data exhibit a nontrivial temperature dependence that could be used as support for a broad transition near 20–25°C. The thermal expansion data (Fig. 1) are consistent with a weak maximum near 20°C and the C_P data may be interpreted as having a broad endotherm centered near 25°C, superimposed upon a background C_P that is increasing steadily with temperature (Figs. 3 and 4). If this interpretation is taken, and it is clearly not a compelling one, one can make very rough estimates of the transition enthalpy ΔH by subtracting a linear baseline from the data. For purple membrane in water this yields a ΔH of 0.08 cal/g or 2.8 kcal/mol of bacteriorhodopsin and associated lipid. For purple membrane in buffer this yields a larger ΔH of 0.2 cal/g or 7.0 kcal/mol of bacteriorhodopsin and associated lipid. Considering that ΔH of melting involves about $RT_m/2 \approx 0.3$ kcal/mol for each degree of freedom melted, this marginally discernible specific heat bump could be due to the melting of 10–25 degrees of freedom in each bacteriorhodopsin molecule, or equivalently, in the melting of 1–2.5 degrees of freedom in each of the 10 lipid molecules associated with each protein molecule. For comparison, the ΔH for the chain melting transition in lipid bilayers is more than 10-times larger [22]. It is somewhat surprising that the magnitude of ΔH for the transition in Figs. 2 and 3 would be so much smaller in water than in buffer. This difference may perhaps implicate the lipid head groups as the active molecular groups in this broad transition. This would be consistent with the ^{31}P-NMR results of Haran et al. [15] which indicate that the

narrowing of the NMR headgroup line width ceases near 30°C.

It seems to us that the most significant, although unforeseen, feature of our data is the juxtaposition of a nearly constant α up to about 30°C and a steeply increasing C_P. This behavior closely parallels the calorimetric data [18,19] and the dilatometric data [22,26] of pure phospholipid bilayers in the gel phase and it is quite different from the thermodynamic behavior of lipid bilayers in the fluid liquid crystal phase. An explanation of this thermodynamic behavior has been given for lipid bilayers and will be briefly reviewed here. Intramolecular vibrations of complex molecules are hindered in the compact gel phase of membranes due to collisions with close neighbors. This keeps the vibrational degrees of freedom in the bottoms of the extended harmonic potential wells that they would have if the atoms involved were not surrounded by other atomic groups belonging to the same or other molecules. As the condensed matter system expands with increase of temperature, larger vibrational amplitudes are required before collision; this yields larger average vibrational energies which in turn increases the specific heat as the temperature increases. A quantum mechanical calculation illustrating this effect has been performed [18].

The preceding explanation predicts that the relative rate of change (RRC) in Eqn. 4 should be larger for systems which have larger α, and our purple membrane data below 30°C are consistent with this. The relative rate of change of purple membrane (0.006–0.011/Cdeg) is intermediate between that of phospholipids in the gel phase (0.013/Cdeg) [18] and alkanes in the solid phase (0.0045/Cdeg) [27], and the α of purple membrane ($6–7 \cdot 10^{-4}$/Cdeg) is also between the α of phospholipids in the gel phase ($8–10 \cdot 10^{-4}$/Cdeg) [18] and alkanes in the solid phase ($5 \cdot 10^{-4}$/Cdeg) [28]. This good agreement for the relative rate of change of purple membrane, with over 75% protein and less than 25% lipid, with that of pure lipid bilayers indicates that hindered vibrations are also important for at least one tightly coupled lipid-protein membrane.

Our measured absolute values of C_P for purple membrane are somewhat lower than the C_P values of many gel phase lipids, such as dipalmitoylphosphatidylcholine, at the same absolute temperature. However, as has been emphasized previously for lipids [18], one should compare C_P at the same relative temperature, $T - T_M$, where T_M is the main melting temperature. Let us consider the 'main melting temperature' of purple membrane to be the melting of the regular lattice structure near 75°C [17,25]. This transition is not the melting of the bacteriorhodopsin protein, which denatures near 100°C; rather it is the melting of the ordered protein-lipid structure, which in pruple membrane is most analogous to the gel-to-liquid crystalline transition of lipids. Taking the lattice melting temperature of purple membrane to be 75°C, consider $T - T_M = -30$°C. Figs. 3 and 4 show $C_P = 0.44$ cal/Cdeg per g for purple membrane and [18] gives $C_P = 0.46$ cal/Cdeg per g for lipid bilayers at the same $T - T_M$. The broad conclusion to be drawn from the temperature dependence of the coefficient of expansion and the specific heat is that purple membrane behaves thermodynamically more like the gel phase of lipid bilayers than like the fluid liquid crystal phase. This conclusion is fully consistent with the fact that purple membrane is a two-dimensional crystal and not a fluid membrane [3,4].

