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Spin-label studies on phosphatidylcholine-cholesterol membranes: effects of alkyl chain length and unsaturation in the fluid phase

Akihiro Kusumi [a,c,*], Witold K. Subczynski [b,d], Marta Pasenkiewicz-Gierula [b,d], James S. Hyde [b] and Hellmut Merkle [a]

[a] *Microphotonic Center, and* [b] *National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, WI 53226 (U.S.A.),* [c] *Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606 (Japan) and* [d] *Biophysics Department, Institute of Molecular Biology, Jagiellonian University, Krakow (Poland)*

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Dynamic properties of phosphatidylcholine-cholesterol membranes in the fluid phase and water accessibility to the membranes have been studied as a function of phospholipid alkyl chain length, saturation, mole fraction of cholesterol, and temperature by using spin and fluorescence labelling methods. The results are the following: (1) The effect of cholesterol on motional freedom of 5-doxyl stearic acid spin label (5-SASL) and 16-doxyl stearic acid spin label (16-SASL) in saturated phosphatidylcholine membrane is significantly larger than the effects of alkyl chain length and introduction of unsaturation in the alkyl chain. (2) Variation of alkyl chain length of saturated phospholipids does not alter the effects of cholesterol except in the case of dilauroylphosphatidylcholine, which possesses the shortest alkyl chains (12 carbons) used in this work. (3) Unsaturation of the alkyl chains greatly reduces the ordering effect of cholesterol at C-5 and C-16 positions although unsaturation alone gives only minor fluidizing effects. (4) Introduction of 30 mol% cholesterol to dimyristoylphosphatidylcholine membranes decreases the lateral diffusion constants of lipids by a factor of four, while it causes only a slight decrease of lateral diffusion in dioleoylphosphatidylcholine membranes. (5) If compared at the same temperature, 5-SASL mobilities plotted as a function of mole fraction of cholesterol in the fluid phases of dimyristoylphosphatidylcholine-, dipalmitoylphosphatidylcholine- and distearoylphosphatidylcholine-cholesterol membranes are similar in wide ranges of temperature (45–82°C) and cholesterol mole fraction (0–50%). (6) In isothermal experiments with saturated phosphatidylcholine membranes, 5-SASL is maximally immobilized at the phase boundary between Regions I and III reported by other workers (Recktenwald, D.J. and McConnell, H.M. (1981) Biochemistry 20, 4505–4510) and becomes more mobile away from the boundary in Regions I and III. (7) 5-SASL in unsaturated phosphatidylcholine membranes showed a gradual monotonic immobilization with increase of cholesterol mole fraction without showing any maximum in the range of cholesterol fractions studied. (8) By rigorously determining rigid-limit

* To whom correspondence should be addressed at: Microphotonic Center, Medical College of Wisconsin, P.O. Box 26509, Milwaukee, WI 53226, U.S.A.

Abbreviations: DLPC, L-α-dilauroylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DSPC, L-α-distearoylphosphatidylcholine; DOPC, L-α-dioleoylphosphatidylcholine; egg-yolk PC, egg-yolk phosphatidylcholine; CSL, cholestane spin label; 5-SASL, 5-doxyl stearic acid spin label; 16-SASL, 16-doxyl stearic acid spin label; Tempo, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; Tempo-PC, Tempocholine dipalmitoylphosphatidic acid ester; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine; 12-AS, 12-(9-anthroyloxy)stearic acid..

magnetic parameters of cholestane spin labels in membranes from Q-band second-derivative ESR spectra to monitor the dielectric environment around the nitroxide radical, it is concluded that cholesterol incorporation increases water accessibility in the hydrophilic loci of the membrane. In contrast, 12-(9-anthroyloxy)stearic acid fluorescence showed that water accessibility is decreased in the hydrophobic loci of the membrane.

