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Lipid Interactions in Membranes of Extremely Halophilic Bacteria. I. Electron Spin Resonance and Dilatometric Studies of Bilayer Structure†

William Z. Plachy, Janos K. Lanyi,* and Morris Kates

ABSTRACT: Dispersions of *Halobacterium cutirubrum* polar lipids in buffer and in salt solutions have been investigated over a wide temperature range (but above the phase transition) by volumetric dilatometry and electron spin resonance (esr) spin-labeling with nitroxide-labeled stearic acids and with vanadyl sulfate. The polar lipid fraction is essentially a binary mixture of the diphytanyl ether analogs of phosphatidyl glycerophosphate and of a glycolipid sulfate, in an approximate mole ratio of 2:1. Stearic acid spin-labels were used to study each pure major component as well. The unusual chemical features of these lipids include uniform, highly branched phytanyl hydrocarbon chains, large anionic head groups, and ether linkages to the phytanyl chains. The thermal coefficient of expansion of the lipids in the presence of added salts (4 M NaCl and/or 0.1 M $MgCl_2$) was found to decrease with increasing temperature and appeared to be discontinuous at −11, 11, 23, and 39–45°. There is evidence from spin-labels for a transition occurring in the polar head group of the major component at 23°. In the absence of added salts the lipid fluidity is increased and the head-group transition is shifted to 8°. Some of the stearic acid spin-labels appear to show transverse displacement in the bilayer, reporting on the head-group region at low temperatures and on the hydrocarbon region of the bilayer at high temperatures. The unusual dilatometric properties and the behavior of the labels can be explained by a model that postulates trans-gauche cooperative kinking of densely packed lipid hydrocarbon chains, occurring preferentially at the methyl-branched chain carbons. The head-group behavior is assumed to be a consequence of changes in the packing of the hydrocarbon phase.

The cell envelope of extremely halophilic bacteria, such as *Halobacterium cutirubrum,* contains two classes of lipids: (a) polar lipids (Kates, 1972; Hancock and Kates, 1973) consisting primarily of the diphytanyl ether analogs of phosphatidyl glycerophosphate[1] (55% of the total lipids by weight) and of a glycolipid sulfate (1-*O*-[β-D-galactopyranosyl-3′-sulfate-(1′→6′)-*O*-α-D-mannopyranosyl-(1′→2′)-*O*-α-D-glucopyranosyl]-*sn*-glycerol (32% of the total lipids by weight), and (b) nonionic, or neutral lipids (Tornabene

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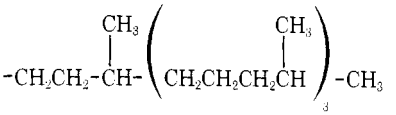
[1] Abbreviations used are: PGP, diphytanyl ether analog of phosphatidyl glycerophosphate; GLS, diphytanyl ether analog of 1-*O*-[β-D-galactopyranosyl-3′-sulfate-(1′→6′)-*O*-α-D-mannopyranosyl-(1′→2′)-*O*-α-D-glucopyranosyl]-*sn*-glycerol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DTBN, di-*tert*-butyl nitroxide.

et al., 1969; Kushwaha *et al.*, 1972) consisting primarily of the red carotenoid pigment, bacterioruberin (3.5% of the total lipids by weight), and squalenes (4.2% of the total lipids by weight). The polar lipids have several properties sufficiently unusual to warrant investigation: (1) a uniform hydrophobic region consisting of highly branched phytanyl chains



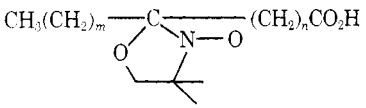
(2) relatively large and highly anionic (at pH 7) polar head groups, and (3) ether linkages between the phytanyl chains and the backbone glycerol of the polar head groups.

The consequences of the unusual features of the lipids on the structure of the membranes of *H. cutirubrum* have been studied by Esser and Lanyi (1973), who used stearic acid type spin-labels to investigate both cell envelopes and vesicles prepared from unfractionated lipids. They found evidence for temperature-induced structural changes in the lipid vesicles of as yet unexplained nature. These workers also observed that the presence of membrane proteins greatly increased the order in the lipid region, and eliminated the temperature-dependent effects in the lipids. Preliminary studies (Chen *et al.*, 1974) showed that *H. cutirubrum* total polar lipids formed stable bilayer vesicles only at low (0.005–0.2 M) ionic concentrations.

We began a study of the polar lipid fraction, essentially a two-component system, in order to further explore these lipid phenomena but in a simpler system. We have also attempted to probe the effect of the unique chemistry of the polar lipids of halophiles on bilayer structure. Earlier work on a pure synthetic phosphatidylcholine with two phytanoyl chains (Redwood *et al.*, 1971) has shown that thin lipid films formed with such highly branched hydrocarbon chains have greater mechanical stability and d.c. resistance than their straight chain analogs.

Materials and Methods

Total polar lipids from *H. cutirubrum* were purified according to established procedures (Hancock and Kates, 1973; Tornabene *et al.*, 1969). PGP and GLS components were separated by thin-layer chromatography and precipitated as their ammonium salts. Nitroxide-labeled stearic acids of the general formula



with $[m,n]$ = [12,3], [5,10], and [1,14] were used as obtained (Syva Corp., Palo Alto, Calif.). Vanadyl sulfate, $VOSO_4$ (Alpha Inorganic Chemicals), and Hepes (Aldrich Organic Chemicals) were used without further purification.

