Studies on Phosphatidylcholine Vesicles with Thiocholesterol and a Thiocholesterol-Linked Spin Label Incorporated in the Vesicle Wall*

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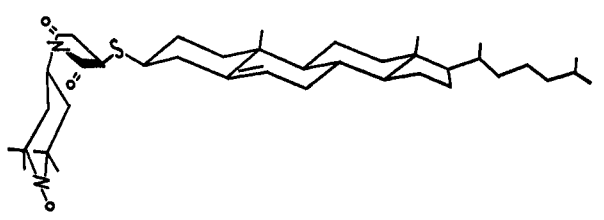
ABSTRACT: Thiocholesterol, the sulfhydryl analog of cholesterol, can be incorporated into single-walled, homogeneous phospholipid vesicles of the type described by C. Huang (*Biochemistry* 8, 344 (1969)). The thiol group provides a convenient chemical measure of the concentration of steroid in the vesicle wall. Under the conditions used, the concentration of thiocholesterol appears to reach a saturation value of about 1 steroid/10 phosphatidylcholine molecules. A maleimide spin label can be introduced into the vesicle system by reaction with the thiol group of the steroid. The details of the electron paramagnetic resonance spectra suggest that the nitroxide group of the spin label is located in the polar head region of the bilayer structure and that the spin-labeled thiocholesterol is undergoing rapid anisotropic motions about an axis that makes an angle of 29° with the nitroxide π-orbital axis.

Recent studies of phospholipid lamellar systems have suggested that certain permeability properties and structural parameters of biological membranes may be directly related to their cholesterol content (Cass and Finkelstein, 1967; Lippe, 1969; de Gier *et al.*, 1969). In studies of this type, the relative chemical inertness of cholesterol, as well as its lack of a convenient chromophoric group, makes it difficult to ascertain the concentration and orientation of cholesterol in the lamellar system. Thiocholesterol, an analog of cholesterol in which the OH group on carbon-3 has been replaced by an SH group, does not suffer from these disadvantages. The chemically reactive thiol group of this steroid not only provides the basis for a direct chemical measure of the absolute concentration of the steroid incorporated in the lamellae, but also provides a convenient means for introducing reporter groups into the steroid-containing bilayer system. This paper describes a method for the incorporation of thiocholesterol into single-walled, homogeneous phospholipid vesicles of the type described by Huang (1969) and the introduction of a maleimide spin label into the vesicle system by reaction with the SH group of thiocholesterol.

The maleimide spin label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide, coupled to thiocholesterol is seen in structure I. The Pauling–Corey–Koltun model of the reporter molecule is shown in Figure 1. Examination of this model indicates that the unique principal hyperfine axis of the large ^{14}N hyperfine splitting, the π-orbital axis, is parallel to the long molecular axis of the steroid. It is also apparent that the NO group in the piperidine ring, which is responsible for the electron paramagnetic resonance signal, is rigidly bound through the maleimide linkage to the SH group of thiocholesterol.



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Materials and Methods

Preparation of Phosphatidylcholine–Thiocholesterol Vesicles. The method of Singleton *et al.* (1965) was followed in isolating phosphatidylcholine from egg yolks. To remove small amounts of lysophosphatide, sphingomyelin, and free fatty acid, the preparation was further purified by silicic acid column chromatography (Rouser *et al.*, 1963). The resultant phosphatidylcholine was dissolved in twice-recrystallized benzene and then lyophilized for 6 hr. The lyophilized material was stored at −20° under a nitrogen atmosphere. Thiocholesterol, obtained from Aldrich Chemical Co. (batch 060571), was three-times recrystallized from ethanol prior to use.