Introduction

Model membranes composed of cholesterol and pure phosphatidylcholines have been the subject of vigorous and controversial research. Key recent papers include Refs. 1–6. On the basis of freeze-fracture electronmicroscopic observation, Lentz et al. [1] proposes that above the main phase transition temperature (T_M) two immiscible liquid phases are formed, designated L_α (a notation of Tardieu et al. [7]) and Γ. Recktenwald and McConnell [2] simply label this Region I (see Fig. 1) and state that fluid-phase–fluid-phase separation probably occurs; and Presti et al. [4] give a similar qualified opinion. Shimshick and McConnell [8] and Lentz et al. [1] show another phase boundary in the Region I which is indicated in a very approximate manner in Fig. 1 by a dotted line.

Most methods agree with the location of the boundaries of Region II (Fig. 1). 'Breakpoints' can be detected by a variety of techniques at these boundaries. The location of the boundary between Regions I and III has been more controversial.

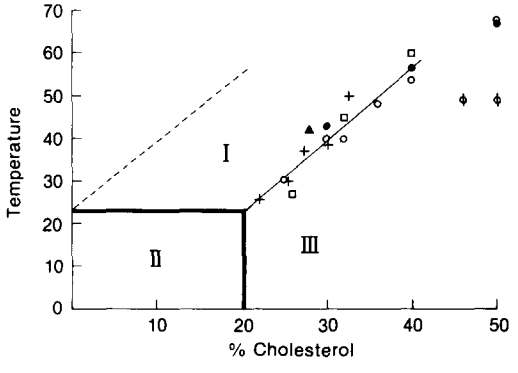

Fig. 1. Phase diagram for DMPC-cholesterol as obtained from: ESR-Tempo partitioning for: +, DMPC [2]; ○, DMPC [8]; ●, DPPC [8]; ESR-stearic acid spin label for: □, DMPC, DPPC and DSPC (this work); NMR for: ▲, DPPC [9] and fluorescence of 1,6-diphenyl-1,3,5-hexatriene for: ∅, DPPC [1].

Shimshick and McConnell [8] and Recktenwald and McConnell [2] give a number of points for both dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) (Fig. 1). One point by Haberkorn et al. [9] using NMR is in agreement with these data. However, using fluorescence probes Lentz et al. [1] give two data points that are far from those described in Ref. 2 and Ref. 8.

In spin-label studies of membranes, two labels, cholestane spin label (CSL) and stearic acid spin label (SASL), have been widely used as analogues for cholesterol and phospholipids. We describe in the present report our studies using cholestane spin label and stearic acid spin label of the lipid motion with special attention to the proposed boundary areas between Regions I and III. It must be remembered that the experiments carried out with probe molecules necessitate due caution in interpreting the results. For example, because of the lack of a 3-β-OH group in cholestane spin label, cholestane spin label cannot be expected to mimic all properties of cholesterol although overall similarity of the phase behavior of phosphatidylcholine-CSL membranes and phosphatidylcholine-cholesterol membranes has been reported [10].

Kusumi and Hyde [11] have previously studied the effect of lipid alkyl chain length and saturation on the tendency for transient portein association in membranes. It was interesting to consider the possibility that similar phenomena occur in cholesterol-phosphatidylcholine systems. In the present work, dilauroylphosphatidylcholine (DLPC), DMPC, DPPC and distearoylphosphatidylcholine (DSPC), containing 12, 14, 16 and 18 carbons per alkyl chain, respectively, were used to investigate the effect of chain length. McIntosh [12] from X-ray diffraction studies found that cholesterol increases the thickness of DLPC, DMPC and DPPC bilayers, but decreases the thickness of DSPC. Presti and Chan [3] also varied chain length, reporting differences in their experi-

ments only with the shortest lipid employed (DLPC).

As a further part of our systematic survey above T_M, dioleoylphosphatidylcholine (DOPC) and egg-yolk phosphatidylcholine (egg-yolk PC) were used to investigate how unsaturation of fatty acyl chains alters the effect of cholesterol observed in saturated phosphatidylcholine membranes. In all lipid systems the mole fraction of cholesterol and the temperature were systematically varied using CSL, 5-SASL and 16-SASL probes.