Although our measured absolute values of C_P for purple membrane are between the specific heats of water-soluble proteins below the denaturing transition (about 0.30 cal/Cdeg per g) [29] and those of lipids (0.50 cal/Cdeg per g) [30] a simple additivity formula for purple membrane with 77% protein gives a value of C_P,

$$0.34 \text{ cal/Cdeg per g} = 0.23(0.50 \text{ cal/Cdeg per g}) + 0.77(0.30 \text{ cal/Cdeg per g}) \quad (5)$$

lower than the value 0.44 cal/Cdeg per g that we observed at 45°C. However, at 15°C our C_P values are close to the value in Eqn. 5. Considering our large measured temperature dependence in C_P for purple membrane, such simple additivity formulae do not offer a correct interpretation.

Our thermal expansion results for $5 < T < 30$°C are consistent with the previous value [30], $\alpha = 6.05 \pm 0.82 \cdot 10^{-4}$/Cdeg given as a temperature unresolved average in the temperature range 5–25°C. It was pointed out in Ref. 30 that this value of α for purple membrane is a simple mass

weighted average of the α of typical lipids ($9 \cdot 10^{-4}$/Cdeg) and the α of proteins ($5 \cdot 10^{-4}$/Cdeg). However, this simple relation does not accommodate the decrease in α that we observe above 30°C. It is interesting to note that extracted polar lipids from *Halobacterium cutirubrum* which are similar to those of purple membrane have an α in 4 M NaCl that is close to $6 \cdot 10^{-4}$/Cdeg below 23°C and decreases at higher temperatures [31]. However, undue emphasis should not be placed upon this similarity, since two investigations have indicated the extracted lipids are in the fluid liquid crystal bilayer phase [32,33].

It is of interest to attempt to account for the measured value of the apparent specific volume of the purple membrane, 0.851 ± 0.003 cm^3/g at 20°C, from its constituent components, using a formula such as

$$v_{PM} = x v_{BR} + (1-x) v_L + v_{INT} \quad (6)$$

where $x = 0.77$ is the weight fraction of bacteriorhodopsin (BR) protein [5] (also Fisher, K.A., unpublished data), v_{BR} is the specific volume of protein, v_L is the specific volume of the lipid and v_{INT} is the specific volume of interaction. From structural studies, estimates of the average volumes of amino acids in proteins have been made [34–36]. These have been reproduced in Table I together with the numbers of amino acids in bacteriorhodopsin from the amino acid sequence data [37]. From the information in Table I it is straightforward to estimate that $v_{BR} = 0.794$ cm^3/g; this is somewhat larger than the mean value 0.74 ± 0.04 cm^3/g estimated for crystals of small molecules and water-soluble proteins [36]. This difference is due entirely to the amino acid composition of bacteriorhodopsin, which, being an integral membrane protein, has more lightweight hydrophobic residues (68.5%) than the average water-soluble protein. The specific volume of simple phospholipids, v_L, ranges from about 0.94 cm^3/g in the gel phase at 20°C to about 0.99 cm^3/g in the liquid crystal phase [22]. Since our previous discussion indicates that the membrane behaves like a gel phase, let us take $v_L = 0.94$ cm^3/g. Then, at 20°C,

$$0.851 \text{ cm}^3/\text{g} = 0.77(0.794 \text{ cm}^3/\text{g}) + 0.23(0.94 \text{ cm}^3/\text{g}) + v_{INT} \quad (7)$$

Eqn. 7 yields $v_{INT} = +0.023$ cm^3/g which is about 11% of the lipid volume given by the second term on the right hand side of Eqn. 7 or 4% of the protein volume given by the first term on the right hand side of Eqn. 7. In order to obtain $v_{INT} = 0$ in Eqn. 7 $v_L = 1.04$ cm^3/g is required which is even larger than v_L for phosphatidylcholines in the fluid, liquid crystal phase [22]. Our conclusion that v_{INT} is positive seems reasonable because of the difficulty of packing any lipid closely together with the helical protein structures with its irregular hydrophobic side chains, although the diphytanyl

TABLE I

CALCULATION OF ESTIMATED SPECIFIC VOLUME OF BACTERIORHODOPSIN

Total for molecular weight in polypeptides is the sum of the products of the first two columns. The total molecular weight of bacteriorhodopsin is 27048 g obtained by adding 266.4 g for the molecular weight of retinal in the protein. Total specific volume of bacteriorhodopsin (minus retinal) is obtained by summing the products of all three columns and dividing by 26782 g.