To prepare the phosphatidylcholine–thiocholesterol vesicles with various molar ratios of the two lipids, 300 mg of lyophilized phosphatidylcholine and an appropriate amount of thiocholesterol were suspended in 8 ml of buffer solution. The suspension was ultrasonically irradiated (20 kc) under nitrogen at 2° for 2.5 hr and then centrifuged at 105,000*g*, at 4°, for 60 min. The resulting supernatant was subjected to gel filtration on a Sepharose-4B column. The detailed procedures for sonication and gel filtration have been reported elsewhere (Huang, 1969). The buffer solution used in this work was NaCl (0.1 M) in Tris-HCl (0.01 M), adjusted to pH 8.0.

Determination of Phosphate and Thiol Concentrations. Phosphatidylcholine concentration in the vesicle solution is expressed in terms of lipid phosphorous (P_i) as determined

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by the method of Gomori (1942). Thiocholesterol concentrations in vesicle solutions were calculated from the sulfhydryl (SH) content determined in 72% (v/v) ethanol–buffer mixture and in buffer. Sulfhydryl content was determined by titration with DTNB[1] reagent, as follows (Ellman, 1959): 0.5 ml of pH 8.0 vesicle solution (2–4 μmoles of P_i) in 2 ml of absolute ethanol (or buffer) was mixed with 0.3 ml of freshly prepared 0.01 M DTNB (pH 8.0) in a 3-ml cuvet. The course of the reaction was followed at 412 nm with a Zeiss PMQII spectrophotometer equipped with recorder. A molar extinction coefficient of 13,600 M^{-1} cm^{-1} for the anion of thionitrobenzoic acid was used to determine the number of moles of SH group reacted. Blank experiments were carried out under the same conditions, except that 0.5 ml of buffer was used in place of the vesicle solution. All measurements were made in triplicate.

Preparation of Spin-Labeled Vesicles. Spin-labeled vesicles were prepared from phosphatidylcholine–thiocholesterol vesicles and maleimide spin label (*N*-2,2,6,6-tetramethylpiperidine nitroxide, maleimide) as follows: 1 mg of maleimide spin label was added to 1 ml of vesicle solution containing 6 μmoles of P_i with a SH:P_i molar ration of 8.44×10^{-2}. The reaction mixture was stirred in the dark at 4° for 4 hr. The vesicle solution was then dialyzed exhaustively against buffer to remove unreacted spin labels. Maleimide spin label was a gift from Varian Associates.

Electron Paramagnetic Resonance Measurements. Electron paramagnetic resonance measurements were performed with a Varian X-band spectrometer (V-4502) with 100-kHz field modulation and Fieldial regulation of the magnetic field. The magnetic field strength was monitored by a Varian F-8 NMR fluxmeter and the microwave frequency was measured with a Sperry Model 12-X1 frequency meter. Measurements were carried out in a flat quartz flow cell. A *g* value of 2.0028 was obtained for diphenylpicrylhydrazyl at 20°.

Results and Discussion

Elution Diagram of Phosphatidylcholine–Thiocholesterol Vesicles. Figure 2A is a typical elution diagram of a phosphatidylcholine–thiocholesterol dispersion on a Sepharose-4B column. This elution diagram consists of two distinct fractions (I and II) with fraction I corresponding to the void volume of the column. Due to overlap between the elution peaks, only those portions of fraction II in tubes 18–22 were utilized in these studies. Figure 2B is a plot of the absorbance *vs.* lipid phosphorus of the same elution profile. The number adjacent to each data point is the tube number plotted in Figure 2A. It is apparent that the absorbance of those portions of fraction II used in the present study is directly proportional to the concentration of lipid phosphorous and that the relevant linear regression line passes through the origin. The vesicles in these portions of fraction II have been previously shown to be homogeneous in size and shape (Huang, 1969).

Vesicle Content of P_i and SH. Figure 3A gives the concentration of P_i and SH of the vesicle solution in each tube of the same column elution shown in Figure 2. The relative molar ratio of SH to P_i in individual tubes is presented in Figure 3B. It should be noted that in those tubes containing homogeneous vesicles, the SH:P_i ratio is constant.