All lipid suspensions were prepared in Hepes buffer (0.05 M, pH 7.0) by evaporating a chloroform–methanol lipid solution to dryness under a nitrogen stream (spin-labeled samples) or by using a rotary evaporator (dilatometry samples), evacuating the vessels to remove trace solvent, and adding the buffer solution with vigorous mixing on a vortex mixer for at least 2 min. Stearic acid spin-labeled samples were "doped" with a chloroform solution of the appropriate label, at a 100:1 mole ratio (lipid to label), before solvent removal. Salts were added to the suspensions in buffer as the solid (NaCl) or solutions ($MgCl_2$, $VOSO_4$), and again mixed on the vortex mixer. The final lipid concentrations were 5.8% (w/v) and 6.9% (w/v) for dilatometry, 1.5% (w/v) for VO^{2+} labeling, and 1% (w/v) for stearic acid spin-labeling. Measurements were made on all samples shortly after preparation. Some of the lipids in samples containing only added NaCl appeared to settle out after a few days storage, while samples containing $MgCl_2$ gave visual evidence for such instability after several hours. VO^{2+}-labeled samples were stable for several days in the absence of added salts.

Aqueous solutions of VO^{2+} are quite stable below pH 3, but at higher pH the vanadyl cation is subject to air oxidation and hydroxide precipitation (Selbin, 1965). For this reason VO^{2+}-labeled samples required somewhat different handling. A stock 0.01 M $VOSO_4$ solution was prepared in 10^{-3} M HCl; the solution was degassed and stored in a serum-cap vial where it was quite stable. Equal volumes of this solution and a 3% (w/v) suspension of polar lipids in 0.05 M Hepes buffer were mixed on a vortex mixer under reduced pressure (*ca.* 100 Torr). NaCl and/or $MgCl_2$ was added before the final mixing, as required. The mole ratio of lipid to VO^{2+} was 3:1.

Dilatometric analysis was performed on two moderately concentrated suspensions of polar lipids, 5.8% lipids in 4.0 M NaCl and 6.9% lipids in 0.10 M $MgCl_2$. The dilatometer bulb volume was 2.238 ± 0.002 ml and the 25-cm long capillary attached had a mean cross-section of (1.73 ± 0.01) × 10^{-3} cm^2. The dilatometer bulb was filled with the outgassed lipid sample through a small stopcock and was placed in a glass-walled bath with the capillary in a vertical position. The bath contained a 50% ethylene glycol solution and could be regulated from −17 to 60° with a stability of ±0.01°. The position of the capillary meniscus was read with a cathetometer at each temperature, to an accuracy of ±0.02 cm. This combination of dilatometer and cathetometer gave a volume-change resolution of two parts in 10^5 which was approximately equal to the density fluctuations caused by the finite thermal stability of the bath. The bath temperature was read to ±0.05°.

Preliminary runs with the dilatometer filled with 4.0 M NaCl or with 0.10 M $MgCl_2$, over temperature ranges in excess of those used for the lipid sample runs, allowed us to fit an eighth order polynomial relating meniscus height to the temperature for each solvent, using least-squares techniques. The solvent expansion data obtained in this manner were used to calculate the specific volume, $\bar{V}(T)$, for the lipids at each temperature, relative to the specific volume at a reference temperature, $\bar{V}(T_0)$, corrected for the volume of the solvent after Träuble and Haynes (1971) using the equation

$$\bar{V}(T) - \bar{V}(T_0) = (1.73 \times 10^{-3}/m_l)[b - b_0 - (m_s/m_0)(l - l_0)] \quad (1)$$

where b_0 is the meniscus height at T_0, b is the meniscus height at T, l_0 is the calculated meniscus height for the pure solvent at T_0, l is the calculated meniscus height for the pure solvent at T, m_0 is the mass of the solvent in the pure solvent run, m_s is the mass of the solvent in the lipid sample, and m_l is the mass of the lipid. When displacements are measured in centimeters and mass is measured in milligrams the units of $\bar{V}$ are ml/g. The value of $\bar{V}(T_0)$ where T_0 is room temperature (17 or 20°) was assumed to be 1.0 ml/g.

In order to span the temperature ranges −16 to 50° and

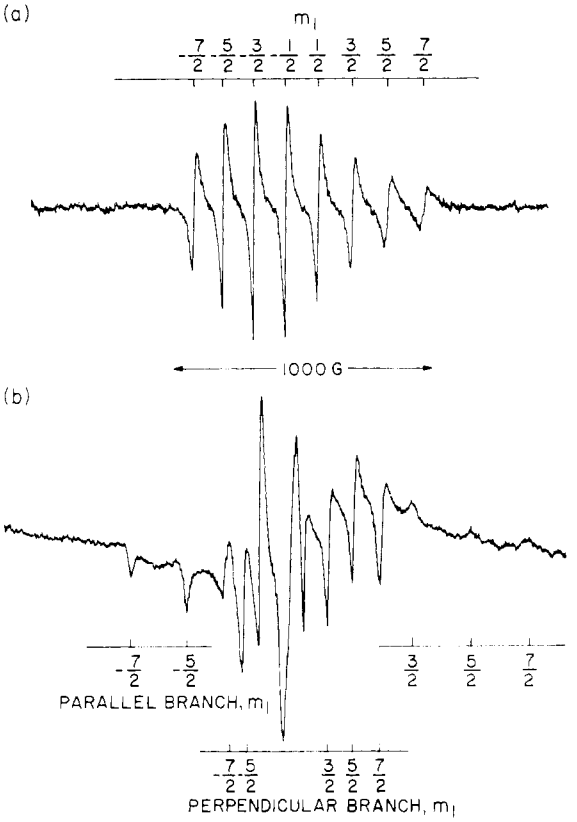


FIGURE 1: (a) Esr spectrum of 10^{-2} M $VOSO_4$ at pH 3. (b) Esr spectrum of 5×10^{-3} M $VOSO_4$ with 1.5% (w/v) polar lipids at pH 7. Spectra were averaged over 64 scans.

