# Lipid Interactions in Membranes of Extremely Halophilic Bacteria. II. Modification of the Bilayer Structure by Squalene†

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ABSTRACT: The main components of the polar lipids of the extremely halophilic bacterium, *Halobacterium cutirubrum*, are di-*O*-phytanyl phosphatidyl glycerophosphate and a glycolipid sulfate. Nonionic lipids, such as squalene and carotenoid pigments, are present in approximately 1:10 ratio to polar lipids. Vesicle suspensions were made from total polar lipids plus increasing amounts of squalene. In the presence of this unsaturated hydrocarbon component, (a) the partitioning of the mobile spin-label di-*tert*-butyl nitroxide, was found to be shifted toward being less in favor of the lipid phase, (b) the thermal motion of the fluorescent probe, perylene, was increased, and (c) in the presence of $MgCl_2$ the rotational activation energy for perylene exhibited a maximum at about 1:6 mole ratio of squalene to polar lipids. These results indicate that squalene is accommodated in the hydrocarbon region of the bilayers, probably perpendicularly to the plane of the membrane, and at the expense of interstitial space in the matrix which is then no longer available to small mobile probes. Unlike acidic lipids from other sources, *H. cutirubrum* polar lipids did not show aggregation and flocculation with $Mg^{2+}$ or $Ca^{2+}$ unless squalene was also present in the bilayers at or above a 1:6 mole ratio. Flocculation under these conditions occurred at a critical temperature which was dependent on the squalene–polar lipid ratio, the concentration of divalent and monovalent cations, and pH. The results obtained are consistent with the hypothesis that the aggregative properties of these lipids are governed by the permeability of the headgroup regions to $Mg^{2+}$ or $Ca^{2+}$ and the role of squalene in the aggregation phenomenon is to space the lipid molecules sufficiently apart to permit the entry of the divalent cations to the inner charged phosphate groups. Since the effects observed occurred at NaCl and $MgCl_2$ concentrations and temperatures within the physiological range for these organisms, the interaction of the lipids with the salts may play a role in the well-described salt-dependence of the membranes of extremely halophilic bacteria.

The cell envelope of extremely halophilic bacteria has been found (Brown, 1963; Kushner *et al.*, 1964; Onishi and Kushner, 1966; Lanyi, 1971) to depend on high salt concentrations for integrity. When NaCl concentration is gradually lowered below 3 M, the membranes disintegrate and yield slowly sedimenting particles, at first containing mostly protein but at <1 M NaCl containing lipids as well (Lanyi, 1971). During this process membrane-bound enzymes become inactivated (Lanyi, 1969, 1972; Lanyi and Stevenson, 1970; Lieberman and Lanyi, 1971). The basis for this peculiar behavior has been thought to be the response of the membrane proteins to salts (Larsen, 1962, 1967; Kushner, 1968; Lanyi, 1974a).

Over the last several years, however, Kates and collaborators have shown that extremely halophilic bacteria contain very unusual lipids also. The lipids were divided into two classes: (1) polar lipids, consisting of di-*O*-phytanyl phosphatidyl glycerophosphate and a lesser amount of a glycolipid (Kates *et al.*, 1965; Kates, 1972), both highly acidic with ether-linked branched-chain hydrocarbon tails, and (2) nonionic lipids (8–10% of the total), consisting of the red pigment, bacterioruberin (Kelly *et al.*, 1970), and squalenes (Tornabene *et al.*, 1969; Kramer *et al.*, 1972). We felt that it would be of interest in our understanding of the membrane structure of these bacteria to determine whether some correlation between the unusual chemistry of the lipids and the salt-dependent behavior of the membranes could be found.

The consequences of the branching of the hydrocarbon chains on the properties of bilayers of *H. cutirubrum* lipids are described in the preceding paper (Plachy *et al.*, 1974). We have found previously, in studies of the fluorescent lipophilic probe, perylene (Lanyi, 1974b), that when the neutral lipids are included with the polar lipids in the lipid dispersions, the influence of various salts on the motion of the probe in the bilayers is greatly increased. In the study reported here we have attempted to explore the effect on nonionic lipids on the structure of bilayers, using a simpler system containing only the polar lipids of *H. cutirubrum* and squalene.

## Materials and Methods

The isolation and purification of *H. cutirubrum* total polar lipids have been described before (Kates, 1972). (DDD-DDL)-Dihydrophytol was prepared by hydrogenation of phytol. Perylene (Gold Label), pristane (2,6,10,14-tetramethylpentadecane), and 1-hexadecanol were purchased from Aldrich Chemicals, squalene from Sigma Chemicals, and *n*-dodecane from Humphrey Chemical Co. Pristane was purified by treatment with concentrated sulfuric acid and vacuum distillation; the other reagents were used without further treatment. DTBN[1] was a gift of R. Fredrickson. Solutions of lipids and reagents were kept in the dark at either 5° or at −15°.

