Phase Behavior of Lipids from *Halobacterium halobium*

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ABSTRACT: Mixtures of dipalmitoylphosphatidylcholine with purple membrane lipids, red membrane lipids, or total lipids of *Halobacterium halobium* have been studied with differential scanning calorimetry. A comparison of red and purple membrane lipids reveals no difference in their phase behavior, indicating that lipid phase behavior plays no role in the in vivo separation of red and purple membranes. The effects of variation of the salt content of the suspending solution have also been examined. Studies of the melting behavior of these mixtures as *H. halobium* lipid content is varied suggest that the gel to liquid-crystal transition does not occur in the lipids of *H. halobium*.

The purple patches of membrane which are produced by the extreme halophile, *Halobacterium halobium*, constitute an excellent example of specialized domains within a cell membrane. The red and purple regions form separate membrane phases (Oesterhelt & Stoeckenius, 1971) with different lipid and protein components (Vaver, 1978; Kushwaha et al., 1975). The existence of specialized regions with different structures and chemical compositions within biological membranes is of general importance and raises interesting thermodynamic and molecular questions.

It is quite possible that the lipids play an important part in this phenomenon. There are many binary mixtures of synthetic lipids which display the coexistence of two gel phases or of gel and liquid-crystal phases over a range of composition and temperature (van Dijck et al., 1977; Mabrey & Sturtevant, 1976; Wu & McConnell, 1975). The capacity of lipid mixtures to form separate phases may assist in the formation of separate regions of distinctive composition and structure in biological membranes. This consideration prompted us to use differential scanning calorimetry (DSC)[1] to examine the phase behavior of the lipids of the red and purple membranes of *H. halobium*. A calorimetric study of purple membranes has been previously undertaken (Jackson & Sturtevant, 1978).

The chemical differences between the red and purple membranes of *H. halobium* are entirely in the polar groups with the purple membranes having a higher proportion of sulfate. Other differences are seen in the glycolipids, but all the hydrocarbon moieties are dihydrophytol (Kushwaha et al., 1975; Vaver, 1978). The lipids of *H. halobium* are quite unusual in themselves, with highly branched dihydrophytol groups in ether links to the glycerol backbones as opposed to the more common straight-chain fatty acids esterified to glycerol (Kates et al., 1965; Kates, 1972). Furthermore, the polar groups are strongly acidic and charged at neutral pH.

The medium employed for growth of this organism has a salt concentration of 4.3 M. When the salt concentration is lowered, the membrane disintegrates (Stoeckenius & Rowen, 1967). The polar groups of the highly charged lipids are probably strongly affected by the ionic strength.

An obstacle to our studies was encountered when it was found that neither red membrane lipids, purple membrane lipids, nor total *H. halobium* lipids form stable suspensions in basal salt (25% NaCl, 2% $MgSO_4 \cdot 7H_2O$, 0.2% KCl), a salt solution with the same composition as the growth medium. In addition, using DSC, no transitions were seen in suspensions of any of these lipids in distilled water or dilute buffer in the range 0 to 95 °C (Jackson & Sturtevant, 1978). Chen et al. (1974) detected transitions in these lipid suspensions in water or dilute salt well below 0 °C, and they express doubts as to whether the lipids form bilayers in basal salt.

In order to surmount these obstacles, we used DSC to study the transition behavior of mixtures of dipalmitoylphosphatidylcholine (DPPC) with the various fractions of *H. halobium* lipids. Studies of these mixtures can indirectly yield information about the properties of their components. Care must be taken in interpreting these results, but comparisons between mixtures of different components in the same proportion, and of several different compositions allow some useful conclusions to be made.

Materials and Methods

Purple membranes were isolated by a modification of the method published by Becher & Cassim (1975). This method involves the sudden addition of water to freshly harvested cells followed by repeated centrifugation at 50 000*g*. The pelleted purple membranes were purified on a 30–50% sucrose gradient at 100 000*g* for 15 h. The purple membrane band was well separated from the trace amounts of red membranes. Sucrose was removed by repeated washings in water.

Red membranes were prepared from the red supernatant after the first step in the purple membrane preparation. The supernatant was centrifuged twice at 50 000*g* to remove most of the remaining purple membrane and then centrifuged at 100 000*g* for 5 h to pellet the red membranes. A sucrose gradient identical with the one employed in the purple membrane preparation was then used, followed by washes with water to remove the sucrose.