3 to 54° it was necessary to make three overlapping runs on each sample and to adjust the sample volume in the dilatometer for each run. All runs on each sample showed the same breaks in the regions of overlapping temperatures (as discussed below), however, the absolute magnitude of $\bar{V}$ differed from one run to the next by 10–20% presumably due to uncertainty in the true values of m_s and m_I. The complete $\bar{V}$ *vs.* T plot for each sample was obtained by normalizing the low and high temperature runs to the middle temperature run. This procedure gave an uncertainty of approximately ±20% in the absolute magnitude of $\bar{V}$ and in the slope of $\bar{V}$ *vs.* T, but did not affect any conclusions about the temperatures at which the slopes change.

Electron spin resonance (esr) spectra were recorded with a Varian V-4502 X-band spectrometer equipped with a 9-in. electromagnet. A spectrum computer (Nicolet, Model 1072) was used for signal-to-noise enhancement, spectral digitization, and for spectral integrations, as required. Samples were sealed in glass capillary tubes of 1.0 mm i.d. and 1.8 mm o.d., and were held concentrically with the Varian variable-temperature accessory dewar in the microwave cavity by a quartz tube of 2.0 mm i.d. The quartz tube was sealed on one end and contained oil to provide efficient heat transfer to the sample. The temperature was monitored immediately above and below the sample with small thermocouple junctions and was read with a meter (Omega Engineering, Model T-5, Stamford, Conn.). The mean of the two readings, which rarely differed by more than 1°, was recorded as the sample temperature. The microwave power was maintained at 4 mW for the nitroxide labels and at 20 mW for the vanadyl label. The 100 kHz field modulation amplitude was maintained at less than 20% of the narrowest peak-to-peak line width in order to avoid line shape distortion. The magnetic field sweep was calibrated relative to Fremy's salt in dilute aqueous solution (a_N = 13.09 G) (Farber and Fraenkel, 1967) for narrow sweeps and relative to vanadyl acetylacetonate in toluene ($7a_N$ = 750 G) (Wilson and Kivelson, 1966) for broad sweeps.

Motional information was extracted from esr spectra in two ways, depending on the nature of the motion. When the paramagnetic *N*-oxyloxazolidine ring of the stearic acid spin-label experienced rapid ($\tau < 3 \times 10^{-9}$ sec) and essentially isotropic reorientational motion, a reorientational correlation time, τ, was calculated following Keith *et al.* (1970)

$$\tau = 6.5 \times 10^{-10} W_0 ((h_0/h_{-1})^{1/2} - 1) \qquad (2)$$

where W_0 and h_0 are the width and height of the center ($m_I = 0$) hyperfine line, respectively, and h_{-1} is the height of the high field ($m_I = -1$) hyperfine line. The hyperfine splitting parameter, a, was measured directly from the separation of the hyperfine lines. When the *N*-oxyloxazolidine ring of the spin-label underwent anisotropic reorientation relatively rapidly only about the axis essentially perpendicular to the bilayer plane, it was usually possible to calculate an order parameter, S, from the observed hyperfine splitting of the parallel, $T_\parallel'$, and the perpendicular, $T_\perp'$, branches of the resulting powder spectrum in the manner proposed by Hubbell and McConnell (1971) and by Seelig (1970)

$$S = \frac{T_\parallel' - T_\perp'}{T_{zz} - \frac{1}{2}(T_{xx} + T_{yy})} \frac{a_0}{a'} \qquad (3)$$

where $a_0 = (1/3)(T_{zz} + T_{xx} + T_{yy})$ and $a' = (1/3)(T_\parallel' - 2T_\perp')$. The values of a', calculated using these relations, was increased by 0.5 G to correct for the systematic error in the determination of $T_\perp'$, as suggested by Hubbell and McConnell (1971). The principal values of the nitroxide hyperfine tensor, T_{zz}, T_{xx}, and T_{yy}, were taken from the work of R. C. McCalley (cited by Esser and Lanyi, 1973). Sample spectra from nitroxide-labeled stearic acids in lipids from *H. cutirubrum* are given in the work of Esser and Lanyi (1973).

The esr spectra of VO^{2+}, a ^{1}d ion, in an aqueous solution and in a polar lipid suspension are given in Figure 1a and b, respectively. The spectrum of VO^{2+} in water shows eight hyperfine lines as expected for the interaction of the unpaired electron with the ^{51}V ($I = 7/2$) nucleus in the limit of rapid reorientation. The separation of the eight hyperfine lines is not constant due to second-order effects (Wilson and Kivelson, 1966). The VO^{2+} esr spectrum obtained in the presence of excess polar lipids is a powder spectrum consistent with all the VO^{2+} being strongly immobilized ($\tau \gg 10^{-9}$ sec) on the anionic vesicle surface, with no evidence for any VO^{2+} in solution. Several of the lines in Figure 1b are well resolved from both the parallel branch (external magnetic field along the V–O bond) and the perpendicular branch, and are indexed with their nuclear spin quantum number, m_I (Adkins and Symmons, 1967).

