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IRREGULAR BILAYER STRUCTURE IN VESICLES PREPARED FROM *HALOBACTBRIUM CUTIRUBRUM* LIPIDS

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SUMMARY

The behavior of the fluorescent probes, perylene and 8-anilinonaphthalenesulfonic acid, was studied by determining fluorescence polarization in vesicles prepared from *Halobacterium cutirubrum* polar lipids and unfractionated lipids. In the latter case, when the non-polar lipids of this organism are included (carotenoids and squalenes, comprising 8 % of the total), the environment of perylene is more fluid than in polar lipids alone. Studies of the fluorescent emission spectra of ANS and the effect of chaotropic perturbants on the motion of perylene suggest that the bilayer structure in vesicles of unfractionated lipids is distorted in such a way as to allow for the penetration of more water molecules near the hydrophobic region or to induce the probes to be nearer to the aqueous phase than is the case for the polar lipids alone. In buffers containing 100 mM $MgCl_2$, and especially in the presence of high concentrations of NaCl as well, an irreversible thermal transition of the liquid crystalline matrix was observed in the region occupied by perylene for vesicles of unfractionated lipids. Vesicles prepared from polar lipids alone do not show such transition, and the temperature at which the transition occurs depends on the amount of non-polar lipids included. It is likely that the irregularity of the bilayer structure and the thermal breakdown are both caused by the disruptive effect of the non-polar lipids.

Cell envelopes of *H. cutirubrum* do not show the above transition, which occurs in the lipid vesicles in ionic environments and at temperatures which are physiological for these organisms. This finding is consistent with our previous suggestion, based on spin label studies, that in *H. cutirubrum* the membrane proteins immobilize most or all of the lipid phase.

INTRODUCTION

The structure of biological membranes is governed by interactions among lipid and protein molecules. In a recent review Singer and Nicholson [1] proposed a model for membranes in which structural stability is derived from a lipid bilayer while pro-

Abbreviations: ANS, 8-anilinonaphthalenesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

teins are either bound to the polar headgroups of lipids or are intercalated among the hydrocarbon tails. In membranous structures of low lipid content, such as reconstituted cytochrome oxidase vesicles [2] and the isolated purple membrane of *Halobacterium halobium* (Stoeckenius, W. and Hubbell, W., personal communication), the presence of proteins reduces the flexibility of the hydrocarbon chains considerably from that predicted from the behavior of lipid bilayers. We found a similar situation in the cell envelopes of *H. cutirubrum* [3], where the membrane structure appeared to be dominated by the organization of lipids by membrane proteins. The above studies utilized stearic acid-type spin labels. Fluorescent molecules, both non-polar and amphophilic, have also been used to investigate the structure of membranes, artificial and natural [4]. This report is concerned with the use of fluorescent labels to extend our studies of the cell envelope of *H. cutirubrum* and, in particular, to explore the effect of the heterogeneity of the lipids in this organism [5, 6] on the structure of the bilayers.

MATERIALS AND METHODS

Buffers contained *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at 0.05 M and pH 7.0. The term "basal salts" refers to salts other than NaCl, which were present in the growth medium, and consist of 20 g/l $MgCl_2 \cdot 6H_2O$, 2 g/l KCl and 0.2 g/l $CaCl_2 \cdot 2H_2O$.

Growth and harvesting of the extremely halophilic bacterium, *H. cutirubrum*, has been described before [7]. Envelope vesicles were prepared in buffer containing 3.4 M NaCl and basal salts by passing a suspension of cells through a Yeda Press at 105 kg/cm^2 (of nitrogen), followed by digestion with DNAase (Sigma Chemical, Type I, at approx. 0.1 mg/ml) at 30 °C for 15 min, and centrifugation at $2000 \times g$ for 20 min. Cell envelopes were recovered from the supernatant by centrifugation at $35\,000 \times g$ for 30 min. The preparations were stored at 4 °C for no longer than 3–4 days. Lipids were isolated from centrifuged cells by extracting 5 times with chloroform–methanol (3 : 1, v/v), evaporating the extract in vacuo, redissolving in chloroform, washing the organic phase with water, and drying by evaporation after adding ethanol and benzene. Polar lipids were precipitated with cold acetone [6] and both the precipitate and the red residue, containing the neutral lipids, were finally dissolved in chloroform. The compositions of the lipid fractions were assumed to be similar to those reported by Kates [5] for analogous preparations. The lipids were stored at −15 °C in the dark.