Visible absorption spectra (taken in a Cary 14 spectrophotometer) of the purified red and purple membrane fractions were identical with previously reported spectra (Kushwaha et al., 1975). In the case of the purple membranes, the absence of a shoulder at approximately 500 nm, which is caused by the carotenoid pigments of red membranes, indicates that there is no red membrane impurity in the purple membrane fraction.

Total lipids were isolated from *H. halobium* by the proce-

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[1] Abbreviations used: DSC, differential scanning calorimetry; DPPC, dipalmitoylphosphatidylcholine.

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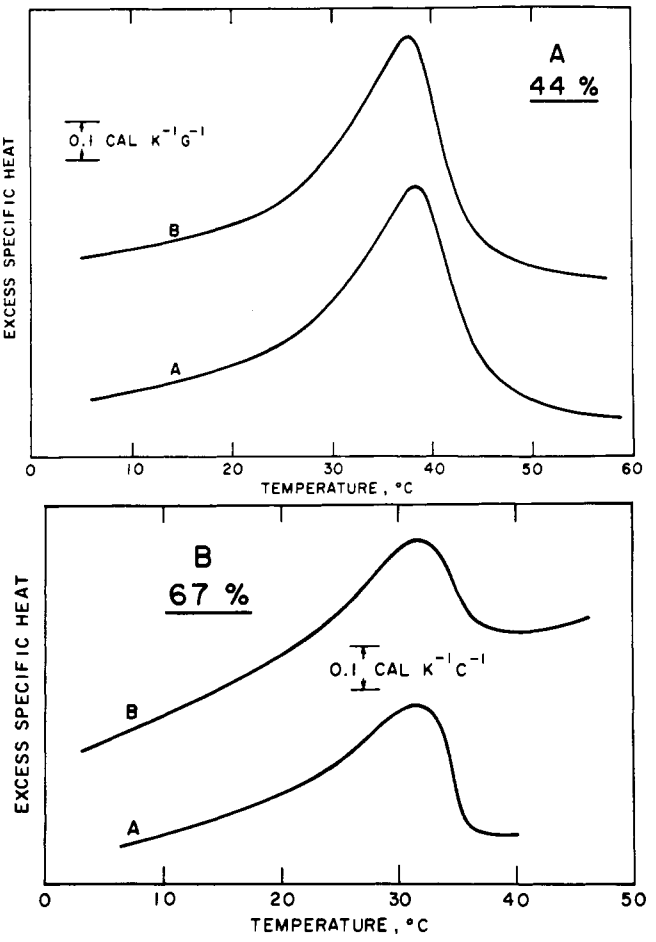

FIGURE 1: Calorimetric scans of mixtures of DPPC with purple or red membrane lipids in basal salt. (A) Scans of mixtures containing 44% purple (curve A) or red (curve B) membrane lipids, total lipid concentration 2.47 mg/mL. (B) Scans of 67% purple (curve A) or red (curve B) membrane lipids, total lipid concentration 2.28 mg/mL.

dure of Kates et al. (1965). Lipids were extracted from red and purple membranes by the method of Kushwaha et al. (1975). The red membrane lipids were red in color, due to carotenoids, and the purple membrane lipids were yellow due to retinal. Lipid solutions in chloroform of known concentration were mixed and the solvent was removed under vacuum. DPPC purchased from Calbiochem was used without further purification. Its transition behavior in multilamellar dispersions indicated it to be of satisfactory purity (Mabrey & Sturtevant, 1976).

Lipid suspensions were made by adding the appropriate aqueous solution to the dried lipids, heating to 50–60 °C, and vortexing. In the case of 2:1 red or purple membrane lipids and DPPC suspended in basal salt, brief sonification (less than 3 min) in a bath sonicator was necessary. All suspensions used in this study were turbid, homogeneous, and stable. We were unable to make stable suspensions in basal salts with higher than a 2:1 ratio of *H. halobium,* red membrane, or purple membrane lipids to DPPC. All compositions are by weight.

Calorimetry was performed in a Privalov calorimeter, the output of which is the excess specific heat as a function of temperature (Privalov et al., 1975). The scan rate was 1 °C/min for all scans reported here. Lower scan rates yielded identical results. Reheats gave nearly the same results as the first heating. Since the expansion of aqueous solutions on freezing would distort or rupture the cells of the calorimeter, scans could not be started below the freezing point of the suspending medium.