We will also report the effects of cholesterol on the interface between membrane and aqueous phases, particularly on water accessibility to the hydrophilic loci of the membrane. Interaction of water and lipid molecules at the interface between membrane and aqueous phases plays critical roles in determining membrane properties and membrane formation itself [13]. Our knowledge on effects of cholesterol on the properties of membrane-water interface regions is rather poor despite the importance of the subject.

A number of studies indicated that the permeation of water through membranes is decreased with incorporation of cholesterol (see, for example, Ref. 14). This result implies decrease of water accessibility to the hydrophobic loci of the membrane, which is the primary barrier for water permeation through the membrane. However, the literature that might give clues to water accessibility (penetration) to the hydrophilic loci of the membrane is sparse. Oldfield et al. [15] made ^{2}H-NMR studies on the mobility of the phospholipid head group using DPPC labeled with a deuteriomethyl group in the choline head group. The effect of cholesterol on the quadrupole splitting is complex. Above 20 mol% cholesterol in the fluid state membrane, the main effect was an increase of choline head group mobility, the opposite effect to that observed with hydrocarbon chains.

Rubenstein et al. [16] made ESR studies on the behavior of the phosphatidylcholine spin-labeled at the head group in binary mixtures of cholesterol and DMPC. The ESR spectra showed complicated changes throughout the temperature (0–40°C) and composition (0–50 mol% cholesterol) regions studied.

We will show in this report that accessibility of water into hydrophilic loci of the membrane increases with inclusion of cholesterol while accessibility into hydrophobic portion of the membrane decreases.

Materials and Methods

5-SASL, 16-SASL, and cholestane spin label (CSL) were obtained from Syva (Palo Alto, CA) and 12-(9-anthroyloxy)stearic acid (12-AS) from Molecular Probes (Junction City, OR). Tempo-PC is a gift from Dr. S. Ohnishi, Kyoto University. Phospholipids were purchased from Sigma (St. Louis, MO), and cholesterol (crystallized) from Boehringer-Mannheim (Indianapolis, IN). The buffer used for the study with 5-SASL, 16-SASL or 12-AS was 0.1 M borate at pH 9.5. To ensure that all probe carboxyl groups are ionized in phosphatidylcholine membranes, a rather high pH was chosen [17–19]. Androstane spin label and cholestane spin label, which do not have any ionizable group, did not show spectral change in phosphatidylcholine membranes between pH 4.5 and pH 9.5, indicating that the structure of phosphatidylcholine membranes is not influenced by pH in this range [19]. The phase transition temperature [20] and electrostatic properties [21] of phosphatidylcholine membranes are the same in this pH range. For experiments with cholestane spin label, 0.1 M sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes) (pH 7.4) was used as a buffer.

The membranes used in this work were multilamellar dispersions of lipids prepared in the following way: A mixture of lipid (phosphatidylcholine and cholesterol; $1.0 \cdot 10^{-5}$ mol) and spin label ($2.5 \cdot 10^{-8}$ mol) or 12-AS ($1.0 \cdot 10^{-8}$ mol) in chloroform was dried with a stream of nitrogen and further dried under a reduced pressure (approx. 0.1 mmHg) for at least 12 h. The buffer (1 ml) was added to dried lipid at about 20°C above the phase transition temperature of the phospholipid membranes and vortexed vigorously. The lipid dispersion was centrifuged briefly and the loose pellet was used for ESR measurement. Dilution of the pellet by resuspension in 10 vols. of the buffer did not change the ESR spectra. Therefore, the loose pellets were used as samples to obtain better signal-to-noise ratio in the ESR spectrum. A portion

of the pellet was then transferred to a capillary (0.9 mm i.d.) made of gas-permeable methylpentene polymer known as TPX [22]. This plastic is permeable to nitrogen, oxygen, and carbon dioxide and is substantially impermeable to water. ESR spectra were obtained with a Varian E-109 X-band spectrometer with Varian temperature control accessories and an E-231 Varian multipurpose cavity (rectangular TE_{102} mode). The TPX sample tube was placed inside the ESR dewar insert and equilibrated with nitrogen gas that was used for temperature control. The sample was thoroughly deoxygenated, yielding correct ESR line shapes and preventing possible oxidation of lipids.