The esr spectra of VO^{2+} immobilized on vesicle surfaces is temperature sensitive and an order parameter, S, could be calculated using eq 3 and the analysis of Wilson and Kivelson (1966) to correct for second-order effects. We found it convenient, however, to routinely scan only the spectral region which included the $m_I = -5/2$ parallel line and the $m_I = -7/2$ perpendicular line as a function of temperature. These two peaks are well resolved at all temperatures and their separation, which decreases with increasing motion,

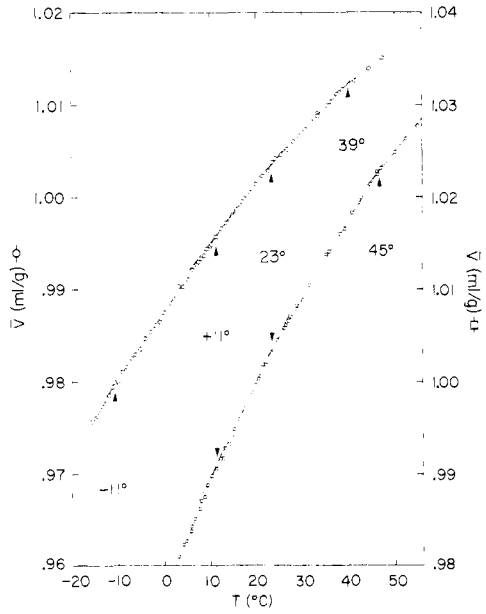


FIGURE 2: Specific volume of total polar lipids in 4 M NaCl (left-hand axis) and 0.1 M $MgCl_2$ (right-hand axis) *vs. T*. $\bar{V}$ at room temperature is assumed to be 1.00 ml/g. (⊙) 5.8% (w/v) polar lipids in 4 M NaCl; (⊡) 6.9% (w/v) polar lipids in 0.1 M $MgCl_2$.

could be measured to a relative accuracy of ±1 G. This peak separation is linearly related to the order parameter, *S*, as long as the *g* and *a'* values of the VO^{2+} complex do not significantly change; no changes in these parameters were detected between −60 and +50°. The coefficients relating the measured peak separation to *S* were determined by independently determining the true value of *S* at several temperatures using the entire powder spectrum and eq 3.

Immediately after sample preparation a broad, featureless background signal was observed in the esr spectra of VO^{2+}-labeled lipid that diminished in intensity after several hours. This signal was presumably due to strong spin-spin interactions in regions with an initial local excess of VO^{2+}. In samples containing NaCl or $MgCl_2$, additional lines, which could not be attributed to free VO^{2+}, appeared on a time scale similar to the lipid instability in the presence of these salts mentioned above. It thus appears that the VO^{2+} probe on the outer vesicle surface is sensitive to agglomeration of the vesicles.

Results

Dilatometry of Lipid Dispersions. The temperature dependence of the specific volume of dispersions of total polar lipids from halophiles in 4 M NaCl and in 0.1 M $MgCl_2$ is given in Figure 2. The specific volume of the lipids increases with increasing temperature with a mean slope (mass coefficient of thermal expansion) of 0.62×10^{-3} ml per g per deg in 4 M NaCl and 0.96×10^{-3} ml per g per deg in 0.1 M $MgCl_2$. These mean values are in good agreement with dilatometric results for pure liquid crystals (for example, see Price and Wendorff, 1972) and with X-ray data on lipid bilayers (Chapman *et al.*, 1967). The mass coefficients of expansion data obtained by Melchior and Morowitz (1972) for vesicles of three different lecithins in water, however, are larger than our values by a factor of about 4. They attribute their anomalous values to the temperature dependence of the extent of ordered water at the vesicle surface. Our results obtained at moderate to extreme salt concentrations should have a much smaller contribution from ordered

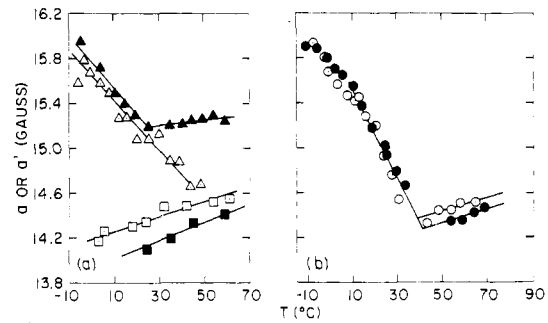


FIGURE 3: Hyperfine parameter, *a* or *a'*, *vs. T* for three spin-labeled stearic acids in polar lipids: (△ and ▲) *a'* data for the [12-3] label; (□ and ■) *a* data for the [1-14] label; (○ and ●) *a'* data below 30° and *a* data above 30° for the [5-10] label. Filled symbols are for 3 M NaCl and unfilled symbols are for buffer only.

water, although the increased mean slope we find at moderate salt concentration (0.1 M $MgCl_2$) relative to that at extreme salt concentration (4 M NaCl) may be due to a residual effect of ordered water.

The data in Figure 2 can be fit by a series of straight lines which intersect at characteristic temperatures, or break points, that are common to both samples. The 4 M NaCl sample shows breaks at −11, +11, +22, and +39° while the 0.1 M $MgCl_2$ sample (not investigated below +3°) shows breaks at +11, +24, and +44°. The uncertainty in these temperatures is about ±2°. Furthermore, the slope change at each break point is in the direction of a less positive slope as the temperature is raised. Alternately, the data in Figure 2 can be fit by smooth curves of decreasing slopes with increasing temperatures.

It is likely that the temperature region investigated lies above the gel to liquid crystalline phase transition temperature (Nagle, 1973a; Linden *et al.*, 1973) for these lipids. Spin-label data, discussed below, are consistent with phase separation at about −10°. Chen *et al.* (1974) have performed differential thermal analysis on these polar lipids, in 50% water and also in 50% 2 M NaCl, and observed no enthalpy changes characteristic of a phase transition over the temperature range −35 to +80°. However, they found evidence for a broad phase transition for the polar lipids in 50% water centered about −49° and a thermal transition for anhydrous PGP and GLS between − 35 and −10°. A very low transition temperature for these highly branched lipids is suggested by the fact that we found the melting point of neat dihydrophytol to be in the range −50 to −60°, since this branched-chain alcohol is analogous to the polar lipids of *H. cutirubrum* in the same sense that 1-hexadecanol is analogous to dipalmitoyl phospholipids, both of the latter showing a phase transition near +40°.