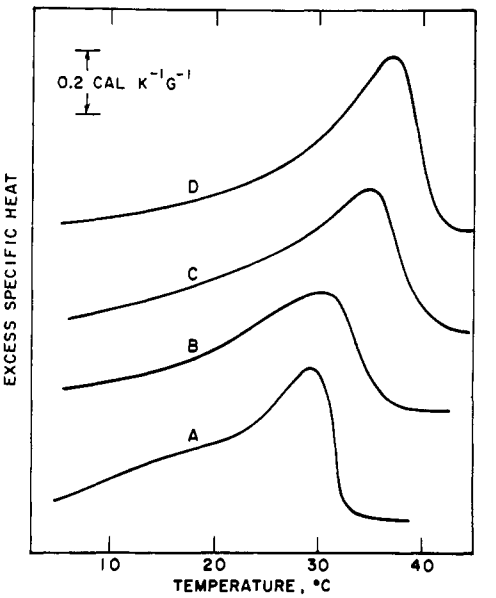

FIGURE 2: Calorimetric scans of mixtures of 50% total *H. halobium* lipids, and 50% DPPC in distilled water and in various salt solutions. (Curve A) Distilled water; (curve B) 2% $MgSO_4 \cdot 7H_2O$; (curve C) 25% NaCl; (curve D) basal salt. All suspensions contained 2.03 mg of lipid per mL.

The heats of the transitions were determined from the area under the specific heat traces. Linear baselines were extended from below and above the transition. When the onset of a transition occurred at a temperature which was too low to establish the initial baseline, the baseline was extrapolated from above the transition, back through the transition to its onset. This resulted in a larger uncertainty in the heats obtained for transitions of mixtures with less than 50% DPPC.

Results

DSC scans are presented in Figure 1 for suspensions of mixtures of red or purple membrane lipids with DPPC in basal salt. Mixtures containing the same proportion of red or purple membrane lipids have essentially identical melting behavior. The curves in Figure 1 are for mixtures containing 44% and 67% red or purple membrane lipids. Similar identical behavior was also observed for mixtures containing 18%, 33%, and 50% red or purple membrane lipids. Differences in the temperatures at which the maximum in excess specific heat occurred (referred to as the melting or transition temperature) for mixtures of the same amount of red or purple membrane lipids with DPPC were less than 1 °C. These variations are not significant and no trend was observed. Studies of suspensions in water showed that mixtures with red membrane lipids melted at slightly higher temperature than mixtures with the same fraction of purple membrane lipids (data not shown).

The results of studies of the melting behavior of suspensions of 1:1 mixtures of total *H. halobium* lipids with DPPC in various salt solutions are shown in Figure 2. The mixture in water has the lowest melting temperature. Adding 2% $MgSO_4 \cdot 7H_2O$ (81 mM $MgSO_4$) raises the transition temperature about 1 °C. Adding 25% NaCl (4.3 M NaCl) raises the transition temperature about 5 °C, and adding the two salts plus 0.2% KCl (27 mM KCl), making it the basal salt solution, raises the transition temperature approximately 9 °C above that observed in water. The differences caused by the salts increase slightly with the proportion of *H. halobium* lipids. The heats of the transitions, as determined from the areas under the excess specific heat curves, do not appear to vary significantly with the salt concentration.