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The preparation of aqueous suspensions of *H. cutirubrum* lipids and the incorporation of perylene have been described before (Esser and Lanyi, 1973; Lanyi, 1974b). Typically, the lipid suspensions contained 0.4 mg/ml of lipid, and, when the label was present, perylene at $10^6$ daltons of lipid/mol. Neat samples contained 1/10th that concentration of perylene and were placed in capillaries centered in 1 × 1 cm cuvets. For spin-label partitioning the lipid dispersions were prepared as described in the preceding paper (Plachy *et al.*, 1974), at $2 \times 10^5$ daltons of lipid/mol of probe.

Fluorescence intensity and polarization were determined as before (Lanyi, 1974b). For the measurements in this report a cooled circulating thermostat was added and the sample chamber was flushed with dry nitrogen to avoid condensation at temperatures below 15°. The thermostated sample compartment of the spectrofluorimeter was used also for studying the temperature-dependence of the flocculation of the lipids. As in the fluorescence measurements the cuvet was allowed to equilibrate for 10 min or more at a new temperature. Fluorescence excitation lifetimes were determined as previously (Lanyi, 1974b).

Electron spin resonance (esr) spectra were obtained as described in the preceding paper (Plachy *et al.*, 1974). In the nonlipid partitioning experiments the two-phase mixtures were equilibrated by rapid stirring while in contact with the thermostated bath. The stirring was stopped and small samples of each phase were analyzed for DTBN content using either absorbance at 236 nm or esr signal intensity.

Unless otherwise mentioned, the buffers used contained Hepes at 0.05 M and pH 7.0. In pH-dependence studies below pH 6.5 NaOAc buffer was used, at 0.1 M concentration. The pH of the buffers was determined with a Corning low Na-error electrode, after dispersing the lipids and adding salts as required. pH changes were less than 0.04 after heat treatment of the lipid suspensions.

## Results

*Rotational Motion of Perylene in Lipid Bilayers.* Rotational relaxation times of fluorescent molecules can be obtained from fluorescence polarization and excitation lifetime according to the following equation (Weber, 1953)

$$\frac{(1/p) - (1/3)}{(1/p_0) - (1/3)} = 1 + (3\tau/\rho_h) \qquad (1)$$

where $p$ is the measured polarization of fluorescence (Lanyi, 1974b), $p_0$ is the limiting polarization obtained for a completely immobilized probe, taken as ½, $\tau$ is the fluorescence excitation lifetime, and $\rho_h$ is the harmonic mean of the relaxation times of the probe in the various possible modes of tumbling.

Previously we found that the excitation lifetime of perylene in dispersions of *H. cutirubrum* total polar lipids was 6.6 ± 0.7 and 6.2 ± 0.8 nsec, in buffer alone and in the presence of 3.4 M NaCl, respectively (Lanyi, 1974b). Fluorescence excitation lifetimes were determined in this study also after adding 16% (w/w) squalene to the polar lipids and the values obtained were 6.3 ± 0.8 and 6.4 ± 0.9 nsec, respectively, in the above two buffer solutions. It appears, therefore, that neither the presence of squalene in the vesicles nor the addition of salts to the buffer affects the excitation lifetime significantly. An average value of $\tau$ 6.4 nsec was used in all calculations.

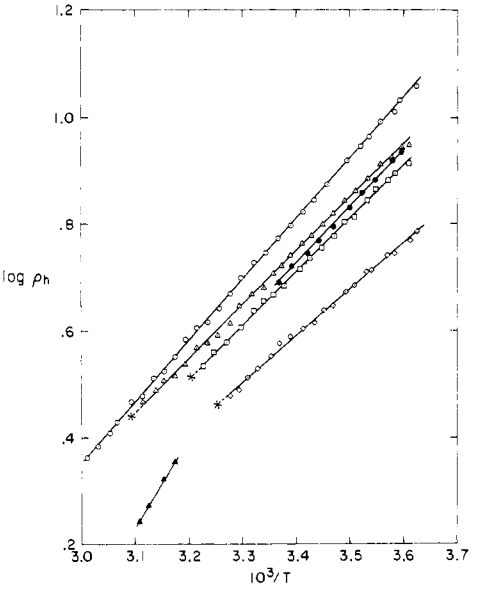


FIGURE 1: Arrhenius plots of the rotational relaxation time of perylene (in nsec) in different environments. Open symbols, vesicles prepared from *H. cutirubrum* total polar lipids in 3 M NaCl *plus* 0.1 M $MgCl_2$, with squalene content (w/w): ○, zero; △, 6.7%; □, 11.0%, ◇, 21.8%. The asterisks represent temperatures at which the lipids exhibited sudden aggregation and flocculation. (●) Neat dihydrophytol; (▲) neat hexadecanol. The latter compound has a melting point of 41–42°. The slopes of the last two curves gave $\Delta E_a$ values of 4.9 and 7.7 kcal/mol, respectively.

