matic); MS *m/e* (rel intensity) 290 (parent, 85), 258 (20), 216 (88), 190 (100), 161 (28), 151 (25), 59 (29). Anal. Calcd for $C_{15}H_{14}O_6$: C, 62.07; H, 4.86. Found: C, 61.91; H, 4,86.

Treatment of Chromone 36 with NaOMe. Chromone **36** (0.0656 g, 0.226 mmol) was treated with excess NaOMe in refluxing MeOH for 29 h to give, after dilution with H_2O, extraction into EtOAc, and chromatography, 0.0082 g (19%) of 7-hydroxy-2,5-dimethylchromone: mp 243–248 °C (lit.[22] mp 253–255 °C); IR (KBr) 1660, 1620, 1570–1545 cm^{-1}; UV 242 nm (ϵ 14 900), 251 (16 500), 292 (10 000), 340 (1400); NMR (CD_3COCD_3) 2.29 (s, 3, CH_3), 2.72 (s, 3, CH_3), 5.93 (s, 1, vinyl), 6.67 (s, 2, aromatic).

Acknowledgment. The authors thank Dr. T. T. Howarth for technical assistance and helpful discussions. Generous support by the Tennessee Eastman Company (fellowship to J.V.H.) and the National Institutes of Health (Research Grant GM-12848) is gratefully acknowledged.

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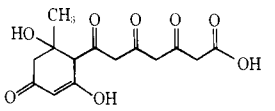

i

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Clustering of Nitroxide Spin Labels in Lipid Bilayer Membranes

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Abstract: Three new nitroxide (spin-label) biradicals have been prepared: *N,N'*-dipalmitoyl-*N,N'*-bis(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)-1,10-diaminodecane (**5**); *N,N'*-dimethyl-*N,N'*-dihexadecyl-*N,N'*-bis(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)-1,10-diammoniumdecane diiodide (**7**); *N,N'*-dipalmitoyl-*N,N'*-bis[*N*-(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)acetamide-2-yl]-1,10-diaminodecane (**9**). It is shown that these biradicals bind strongly to phosphatidylcholine–cholesterol bilayer membranes and exhibit a degree of clustering in the plane of the membrane that depends on the particular biradical, the concentration of the biradical in the plane of the membrane, the lipid composition of the membrane, and the temperature. These results illustrate one approach to controlling the lateral distribution and motion of membrane components that is relevant to current studies of membrane immunochemistry.

The paramagnetic resonance spectra of amphipathic nitroxide radicals (spin labels)[1] bound to model membranes[2] and to biological membranes can sometimes provide significant biophysical information on local molecular motion, orientation, and lateral diffusion.[3-7] In recent work we have undertaken a study of the immunochemistry of model membranes containing low concentrations of lipid haptens[8-10] and have employed antibodies specifically directed against nitroxide free radical groups such as the 2,2,6,6-tetramethyl-*N*-oxylpiperidine ring.[9-12] The thrust of these studies is to relate the structure of membrane-bound haptens, and their lateral distributions and motions, to various immunochemical reactions. It is possible that such relationships are significant for some of the more subtle membrane recognition problems in cellular immunology. Spin-labeled lipids and other amphipathic molecules are ideally suited for some of these studies, since their resonance spectra provide direct quantitative information on structure, distribution, and motion, and the *same* molecules can interact specifically with components of the immune system. The initial purpose of the present work was to prepare divalent haptens (nitroxide biradicals) to complement our earlier studies using monovalent haptens, particularly spin-

labeled lipids;[9-11] however, during the course of this investigation we discovered a particularly interesting effect, namely a highly specific clustering of nitroxide biradicals in bilayer membranes that exhibits a strong dependence on the chemical structure of the biradical, the lipid composition of the host membrane, the concentration of the biradical in the plane of the membrane, and the temperature. The immunochemical properties of these biradicals in membranes will be reported elsewhere.