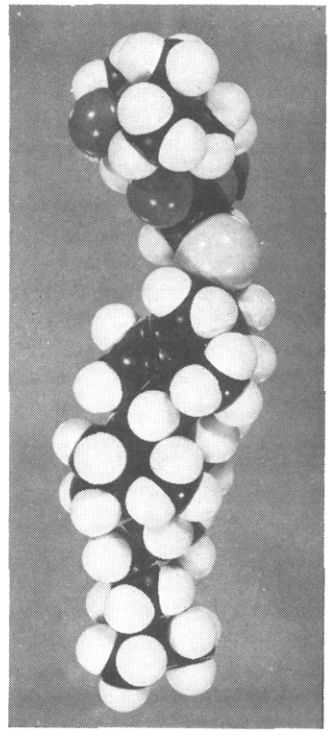

FIGURE 1: Photograph of a Corey–Pauling–Koltun space-filling model of *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)maleimide–thiocholesterol (spin-labeled reporter molecule).

The SH:P_i molar ratio in the homogeneous vesicle solution obtained with different initial ratios of thiocholesterol and phosphatidylcholine in the sonication vessel are given in Figure 4. The correlation between the molar ratio of SH:P_i in solution to that in the sonication vessel appeared to approximate a saturation curve, if conditions of sonication were kept constant. When more than 40 mg of thiocholesterol were sonicated for 2.5 hr with 300 mg of phosphatidylcholine in a volume of 8 ml, the resultant dispersion was very cloudy. The undispersed material, removed by centrifugation, was found to be mainly thiocholesterol.

Figure 5A shows the time dependence of the reaction of DTNB with thiocholesterol–phospholipid vesicles in 72% (v/v) ethanol–buffer mixture and in buffer solution. It is apparent from Figure 5B that two kinetically distinguishable classes of reactive thiol groups are present in the system. Although not shown in Figure 5B, in the ethanol–buffer system, however, only one class of rapidly titrating group is seen. The content of rapidly titrating SH groups in thiocholesterol–phospholipid vesicles in aqueous buffer was found to be smaller by a factor of 0.73 ± 0.04 than that determined in 72% ethanol–buffer (v/v). Electron paramagnetic resonance evidence presented below as well as the data of Tinker and Saunders (1968) indicate that the vesicles present in aqueous buffer disintegrate in the 72% ethanol–buffer mixture. Thus, the apparently smaller content of easily titrated thiol groups in the vesicle in the aqueous buffer could be due to the fact that only that fraction of thiol groups