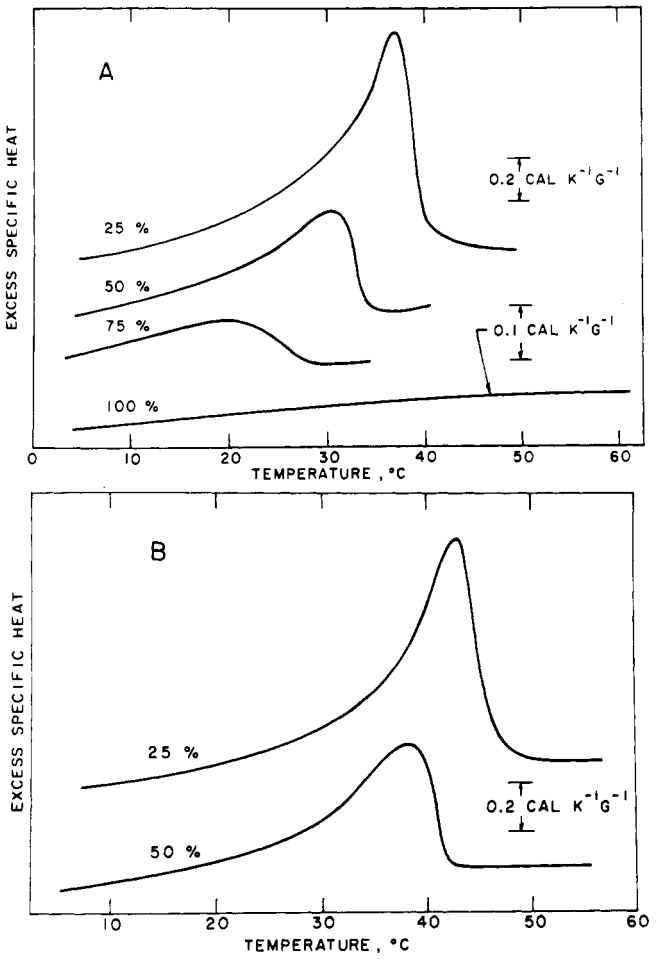

FIGURE 3: Calorimetric scans of mixtures of DPPC and total *H. halobium* lipids of various compositions suspended in distilled water (A) and basal salt (B). All suspensions of mixtures contained 2.03 mg of lipid per mL. A also shows a scan of *H. halobium* lipids alone (100%) in water with a concentration of 5.07 mg/mL.

Seven percent of the lipids of *H. halobium* are nonpolar and can be removed by acetone precipitation (Kates, 1972). Mixtures of the remaining polar lipid fraction with DPPC have a transition which is only about 1 °C lower in temperature than the transition of a mixture of total *H. halobium* lipids with DPPC in the same proportion (data not shown). The polar lipids alone, suspended in water, showed no transition in the range 0 to 95 °C (Jackson & Sturtevant, 1978).

An investigation of the changes in melting behavior of suspensions of mixtures as a function of composition was undertaken. The results of studies in basal salt and in water are shown in Figure 3. Stable suspensions of any composition can be prepared in water. The transition becomes broader and lower in temperature as the fraction of DPPC decreases. The transition of the 75% *H. halobium* lipid mixture is very broad and the specific heat never rises much above the baseline, although the total lipid concentration in all of these mixtures is the same (2.03 mg/mL). A scan of a suspension of *H. halobium* lipids alone in water with a relatively high lipid concentration (5.07 mg/mL) shows no transition at all, as was previously reported (Jackson & Sturtevant, 1978). Since we cannot make measurements below 0 °C, we cannot look for the transition reported by Chen et al. (1974) at −45 °C.

In basal salt, studies were limited to mixtures with a maximum of 67% total *H. halobium* lipids. These mixtures also show a broadening and lowering in temperature as the fraction of DPPC is lowered.

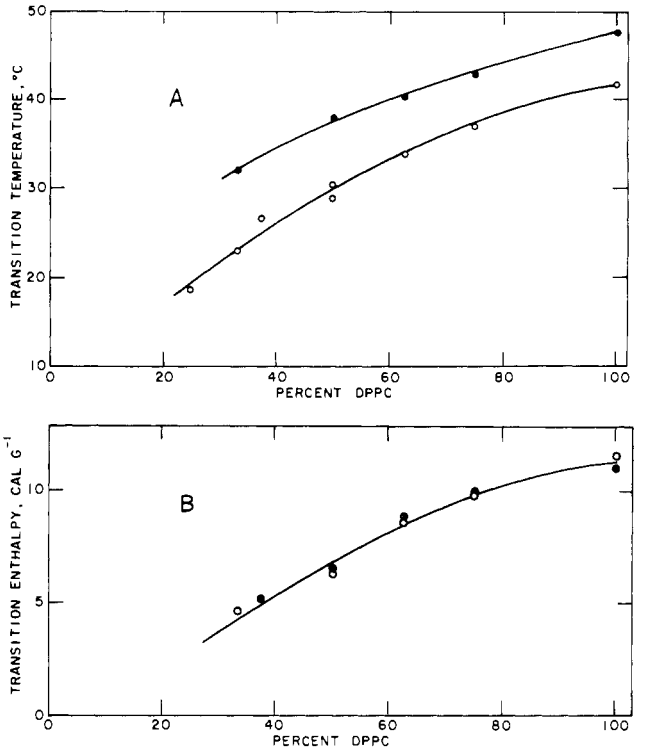

FIGURE 4: Plots of transition temperature (temperature of maximum specific heat), A, and transition heat (in calories per gram of lipid), B, vs. % DPPC in the mixture. (●) Suspensions in basal salt; (○) suspensions in distilled water.