The rotational relaxation time, $\rho_h$, was studied, as a function of temperature, in vesicles prepared from *H. cutirubrum* polar lipids containing increasing amounts of squalene. Figure 1 shows some of the data obtained, as Arrhenius plots. The presence of squalene in the lipid vesicles is seen to cause changes both in rotational relaxation times, $\rho_h$, at a single temperature and in rotational activation energy, $\Delta E_a$ (Shinitzky *et al.*, 1971; Cogan *et al.*, 1973; Papahadjopoulos *et al.*, 1973), obtained from the slope of the lines over the temperature range studied. Figure 2 shows values of $\rho_h$ at 5°, in the presence of various salts, as functions of the squalene content of the lipid dispersions. The salts used in these experiments, NaCl at 3 M and $MgCl_2$ at 0.1 M concentration, approximate the salt composition of the growth medium for halophilic bacteria (Larsen, 1962, 1967). In all cases squalene is seen to decrease considerably the rotation-

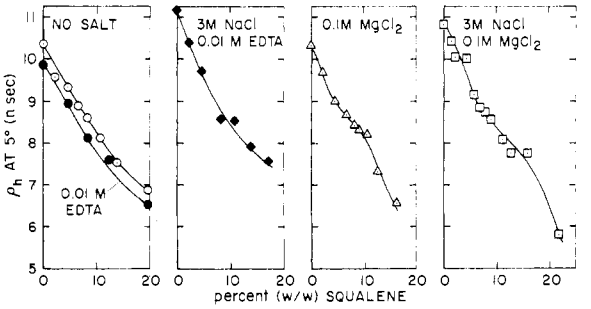


FIGURE 2: Rotational relaxation times of perylene at 5° in vesicles of *H. cutirubrum* polar lipids containing various amounts of squalene. Suspending buffer solutions contained the following added salts: (○) none; (●) 0.1 M EDTA; (♦) 3.0 M NaCl *plus* 0.01 M EDTA; (△) 0.1 M $MgCl_2$; (□) 3.0 M NaCl *plus* 0.1 M $MgCl_2$.

[1] Abbreviations used are: DTBN, di-*tert*-butyl nitroxide; Tempo, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

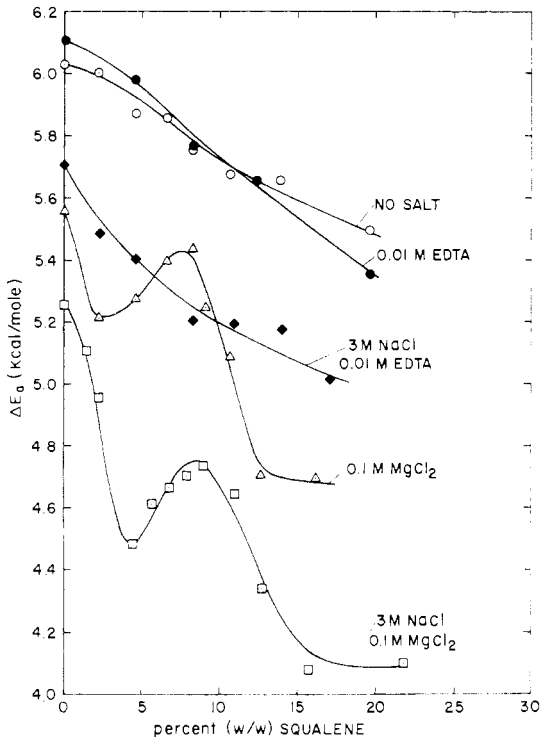


FIGURE 3: Rotational activation energy of perylene in vesicles of *H. cutirubrum* polar lipids containing various amounts of squalene. Symbols as in Figure 2.

al relaxation time of perylene, indicating that the apparent local viscosity near the probe is decreased. In the presence of $MgCl_2$ there is an inflection in the curves near 6–8% squalene.

The slopes of the curves in Figure 1 yielded rotational activation energies for the probe under various experimental conditions. These were calculated from

$$\Delta E_a = 2.303R[\mathrm{d} \log \rho_h/\mathrm{d}(1/T)] \quad (2)$$

The rotational activation energy reflects the temperature dependence of the reorientation motion rate of the probe. When a small molecule, such as perylene, experiences thermal motion in a lipid bilayer lattice, the rotational activation energy of the probe depends on how strongly its motion is coupled to the motion of the hydrocarbon chains. In the extreme case, when little or no independent motion is possible in the matrix, the rotational activation energy of the probe is equal to the motional activation energy of the entire system. This is the situation usually when the probe is in a straight-chain hydrocarbon environment. Results obtained with perylene in lipids of this type (Cogan *et al.*, 1973; Papahadjopoulos *et al.*, 1973), as well as in neat 1-hexadecanol (Figure 1), give $\Delta E_a$ = 7–9 kcal/mol, somewhat higher than the value predicted for the activation energy of flow for hydrocarbon chains in isotropic systems (Kauzman and Eyring, 1940). In the other extreme case there is zero coupling between the probe and the lattice and the probe molecule rotates entirely independently from the matrix. The apparent activation energy of such motion, calculated from the temperature dependence of the kinetic energy, is extremely low (<0.2 kcal/mol near room temperature). Perylene in neat dihydrophytol phase exhibits lowered rotational activation energy ($\Delta E_a$ = 4.9 kcal/mol in Figure 1), consistent with the expectation that the four methyl branches on these chains give rise to gaps and spaces which perylene can occupy and thus execute motions somewhat uncoupled from those of the lattice.