Lipid vesicles were prepared by evaporating 0.5 ml lipid solution (5–10 mg/ml, determined gravimetrically) in a 250-ml flask and shaking with 5 ml buffer [3]. When NaCl or other salts were to be included, these were added after the lipids were resuspended in a smaller volume of buffer and the solution was brought up to a final volume of 5 ml. Perylene (Aldrich Chemical) was added to the chloroform solutions before evaporation, typically in the ratio of $1 \cdot 10^6$–$2 \cdot 10^6$ daltons of lipid per mole of probe. 8-Anilinonaphthalenesulfonic acid (ANS) (Sigma Chemical) was added to the final aqueous suspension to give 0.1–0.2 times the above ratio. Cell envelopes and bovine serum albumin were labeled by incubating overnight at 4 °C in 3.4 M NaCl plus basal salts (1–2 mg/ml protein) with perylene-coated glass beads [8, 9] under a nitrogen atmosphere, in the dark.

Fluorescence was measured in a Schoeffel Model 1000 spectrofluorimeter, which consisted of a 1000 W xenon lamp, an infrared filter, an excitation monochromator, a thermostated cuvette holder for 1 cm × 1 cm cuvettes with right-angle geometry, equipped with stirring, an emission monochromator and a thermoelectrically cooled photomultiplier. For fluorescence polarization measurements a Glan prism and a Pola-coat filter were used, before and after the sample cuvette, respectively. Variations in lamp energy were corrected by placing a beam-splitter before the sample and measuring the ratio of the sample fluorescence and the quantum yield of a Rhodamine B reference solution. Excitation and emission bandwidths were 6.6 nm. Fluorescence intensities were either corrected for light-scattering contribution [8] or a No. 3 (yellow) filter was inserted after the sample cuvette, which eliminated the need for such correction. In the temperature-dependence studies readings were taken after thermal equilibration, generally within 10–15 min; the sample was protected from light between readings. The value of the polarization (p) was calculated from the following relationship:

$$p = \frac{I_{vv} - GI_{vh}}{I_{vv} + GI_{vh}} \tag{1}$$

where I_{vv} is the light intensity measured with both exciting and emitted light vertically polarized and I_{vh} is the intensity measured with vertically polarized exciting light and horizontally polarized emitted light. G is an instrument factor arising from transmissivity differences for vertically and horizontally polarized light [10].

Rotational relaxation times for the probes were calculated from the following formula [11]:

$$\frac{1/p - 1/3}{1/p_0 - 1/3} = 1 + \frac{3\tau}{\rho_h} \tag{2}$$

where p_0 is the limiting polarization for a completely immobilized probe and approaches 1/2 when excitation is at the absorption peak of lowest energy. τ is the fluorescence lifetime and ρ_h is the harmonic mean of the relaxation times of the probe molecule in the various possible modes of tumbling.

Fluorescence lifetimes were determined with a TRW Model 75A decay time fluorimeter system, using No. 405 and 8 filters for excitation and emission, respectively, for both perylene and ANS.

RESULTS

Location of probes in lipid vesicles and cell envelope membranes

Fluorescence spectra can often be used to determine the polarity or polarizability of the immediate environment of the emitting molecules [4, 12, 13]. The solvent dependence of emission spectra can be analyzed according to the following relationship [14]:

$$\nu_{abs} - \nu_{em} = \text{const.} + \frac{(m_e - m_g)^2}{a^3 h}\left[\frac{2(D-1)}{2D+1} - \frac{2(n^2-1)}{2n^2+1}\right] + \text{higher terms} \tag{3}$$

where ν_{abs} and ν_{em} are frequencies of the maxima of absorption and emission peaks,

m_e and m_g are the dipole moments of the fluorescent molecule in the excited and ground states, a is the effective cavity radius, and D and n are the dielectric constant and refractive index of the solvent. Excitation and emission maxima were determined accordingly for perylene [18] in various solvents and the data are plotted in Fig. 1a. The invariance of $\nu_{abs}-\nu_{em}$ in a wide range of solvents indicates that the dipole moment of perylene is unchanged on excitation, with a consequent lack of solvent rearrangement. It might be expected therefore, that immobilization of solvent molecules does not affect the fluorescence spectra of perylene in a manner described for other molecules [14, 15]. Indeed, the second emission peak of perylene was determined to be constant in glycerol (473.8±0.5 nm) at p values between 0.33 and 0.08, for temperatures between 14 and 50 °C. Any solvent dependence of emission peaks observed is therefore due to the ground-state dipole moment of perylene. These shifts, which can be expected to occur for both absorption and emission peaks, are proportional [16] to $(n^2-1)/(2n^2+1)$. The frequency of the second emission peak is plotted against this function in Fig. 1b, and the expected solvent polarizability dependence is