Plots of the transition temperature and of the heat of transition vs. composition for mixtures suspended in either water or in basal salt are shown in Figure 4. The difference between the plots of transition temperature for suspensions in basal salt and water again illustrates the effect of salt and the increase of this effect as the fraction of DPPC decreases. The heat of transition is seen to be the same in basal salt and in water. Heats of transition for the suspensions in water cannot be determined when the fraction of *H. halobium* lipids is greater than about 60% because the transition starts at too low a temperature.

Discussion

The studies comparing red and purple membrane lipids in basal salt show that there is no difference in the phase behavior of mixtures of these lipids with DPPC in the physiologically relevant basal salt solution. Mixtures of red membrane lipids with DPPC in water have slightly higher melting temperatures than mixtures of purple membrane lipids, but this result has no direct bearing on the phase behavior in basal salt where no differences are seen. A greater charge in the polar groups of the purple membrane lipids would increase the polar group repulsion and lower the transition temperature relative to the equivalent red membrane lipid mixture. In basal salt the electrostatic repulsion is screened by the ions in solution, effectively abolishing that effect (discussed in greater detail below). The lowering of the transition temperature as *H. halobium* lipids are added to DPPC shows that *H. halobium* lipids have a lower free energy of mixing with liquid-crystal DPPC than with gel DPPC. The identical behavior of red and purple membrane lipids mixed with DPPC indicates that these lipids have an equal preference for the liquid-crystalline state over the gel state.

ESR (Chignell & Chignell, 1975) and NMR (Sillerud & Jackson, unpublished data) studies indicate that the lipids in

purple membranes are highly immobile. X-ray experiments attest to the order of purple membranes (Unwin & Henderson, 1975). There is no evidence of such order in purified red membranes and the degree of mobility has not been determined. If the separation of these two membrane regions is due to a tendency of the different lipids to form two phases of either gel and liquid-crystal or two gel phases, then the lipids of these two regions should express different relative affinities for the gel and liquid-crystal phases of a DPPC mixture. The fact that no difference is seen in these studies of mixtures with DPPC indicates that the red and purple membrane system of *H. halobium* is not analogous to phospholipid mixtures which show phase separation. We can conclude that no difference in the phase behavior of the lipids of red and purple domains contributes to their in vivo segregation in the membrane of *H. halobium*. This leaves protein-protein and protein-lipid interactions as the remaining factors which can give rise to the observed formation of separate domains in this case.

The membrane of *H. halobium* is highly unusual and these findings are not of general applicability to domain separation in biological membranes. However, there may be some parallel between the crystallinity of these membranes and that of acetylcholine receptors in *Torpedo* and *Electrophorus* electroplax membrane (Dupont et al., 1974; Raftery et al., 1975) as well as some relation to the immobility of junctional acetylcholine receptors in cultured embryonic muscle cells (Axelrod et al., 1976).

Addition of salts raises the transition temperature of the lipid mixtures studied here a significant but not dramatic amount. The effect of $MgSO_4$ is greater than the effect of NaCl when scaled to the amount added. These effects do not appear to be completely additive. KCl is present in such small amounts in basal salt relative to the other salts, that its effect was not studied.

The raising of the transition temperature as salt is added results from a reduction in the polar group repulsion of the charges of the head groups. Theoretical studies indicate that, if a term in the intermolecular potential representing the polar group repulsion is reduced by a factor of two, the transition temperature is raised by 14 °C (Jackson, 1976). Experimental studies show that the transition temperature is indeed raised if the charge on the polar head groups is decreased (Jacobson & Papahadjopoulos, 1975).

Although the phosphatidylcholine head group is zwitterionic, there must be dipolar repulsive interactions which are weakened in the presence of salt, since the transition temperature is higher in basal salt. Adding *H. halobium* lipids with their charged head groups to DPPC adds electrostatic charge to the surface and increases the sensitivity of the transition to salt as these studies have shown. Salt also raises the temperatures of other non-lipid transitions in purple membranes (Jackson & Sturtevant, 1978).

It is unfortunate that studies of mixtures of DPPC with total *H. halobium* lipids in basal salt are not possible at compositions with very small amounts of DPPC. The plot of transition heat vs. composition appears to extrapolate to zero as the fraction of DPPC goes to zero, for suspensions in either water or basal salt. For the 75% *H. halobium* lipid mixture suspended in water, the transition centered at 19 °C is very small, and if *H. halobium* lipids alone undergo a transition it would have to be completed by 3 °C, since in our DSC scans, which start at this temperature, no heat absorption is seen. In basal salt, the temperature plot (Figure 4A) extrapolates to between 10 and 20 °C at zero DPPC content and yet no transition is seen near those temperatures with purple membranes, red membranes, or whole membrane envelopes in basal salt (Jackson & Sturtevant, 1978). In purple membranes, the proteins appear to immobilize the lipids (Chignell & Chignell, 1975), but, in red membranes which have higher lipid contents (Kushwaha et al., 1975), this is probably not the case.