Fluorescence photobleaching recovery experiments were performed to observe lateral diffusion of *N*-(7-nitrobenz-2-oxa-1,3-diazo-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) in the membrane as described previously [23]. NBD-PE is a gift of Dr. K. Machida at Kyoto University.

Principal values of *g*-tensor and hyperfine tensor of cholestane spin label in various membranes were determined by simulating Q-band (35 GHz) ESR second derivative spectra of the frozen sample measured at −120°C [24,25]. The rigid-limit spectrum (motional effects were effectively suppressed) was obtained at this temperature. Q-band second derivative spectra have more features than conventional X-band first derivative spectra, and this facilitates computer simulation [25]. The spectrum was taken with a Varian E-9 Q-band spectrometer with Varian temperature control accessories and a Varian cylindrical cavity.

The second derivative spectrum was obtained with 50 kHz magnetic field modulation and 100 kHz in-phase (second harmonic) phase-sensitive detection. The ESR signal is stored in a PDP11-34 computer interfaced to the spectrometer. Signal averaging (typically 10 scans) was employed. The field scan was calibrated by a direct reading magnetometer (Radiopan, Poznan, Poland) and by a direct reading microwave frequency counter (EIP Microwave, Santa Clara, CA). The linearity of the field scan was carefully checked. Considerable care was taken to avoid dispersive admixture to the detected absorption signal.

For fluorescence experiments, the final lipid concentration was adjusted to 1 mM. The fluorescence emission spectrum (2 nm bandwidth) was measured on a Aminco-Bowman SPF-500 spectrofluorometer with excitation at 355 nm (2 nm bandwidth). Molecular oxygen was replaced with argon gas by slow bubbling for 30 min.

Results

Effects of cholesterol on saturated phosphatidylcholine membranes

Fig. 2 shows typical ESR spectra of 5-SASL and CSL in fluid-phase membranes. Maximum splitting values have been used as a convenient parameter to monitor rotational motional freedom of the nitroxide radical group of these probes. All ESR spectra of 5-SASL obtained in this study can be analyzed by the method of Hubbell and McConnell [26], in which the maximum splitting value is directly related to the order parameter of 5-SASL [27]. The maximum splitting value increases with increase of alkyl chain order. For brevity, we describe this observation as the decrease of 5-SASL mobility. It is noted that CSL has a fairly rigid structure and the spin label reports the motion of the molecule as a whole, whereas segmental motions of the alkyl chain (*gauche-trans* isomerism) play important roles in

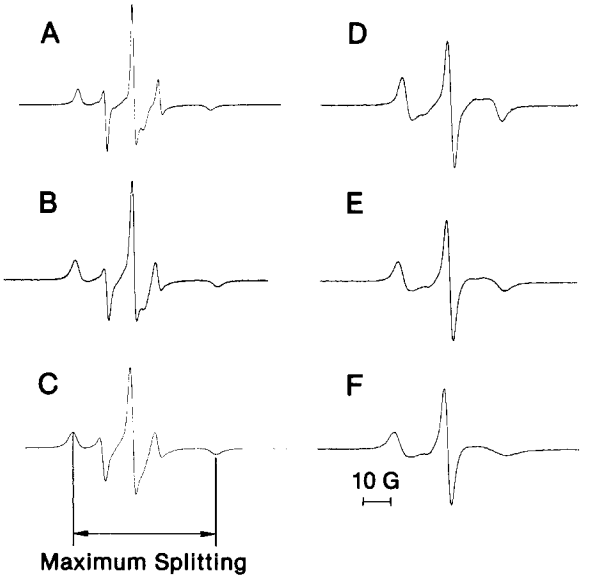

Fig. 2. ESR spectra of 5-SASL (A, B, C) and CSL (D, E, F) in membranes made from various mixtures of DPPC and cholesterol at 45°C. The mole fraction of cholesterol is A 0.01%, B 30%, C 50%, D 0%, E 20% and F 50%.