Experimental Section

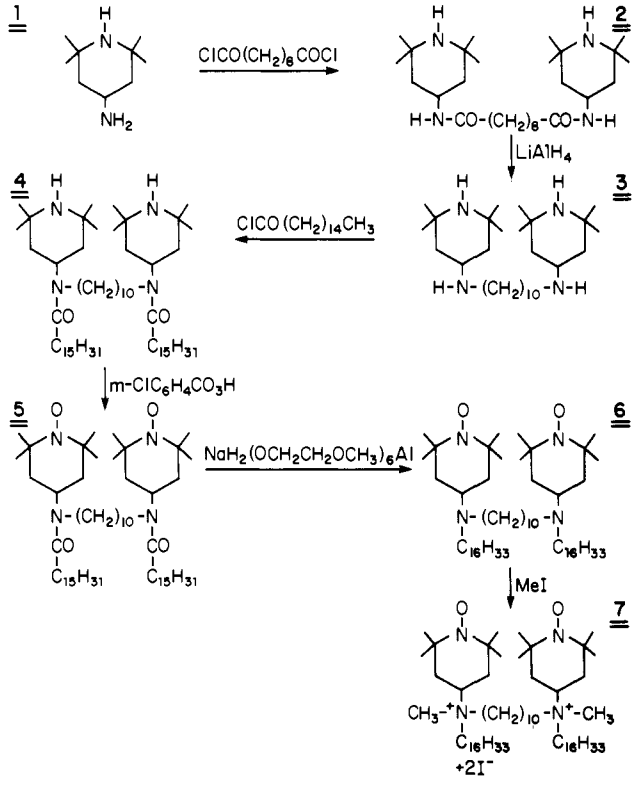
Reagents. The following materials were obtained from Calbiochem and used without further purification: L-α-dipalmitoylphosphatidylcholine(DPPC), L-α-dimyristoylphosphatidylcholine (DMPC). Cholesterol was obtained from the same source and twice recrystallized from ethanol, mp 148–148.5 °C.

Sample Preparation. Most paramagnetic resonance spectra were obtained using liposomes prepared as described below. The appropriate proportions of stock solutions of phospholipid, cholesterol, and biradical in chloroform were mixed and evaporated to dryness on a rotatory evaporator. The samples were either kept under oil pump vacuum overnight, or repeatedly pumped with an oil pump and flushed with oxygen-free nitrogen. Samples were usually hydrated by mixing 13.4 μM of sample and 500 μL of water, followed by gentle shaking. A few oriented samples with egg lecithin were prepared as discussed later.

Measurements. Paramagnetic resonance spectra were obtained at 9.3 MHz with a Varian E-12 spectrometer. The spectrometer was coupled to a Digital PDP8 computer so that spectra could be stored, averaged, integrated, normalized, and mixed (linear combinations of normalized spectra). Temperatures were measured with a copper–constantan thermocouple connected to a microvoltmeter. Nuclear magnetic resonance spectra were obtained with a Varian T60 spectrometer.

Synthesis of Spin Labels.[13] The synthesis of two biradicals (**7**) and (**5**) were carried out as indicated in Scheme I.

Scheme I



***N,N'*-Bis(2,2,6,6-tetramethylpiperidin-4-yl)sebacoyldiamide (2).** To a solution of 2.250 g of 4-amino-2,2,6,6-tetramethylpiperidine (Aldrich) (**1**) in 100 mL of ethyl ether, at 0 °C, was added dropwise a solution of 1.694 g of sebacoyl dichloride in 50 ml of ethyl ether. The precipitate formed was filtered off, dissolved in a solution of NaOH (1 N; 50 mL), and the resulting solution extracted with methylene chloride. The yield was 2.995 g of a colorless oil which crystallized slowly (87%): NMR ($CDCl_3$, Me_4Si) δ 4.21 (m, 2 H, C(4)H), 2.3–0.8 (m, 12 H, C(3) and C(5) H's, $NCOCH_2$–), 1.31 (br s, 12 H, $COC(CH_2)_6$), 1.25 (s, 12 H, Me), 1.11 (s, 12 H, Me); IR (Nujol, cm^{-1}) 3265 (γ-NH), 1648 (γ-CO).

***N,N'*-Bis(2,2,6,6-tetramethylpiperidin-4-yl)-1,10-diaminodecane (3).** Diamide **2** (2.4 g) was placed in a Soxhlet extractor and extracted with ether, and refluxed with a suspension of 2 g of $LiAlH_4$ in 300 ml of anhydrous ethyl ether for 5 days. The following was then added to the cold suspension: 2 mL of water, 2 mL of sodium hydroxide solution (15% w/w), and 6 mL of water. The yield was 2.020 g (90%) of white crystalline material, mp 61 °C: NMR ($CDCl_3$, Me_4Si) δ 2.90 (m, 2 H, C(4)H–N), 2.63 (m, 4 H, >C–NCH_2), 2.1–0.8 (m, 8 H, C(3) and C(5)H's), 1.31 (br s, 16 H, $NC(CH_2)_8$), 1.16 (s, 12 H, Me), 1.11 (s, 12 H, Me); IR (Nujol; cm^{-1}) 3250 (ν-NH).