Using differential thermal analysis, Chen et al. (1974) observed a broad transition in a total cellular lipid-water dispersion centered at −45 °C. Electron spin resonance studies by Esser & Lanyi (1973) with fatty acid spin labels incorporated into *H. halobium* lipids dispersed in basal salt solutions indicate transitions at various temperatures between 5 and 35 °C. We are unable to explain our failure to disperse these lipids by the procedure used by Esser & Lanyi or by other procedures as was previously mentioned (Jackson & Sturtevant, 1978). The transition temperature that Esser & Lanyi observed was higher if the spin label was closer to the carboxyl group of the labeled fatty acid chain.

Although the mixture of DPPC with dihydrophytol glycerol ether phospholipid analogues is probably nonexistent in nature, extrapolations to zero DPPC content raise some interesting questions about the phase behavior of *H. halobium* lipids. Our temperature plots would have to curve sharply downward to have intercepts at approximately −45 °C, the transition temperature reported by Chen et al. (1974). In addition, these workers did not detect transitions in aqueous dispersions of phosphatidyl glycerol phosphate dihydrophytol ether (the single most abundant lipid species in *H. halobium* and *H. cutirubrum* membranes) from −80 to +80 °C. Transitions were also not seen in the phosphatidyl glycerol sulfate analogue or in the total polar lipids in 2 M NaCl. On the other hand, our temperature plots appear to come closer to the region in which Esser & Lanyi (1973), Lanyi (1974), and Plachy et al. (1974) observed transitions. All in all the literature concerning the phase behavior of these lipids is confusing and contradictory.

It may well be that these lipids do not undergo a cooperative transition analogous to the hydrocarbon chain, gel to liquid-crystal transition which occurs in most phospholipids. Our inability to see a lipid transition in aqueous lipid dispersions and in purified red membranes (in basal salt or water) which perhaps have some lipids which are not immobilized by proteins, together with the trends in transition temperature, transition breadth, and heat of the transition as the DPPC content approaches zero (Figure 4), suggest this. We propose that a noncooperative, continuous increase in the disorder of the dihydrophytol chains occurs as the temperature is increased. This is similar to what was suggested by Plachy et al. (1974) based on ESR data and a gradual, broadly curved plot of partial specific volume vs. temperature. The four methyl groups on each chain are strong perturbants which create vacancies which could allow kinks (Jackson, 1976) and other disordered configurations to occur without the cooperative expansion of the bilayer. Although this is speculation on our part, it seems to resolve much of the apparent disagreement. The various broad transitions seen by Esser & Lanyi (1973) and by Lanyi (1974) could be the gradual spreading of disorder outward toward the polar head groups with the time scale of motion becoming rapid enough to allow its detection by electron spin resonance at higher temperatures for labels closer to the head groups. This, however, does not explain the broad transitions seen by Chen et al. (1974) at much lower temperatures in some but not all preparations.

The order of the gel to liquid-crystal transition has been frequently discussed. Highly purified DPPC has a transition with a width at half maximum specific heat of under 0.1 °C (Albon & Sturtevant, 1978) and is clearly first order. Phosphatidylethanolamines may have second-order transitions

Jackson & Sturtevant, 1977). Nagle has suggested that lipid bilayers cross a first-order transition line near a critical point (Nagle, 1976). *H. halobium* lipids and dihydrophytol glycerol ethers may be well beyond that critical point so that no thermally induced transition can be seen. Studies with the appropriate synthetic lipid analogues may be helpful in further analysis of the situation.

Conclusion

Studies of the melting behavior of mixtures of DPPC with total *H. halobium* lipids, red membrane lipids, and purple membrane lipids have led us to two conclusions which we wish to emphasize.

(1) There is no difference between red and purple membrane lipids in their tendency to partition between the gel and liquid-crystal phases of DPPC, making it doubtful that the phase behavior of the lipids of red and purple membranes plays a role in the separation of the red and purple regions of the membrane of *H. halobium*.

(2) The melting behavior of mixtures of DPPC and total *H. halobium* lipids suggests that total *H. halobium* lipids alone do not undergo a phase transition from the gel state to the liquid-crystal state.

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