Stearic Acid Spin-Labels in Lipid Dispersions. The polarity gradient across a lipid bilayer (Griffith *et al.*, 1974) (approximating an ionic aqueous solution in the strongly anionic polar head group and a hydrocarbon-like milieu in the middle of the bilayer), together with the sensitivity of ^{14}N hyperfine parameter, *a* or *a'*, of the paramagnetic *N*-oxyloxazolidine ring of the stearic acid spin-labels to the polarity of its immediate environment (Seelig, 1970; Seelig *et al.*, 1972), were expected to provide evidence for the location of these probes in the bilayers under different conditions. Recent ^{13}C nuclear magnetic resonance relaxation data on egg yolk phosphatidylcholine with incorporated stearic acid spin-labels enabled Godici and Landsberger (1974) to assign unique locations to the spin-labels, which were determined primarily by the need to solvate the ionic

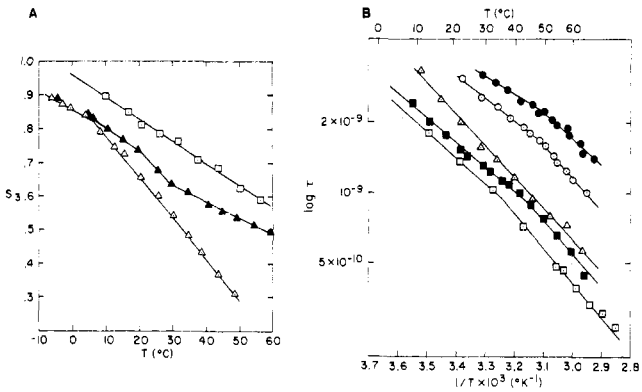


FIGURE 4: (A) Order parameter, S, for the [12-3] stearic acid spin-label *vs.* T for total polar lipids and GLS. Data for pure PGP lipid was within experimental error of the total polar lipid data. (▲) polar lipids in buffer without added salt; (▲) polar lipids in 3 M NaCl; (⊡) GLS in 3 M NaCl. Lipids 1% w/v. (B) Log τ *vs.* $1/T$ for the [1-14] and [5-10] stearic acid spin-labels in polar lipids and GLS. Data for the pure PGP component was within experimental error of the polar lipid data. (□ and ⊙) labels [1,14] and [5,10], respectively, in polar lipids, in the absence of added salts; (■ and ●) labels [1,14] and [5,10], respectively, in polar lipids, in 3 M NaCl; (▲) label [1,14] in GLS, in 3 M NaCl. Lipids, 1% w/v.

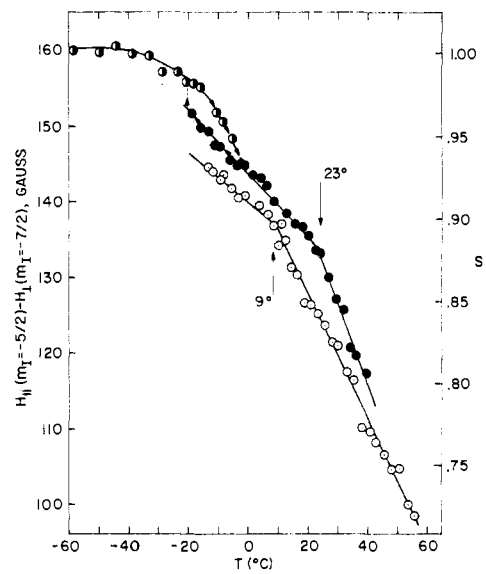


FIGURE 5: Motion of the VO^{2+} probe *vs.* T for total polar lipids. Left-hand axis is the measured peak separation of the $m_I = -5/2$ parallel line and the $m_I = -7/2$ perpendicular line. Right-hand axis is the equivalent S values (see text) (⊙) buffer without added salt; (●) 3 M NaCl; (◑), 3 M NaCl, frozen state. Lipids, 1.5% w/v. $VOSO_4$ concentration, 5 mM.

carboxyl groups. The data in Figure 3, showing the dependence of a and a' on temperature for three stearic acid spin-labels, however, suggest that in the polar lipids of *H. cutirubrum* the location of the spin-labels [12-3] and [5-10] change significantly with temperature. At low temperatures both labels have a' values in excess of that expected for aqueous solutions, such as would originate in the strongly anionic head-group region (Griffith *et al.*, 1974). Thus it is likely that at low temperatures the nitroxide dipole of both these labels is positioned in proximity to the head group anions. As the temperature is raised to approximately 20°, a' is reduced, probably due to lateral expansion of the lattice and the increased thermal motion which allows the average nitroxide-head-group separation to increase. Further increase in temperature for the [12-3] label in the presence of 3 M NaCl causes no further change in a' and the *N*-oxyloxazolidine ring remains in a region of intermediate polarity, typical of the backbone glycerol region. In the absence of salt a' is reduced further at higher temperatures and the [12-3] *N*-oxyloxazolidine ring evidently enters the hydrocarbon region. As shown in Figure 3, the *N*-oxyloxazolidine ring of the [5-10] probe continues to approach the hydrocarbon region with increasing temperature, independent of the salt concentration, and above 30° occupies a region of high fluidity with a hydrocarbon-like polarity very much like that experienced by the *N*-oxyloxazolidine ring of the [1-14] probe. The latter probe appears to report on an essentially hydrocarbon-like region (hyperfine parameter about 14.2 G) at all temperatures. The small upward slope of the hyperfine parameter of the [1-14] probe at all temperatures and for [5-10] probe above 30° may be due to increased water permeability into the hydrocarbon region at high temperatures as suggested by Griffith *et al.* (1974).