***N,N'*-Dipalmitoyl-*N,N'*-bis(2,2,6,6-tetramethylpiperidin-4-yl)-1,10-diaminodecane (4).** To a solution of 0.950 g of tetraamine **3** in 100 mL of ethyl ether was added dropwise a solution of 1.223 g of palmitoyl chloride in 20 mL of ethyl ether. The mixture was allowed to react for 15 h at room temperature. Then 20 mL of aqueous sodium hydroxide (N) were added. The organic layer was dried and evaporated to obtain 1.840 g of a colorless, waxy material (95%): NMR ($CDCl_3$, Me_4Si) δ 4.17 (m, 2 H, C(4)H–N), 3.38 (m, 4 H, NCH_2–), 2.40 (m, 4 H, $COCH_2$), 2.10–0.8 (m, 8 H, C(3) and C(S)H's), 1.21 (br s, 84 H, $(CH_2)_{42}$, 1.18 (s, 12 H, Me), 1.10 (s, 12 H, Me); IR (Nujol, cm^{-1}) 3310 (ν-NH), 1643 (ν-CO).

***N,N'*-Dipalmitoyl-*N,N'*-bis(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)-1,10-diaminodecane (5).** Compound **4** (500 mg), 500 mg of potassium carbonate, and 20 mL of anhydrous ethyl ether were mixed and cooled down in an ice bath. To this suspension was added dropwise a solution of 250 mg of *m*-chloroperbenzoic acid in 20 mL of ethyl ether. After 3 h 20 mL of NaOH (N) was added. The ethereal layer was washed exhaustively with NaOH (N) (five 10-ml portions). After drying and evaporating, 428 mg of red crystals were obtained. Thin layer chromatography (SiO_2–ethyl ether 100%) yielded 337 mg of pure binitroxide **5** (65%; mp 75–76 °C): IR (Nujol, cm^{-1}) 1641 (ν-CO); ESR ($CHCl_3$, 10^{-3} M) five lines a_N = 15.85 G. Anal. Calcd for $C_{60}H_{116}N_4O_2$: C, 75.25; H, 12.20; N, 5.85; O, 6.68. Found: C, 75.54; H, 12.36; N, 5.59. NMR of the corresponding hydroxylamine: 20 mg of **5** was reduced using 10 mg of 1,2-diphenylhydrazine in 300 μL of $CDCl_3$ in an NMR sample tube: δ 4.15 (m, 2 H, >C–NCO), 3.30 (m, 4 H, NCH_2–), 2.35 (m, 4 H, $COCH_2$–), 2.10–1.0 (m, 8 H, C(3) and C(5) H's), 4.23 (br s, 84 H, $(CH_2)/n$); 1.12 (br s, 24 H, Me).

***N,N'*-Dihexadecyl-*N,N'*-bis(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)-1,10-diaminodecane (6).** Binitroxide **5** (150 mg) was dissolved in 20 mL of anhydrous ethyl ether and the reaction flask flushed with dry nitrogen and cooled to 0 °C. Then 0.5 mL of sodium bis(2-methoxyethoxy)aluminum hydride standard benzene solution (Aldrich) was added dropwise. After 1 h, 2 mL of NaOH (N) was added and the organic layer dried and evaporated. A yield of 222 mg of red oil was obtained which, after thin layer chromatography (SiO_2–pentane 55%–ethyl ether 45%) yielded 68 mg (47%). Two other paramagnetic compounds were obtained and have not been identified: IR (Nujol), no characteristic band; NMR of the corresponding hydroxylamine (obtained by the same procedure as for **5**): δ 2.85 (m, 2 H, C(4)H–N–), 2.60–2.20 (m, 8 H, NCH_2–), 2.10–1.0 (m, 8 H, C(3) and C(5) H's), 1.25 (br s, 78 H, $-(CH_2)/n-$), 1.12 (br s, 24 H, Me).

***N,N'*-Dimethyl-*N,N'*-dihexadecyl-*N,N'*-bis(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)-1,10-diammoniumdecane Diiodide (7).** Methyl iodide (0.1 mL), 50 mg of binitroxide **6**, and 1 mL of acetone were allowed to react at room temperature. After 20 h volatile materials were removed by flushing with dry nitrogen. Then 2 mL of anhydrous ethyl ether was added and the precipitate collected and washed several times with anhydrous ethyl ether. The yield was 38 mg of orange crystals (59%; mp 89–92 °C). On thin layer chromatography (SiO_2–$CHCl_3$ 70%–CH_3OH 30%), there was only one spot. IR, no characteristic band above 700 cm^{-1}. Anal. Calcd for $C_{62}H_{126}I_2N_4O_2$: C, 61.32; H, 10.46; N, 4.62. Found: C, 60.92; H, 10.34; N, 4.53.