[1] Abbreviation used is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

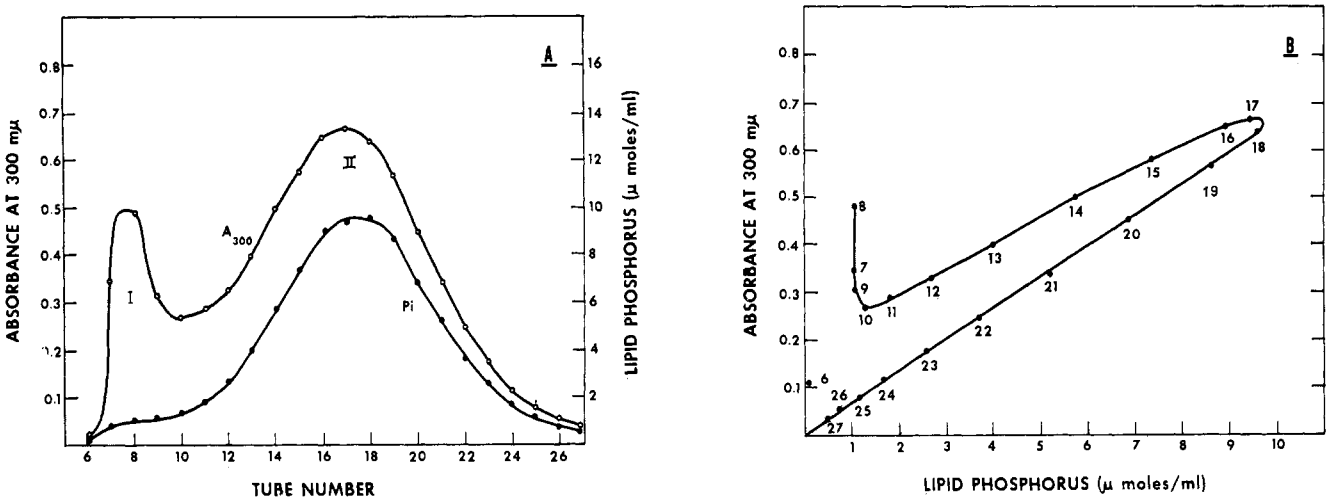

FIGURE 2: (A) Elution patterns of phosphatidylcholine–thiocholesterol vesicles. The open dots indicate the absorbance at 300 nm of each individual tube eluted from the Sepharose-4B column after storage under a nitrogen atmosphere at 4° for 1 week. The solid dots indicate the lipid phosphorus content (P_i) of each of the tubes. (B) Absorbance at 300 nm *vs.* lipid phosphorus. A linear regression correlation coefficient of the line covering samples 18–27 was calculated to be 0.99989.

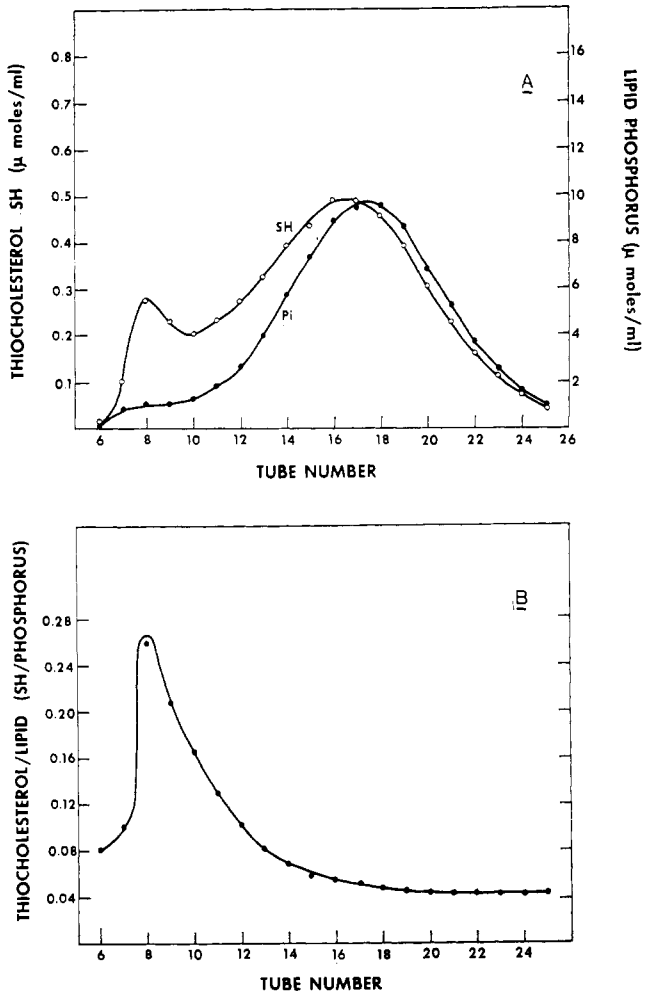

FIGURE 3: (A) Thiocholesterol contents of the vesicle solutions. The SH measurement was carried out in ethanol–buffer mixture as described in the text. (B) Molar ratio of incorporated thiocholesterol to phosphatidylcholine in the vesicle solution. The peak corresponds to fraction I of Figure 2A.

on the external surface of the vesicle wall are titrated, whereas in the ethanol–water solvent, all thiol groups are accessible to DTNB. The second class of slowly titrating SH groups seen in the vesicle system, then, would represent the thiol groups on the internal face of the wall which are essentially unavailable to the DTNB. If this is the case, then the factor 0.73 should represent the ratio of the area of the outer face of the vesicle wall to the total area of both the inner and outer faces.