In Figure 4A the S value for the [12-3] probe in *H. cutirubrum* polar lipids is plotted. This parameter is seen to be salt dependent at most temperatures, with 3 M NaCl or 0.1 M $MgCl_2$ (not shown) raising the S values significantly. Since the [1-3] probe carries the *N*-oxyloxazolidine ring close to (or in) the head-group region this observation is consistent with the counterions causing an increase in rigidity of the anionic sites. In the presence of 3 M NaCl the S plot shows a discontinuity centered about 25°, while in the absence of added salt a break near 7° is observed. The pure PGP component labeled with [12-3] shows essentially identical behavior to that of the total polar lipids whereas the GLS component in the presence of 3 M NaCl is significantly more ordered at each temperature and displays no discontinuity at any temperature. Below 25° an S plot for the [5-10] probe in total polar lipids or in PGP shows slightly lower S values at each temperature and a similar salt effect.

The *N*-oxyloxazolidine ring of the [1-14] probe, well removed from the head-group region, gives a linear Arrhenius plot of log τ *vs.* $1/T$ (Figure 4B) up to 40° for both the polar lipid mixture and for PGP. Above this temperature the curves from both lipid samples show a break to a slightly greater slope. The *N*-oxyloxazolidine ring of the [5-10] probe above 30° has sufficiently rapid motion to allow a τ value to be calculated and these data are also included in Figure 4B. The [5-10] probe data show a similar break toward greater mobility to that found for the [1-14] probe but the temperature of the break is about 47°. The slopes of the Arrhenius plots are nearly completely independent of the label used, and give an activation energy of 4.9 kcal/mol below the break and 6.0 kcal/mol above the break. Added salts increase the τ values for both labels, but the effect is greater for the [5-10] probe. When dispersions of GLS were labeled with the [1-14] probe in the presence of 3 M NaCl the Arrhenius plot (Figure 4B) did not show the break and gave an activation energy of 6.0 kcal/mol. Thus, there appears to be a weak head-group dependent effect on the motion of the [1-14] and the [5-10] labels in PGP, which gives rise to the slope change above 40°.

The [1-14] label showed considerable line broadening, indicative of spin–spin interactions, below −10°, suggesting that in this temperature region pooling of the label becomes extensive. Such pooling of lipid probes has been found (McConnell *et al.*, 1972) to take place concurrently with phase transitions.

VO^{2+} Spin-Label in Lipid Dispersions. The S *vs.* T plot for VO^{2+}-labeled polar lipid vesicles (Figure 5) indicates

that the V-O bond is experiencing only low amplitude reorientational motion at all temperatures ($S > 0.7$ or $\bar{\theta} < 20°$). The effect is similar to the ordering of the head group by counterions observed with the [12-3] probe and probably reflects the binding together of adjacent polar head groups by the VO^{2+} ion through coulombic attractions. However, a temperature-induced transition toward a greater slope with increasing temperature is seen in Figure 5 at 9° in the absence of added salt and at 23° in 3 M NaCl. The close equivalence of these temperatures for the VO^{2+} probe and the break and discontinuity, in the absence and presence of salt, respectively, observed for the [12-3] probe, together with the observation that both probes report on the head-group region, is evidence for a salt dependent head-group transition occurring at about 9° in the absence of salt and about 23° in 3 M NaCl.

The order parameter, S, for the VO^{2+} probe is also sensitive to freezing of the aqueous phase, as shown in Figure 5. This probe thus appears to respond to head-group changes and to the interactions of vesicles with their surroundings.

Discussion

Structure and motions in nonbranched lipid hydrocarbon chains have been the subject of several theoretical studies, for example, Nagle (1973b) and Seelig *et al.* (1973), and experimental investigations, for example, Horwitz *et al.* (1973), Träuble and Haynes (1971), Steim *et al.* (1969), and Hubbell and McConnell (1971). The dominant mode of motional freedom appears to be rotations about hydrocarbon C-C bonds. Seelig *et al.* (1973) suggested that the barrier to rotation about these bonds is composed of two parts: an intrachain activation energy for bond rotation (*ca.* 3 kcal/mol, Lowe, 1968), and a structure factor due to interchain steric interactions providing additional activation energy, estimated to be about 1 kcal/mol for normal hydrocarbon chains by Seelig *et al.* (1973). The chemical composition of the hydrocarbon region greatly influences the interchain contribution. Finer and Phillips (1973) have pointed out that a uniform hydrocarbon region, as in the polar lipid fraction from *H. cutirubrum*, is expected to result in greater cooperativity, better packing, and enhanced mixing of the components.

The relative intrachain energy of the various rotational configurations for normal and branched hydrocarbon chains is seen with the aid of Newman projections (Newman, 1955). For rotation about a bond between two nonbranched carbons (Figure 6a) the conformation having chain fragments R_1 and R_2 in trans relationship is lowest in energy, while the two gauche conformations are equivalent. When one of the bonded carbons has a methyl branch (Figure 6b) the trans conformation is similar in energy to one of the gauche conformations, while the second gauche conformation is higher in energy. Thus, according this simple picture, the equilibrium distribution of rotational isomers involving a branched carbon contains essentially equal contributions from both the trans and favored gauche conformations.