The synthesis of the biradical **9** was carried out as shown in Scheme II.

Results

Under some conditions the paramagnetic resonance spectra

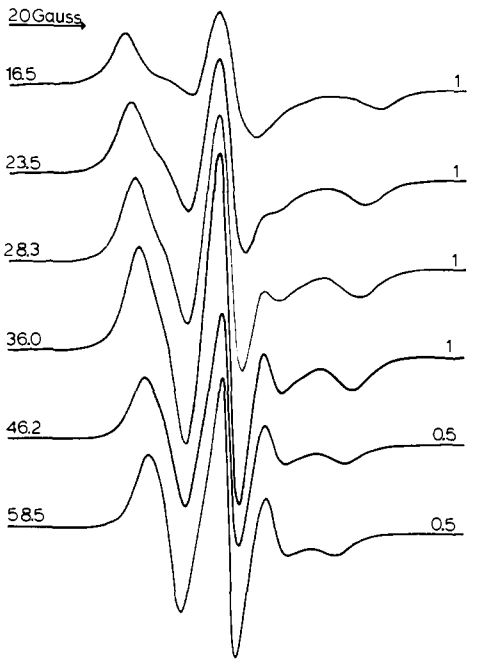
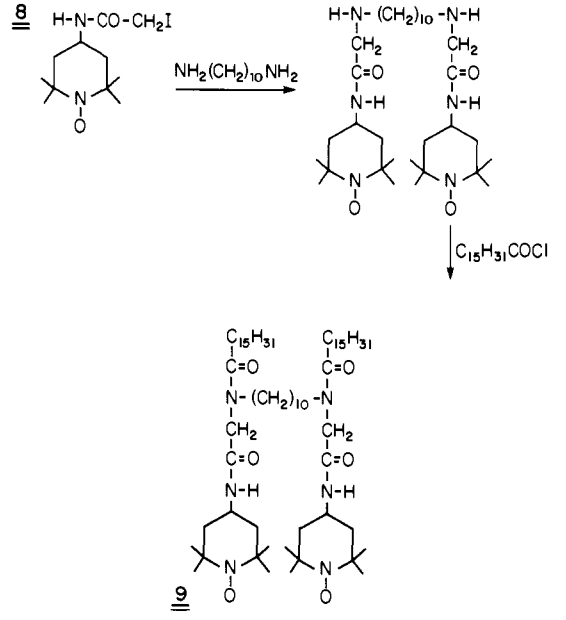


Figure 1. The paramagnetic resonance spectra of the nitroxide biradical **7** (0.9 mol %) in a bilayer membrane composed of DMPC (80%) and cholesterol (20%) at various temperatures (left-hand numbers) and relative spectrometer gain settings (right-hand numbers).

Scheme II



of biradicals **7**, **5**, and **9** are typical of those observed for a number of spin-labeled amphiphilic molecules bound to lipid membranes.[1,2] These biradicals are insoluble in water in the absence of host lipid. Thus the resonance spectra under discussion reflect the spectra of **7**, **5**, and **9** bound to lipid bilayers. Additional evidence for this binding was found in a few preliminary experiments in which the anisotropy of the resonance spectra was observed when **5** was incorporated in oriented multilayers of egg phosphatidylcholine, following procedures discussed extensively elsewhere.[14] In these experiments the observed hyperfine anisotropy was such as to indicate that the preferred orientation of the nitroxide π orbital was predominantly in the plane of bilayers.[1,2,14] In these studies the hyperfine splittings were quite well resolved, and there was no detectable intramolecular spin-spin splitting (exchange or dipolar).[1]

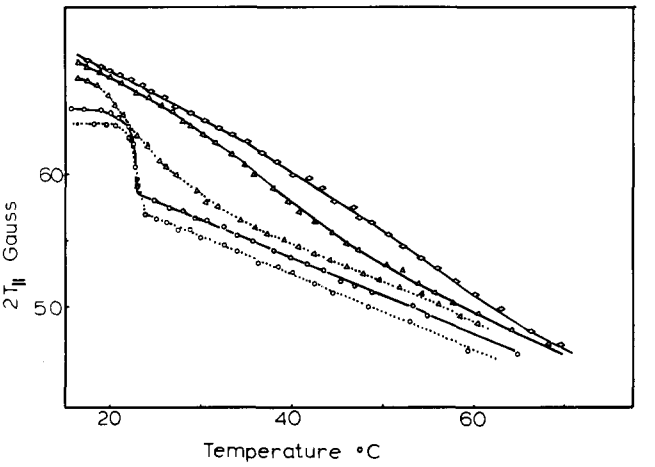


Figure 2. The nitrogen hyperfine splitting $2T_{\parallel}$ as a function of temperature for biradical **7** (0.9 mol %) in DMPC-cholesterol bilayer membranes: 0% (O···O), 10% (O—O), 20% (Δ···Δ), 30% (Δ—Δ), 40% (□—□). See text and ref 1.