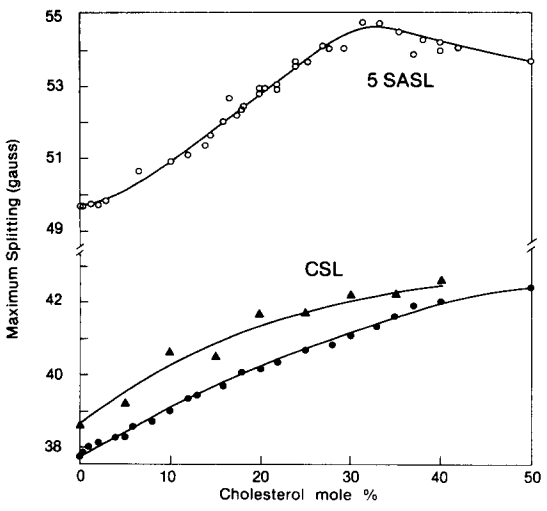

Fig. 3. Maximum splitting of 5-SASL (○) and CSL (●, ▲) in DPPC-cholesterol membranes plotted against mole fraction of cholesterol. The mole ratio of 5-SASL/lipid = 1 : 400 (○), CSL/lipid = 1 : 400 (●) and 1 : 9 (▲).

determining the ESR spectra of stearic acid spin labels. These two types of labels may also exhibit different sensitivity to the tilt angle of the phospholipid long axis (approx. 30° has been observed near the surface of the membrane) [28,29].

Fig. 3 shows the maximum splittings of 5-SASL and CSL in DPPC-cholesterol membranes at 45°C (liquid-crystalline phase) as a function of the mole fraction of cholesterol. Because of structural similarities, it seems apparent as noted previously that 5-SASL is a probe of alkyl chain mobility of phospholipids and that CSL is a probe of cholesterol mobility. There are three remarkable features in this figure: (1) The profile of 5-SASL shows an extremum in the maximum splitting value at 32 mol% cholesterol; the splitting value decreases with further increase of cholesterol in the membrane. This extremum lies on the Region I/Region III interface proposed by McConnell's group and others. (2) The mobility of the cholestane spin label decreases monotonically as the mole fraction of cholesterol increases. (3) The behavior of membranes containing 10 mol% (cholestane spin label is similar to that of membranes containing 10 mol% cholesterol. Observation (3) is consistent with previous results that cholestane spin label is likely to simulate the behavior of cholesterol [10,30,31]. At 10% cholestane spin label, some increase in the linewidth of the ESR spectrum was observed. Since the lines that determine the maximum splitting are composites of lines with all orientations of principal axes and unresolved proton hyperfine lines, the observed line broadening by spin-spin interaction would not seriously change the maximum splitting.

The influence of alkyl chain length on cholesterol effects on 5-SASL mobility in saturated phosphatidylcholine membranes is shown in Fig. 4. It is possible that mismatch of the hydrophobic length between phospholipid and cholesterol leads to lateral phase separation in membranes as in other systems [11,32]. Fig. 4A shows that there is indeed a chain length dependence. The profiles of DLPC-cholesterol and DMPC-cholesterol are very different at the same temperature, suggesting different phospholipid-cholesterol interaction in these membranes *. As was shown by McIntosh [12], the alkyl chain of DLPC may be too short to accommodate cholesterol easily.

Fig. 4B, however, shows that the mobility of 5-SASL is similar in DMPC-cholesterol, DPPC-cholesterol, and DSPC-cholesterol membranes if they are compared at the same temperature in Regions I and III.