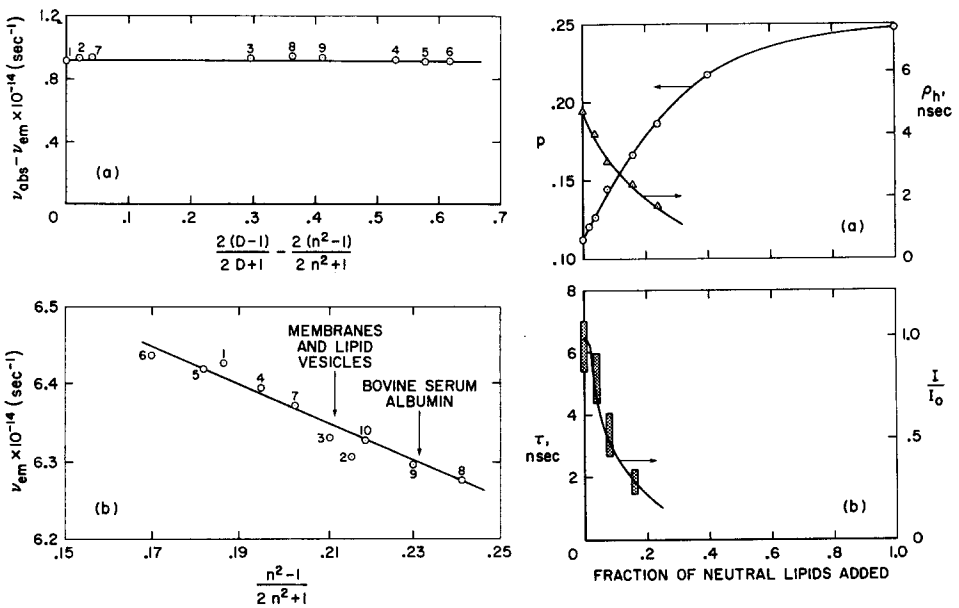


Fig. 1. Spectral shifts of the fluorescence of perylene in various environments. (a) Frequency of absorption (excitation) peaks minus frequency of the second emission peaks in selected solvents. (b) Frequency of the second emission peaks in selected solvents, cell envelope membranes and lipid vesicles in 3.4 M NaCl plus basal salts, as well as in the presence of bovine serum albumin. Solvents used: (1) hexane, (2) carbon tetrachloride, (3) chloroform, (4) *n*-butanol, (5) ethanol, (6) methanol, (7) dioxane, (8) phenol, (9) pyridine and (10) glycerol.

Fig. 2. Fluorescence parameters for perylene in lipid vesicles prepared from mixtures of *H. cutirubrum* polar and neutral lipids. The abscissa refers to the fraction of the original content of non-polar lipids restored (1.0 corresponds to unfractionated lipids, which contain 8 % neutral lipids). (a) Polarization of fluorescence, p (○) and rotational relaxation time, ρ_h (△) as functions of lipid composition. (b) Measured fluorescence lifetimes, τ (vertical bars, representing two standard deviations), and fluorescence yields relative to that measured in vesicles of polar lipids alone (——) as functions of the lipid composition. Measurements at 22 °C; the buffer contained 3.4 M NaCl plus basal salts.

indeed obtained. As indicated in Fig. 1b, the frequency of the emission peak of perylene was similar to that seen in chloroform when placed either in membranes or lipid vesicles prepared from unfractionated lipids or from polar lipids alone. It is interesting that a similar analysis of the absorption spectrum of bacterioruberin, a lipid-soluble carotenoid found in membranes of halophiles [17] also indicated a polarizability in the lipid phase similar to that of chloroform [7]. The refractive index (and polarizability) of chloroform is very similar to those of various oils and paraffin (Handbook of Chemistry and Physics, 1967–1968). In contrast to the results with cell envelope membranes, perylene adsorbed to bovine serum albumin shows an emission peak at longer wavelengths, between the frequencies observed in pyridine and phenol (Fig. 1b).

Since the lipid phase of *H. cutirubrum* cell envelope contains carotenoid pigments, most notably bacterioruberin [17], which absorb light at the wavelengths of the emission peaks of perylene, it might be expected that energy transfer could take place between these species, resulting in lowered fluorescence yields and decreased lifetimes [18]. To test this possibility lipid vesicles were made from the polar lipids of *H. cutirubrum* and from polar lipids with increasing amounts of the non-polar lipids (which include the carotenoids) added back until the composition of the unfractionated lipid preparation was reached. Measured values of p, relative fluorescence intensity and fluorescence lifetime τ, as well as calculated values of the rotational relaxation times of perylene, ρ_h, from Eqn 2, are shown in Figs 2a and 2b. As expected [18], the determined values of the excited state lifetimes appear to be proportional, within the accuracy of the method, to the relative quantum yield. Thus, despite the increasing magnitude of the polarization p, with increasing amounts of neutral lipids, the calculated values of the rotational relaxation times exhibited a decrease in these lipid mixtures.