Detailed physical studies of these phosphatidylcholine vesicles have led to the conclusion that the wall thickness is about 50 Å and the vesicle radius about 125 Å (Huang, 1969). Using this information the ratio of outer face area to total wall area can be calculated to be 0.73. This value is in good agreement with the thiol titration data.

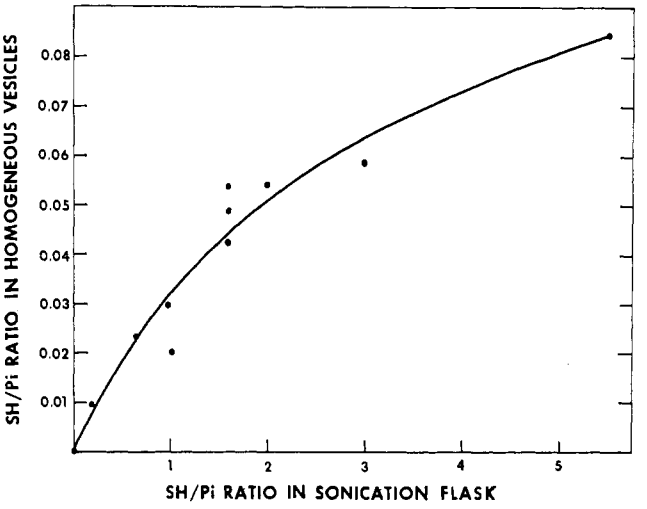

FIGURE 4: The SH:P_i molar ratios in the homogeneous vesicle solution *vs.* the initial molar ratios of thiocholesterol and phosphatidylcholine in the sonication vessel. Sonication time: 2.5 hr.