Recent ^{13}C Fourier transform nmr relaxation data on related systems provide information on bond rotations in straight and branched chain systems. T_1 relaxation times for each carbon in the straight chains of dipalmitoyllecithin suspensions in D_2O have been obtained by Levine *et al.* (1972). Similar data have been obtained on inverse micelles of neat 1-decanol (Doddrell and Allerhand, 1971). The relaxation data for the carbons in these systems show a gradient of rotational freedom along the chain, with rapid motions at the terminal methyl end and least motion at the ordered end (ester or alcohol, respectively), similarly to spin-label results on (Hubbell and McConnell, 1971). Goodman *et al.* (1973) have made nmr measurements on neat phytol, an inverse micelle system that is a good analog to an ordered phytanyl lattice. The phytol data indicate greatest motion at the free end and least motion at the alcohol end, but do not show a *smooth* gradient of motional freedom. Instead, the carbons with branching methyls were observed to rotate more slowly than either of the adjacent chain carbons which is consistent with our expectations based on Newman projections described above.

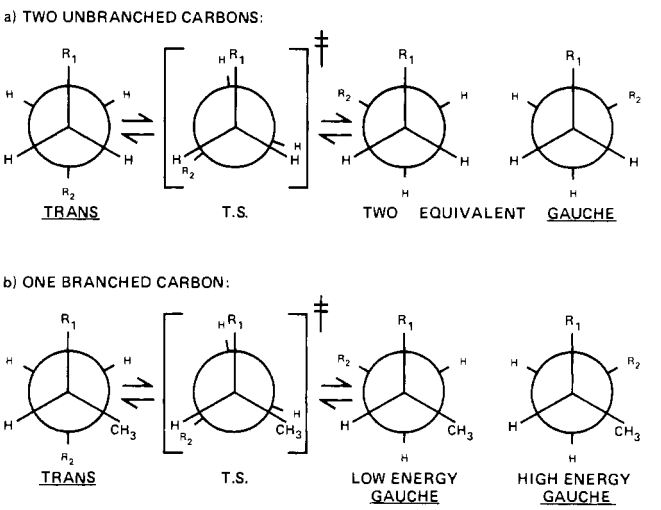


FIGURE 6: Newman projections for C-C bond rotation in hydrocarbon chains. R_1 and R_2 are chain fragments. T.S. denotes a transition state. (a) Both carbons adjacent to the rotated bond are unbranched. The conformation having R_1 and R_2 in trans relationship is of lowest energy since the R groups only experience protons on the opposite carbon. Two equivalent higher energy conformations with R_1 and R_2 in gauche relationship are found in straight-chain lipid bilayers above the crystalline-liquid crystal phase transition (Nagle, 1973b). The transition state between the trans and gauche conformations is of higher energy due to eclipsing of carbons with hydrogens on the opposite carbon. (b) One carbon adjacent to the rotated bond has a branching methyl. Now the R_1 trans to R_2 conformation is approximately equal in energy to the gauche conformation that has the R_2 fragment trans to the branching methyl. The second gauche conformation is higher in energy by an amount similar to the energy difference between the trans and gauche conformation of a straight chain (Verma *et al.*, 1974). This high energy gauche conformation can only be achieved through a transition state requiring two carbons to be eclipsed (not shown).

The interchain barrier to rotation will modify the rotational effects described above. From model-building studies, the interchain barrier to rotation for the branched lipid chains appears to be larger than for normal lipid chains. The branching methyl groups can pack rather well if the lipid chains are not perpendicular to the bilayer surface, but tilted about 20° from the normal (Figure 7, top) in the manner suggested by McFarland and McConnell (1971). Alternatively, methyl group packing is facilitated if the lipid molecules are staggered, one above the mean plane and the next below, etc. In either of these packing arrangements the interchain steric effects favor the all-trans conformation over a mixture of trans and low energy gauche conformations since the latter, even though approximately equal in energy from intramolecular considerations, would disrupt the methyl packing.

The interchain steric effects should also increase the importance of cooperative motions, or "cooperative kinking"

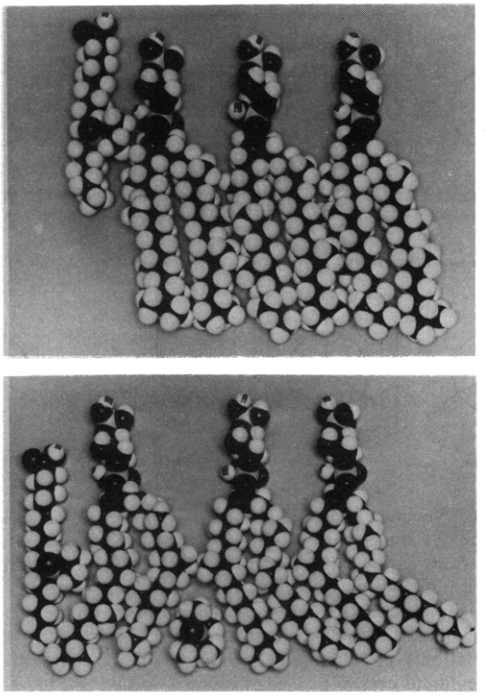


FIGURE 7: Model studies of PGP bilayers. Indicated protons are ionized at pH 7. Top: proposed low temperature structure, tilted chains facilitate methyl packing and the [5-10] stearic acid spin-label (left) occupies a polar site in the head group. Bottom: proposed higher temperatures (*ca.* 30°) structure. Note cooperative kinking of the four right-hand lipid chains, and free volume sites in the hydrocarbon milieu occupied by the [5-10] *N*-oxyloxazolidine ring (left) and by di-*tert*-butyl nitroxide (between the phytanyl chains in the center) (Lanyi *et al.*, 1974).

of the phytanyl chains at the expense of motions by individual chains. The resulting local order, or instantaneous structure, with a mean lifetime of at least 10^{-8} sec (McFarland and McConnell, 1971) would consist of clusters of lipid chains all with single kinks, as in Figure 7, bottom (low energy gauche conformations on the same branched carbon) or multiple kinks separated by four or eight carbons. At the edge of each cluster would exist defects, or free volume pockets, that will accommodate a small molecule such as DTBN (Figure 7, bottom). This mechanism of DTBN solubilization may explain the enhanced solubility of DTBN in these lipids over that in bulk pristane and in other similar liquids (Lanyi *et al.*, 1974). Such a mechanism does not require more free volume, but concentrates the available free volume in defect sites of approximately the correct size for the solute, in contrast with an isotropic liquid, where the free volume is distributed essentially randomly over the liquid phase, with only occasional fluctuations reaching the size of solute dimensions.