Experimental values of $\Delta E_a$ obtained with a standard error of 4–5% (by linear regression analysis) from data like those shown in Figure 1 are plotted in Figure 3 as functions of the squalene content of the lipid suspensions. It is apparent in Figure 3 that the presence of squalene in the branched-chain matrix decreases even further the rotational activation energy for the motion of perylene. Both Figures 2 and 3 show that, in the absence of salts, in the presence of 0.01 M EDTA or in the presence of 3 M NaCl *plus* 0.01 M EDTA the measured parameters decrease monotonously with increasing squalene concentration. In contrast, in both figures, the presence of 0.1 M $MgCl_2$ appears to give rise to anomalous curves, resulting in $\Delta E_a$ maxima at about 8% squalene. Since the molecular weight of squalene is about half that of the average for *H. cutirubrum* polar lipids, the approximately 1:12 weight ratio, where the anomalous behavior is observed, corresponds to a 1:6 mole ratio.

It is indicated in Figure 1 that at squalene concentrations higher than 6–7% (w/w) sudden precipitation or flocculation of the lipid suspension was observed at elevated temperatures. This finding was reproducible and will be discussed in more detail below.

*Partitioning of Di-tert-butyl Nitroxide between the Lipid Phase and Water.* In a lipid suspension in water, the esr spectrum of a spin-label partitioning probe is observed to be the sum of two spectra, one from the lipid phase and one from the aqueous phase (Hubbell and McConnell, 1968). The relative intensity of the two signals depends on the relative volumes of the two phases and on the partition coefficient, $K_{par}$, which is the ratio of the activities of the nitroxide label in the two environments. The partition coefficient should be sensitive to changes in the nature of either region. Most authors assume that $h_0/h_{aq}$ is proportional to $K_{par}$ and thus plot log $(h_0/h_{aq})$ *vs.* $1/T$, where $h_0$ and $h_{aq}$ are the peak-heights of the organic and aqueous signals, respectively (Figure 4a). This analysis has been successful, for example, using Tempo as a probe of lipid phase equilibria (Shimshick and McConnell, 1973), in spite of the fact that the height ratio is sensitive to line-width changes and to overlap of the two signals. Esr partitioning experiments have been reviewed by Keith *et al.* (1973).

We have performed partitioning experiments on 1% (w/v) suspensions of polar lipids in 3 M NaCl with DTBN as the partitioning probe. The physical and chemical properties of DTBN and Tempo are virtually identical, but the observed intrinsic esr line width of DTBN is about half that of Tempo in most solvents. In Figure 4a the enhanced resolution of the signals from the two sites for the DTBN probe, as compared to Tempo, is seen. The reduced overlap allowed us to double integrate the high-field line, and, after correcting for the $^{13}C$ satellite lines, to evaluate the ratio of the spin-labels in each environment to an accuracy of ±15% following the method of Dix *et al.* (1974). This ratio is independent of line-width effects and is simply related to $K_{par}$ by the volume ratio of the two environments. DTBN was used in partitioning experiments by Bales and Baur (1970) and more recently by Dix *et al.* (1974) and Griffith *et al.* (1974).

The results obtained with DTBN are shown in Figure 4b, where it is seen that the plot of log $K_{par}$ *vs.* $1/T$ shows a sharp break toward a temperature-independent partition constant near 42°. When the same data are treated in the manner of Hubbell and McConnell (1968), as shown in the