Biradical 7. Typical paramagnetic resonance spectra of 0.9 mol % **7** in an isotropic sample of lipid (DMPC-cholesterol, 0.80:0.20) at various temperatures are shown in Figure 1. The line shapes of these spectra are dominated by the molecular motion of the individual nitroxide groups. A simple and convenient measure of the amplitude of this motion is the separation of the outer hyperfine extrema in Figure 1. This splitting is commonly designated $2T_{\perp}$.[1,2,15]

Figure 2 shows a plot of $2T_{\perp}$ for **7**(0.9 mol %) as a function of temperature for various DMPC-cholesterol mole ratios. The only abrupt change in $2T_{\perp}$ occurs at about 23 °C for 0–10 (or 20) mol % cholesterol. These spectral changes are related to the known chain-melting transition temperature in DMPC at 23 °C, and to the nature of the phase diagram for DMPC-cholesterol mixtures.[16] The distinctive feature of all the observed spectra for **7** (all at 0.9 mol %) is that irrespective of cholesterol concentration (0–50 mol % cholesterol) and temperature (15–65 °C) there is no evidence whatever for significant spin-spin interactions arising from interactions between radicals in the plane of the membrane. In marked contrast to this result, radicals **5** and **9** show clear evidence for strong spin-spin interactions between radicals due to clustering.

Figure 3 gives the paramagnetic resonance spectrum of **5** (0.9 mol %) in DMPC-cholesterol (0.80:0.20) at various temperatures. These spectra, particularly at the lower temperatures, show strong spin-spin broadening which, as discussed in detail below, can be attributed to a clustering of **5** in the plane of the membrane. First, however, let us compare the spectra of **5** and **7** under conditions where there is no clustering.

Figure 4 (upper) shows the spectra of **7** (left) and **5** (right) in DMPC (0.9 mol %) below the chain-melting transition temperature (23 °C). It will be seen that the spectra are quite similar, corresponding to nearly "strongly immobilized" signals. There is no evidence for intra- or intermolecular spin-spin interaction.[1] In Figure 4 (lower) the spectra of **7** (left) and **5** (right) (0.9 mol %) in DMPC are compared a few degrees above the chain-melting transition temperature. At these temperatures the resonance spectra of **5** and **7** are different in that **7** remains nearly "strongly immobilized", whereas **5** is more nearly "weakly immobilized".[1] Again, there is no indication of intra- or intermolecular spin-spin interaction. These results may be contrasted with results obtained with DPPC (not described here in detail). The spectrum of **7** (1 mol %) in DPPC below the chain-melting temperature (42 °C) is similar to that in DMPC below 23 °C; the spectra of **7** just above the chain-melting temperatures are also similar in the two cases.

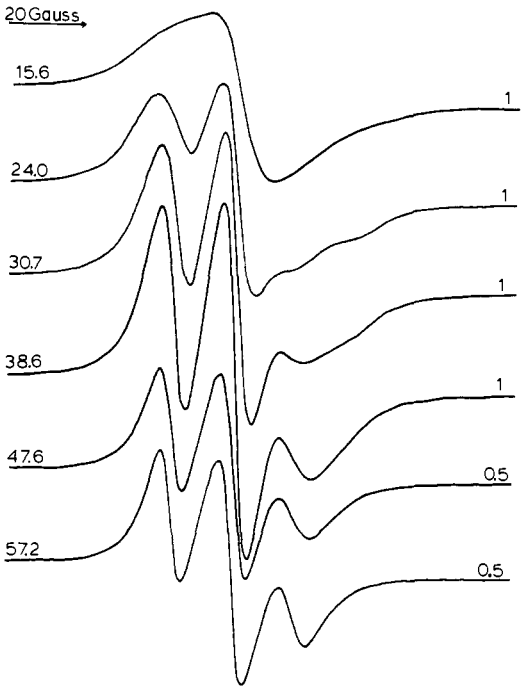


Figure 3. The paramagnetic resonance spectra of the nitroxide biradical **5** (0.9 mol %) in a bilayer membrane containing 80% DMPC and 20% cholesterol at the various temperatures indicated on the left, and relative spectrometer gain settings indicated on the right.