An idealized model, postulating preferential kinking at branched carbons, high cooperativity of chain motions and close packing of chains at low temperatures, can be used to interpret the dilatometric data in Figure 2. The rotation about the isopropyl branched carbon (carbon number 15 from the ether linkage) is assumed to be relatively free even at low temperatures. At about −11° cooperative kinking from the trans to the low energy gauche conformation begins to occur primarily about carbon number 11. The molar volume increases sharply with increasing temperature, up to 11°, as more and more chains link at carbon 11 and experience more free volume by disrupting the methyl group packing below carbon 11. Above 11° carbon 11 is rotating quite freely and carbon number 7 begins to undergo increased trans to low energy gauche cooperative kinking. This process continues up to about 24° where cooperative kinking about carbon number 3 begins to be facilitated. The slope of the $\bar{V}$ *vs.* T plot (Figure 2) at each break point should be less above the break temperature than below, because of the effect of multiple kinks, for example at carbons 7 and 11, which require less free volume than two single kinks. Above 42° rotational isomerization is available to all the branching carbons. The slope above 42° should now be free from breaks caused by the disruption of methyl group packing described above.

The overlapping of the temperature regions in which subsequent segments acquire motional freedom would tend to eliminate sharp breaks in the dilatometry curve. In fact, with the possible exception of the break at 22–24°, the data in Figure 2 may be viewed as curved lines. Thus, the propagation of disorder in these bilayers toward the head groups in a purely segmental and sequential fashion, as described above, is probably too idealized a picture. Rather, it is likely that these effects predominate locally in the bilayer.

If cooperative kinking is important in *H. cutirubrum* lipids, as suggested above, perturbation of the bilayers by any membrane protein which projects into the hydrocarbon phase would continue beyond the boundary lipid layer (Jost *et al.*, 1973). The extensive immobilization of the stearic acid spin-labels in cell envelopes of *H. cutirubrum*, as compared to that in lipid vesicles (Esser and Lanyi, 1973), may originate from this effect. Conversely, preferential kinking at the branching points on the hydrocarbon chains may provide less favorable steric conditions for the accommodation of proteins in the bilayer. Halophilic membranes are indeed highly unstable in the absence of $MgCl_2$ or without additional hydrophobic stabilization, such as obtained in the presence of several molar NaCl (Stoeckenius and Rowen, 1967; Lanyi, 1971).

The head-group structure of the PGP lipid can be understood if the data are viewed in light of the above model for the chain motions. Below 25° the a' values for the [5-10] probe (Figure 3) indicate that the *N*-oxyloxazolidine ring of this probe is in a polar environment. Since the ionized carboxyl group in this case, 14–15 Å removed from the *N*-oxyloxazolidine ring for a straight chain, is almost certainly also in the polar region, it is reasonable to assume that the PGP head group is in an extended configuration, since in this way the head group (about 13 Å long) can accommodate both functional sites of the [5-10] probe with the stearic acid chain perpendicular to the bilayer. The relative sizes and arrangement of the head groups and the [5-10] label are shown with molecular models at the top of Figure 7.

According to the lipid-chain model presented above, at about 24° in the presence of salt, kinking is extensive in the bilayer, but branched carbon number 3, closest to the head groups, is only beginning to undergo kinking motions. Kinking motions about this carbon atom should be expected to have a significant effect on the lateral spacing of the head groups, as disorder and free volume begin to appear in this region. With these assumptions the various probes are expected to behave above 24° and in the presence of salt in the following manner: the [5-10] *N*-oxyloxazolidine ring can now occupy the fluid hydrocarbon milieu, while the [5-10] carboxyl group remains in the head group near the backbone glycerol (Figure 7, bottom); the [12-3] *N*-oxyloxazolidine ring moves from near the aqueous interface into

the backbone glycerol region where it experiences the intermediate polarity (Griffith *et al.*, 1974) and rigidity (Levine *et al.*, 1972) characteristic of this region; and the slope of the S plot for the VO^{2+} probe assumes increased temperature dependence as the phosphate oxygen ligands, coordinated to the VO^{2+} probe, separate with an enhanced temperature dependence.

In the absence of added salt the head-group spacing is relatively extended even at lower temperatures, due to interlipid ionic repulsions largely unshielded by counterions. Under these conditions the S plots for the [12-3] and the VO^{2+} probes show a break toward increased temperature dependence already at 9° (Figures 4a and 5), and even the [12-3] N-oxyloxazolidine ring is now able to enter the hydrocarbon milieu of the expanded lattice, as evidenced by the a' data in Figure 3.

Alternatively, the head-group events detected by the spin probes may be the consequence of a head-group structural transition which occurs at 9° in the absence of salt and at 24° in the presence of salt. The low-temperature structure is probably the extended conformation, as suggested above. A possible high-temperature conformation might include a bent head group between the two phosphate groups. Extended and bent head-group conformations have been considered for phospholipids by Horwitz *et al.* (1973).

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