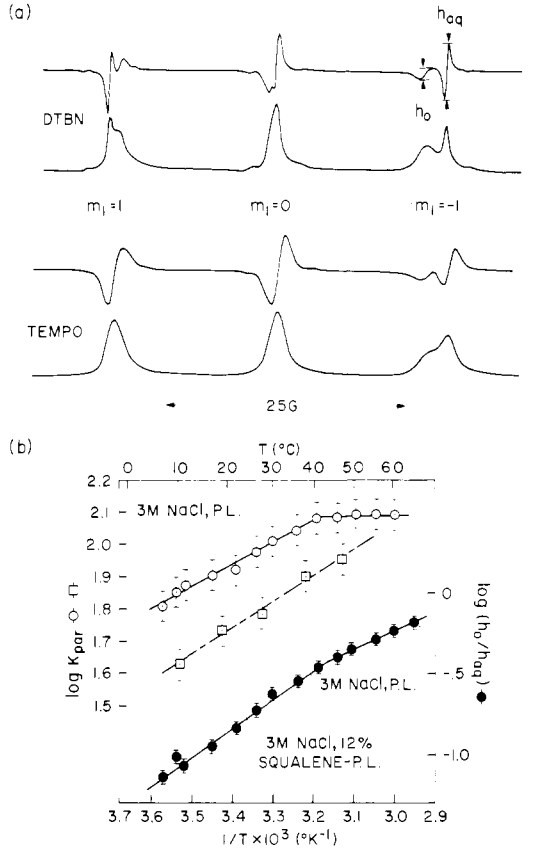


FIGURE 4: Partitioning of DTBN and Tempo between lipid and water phases. (a) Derivative and absorption (first integral) esr spectra for DTBN (upper two traces) and Tempo (lower two traces), partitioned between *H. cutirubrum* polar lipids, 1% (w/v), and 3 M NaCl. Note the greatly reduced overlap of the absorption signals from the two phases on the high field, $m_I = -1$, line for DTBN. Label concentration was $5 \times 10^{-5}$ M, temperature 21° in both cases. (b) Dependence of the partitioning coefficient, $K_{par}$, on lipid composition and temperature. (○) log $K_{par}$ *vs.* $1/T$ for DTBN between *H. cutirubrum* polar lipids and 3 M NaCl; (△) log $K_{par}$ *vs.* $1/T$ for DTBN between polar lipids with 12% (w/w) squalene added and 3 M NaCl; (●) log $h_0/h_{aq}$ *vs.* $1/T$ (right-hand axis).

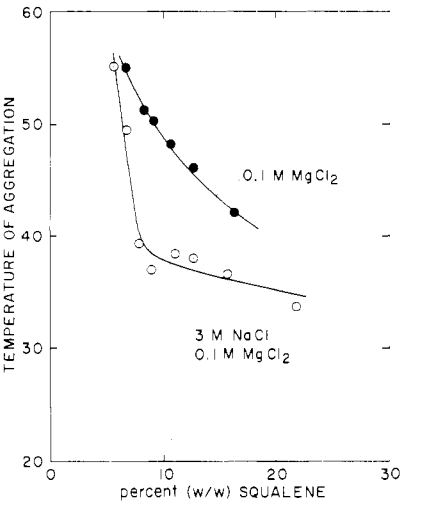


FIGURE 5: Dependence of the flocculation temperature on the squalene content of the lipids and on the salts added to the suspending medium. The lipid suspensions were equilibrated at increasing temperatures (in approximately 2° steps) until sudden, massive aggregation could be seen. (○) 3.0 M NaCl *plus* 0.1 mM $MgCl_2$; (●) 0.1 M $MgCl_2$.

lower curve in Figure 4b, the break appears also at 42°. This temperature is in good agreement with the highest temperature break-point observed dilatometrically, at 39–44° for these lipids (Plachy *et al.*, 1974).

The esr hyperfine coupling parameter, *a*, observed for DTBN in the lipid phase suggested that the spin-label in the lipid is primarily in a hydrocarbon-like environment. For this reason we have independently determined the partitioning coefficient for DTBN between 3 M NaCl and pristane, a saturated hydrocarbon with four methyl branches like the lipid phytanyl chains, and for DTBN between 3 M NaCl and *n*-dodecane, a straight-chain hydrocarbon. The data showed that the partition coefficients are essentially the same for the two hydrocarbons, but the value of $K_{par}$ in these systems is about 50% smaller than $K_{par}$ for the lipids at most temperatures. This indicated that the relatively ordered hydrocarbon chains of the lipid bilayer are better solvents for this small spherical molecule than either branched-chain or straight-chain pure hydrocarbons. As seen in Figure 4b, however, the presence of 12% (w/w) squalene causes the partitioning of DTBN in favor of the lipid phase to be considerably decreased.

*$Mg^{2+}$ or $Ca^{2+}$ Dependent Flocculation of Lipid Vesicles.* Similarly to the behavior of other phospholipids (Abramson *et al.*, 1964, 1968), suspensions of vesicles prepared from *H. cutirubrum* total polar lipids become turbid on addition of divalent cations. The light-scattering increase occurs mainly in the forward direction (J. K. Lanyi, unpublished experiments), indicating that the size of the scattering particles is increased. In contrast with phosphatidylinositol (Abramson *et al.*, 1968), however, flocculation of the *H. cutirubrum* polar lipids with $MgCl_2$ or $CaCl_2$ does not occur unless squalene is also added to the lipid preparations. Under these conditions flocculation is observed suddenly, at a particular temperature, which can be determined with an accuracy of ±1–2° (Figure 1). This parameter is plotted in Figure 5 as a function of squalene concentration in the lipid vesicles, in the presence of 0.1 M $MgCl_2$ or 0.1 M $MgCl_2$ *plus* 3 M NaCl. As seen in Figure 5, aggregation is observed only when squalene concentration is more than 6–7% (w/w) and under these conditions the temperature of flocculation approaches a limiting value of about 35°. The aggregation process appeared to be cooperative in its sharp temperature dependence, as well as in its squalene concentration dependence, at high NaCl concentrations. The aggregation temperature was unaffected by varying the total amount of lipids in the suspension between 0.13 and 1.2 mg/ml. Neither was the aggregation temperature changed when the main polar lipid, di-*O*-phytanyl phosphatidyl glycerophosphate (Kates, 1972), was tested, under the same conditions, in place of the mixed polar lipid preparation.