This result is surprising in the light of the general belief that the membrane is more fluid when the alkyl chain length is shorter (for a review, see Ref. 33). Fig. 4B indicates that in very wide ranges of temperature and mole fraction of cholesterol, 5-SASL motion is independent of the length of phospholipid lakyl chains.

In Fig. 4B, we note another characteristic effect of cholesterol on saturated phosphatidylcholine membranes in addition to three features displayed in Fig. 3: the cholesterol content that gives the largest maximum splitting increases as the temperature is raised.

* Because a small difference in $2T'_{\parallel}$ between DLPC and DMPC membranes is observed at 0% cholesterol, the difference between DLPC-cholesterol and DMPC-cholesterol membranes cannot solely be ascribed to the mismatch of lengths of DLPC and cholesterol molecules. However, the difference is much larger in the presence of approx. 25% cholesterol.

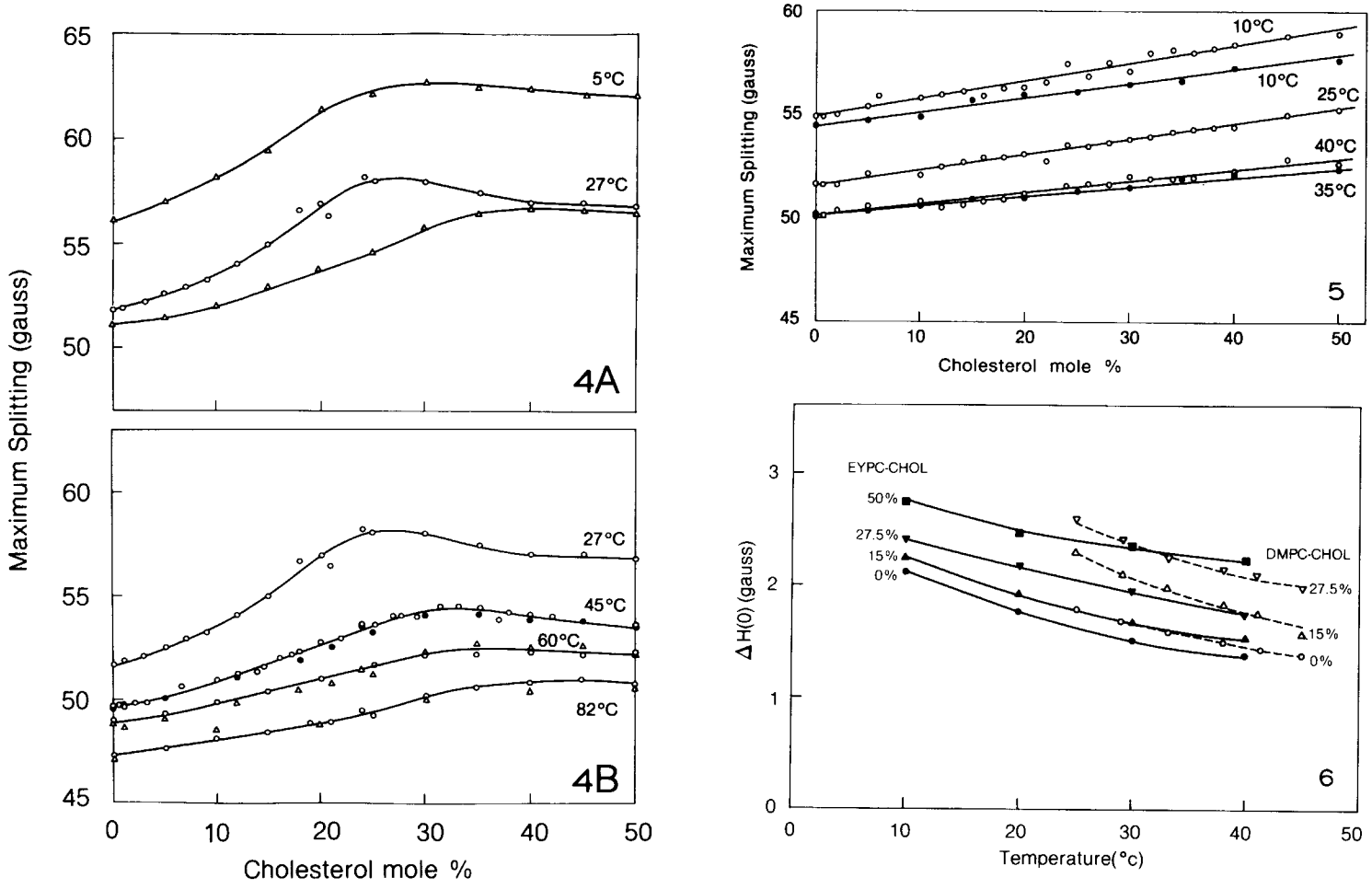

Fig. 4. Maximum splitting of 5-SASL in (A) DLPC-cholesterol (△) and DMPC-cholesterol (○) membranes and (B) DMPC-cholesterol (○), DPPC-cholesterol (●), and DSPC-cholesterol (△) membranes plotted against mole fraction of cholesterol at various temperatures (above the phase transition temperatures).

Fig. 5. Maximum splitting of 5-SASL in egg-yolk PC-cholesterol (○) and DOPC-cholesterol (●) membranes plotted against mole fraction of cholesterol at various temperature.

Fig. 6. Peak-to-peak linewidth of the central line ($\Delta H(0)$) of 16-SASL in egg-yolk PC-cholesterol (closed keys) and DMPC-cholesterol (open keys) membranes plotted against temperature at various cholesterol concentrations.

Alkyl chain unsaturation moderates cholesterol effects

In Fig. 5, results on unsaturated phosphatidylcholine membranes are reported. ESR spectra of 5-SASL in egg-yolk phosphatidylcholine-cholesterol and dioleoylphosphatidylcholine (DOPC)-cholesterol membranes were studied as a function of cholesterol content and temperature. These measurements show that the maximum splitting always increases monotonically with increase in cholesterol content, indicating that unsaturation of the alkyl chains eliminates the maxima in the profiles. The effect of cholesterol is much smaller in unsaturated phosphatidylcholine membranes.