A summary of the data obtained which relate to the behavior of perylene in lipid vesicles and in cell envelopes at room temperature, in the presence or absence

TABLE I

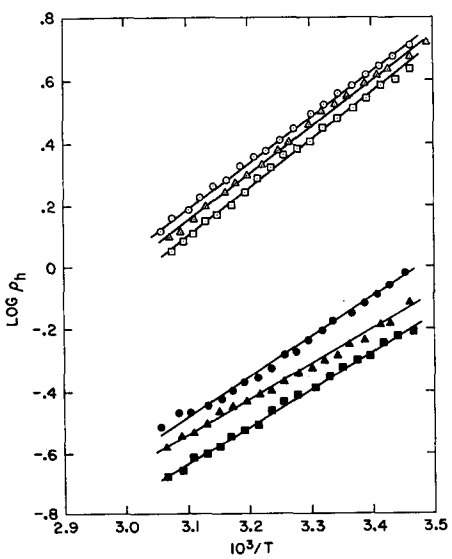
FLUORESCENCE PARAMETERS OF PERYLENE IN VARIOUS ENVIRONMENTS AT 22 °C

System	Salts added	p	τ (ns)	ρ_h (ns)
Unfractionated lipids of *H. cutirubrum*	None	0.2131		0.5
	Basal salts	0.2495	0.25*	0.6
	3.4 M NaCl	0.2743		0.8
	3.4 M NaCl plus basal salts	0.2881		0.9
Polar lipids of *H. cutirubrum*	None	0.1035	6.6±0.7	4.3
	3.4 M NaCl plus basal salts	0.1124	6.2±0.8	4.5
H. cutirubrum envelope membranes	3.4 M NaCl plus basal salts	0.2278	2.85*	6.0
Bovine serum albumin	None	0.3640	6.4**	42.8

* Calculated from fluorescent yield. The amount of label in membranes was determined by extraction with chloroform–methanol (as described under Materials and Methods) and determining fluorescence in chloroform solution.

** Assumed value.

of various salts added to the growth medium, is given in Table I. For comparison, some results obtained with bovine serum albumin are also included. The measured values of fluorescence lifetimes are given with standard deviations calculated from seven determinations. As expected on the basis of the spectral properties of perylene, the value of τ in vesicles of polar lipids (where there is no quenching) was similar to that in ethanol, found to be 5.6 ± 0.8 ns. The τ values reported in these environments are compatible with previously published results [8, 19]. In vesicles prepared from unfractionated lipids and in cell envelopes the fluorescence lifetimes were calculated from relative fluorescence yields, which could be determined with reasonable accuracy even at the low values (Fig. 2b), on the assumption that these are proportional to τ ([18] and Fig. 2b). It is apparent from the fluorescence lifetimes that energy transfer in cell envelopes is considerably less efficient than in vesicles of lipids alone. In addition, the calculated values of the rotational relaxation times of perylene, ρ_h, indicate that the inclusion of non-polar lipids in vesicles greatly increases the freedom of motion for this probe. The response to added monovalent and divalent salts, all of which apparently cause the lipid phase to be less fluid, is also greater in the case of unfractionated lipids than for polar lipids alone. In cell envelopes the probe is much more restricted than in vesicles of unfractionated lipids, but even in the envelopes the region sampled is highly fluid compared to the site of adsorption on serum albumin, which is thought to bind perylene hydrophobically but with a geometry restricted by the tertiary structure of the protein. Such increased immobilization of the lipid phase



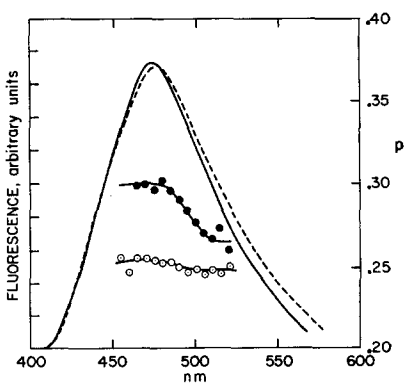


Fig. 3. Arrhenius plots of the rotational relaxation times of perylene (in ns) in vesicles prepared from the polar lipids (open symbols) and from unfractionated lipids (closed symbols) of *H. cutirubrum*. Salts present, at 3.4 M concentration, NaCl (○ and ●), $NaNO_3$ (△ and ▲), KSCN (□ and ■).