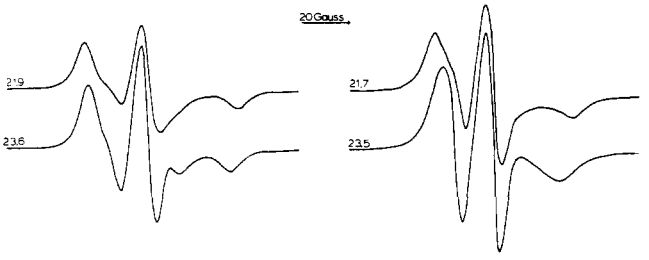


Figure 4. Upper: the paramagnetic resonance spectra of **7** (left) and **5** (right) in pure DMPC at temperatures (21.9 and 21.7 °C) just under the chain-melting transition temperature of DMPC (23 °C). Lower: the paramagnetic resonance spectra of **7** (left) and **5** (right) in pure DMPC at temperatures (23.6 and 23.5 °C) just above the chain-melting transition temperature of DMPC (23 °C). In all cases **7** and **5** are present at a concentration of 0.9 mol % in the host lipid.

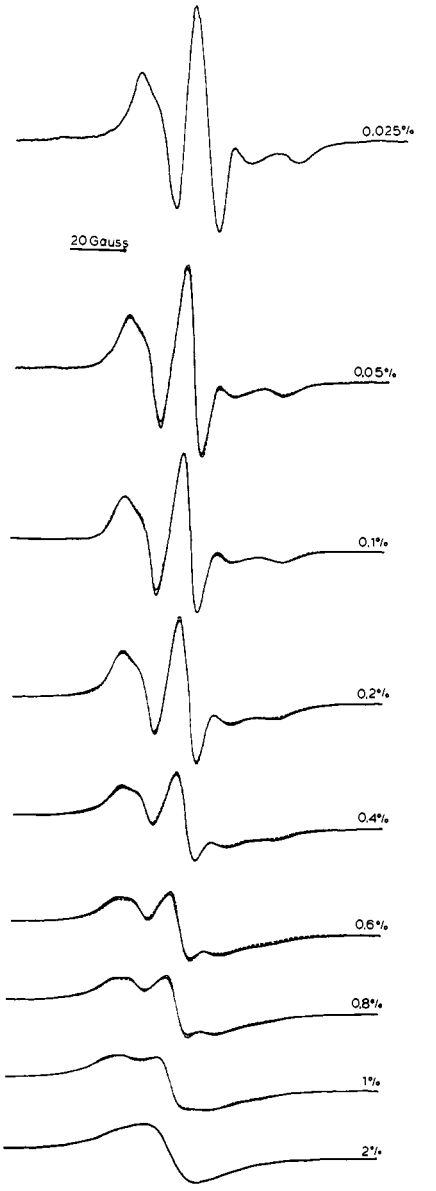


Figure 5. The normalized spectra of **5** in a host bilayer membrane containing DMPC (70 mol %) and cholesterol (30 mol %) at 30 °C for molar concentrations of **5** indicated by the mole % values given on the right. The solid lines are experimental spectra and the dotted lines are calculated spectra (see text).

In contrast, the spectrum of **5** (1 mol %) shows strong spin–spin interaction in DPPC below 42 °C, and shows some spin–spin broadening of the resonance lines even above 42 °C.

In contrast to **7**, biradical **5** shows a strong concentration and cholesterol-dependent spin–spin broadening in bilayers containing DMPC even above 23 °C. We consider first the concentration dependence of the clustering of **5** in bilayers composed of DMPC and cholesterol (70 and 30 mol %) at 30 °C.