Amino acid	Number in bacterio-rhodopsin [37]	Molecular weight in polypeptides (g) [38]	Specific volume (cm^3/g) [34]
Ile	15	113.15	0.999
Leu	36	113.15	0.894
Val	21	99.13	0.861
Phe	13	147.17	0.832
Arg	7	156.18	0.809 [30]
Lys	7	128.17	0.805
Pro	11	97.11	0.802
Met	9	131.19	0.784
Ala	29	71.07	0.775
Trp	8	186.20	0.769
Gln	3	128.13	0.757
Tyr	11	163.17	0.752
Thr	18	101.10	0.727
Glu	10	129.11	0.724
Asn	3	114.10	0.714
Gly	25	57.05	0.701
Ser	13	87.07	0.686
Asp	9	115.08	0.652
His	0	155.16	0.649
Cys	0	121.16	0.525
Total	248	26782	0.794

chains of the purple membrane lipids are undoubtedly better suited to this task than straight-chain lipids.

Our discussion in the preceding paragraph differs from a recent one [30] which used a measured value of $v_{PM} = 0.820$ cm^3/g (from the measured density, 1.22 g/cm^3), and which assumed that $v_{INT} = 0$. Because we were aware of the discrepancy in the measured values of v_{PM} we checked our procedure of absolute apparent specific volume determination very carefully. Our results are also in fairly close agreement with Stoeckenius and Kunau [39], $v = 0.847$ cm^3/g (from the measured density of 1.18 cm^3/g). It may also be noted that there were only two reported values for v_{PM} at 15°C (Table IB in Ref. [30]) and that these differed by 3%, with the larger one in good agreement with our value. Their values of v_{PM} were obtained using a vibrating densimeter [30] and it is possible that a higher percentage of purple membrane falls to the bottom of the tube, thereby registering a larger apparent specific density.

In general, given the nonexistence of any bulk lipid or bulk protein phases in purple membrane, we think it is unlikely that thermodynamic properties can be understood in terms of the recently proposed [30] simple additivity of protein plus lipid with no interaction term. In particular, the very existence of the sandwiching of the lipids between protein trimers requires, as in antiferromagnetism or stoichiometric binary ordering of alloys [40], that the interaction between lipids and proteins be stronger than the average of the interaction of protein with protein and the interaction of lipid with lipid. Therefore, the lipid-protein interaction must dominate the thermodynamics of the system in the well-ordered phase. It is not surprising that our data are inconsistent with simple additivity formulae involving pure lipid data and pure protein data.

When coupled with the structural diffraction data [3,4], the apparent specific volume enables one to calculate the average thickness h of the purple membrane, using

$$v = AhN/M \tag{8}$$

where M is the molecular weight of bacteriorhodopsin without retinal divided by 0.77 to account for the associated lipid, N is Avogadro's number and $3A$ is the area of a rhombic (hexagonal) (h) unit cell of edge 62.7 Å associated with a trimer of bacteriorhodopsin at 28°C [25]. Using our value of $v = 0.856$ cm^3/g at 28°C yields $h = 43.5$ Å. Since our value for h is an average thickness, the closest approach of two stacked membranes would be at least this large. This is consistent with the repeat distance of 49 Å measured by Blaurock for dried purple membrane [41] (see Fig. 5).

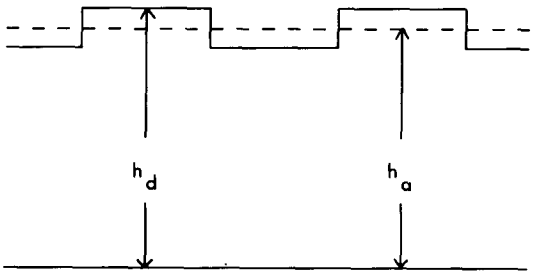

Fig. 5. Sketch showing one of many possible profiles for which the dried stacking repeat distance, h_d, is larger than the average thickness, h_a, of purple membrane. (The hexagonal in-plane symmetry prohibits perfect interdigitation of the protuberances of oppositely oriented membranes).

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