Fig. 4. Uncorrected emission spectra and fluorescence polarization of ANS in vesicles prepared from the polar lipids (——, and ○) and from unfractionated lipids (- - -, and ●) of *H. cutirubrum*, at 22 °C. The buffer contained 3.4 M NaCl plus basal salts. Excitation was at 370 nm; the intensities of scattered light, determined without added ANS, were subtracted. For the sake of comparison the peak heights were adjusted to be approximately equal.

in the cell envelopes is consistent with previous results, using stearic acid-type spin labels, in this system [3].

The greater influence of external salts on the motion of perylene in unfractionated lipids led us to expect that chaotropic agents would perturb vesicles of unfractionated lipids more than vesicles prepared from polar lipids alone. Three salts, NaCl, $NaNO_3$ and KSCN, were taken as representatives of the entire range of the Hofmeister series [20]. The fluorescence polarization of perylene was followed in vesicles of unfractionated lipids and of polar lipids as a function of temperature in 3.4 M solutions of the above salts. The results are shown in Fig. 3 in the form of Arrhenius plots of ρ_h. These curves show that the motion of the label in polar lipids is not greatly influenced by anion effects. In vesicles of unfractionated lipids the environment of perylene is somewhat more affected, however, and is seen to be progressively changed with the increasing chaotropic character of the salt added.

Unlike perylene, which is a simple non-polar molecule, ANS is an anion with various possibilities of intramolecular motion and polarizability. In fact, ANS shows both solvent-dependence of the separation between the excitation and emission peaks with a resulting variation in quantum yield [12] and so-called "constraint shift" [14, 15] due to immobilization of solvent molecules. It is understood [4, 21] that ANS occupies the polar–nonpolar interphase of lipid bilayers, and the increased quantum yield observed in such an environment in a consequence of the penetration of the chromophore into the glycerol region. Room temperature fluorescence emission spectra of ANS, in vesicles of unfractionated lipids and of polar lipids alone, are shown in Fig. 4. In both cases the emission peak is near 475 nm, similar to that observed in a 90% ethanol–water mixture [12], although in unfractionated lipids there is a 2-nm shift toward longer wavelengths (and toward greater polarity). The emission peak in unfractionated lipids appears to be somewhat broader on the longer wavelength side than the peak for polar lipids. The values of fluorescence polarization, p, determined at wavelengths across the emission peak indicate a single environment for this label in polar lipids, showing a high degree of immobilization, since the fluorescence lifetime of ANS in this system was found to be 11.2 ± 0.8 ns. However, at least two environments are seen in unfractionated lipids, differing in degree of fluorescence polarization and in emission wavelength. While the position of the emission peak is usually indicative of the dielectric properties of the environment of the probe, the lower wavelength of emission for one of the two populations of ANS in unfractionated lipids may be due to the "constraint shift" mentioned above. If this is the case, the average polarity of the site of ANS in this system is considerably higher than it is in polar lipids, implying greater accessibility of water and other mobile polar molecules.

Effect of temperature on the motion of probes in lipid vesicles and cell envelope membranes

The dependence of the relaxation times of perylene on temperature was studied in cell envelopes and in vesicles prepared from polar lipids, unfractionated lipids and mixtures of polar and neutral lipids (as in Fig. 2). The results, obtained in buffer containing 3.4 M NaCl and basal salts, are shown as Arrhenius plots in Fig. 5. It is evident in Fig. 5 that in buffers containing the salts present in the growth medium the addition of non-polar lipids changes the environment of perylene in the lipid phase,