Figure 5 gives the observed paramagnetic resonance spectra of **5** at concentrations between 0.025 and 2 mol %. The spectra have been computer normalized so that they each represent the same number of spins calculated from double integrals of derivative curve spectra. In addition, Figure 5 gives calculated spectra based on the assumption that each experimental spectrum in Figure 5 is some normalized linear combination of two spectra. In other words,

$$S(H) = fS_1(H) + (1 - f)S_n(H) \qquad (1)$$

where $S(H)$ is the derivative curve spectrum as a function of the applied field strength H, $S_1(H)$ is the spectrum of a biradical "monomer" (corresponding to infinite dilution), and $S_n(H)$ is the spectrum of a cluster of biradicals containing (by assumption) exactly n molecules per cluster; $(1 - f)$ is the fraction of all biradicals that are present in clusters of n molecules each. It will be seen that the observed spectra can be represented with a high degree of accuracy using the assumption of eq 1. From the curve fitting in Figure 5 we obtain $(1 - f)$; Figure 6 gives a plot of the "percent cluster" $(100(1 - f))$, the percent of molecules in the form of clusters. The shape of the "percent cluster" curve in Figure 6 suggests a polymerization reaction. If the only cluster formed has a definite stoichiometry, then we may write the equilibrium reaction as,

$$n(\text{monomers}) \rightleftharpoons (\text{monomer})_n \qquad (2)$$

where $(\text{monomer})_n$ is a "cluster". For this reaction we expect a straight line when $\log c(1 - f)$ is plotted against $\log cf$, where c is the number of moles of label per 100 mol of host lipid (c = mol % biradical in the membrane). The slope of this line is n, the number of molecules in the cluster. The deduced value

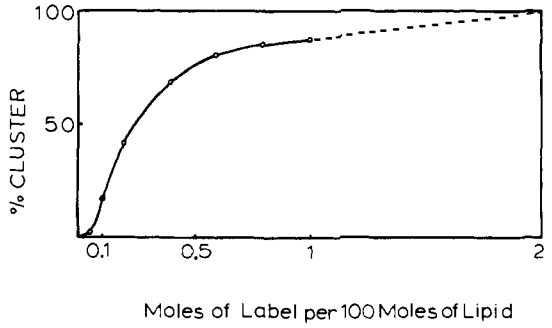


Figure 6. The percent of molecules of **5** ("percent cluster") that are clustered in a host bilayer membrane (70% DMPC and 30% cholesterol) at 30 °C as a function of the concentration c of the biradical in the plane of the membrane.

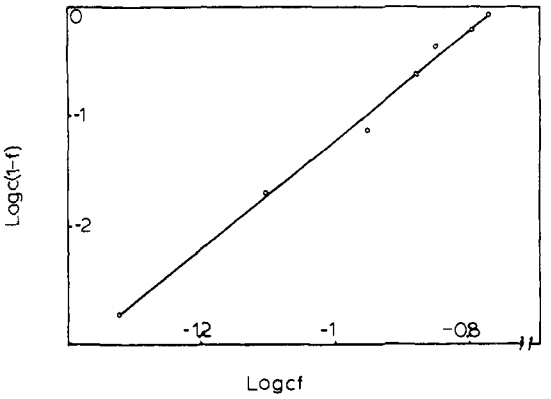


Figure 7. A plot of log $c(1-f)$ vs. log cf. The linearity of this plot shows that the clusters have a unique stoichiometry, the slope ($n = 6$) shows that they are essentially hexamers, see text.

of n is $n = 6 \pm 0.2$, see Figure 7. The experimental data do indeed fall remarkably well on a straight line.

We have also recorded resonance spectra of **5** (0.9 mol %) at other cholesterol concentrations in the range 0–50 mol %, and at different temperatures. All of these spectra show an increase of the fraction of biradicals clustered with increasing cholesterol concentration, and with decreasing temperature. We have not attempted a detailed quantitative analysis (as in Figures 5 and 6) at all these cholesterol concentrations, but the observed spectra do appear to be consistent with the same simple clustering model under all conditions. As an extreme example, all the biradicals appear to be clustered at 50 mol % cholesterol and at all temperatures in the range 15–65 °C.

We have also not as yet attempted a detailed quantitative analysis of the dependence of the resonance spectra of **9** on its concentration in the plane of the membrane, and on the relative concentrations of DMPC and cholesterol in the host membrane. The spectra of **9** are at least qualitatively very similar to those observed for **5**. For example, **9** shows spectra similar to **5** in DMPC above and below the transition temperature (see Figure 4), and shows spectra indicating "complete" clustering in 50 mol % mixtures of DMPC and cholesterol.

Discussion

The present work describes a study of the lateral distribution of nitroxide biradicals in lipid bilayer membranes; these molecules can serve as spin-label haptens in investigations of immunochemical reactions involving model membranes. We have shown that the degree of clustering of the biradicals (haptens) depends on the composition of the lipid host (DMPC, DPPC, or mixtures of these phospholipids with cholesterol) and on the nature of the lipid hapten (**5**, **7**, or **9**). In separate work it has been shown that the lateral mobility (diffusion constant) of (other) lipid haptens depends on the composition of the host lipid at a given temperature.[9] Spin-label studies such as those described here and elsewhere thus constitute an effective approach to studying the dependence of immunochemical reactions on the motion and distribution of membrane components.