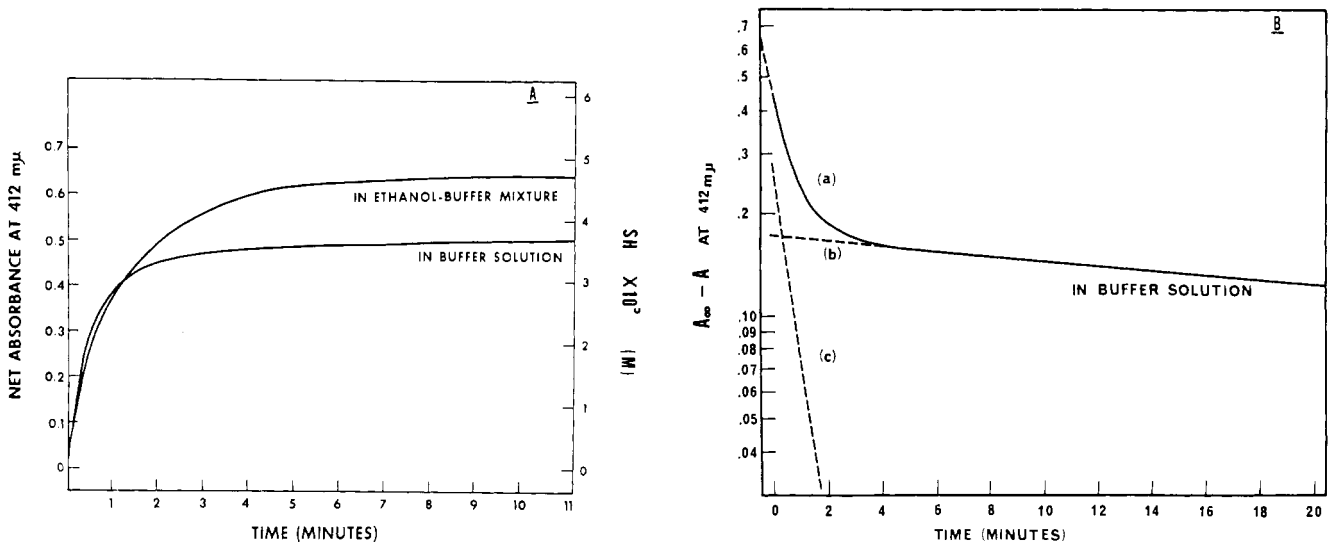

FIGURE 5: (A) Reaction of thiocholesterol-containing vesicles with DTNB at pH 8.0, 22°, in ethanol–buffer mixture (72%, v/v) and in buffer solution. Spectrophotometric titrations were initiated by the addition of a 40-fold molar excess of DTNB to the vesicle mixture. Values of net absorbance at 412 nm were corrected for self-absorption by DTNB and were converted into moles of SH groups using a molar extinction coefficient of 1.36×10^4. (B) Biphasic kinetics of the reaction with DTNB in 0.11 M buffered NaCl solution at pH 8.0, 22°. Curve a, the rate plot of log $(A_\infty - A)$ *vs.* time obtained from experimental data of part A, can be resolved into two straight lines (b and c) in the semi-logarithmic plot. Line b is the straight-line segment of the curve a at the longer times extrapolated to time zero. Line c is the difference between the experimental curve a and the extrapolated line (b). The pseudo-first-order rate constant for the fast reaction, 2.48×10^{-2} sec^{-1}, is 2.303 times the slope of line c and that for the slow reaction, 2.74×10^{-4} sec^{-1}, is 2.303 times the slope of line b.

Spin-Labeled Vesicles. The electron paramagnetic resonance spectrum of the spin-labeled vesicles, shown in Figure 6A, is approximately the same as that recorded for the same spin label attached to the erythrocyte membrane (Sandberg *et al.*, 1969). The line shape in Figure 6A can be attributed to a rapid anisotropic motion of the nitroxide-containing piperidine ring about a fixed molecular axis, **v**, which must be relatively parallel to the unique axis of the static spin Hamiltonian, **Z**. A similar rapid anisotropic motion has been observed for carbon-5 spin-labeled *n*-docosanoic acid in the hydrocarbon core of asolectin vesicles (Hubbell and McConnell, 1969). The apparent splitting between the outermost lines, $2T_{\parallel}'$, in Figure 6A is 56.1 ± 0.4 gauss and that between the inner lines, $2T_{\perp}'$, is 22.2 ± 0.1 gauss. The corresponding apparent g values are $g_{\parallel}' = 2.0025$ and $g_{\perp}' = 2.0061$.

In order to calculate and interpret the mean angular deviation between **v** and **Z** for the nitroxide group in the vesicle, it is necessary to know the parameters of the static spin Hamiltonian for the nitroxide group of the maleimide-linked steroid derivative. In addition, the condition of Trace (**T**) = Trace (**T'**) should be fulfilled (Hubbell and McConnell, 1969). Based on the assumptions that the static spin Hamiltonian of the nitroxide group of the reporter molecule has axial symmetry about the unique principal hyperfine axis ($T_X \simeq T_Y \simeq T_\perp$) and that the isotropic splitting constant, a_N, is approximately equal to $1/3(T_X + T_Y + T_Z) \simeq 1/3(2T_X + T_Z)$, the static Hamiltonian parameters, $T_X(\simeq T_Y \simeq T_\perp)$ and $T_Z(\simeq T_\parallel)$, can be estimated from the experimentally determined values of $T_\parallel$ and a_N. The value of $T_\parallel$, 34.53 ± 0.26 gauss, was calculated from the distance between the two outermost peaks in the powder spectrum (Edelstein *et al.*, 1964) as shown in Figure 6C, and the a_N value was calculated from the mobile spectrum (Figure 6B) to be 16.27 ± 0.02 gauss. These gave the following approximate values for the parameters of the static spin Hamiltonian: $T_X \simeq T_Y \simeq 7.14$ ± 0.10 gauss, and $T_Z \simeq 34.53$ ± 0.26 gauss. The reason that Trace (**T**), estimated from $3a_N$, was slightly smaller than Trace (**T'**) by about 1 gauss could be partly due to the different solvents used for the separate measurements.