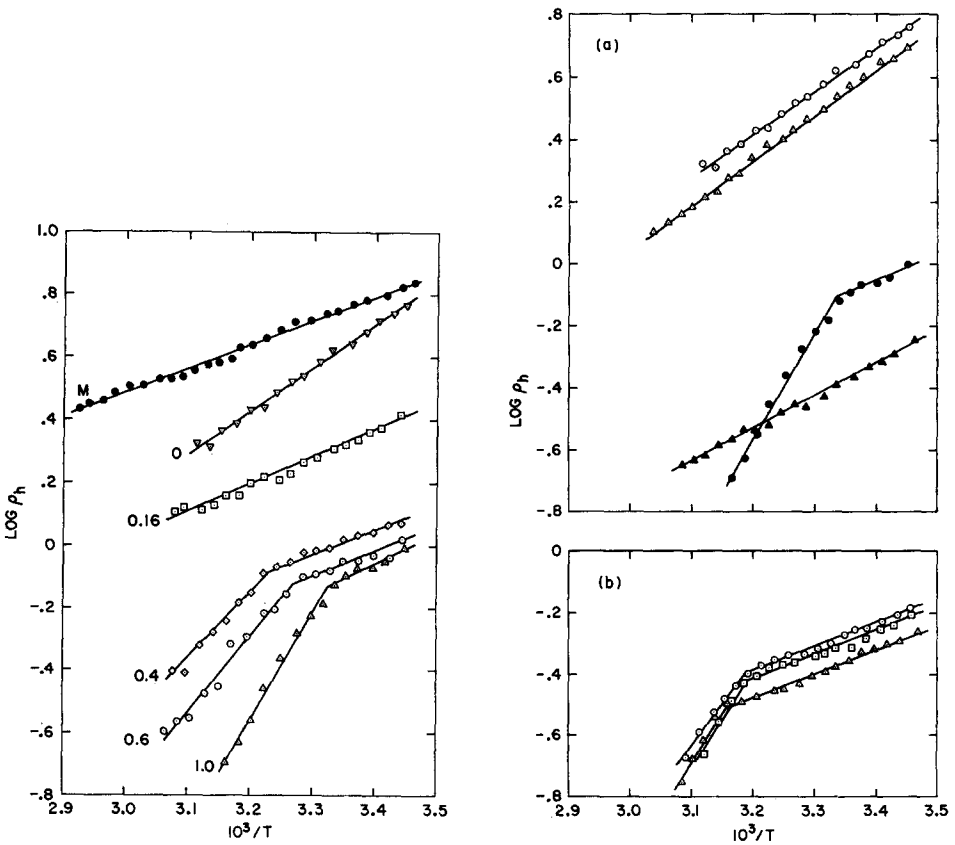


Fig. 5. Arrhenius plots of the rotational relaxation times of perylene (in ns) in cell envelope membranes (●) and in vesicles prepared from polar lipids alone (▽) and mixtures of polar and neutral lipids, containing 16 % (⊡), 40 % (◇), 60 % (○), and 100 % (△) of the neutral lipid content of the unfractionated lipids of *H. cutirubrum*. The buffer contained 3.4 M NaCl plus basal salts.

Fig. 6. Arrhenius plots of the rotational relaxation times of perylene (in ns) in vesicles prepared from *H. cutirubrum* lipids. (a) Vesicles of polar lipids alone, open symbols; those of unfractionated lipids, closed symbols. The buffer contained either 3.4 M NaCl plus basal salts (○ and ●) or no added salts (△ and ▲). (b) Vesicles of unfractionated lipids in the presence of basal salts (○), 100 mM $MgCl_2$ plus 1.4 mM $CaCl_2$ (⊡) and 100 mM $MgCl_2$ (△).

which appears to become more fluid. When more than 16 % of the original amount of non-polar lipids are added, a break is observed in the curves which occurs at progressively lower temperatures, reaching 26–27 °C in unfractionated lipids. Although the results are calculated in Fig. 5 as if the excitation lifetime of the label were unchanged at temperatures above the breaks, this is an unsupported assumption at present. It is possible to conclude, however, that the breaks in Fig. 5 reflect a sudden, if as yet unspecified, change in the environment of perylene. The breaks were accompanied by visible precipitation of some of the lipid.

Cell envelopes do not exhibit the transition described above although the rotational activation energy in this system is similar to that in polar lipids containing

$\geqslant 40\ \%$ of the neutral lipids and in unfractionated lipids, at lower temperatures (3–4 kcal/mole), and unlike that seen in polar lipids alone (5–6 kcal/mole). These values are lower than the 9.0 kcal/mole found in dipalmitoylphosphatidylglycerol vesicles above the melting temperature [22].

Arrhenius plots for the motion of perylene in vesicles of unfractionated lipids and of polar lipids were obtained also in buffers containing the various salts of the growth medium in different combinations. In Fig. 6a data are shown for these two systems in buffer containing no added salt and in buffer containing 3.4 M NaCl and basal salts. The relatively small effect of these salts on perylene in polar lipids in Fig. 6a is contrasted with the case of unfractionated lipids, where the salts have a more dramatic effect, with a break in the curve and the apparent mobility of the probe greatly decreased. Recalling that in the presence of NaCl only no transition was observed for perylene (Fig. 3), it seemed likely that the presence of basal salts, which are mostly divalent cations, is essential for such a transition. In Fig. 6b are shown curves obtained for unfractionated lipids in buffers containing 100 mM $MgCl_2$, 100 mM $MgCl_2$ plus 1.4 mM $CaCl_2$ or complete basal salts. The plots all show a break and the curve for the first solution is similar and that for the second is nearly identical to the curve for the basal salts, indicating that $MgCl_2$, and to a smaller extent $CaCl_2$, are involved in this phenomenon. In Figs 6a and 6b the effect of 3.4 M NaCl is seen to be to decrease the temperature of the transition from 40 to near 26 °C.