The absence of clustering of the charged biradical **7** and the clustering of the uncharged biradical **5** under otherwise identical conditions is doubtless related to Coulomb repulsions between the charged radicals, as well as to radical–host lipid interactions. The enhanced motional freedom of **5** relative to **7** in fluid DMPC membranes may also be related to charge effects involving repulsive interactions between the two positive groups in **7**, as well as to attractive interactions between these positive groups and the negative phosphate groups of the phosphatidylcholine molecules.

A quantitative physical chemical question raised by the above results concerns the nature of the "clusters" formed by **5**. Their well-defined stoichiometry ($n \simeq 6$) under the experimental conditions of Figures 5 and 6 suggests a two-dimensional analogue of the familiar three-dimensional micelle formation in aqueous solutions where one has an equilibrium between monomers and a "cluster" having a rather well-defined size, shape, and number of constituent molecules.[17] Alternatively, the clusters formed by **5** may be looked upon as a special kind of lateral phase separation in membranes.[18] In the present case, with $n = 6$ and low concentrations of **5**, one might imagine that large domains of pure **5** tend to break up into small clusters until the increase of boundary free energy (between cluster and lipid host) equals the decrease of free energy due to entropy gain by domain break up. If this is the case one expects n to be dependent on the composition of host lipid. On the other hand, if a special molecular complex model, or two-dimensional micelle model, is more appropriate, n could be relatively independent of host lipid composition, although the equilibrium constant for reaction 2 might depend strongly on this composition. Additional experiments may possibly resolve these questions.

A second point of special interest is the lifetime of the clusters of **5**. The paramagnetic resonance line widths of the clustered and nonclustered resonance signals differ significantly (cf., Figure 5); this allows one to say that the lifetime τ of the cluster is long compared to the inverse of the paramagnetic resonance line width $1/\pi\Delta\nu \simeq 10^{-8}$ to 10^{-9} s. This conclusion is not particularly surprising, since the jump rate for lateral diffusion in bilayer membranes in already known[3-7] to be of the order of 10^{-7} s, and six molecules could hardly dissociate in a shorter time than this. Indirect evidence for the cluster formation of lipids concurrent with lateral phase separations has been suggested from ^{13}C nuclear resonance data; these data require that the putative lipid clusters be relatively long lived (e.g., milliseconds),[19] but provide no information on their size. In future work we hope to measure the rates of lateral diffusion of molecules of **5** under conditions where the fraction of these molecules in the form of clusters is high; this should establish if the clusters in question do have long lifetimes.

In conclusion, it is of interest to compare briefly the present study of cluster formation with other spin-label studies of cluster formation and lateral phase separations (domain formation) in bilayer membranes. In a number of studies involving the use of spin-labeled lipids in phospholipid membranes, Galla and Sackmann,[20] and Träuble and Sackmann,[5] have analyzed the concentration dependence of electron spin–spin line broadening in order to distinguish diffusion-controlled line broadening, and lateral phase separations that can also give enhanced spin–spin interaction if the labels are concentrated

in some region of the membrane. The clusters described in the present work are far smaller than the "clusters" referred to in their work; moreover, they conclude that their "cluster" size depends on the lipid label concentration (e.g., in the range 3.5–27 mol %). In our opinion it is likely that their "clusters" are in fact similar to the large lipid domains ($\sim$0.1–1 μm^2) that have been observed extensively in freeze-fracture electron microscopy.[18] Ohnishi[21] and collaborators have also carried out a number of analogous experiments demonstrating Ca^{2+} induced lateral phase separations, and again we anticipate domains will be found that are far larger than the clusters reported here. At the opposite extreme, Marsh and Smith have found clear-cut evidence for dimer formation of androstan spin labels in phosphatidylcholine–cholesterol membranes.[22] Lee et al.[23] and Lee[24] have suggested cluster formation on the basis of other types of spin-label experiments in single component lipid bilayers, but their experiments provide no order-of-magnitude estimates of the size of the individual lipid clusters.

In conclusion, the present paper reports the first case of a well-defined small molecular cluster formation in lipid bilayer membranes. Such clusters can be expected to have physical chemical as well as biophysical properties that are distinct from the frequently observed large lipid domains (0.1–10 μm^2) that are visualized with freeze-fracture electron microscopy, and that are in accord with thermodynamic phase diagrams derived from spin-label resonance data, optical data, and calorimetric data.[18]

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