The mean angular deviation between **v** and **Z**, $\cos^{-1}(\bar{\gamma}^2)^{1/2}$, for the nitroxide group in the vesicle can be calculated to be 29° based on the equation (Hubbell and McConnell, 1969): $T_\parallel' = T_X(1 - \bar{\gamma}^2) + \bar{\gamma}^2 T_Z$, where $T_\parallel'$ is the maximum splitting (28.05 ± 0.20 gauss), T_X (7.14 gauss), and T_Z (34.53 gauss) are the static spin Hamiltonian parameters derived above. The motion of the nitroxide group in the vesicle could be expected from the CPK space-filling model (Figure 1) to be dominated by motions of the thiocholesterol part of the labeled molecule. Thus, the calculated 29° can be recognized as the angle between the rapidly rotating axis of the thiocholesterol and the nitroxide π-orbital axis.

Studies of the solvent effect on the electron paramagnetic resonance spectrum of nitroxide spin labels have shown that the isotropic splitting constant, a_N, proportionally increases with increasing polarity of solvent (Briere *et al.*, 1965). It is thus possible from the analysis of electron paramagnetic resonance spectrum to obtain information about the polarity of the environment surrounding the spin label. For maleimide spin labels, the isotropic splitting constant measured in buffer and *n*-tetradecane was found to be 17.04 and 15.32 gauss, respectively (Figure 7A,B). A value of 16.8 gauss was calculated to be the averaged isotropic splitting for the nitroxide of the spin-labeled vesicle in buffer based on the condition Trace (**T**) ≃ Trace (**T'**) and the relation $a_N = 1/3T_\parallel' + 2/3T_\perp'$. The value of 16.8 gauss obtained in the vesicle system suggests that the nitroxide group of the reporter molecule is in a more polar environment than *n*-tetradecane (or the hydrocarbon core of the lamella)

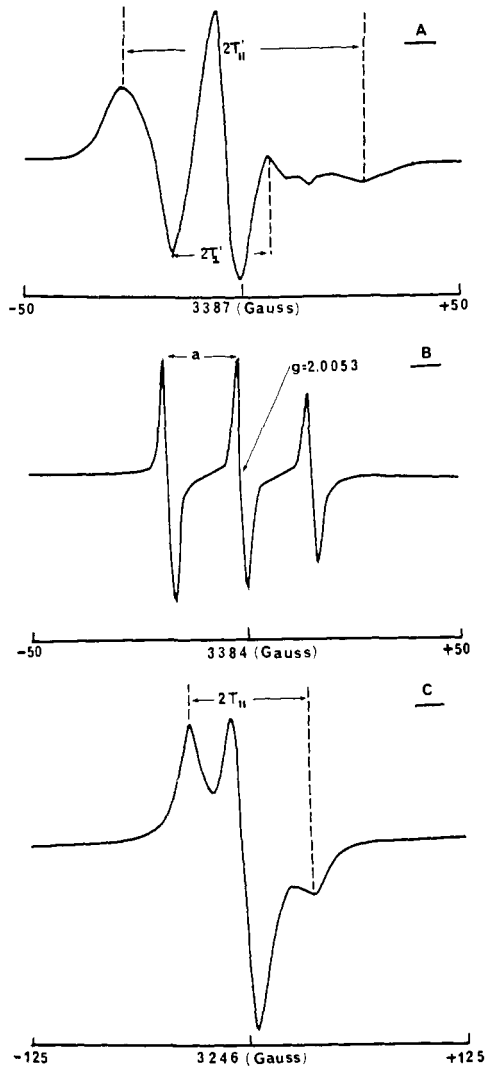

FIGURE 6: Electron paramagnetic resonance spectra of spin-labeled vesicles in (A) 0.11 M buffered NaCl solution (pH 8.0) at room temperature, (B) ethanol–buffer mixture (72%, v/v) at room temperature, (C) 0.11 M buffered NaCl solution (pH 8.0) at liquid nitrogen temperature. Spectra A, B, and C were recorded at frequency 9.496, 9.497, and 9.098 GHz, respectively.

but less polar than the aqueous buffer solvent. It seems that the nitroxide group is located in the polar head region and probably near to the interface between the bilayer and the ambient aqueous phase.