X-Ray diffraction analysis (kindly performed by Dr. G. King on our samples, which contained 9% squalene and were suspended in 0.1 M $MgCl_2$) indicated that the lipid is found in bilayer vesicles both before and after the heat-induced aggregation. It is probable, therefore, that what is visually detected as flocculation consists of the side-to-side, random aggregation of the vesicles.

Vesicle preparations containing 16% (w/w) squalene were incubated in the presence of $MgCl_2$ or $CaCl_2$, at various concentrations, to explore the divalent cation concentration dependence of the aggregation process. The results are shown in Figure 6a. It appears that both $MgCl_2$ and $CaCl_2$ begin to induce flocculation above 40 mM concentration and reach maximal effectiveness near 200 mM. In the pres-

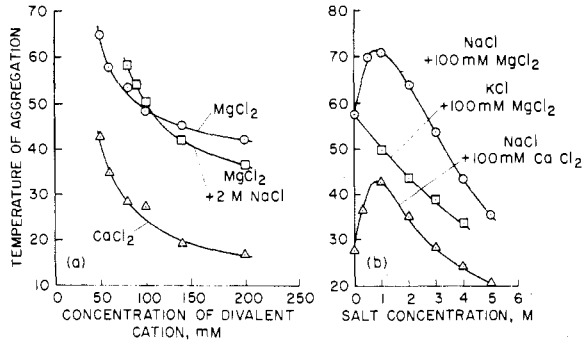


FIGURE 6: Dependence of the flocculation temperature on divalent and monovalent salt concentration in the suspending medium. Vesicles contained *H. cutirubrum* polar lipids and 16% (w/w) squalene (a) or 9% (w/w) squalene (b). (a) Symbols: (○) $MgCl_2$ concentration varying; (△) $CaCl_2$ concentration varying; (□) 2.0 M NaCl added, $MgCl_2$ concentration varying. (b) Symbols: (○) 0.1 M $MgCl_2$ added, NaCl concentration varying; (△) 0.1 M $CaCl_2$ added, NaCl concentration varying; (□) 0.1 M $MgCl_2$ added, KCl concentration varying.

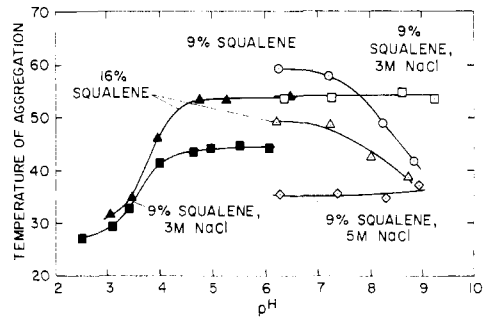


FIGURE 7: Dependence of the flocculation temperature on the pH of the suspending buffer solution. Open symbols, 0.05 M Hepes; closed symbols, 0.1 M acetate buffer. Vesicles of *H. cutirubrum* polar lipids and added squalene were prepared in buffer solutions containing the salts indicated: (○) 9% (w/w) squalene, 0.1 M $MgCl_2$; (△ and ▲) 16% (w/w) squalene, 0.1 M $MgCl_2$; (□ and ■) 9% (w/w) squalene, 0.1 M $MgCl_2$ *plus* 3.0 M NaCl; (◇) 9% (w/w) squalene, 0.1 M $MgCl_2$ *plus* 5.0 M NaCl.

ence of $CaCl_2$ the process occurs 25° below that observed for $MgCl_2$. Addition of 2 M NaCl shifts the concentration-dependence curve to the right and downward, suggesting at least two effects for the monovalent salt: (a) competitive inhibition of the interaction of the lipids with $MgCl_2$ and (b) enhancement of the interaction (*i.e.*, lowered flocculation temperature) at high $MgCl_2$ concentrations.

It is well-known that the presence of 3–5 M NaCl or KCl is essential for the integrity of the membranes of halophilic bacteria (Brown, 1963; Kushner *et al.*, 1964; Onishi and Kushner, 1966; Lanyi, 1971). The effect of high concentrations of monovalent salts on the behavior of the lipids from these organisms was therefore investigated in more detail. Figure 6b shows results obtained with polar lipids containing 9% (w/w) squalene, as flocculation temperatures plotted against monovalent salt concentration, in the presence of 0.1 M $MgCl_2$ or $CaCl_2$. With NaCl, again, two effects are seen: (a) increased temperature for aggregation at <3 M concentration and (b) decreased temperature at >3 M concentration. Potassium chloride, apparently, shows only the latter effect.