Effects of hydrocarbon chain length, unsaturation of the alkyl chains, and incorporation of cholesterol on motional properties of 5-SASL are summarized in Table I. Alkyl chain length and unsaturation hardly affect the maximum splitting value of 5-SASL. These results indicate that the order parameter as detected by 5-SASL is not dependent on alkyl chain length or unsaturation. Cholesterol causes a dramatic change in the maximum splitting value of 5-SASL in phosphatidylcholine membranes with saturated alkyl chains but only a modest change in unsaturated phosphatidylcholine membranes. Thus it is concluded that alkyl chain unsaturation moderates cholesterol effects on the alkyl chain order close to the membrane surface (where the nitroxide group of 5-SASL is located), although unsaturation alone does not change the order at this depth of the membrane.

To examine the influence of alkyl chain unsaturation on cholesterol effects in the membrane interior regions, we performed similar sets of experiments using 16-SASL. Fig. 6 shows the peak-to-peak linewidth of the central line of 16-SASL ESR spectrum ($\Delta H(0)$) as a function of temperature, cholesterol mole fraction and the degree of unsaturation. $\Delta H(0)$ decreases with increase of 16-SASL mobility. The effect of cholesterol is much smaller in egg-yolk PC membranes than in DMPC membranes. Unsaturation (egg-yolk PC) gives small increase in motional freedom of the 16-SASL nitroxide radical in the absence of cholesterol.

Comparative results with the data shown in Table I were obtained by using 16-SASL and are summarized in Table II. Mobility of the 16-SASL nitroxide radical is slightly smaller in saturated phosphatidylcholine membranes with longer alkyl chains. Unsaturation gives only moderate increase of 16-SASL mobility. These data indicate some effects of chain lengths and degrees of unsaturation on motional freedom of 16-SASL in membrane core regions in contrast to very little

TABLE I

MAXIMUM SPLITTING VALUES OF 5-SASL IN VARIOUS MEMBRANES

Maximal measurement errors are estimated to be ±0.25 gauss.