For perylene, in either polar or unfractionated lipids, the effect of heating on the rotational relaxation time was nearly completely reversible under conditions where the break in the Arrhenius plots, described above, was absent. Whenever transition was observed, however, cooling from a temperature above the transition point resulted in only partial (10–30 %) return to the original, higher, rotational relaxation times for the probe.

DISCUSSION

The lipids of *H. cutirubrum* are of two types: polar (acidic) lipids (Fig. 7) and non-polar (neutral) lipids (Fig. 8). The former comprise 92 % of the total [5] and are characterized by a single kind of hydrocarbon tail, *O*-phytanyl chains. The latter type of lipids, which account for the rest, are carotenoids and squalenes, in approximately equal proportion [6]. Spin-label studies of vesicles prepared from the unfractionated lipid mixture isolated from this organism showed a profile of label mobility, perpendicular to the surface of the vesicles, that was consistent with a bilayer structure [3].

The location of perylene in artificial lipid bilayers, above the melting temperature, has been proposed to be in the hydrocarbon phase [19, 22]. The present studies of the emission spectra of perylene indicate that the polarizability of the environment of this label is essentially the same in membranes as in vesicles of polar lipids or unfractionated lipids. It seems reasonable to assume that the free energy of transfer of perylene to the most fluid and least polar region, at the center of the bilayer [3], would be less than that of any other possible location. The fluidity of the environment of perylene in vesicles of the polar lipids of *H. cutirubrum* was estimated, according to Shinitzky et al. [8] to be the equivalent of 203 cP in bulk viscosity at room temperature. In unfractionated lipids the corresponding value was 38 cP. The former viscosity

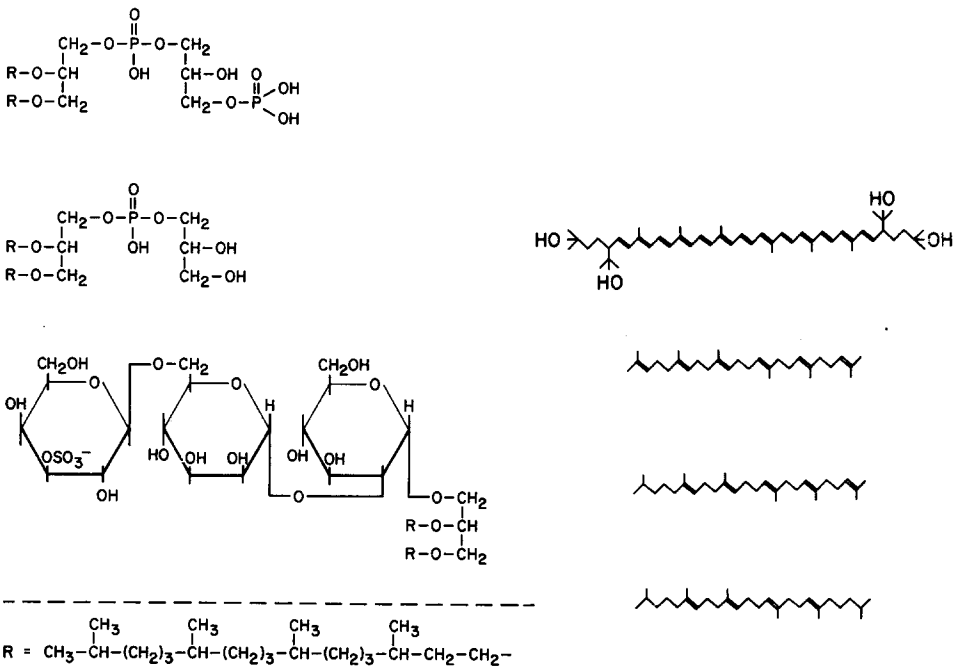


Fig. 7. Polar lipids of *H. cutirubrum* [5]. Diphytanyl ether analogs of (from top) phosphatidylglycerophosphate (60 % by wt of polar lipids), phosphatidylglycerol (≦5 %) and 1-*O*-(*β*-D-galactopyranosyl-3′-sulfate-(1′→6′)-*O*-α-D-mannopyranosyl-(1′→2′)-*O*-α-D-glucopyranosyl)-*sn*-glycerol (35 %).

Fig. 8. Neutral (non-polar) lipids of *H. cutirubrum* [6]. From top, bacterioruberin (major component of carotenoids, which comprise 40–45 % of neutral lipids), squalene, dihydrosqualene and tetrahydrosqualene (50–55 % of neutral lipids). In addition, a K-type quinone was found, amounting to 0.2 % of the neutral lipids.

is like those determined in egg lecithin vesicles and lower than observed in other systems [19], suggesting a highly fluid region in the neighborhood of the probe.