It is interesting to compare the electron paramagnetic resonance spectrum of spin-labeled vesicles in an aqueous medium to that in ethanol–water mixture (72% v/v) (Figure 6A,B). These spectra clearly indicate that the tumbling rates of the reporter molecules are markedly different in aqueous medium and the ethanol–water mixture. The change in correlation time is from 10^{-8} to 10^{-11} sec in the order of magnitude. This result is consistent with the expectation based on the phase diagram of egg phosphatidylcholine–ethanol–water ternary system which indicates that phosphatidylcholine vesicles disintegrate in 72% ethanol–water (v/v) mixture (Tinker and Saunders, 1968).

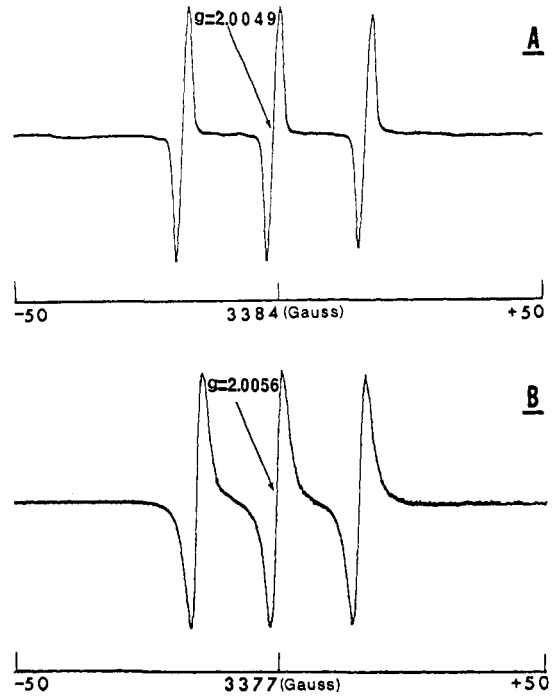

FIGURE 7: Electron paramagnetic resonance spectra of *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide in (A) 0.11 M buffered NaCl solution (pH 8.0) and (B) *n*-tetradecane. Spectra A and B were recorded at room temperature, and at frequency 9.495 and 9.480 GHz, respectively.

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References

Briere, R., Lemaire, H., and Rassat, A. (1965), *Bull. Soc. Chim. Fr. 32*, 3273.

Cass, A., and Finkelstein, A. (1967), *J. Gen. Physiol. 50*, 1765.

de Gier, J., Manderslost, J. G., and van Deenen, L. L. M. (1969), *Biochim. Biophys. Acta 173*, 143.

Edelstein, N., Kwok, A., and Maki, A. H. (1964), *J. Chem. Phys. 41*, 179.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Gomori, G. (1942), *J. Lab. Clin. Med. 27*, 955.

Huang, C. (1969), *Biochemistry 8*, 344.

Hubbell, W. L., and McConnell, H. M. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 20.

Lippe, C. (1969), *J. Mol. Biol. 39*, 669.

Rouser, G., Kritchevsky, C., Heller, D., and Lieber, E. (1963), *J. Amer. Oil Chem. Soc. 40*, 425.

Sandberg, H. E., Bryant, R. G., and Piette, L. H. (1969), *Arch. Biochem. Biophys. 133*, 144.

Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1965), *J. Amer. Oil Chem. Soc. 42*, 53.

Tinker, D. O., and Saunders, L. (1968), *Chem. Phys. Lipids 2*, 316.