The pH dependence of the flocculation behavior was determined with polar lipid preparations containing 9 or 16% (w/w) squalene, in the presence of various salts. Flocculation temperature as a function of pH is plotted in Figure 7. Two kinds of pH-dependent effects are evident in this graph, one at pH 3.7–3.8 and another at pH 8 or above. These values are about 0.5 pH unit higher than the titration data of Kates *et al.* (1965) for the two primary and the single secondary protons on the phosphate groups of phosphatidyl glycerophosphate in methanol–water solution and probably reflect the cooperative shift in p$K$ expected in a multianionic bilayer. If the correspondence between the aggregation data and the ionization properties of the lipids indeed reflects the involvement of the phosphate groups in the aggregation phenomenon, it is apparent that the loss of the two primary protons (at pH 3.7–3.8) raises the aggregation temperature, while the loss of the secondary proton (at pH >8) lowers it. In addition, the data in Figure 7 indicate that the latter effect is abolished when NaCl is added at high concentrations, while the former is unchanged. None of the conditions employed shifted the apparent p$K$ values in Figure 7.

## Discussion

We have previously proposed (Lanyi, 1974b) that the inclusion of the neutral lipids of *H. cutirubrum*, the carotenoids and squalenes, in vesicles prepared from the total polar lipids of this organism, cause an irregularity in the bilayer structure due to the lack of hydrophilic head groups in the neutral lipids, particularly in squalene. In this report we have determined some properties of a simpler system, containing only the polar lipids and squalene. The behavior of the polar lipids, as discussed in the preceding paper (Plachy *et al.*, 1974), reflect largely the properties of their main component, di-*O*-phytanyl phosphatidyl glycerophosphate (Kates *et al.*, 1965). There are two plausible sites for squalene in bilayers formed from such lipids: (a) intercalated between the hydrocarbon chains of the lipids, most likely in a perpendicular direction to the plane of the membrane, and (b) parallel to the plane of the membrane, between the two halves of the bilayer. The partitioning of DTBN was found to be shifted toward the aqueous phase when squalene was present in the lipid vesicles (Figure 4). This observation argues against possibility (b) since the distribution of the small label molecule seems to be primarily dependent on the volume accessible to it in the bilayer (Dix *et al.*, 1974) and if squalene occupied the region between the two halves of the bilayer the thickness of the bilayer would be increased. In fact, squalene competes for sites with DTBN in the lipid phase. We have concluded, therefore, that the squalene molecules probably project into the hydrocarbon matrix, occupying space between the phytanyl chains resulting from thermal motion. Accommodation of the probe in the bilayer under these conditions requires more bending of the hydrocarbon chains (Träuble, 1971), a process limited by the available kinetic energy.

The results obtained with perylene, another small mobile probe, less bulky than DTBN and completely nonpolar, are in agreement with the above model. As a first approximation, the introduction of increasing amounts of squalene is expected to cause increased disorder in the bilayers, which had originally contained a single kind of hydrocarbon chain. Accordingly, the motion of perylene is increased when squalene is added (Figure 2). However, the rotational activation energy for the probe exhibits a peak at about 6:1 mole ratio of polar lipid to squalene suggesting that the motion of perylene is more dependent on the motion of the lipid matrix than at lower or higher ratios.

Squalene is a linear molecule of 28–29 Å contour length, and, if extended, would nearly span the hydrophobic width of the bilayer. It is not possible at this time to determine a

packing geometry which might favor the interaction between the phytanyl chains and squalene with a particular stoichiometry. There is, however, evidence from monolayer pressure–area studies (Tinoco and McIntosh, 1970; Gosh *et al.*, 1971) for the condensation of various saturated and unsaturated hydrocarbon chains in lecithin with cholesterol and it is reasonable to assume that such packing may be possible in the system reported here, as well. No reflections were observed in X-ray diffraction patterns which might indicate increased crystallinity in the presence of squalene, however, and we conclude that the addition of squalene does not cause a net increase of order in the bilayer.

Figures 2 and 3 show that the anomalous behavior in the motion of perylene is obtained only in the presence of $MgCl_2$. In these lipids (Plachy *et al.*, 1974), as well as in others (Butler *et al.*, 1970; Ohnishi and Ito, 1974), the mobility of oriented spin-labels is reduced by divalent cation binding, presumably because of closer packing of the lipid tails when divalent counterions reduce the net anionic charge density. The implication of our results is that the geometrical requirements of the interaction between the phytanyl chains and squalene are satisfied only when the motional freedom of the hydrocarbon phase is reduced.