There is adequate evidence that the location of ANS in lipid bilayers is near the aqueous interface [4, 21]. As expected, in this study ANS was found to be more immobilized than perylene. Significant differences were found in the behavior of both ANS and perylene in vesicles of polar and of unfractionated lipids. The site of ANS in bilayers of polar lipids is apparently homogeneous and equivalent in polarity to a 90 % ethanol–water mixture [12]. In vesicles of unfractionated lipids, however, the ANS sites can be resolved into at least two populations, which differ in the degree of fluorescence polarization; one of these, and probably both, represent regions of greater polarity than found in polar lipids (Fig. 4). In addition to the much greater rotational freedom found for perylene in unfractionated lipids (Table I), externally added chaotropes seem to have greater effect on the probe in this environment than in polar lipids (Fig. 3). All of these observations are consistent with a more inhomogeneous structure in vesicles of unfractionated lipids, with more solvent (and salts) in the vicinity of the probes.

The deviation from coherent structure, suggested by the results above, can

perhaps be understood in terms of the dimensions and polarity of the various lipids of *H. cutirubrum*. The polar lipids (Fig. 7) contain branched-chain hydrocarbons, and ether bonds instead of ester groups. The first of these features, and to a lesser degree, the second, is expected to give rise to less compact packing in bilayers. Bilayers of the polar lipids of *H. cutirubrum*, in fact, exhibit a greater fluidity at the site of perylene than many artificial membranes [19], but are somewhat less fluid than erythrocyte membranes [9]. The low rotational activation energy, calculated for perylene in polar lipids, as compared to the values obtained with other lipids containing single species of straight-chain hydrocarbon tails, probably reflects a low degree of packing that is inherent in the structure of the hydrocarbon tails, rather than one which arises primarily by thermal motion. In fact, the flexibility of these branched chains may be lowered because of increased steric hindrance to *trans–gauche* isomerization.

Addition of the non-polar lipids is expected to alter this bilayer structure in two ways. Firstly, the squalenes, which lack hydrophilic groups, contribute only to the hydrophobic phase and thus may cause a stretching of the polar regions on the surfaces of the bilayer. It is difficult to estimate the magnitude of this effect. Bilayers can accommodate a few percent volume increase on melting [23, 24], although at the expense of large changes in the behavior of the system. On the other hand, squalenes, which have non-conjugated double bonds and a branched chain (Fig. 8) are highly flexible molecules, and may be expected to occupy a disproportionately large volume. Secondly, the hydrocarbon portion of bacterioruberin (Fig. 8) is approximately 28 Å long, shorter than twice the length of phytanyl chains, which, fully extended, add up to 38 Å. Hence, either the polar groups of the carotenoid occupy a region in the hydrophobic core of the bilayer or the thickness of the bilayer is reduced locally in such a way as to accommodate these polar groups on the surface. An interaction between the squalenes and bacterioruberin may be possible but would not circumvent the difficulties of placing these molecules into the bilayer. The suggested effects of squalenes and bacterioruberin on lipid structure, giving rise to a more inhomogeneous bilayer, are consistent with the experimental results described above.

Investigations of the thermal behavior of stearic acid-type spin labels in vesicles of unfractionated *H. cutirubrum* lipids [3] showed a transition temperature near the headgroups, at 25 °C. The cause and nature of the matrix transition observed is by no means certain and caution is needed in comparing these results with those obtained with other probes. Nevertheless, it may not be a coincidence that the average polarization, p, detected with ANS as a probe of the headgroup region of bilayers of unfractionnated lipids, also undergoes a transition near 25 °C (unpublished experiments). The site of perylene has been found in this study to undergo a change also near 25 °C (Fig. 5). This latter effect is clearly not a solid to fluid transition, however, because the motion of perylene indicates a highly fluid environment below the transition temperature. Another puzzling result was that the transition is found only in the presence of divalent cations and occurs at a lower temperature on addition of NaCl (Fig. 6), even though both of these agents ordinarily reduce the motion of lipids in bilayer structures through neutralizing charged headgroups. It is not possible at this time to offer a satisfactory model for the thermal transition observed, except to say that it is probably related to the inhomogeneity of the bilayer structure, apparently also caused by the presence of non-polar lipids, as described above.

Cell envelopes of *H. cutirubrum* do not show the breakdown which is observed

in lipid vesicles in the presence of salts which are physiological for this organism (Fig. 5). This finding reinforces our view, expressed earlier [3], that these membranes are not adequately described by a fluid bilayer model with lipid regions unaffected by membrane proteins. Instead, it is likely that in this system close interaction between protein and lipids [3] leads to immobilization of the lipid chains, which overcomes the instability inherent in the bilayer made from these lipids, described in this report.

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