The interaction of monovalent and divalent cations with acidic lipids has been extensively studied and it has been proposed that $Na^+$ and $K^+$, and particularly $Ca^{2+}$ and $Mg^{2+}$, interact with the head-group regions of the lipid vesicles and decrease net charge density and extent of hydration (Abramson *et al.*, 1964, 1968; Seimiya and Ohki, 1973). Papahadjopoulos (1968) had suggested that $Ca^{2+}$ interacts with dianionic phospholipids to form linear complexes of well-defined stoichiometry. Recently, phosphatidylserine was shown (Ohnishi and Ito, 1974) to separate from mixed bilayers as a separate phase of immobilized Ca-complex when this cation was added. For phosphatidylinositol, flocculation of the lipids is observed at 0.3–0.4 M NaCl or KCl and at 1–4 mM $CaCl_2$ or $MgCl_2$ (Abramson *et al.*, 1968). The polar lipids of *H. cutirubrum* show flocculation with cations, but with some important differences, as follows: (a) flocculation is observed only when squalene is included with the polar lipids in the vesicles at >6–8% (w/w), (b) flocculation occurs at much higher concentrations of divalent cations (>40 mM) and not at all with monovalent cations alone, up to 5 M concentration, (c) flocculation occurs suddenly at a critical temperature which depends on the experimental conditions, and (d) in mixtures of monovalent and divalent cations both competitive effects and enhancement by the monovalent cations are observed.

The aggregation and flocculation of phospholipids by $Ca^{2+}$ or $Mg^{2+}$ have been proposed to proceed through the formation of "bridge" complexes between phosphate groups on opposing bilayers and the divalent cation. As discussed in the preceding paper (Plachy *et al.*, 1974), *H. cutirubrum* lipids have unusually deep head-group regions and carry negative charges. Phosphatidyl glycerophosphate, the main component of these lipids, is a dianion at neutral pH (Kates *et al.*, 1965, Kates and Hancock, 1971), each phosphate group being singly ionized. The negative charges are distributed in two layers, one near the aqueous interface, the other near the hydrocarbon interior, in a region which is incompletely permeable to water and cations requiring a greater degree of desolvation for charge interaction with the cation than does the relatively exposed outer layer. The effect of squalene is assumed to consist of spacing the lipid molecules sufficiently apart to permit the approach of cations to the inner layer of charged phosphate groups. Bilayers of phosphatidyl glycerophosphate thus contain two charged loci, and it is tentatively proposed that flocculation is dependent on the screening of the inner layer of charges as well as on the "bridging" effect, which involves only the outer phosphate groups. The antagonistic effect of monovalent cations at lower $Mg^{2+}$ concentrations and at <3 M NaCl (Figure 6) is obtained in other systems as well (Abramson *et al.*, 1968), and can be understood to originate from competition for the outer charges, involved in the "bridging" effect. The screening of the inner layer of charges, however, is expected to be accomplished by the penetration of both monovalent and divalent cations into the head-group region. At high $Mg^{2+}$ and $Na^+$ or $K^+$ concentrations the divalent cation-induced aggregation effects should be thus enhanced. The preference of the system for $Ca^{2+} > Mg^{2+}$ and $K^+ > Na^+$ is consistent with greater effectiveness for the smaller of the hydrated cations.

It is interesting to note that above pH 8, where the third ionizable proton, on the phosphate group near the aqueous interface, is lost, the temperature at which flocculation occurs is lowered (Figure 7). This effect is eliminated at high NaCl concentrations, as expected if the ionized group involved is fully accessible to the cations. The ionization of the primary phosphate groups, at pH 3.7–3.8, in contrast, results in the rise of the flocculation temperature and this effect is not eliminated by NaCl. It is likely, therefore, that the latter ionization occurs in a region not readily accessible to counterions, as discussed above.

It appears that the inclusion of squalene in the vesicles prepared from *H. cutirubrum* polar lipids, at polar lipid/squalene ratios not very different from that determined for *H. cutirubrum* cell envelopes (Tornabene *et al.*, 1969) profoundly affects both the packing of the phytanyl chains and the arrangement of the head groups, allowing the latter to interact with divalent cations. Such a conclusion could be of importance for the structure of the cell envelopes of these organisms, since both in halophilic membranes (McClare, 1967) and a model system (Bulkin and Hauser, 1973) the binding of some proteins by the phospholipids has been proposed to be mediated by $Mg^{2+}$ or $Ca^{2+}$. The *H. cutirubrum* lipids exhibited much of the effects described in this study under conditions similar to the physiological environment of the extremely halophilic bacteria, which is 3–5 M NaCl *plus* 0.1 M $MgCl_2$ at 37° (Larsen, 1962, 1967). It is possible, therefore, that the binding of some of the proteins in the cell envelope and their release at lowered salt concentrations (Onishi and Kushner, 1966; Lanyi, 1971) is, in part, due to the response of the phospholipids in the membranes to the ionic conditions.

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