Lipid	Maximum splitting value (gauss)			
	27°C	45°C	60°C	82°C
DMPC	51.9	49.7	49.0	47.3
DPPC		49.7	48.7	47.2
DSPC			48.8	47.4
DOPC	52.0	49.1	48.5	47.2
Egg-yolk PC	51.8	49.5	48.8	47.3
DMPC (or DPPC or DSPC) + 30% cholesterol	58.1	54.4	52.2	50.1
DOPC + 30% cholesterol	52.6	50.8	49.3	48.2
Egg-yolk PC + 30% cholesterol	53.3	51.5	49.8	48.5

TABLE II

PEAK-TO-PEAK CENTRAL LINEWIDTHS OF 16-SASL ($\Delta H(0)$) IN VARIOUS MEMBRANES

Maximal measurement errors are estimated to be ±0.03 gauss.

Lipid	$\Delta H(0)$ (gauss)			
	27°C	45°C	60°C	82°C
DMPC	1.58	1.26	1.15	1.06
DPPC		1.35	1.19	1.09
DSPC			1.21	1.08
DOPC	1.47	1.21	1.11	1.01
Egg-yolk PC	1.49	1.21	1.10	1.02
DMPC + 30% cholesterol	2.43	2.05	1.77	1.50
DPPC + 30% cholesterol		2.10	1.88	1.60
DSPC + 30% cholesterol			1.83	1.48
DOPC + 30% cholesterol	1.73	1.42	1.28	1.13
Egg-yolk PC + 30% cholesterol	1.75	1.42	1.26	1.12

TABLE III

EFFECTS OF CHOLESTEROL AND UNSATURATION ON LATERAL DIFFUSION CONSTANT OF NBD-PE

Phospholipid	Lateral diffusion constant (10^{-8} cm^2/s) at 26°C	
	0% cholesterol	30% cholesterol
DMPC	2.2±0.8	0.51±0.14
DOPC	1.9±0.4	1.6 ±0.5

influences on 5-SASL mobilities. It is likely that these results reflect different effects of the chain length and unsaturation at various depths in the membrane. However, since external probe technique is used in this work, due caution is required for this interpretation.

Cholesterol causes larger decreases of 16-SASL mobility in saturated phosphatidylcholine membranes but moderate decreases in unsaturated phosphatidylcholine membranes. Thus it is concluded that alkyl chain unsaturation moderates cholesterol effects on the alkyl chain order in membrane core regions as well as in near-surface regions.

We have also studied effects of alkyl chain unsaturation and cholesterol on lateral diffusion in DMPC-cholesterol and DOPC-cholesterol membranes by using the fluorescene photobleaching recovery technique. The results are summarized in Table III. Unsaturation of alkyl chains hardly affects lateral diffusion of NBD-PE (Compare DMPC and DOPC membranes). Addition of 30% cholesterol to DMPC membranes decreases the lateral diffusion constant by a factor of 4. In contrast, only a little effect of cholesterol is observed in DOPC membranes. Unsaturation of alkyl chains, thus, moderates cholesterol effects on lateral diffusion as well as on rotational motional freedom.

Effects of cholesterol on water accessibility to the hydrophilic loci of the membrane

Griffith and his co-workers [24,34] established a procedure for observation of water accessibility to nitroxide radicals (attached to stearic acid spin labels) in the membrane. This method is based on the dependence of unpaired electron spin density over nitrogen nuclei on solvent polarity. Polar solvents tend to increase the spin density on the nitrogen nucleus and, therefore, increase the isotropic hyperfine constant A_0. Griffith et al. [24] empirically showed that solvent-dependent changes in A_z (z-component of the hyperfine tensor) in frozen solution is proportional to changes in A_0 in homogeneous liquid, and estimated the water accessibility (or the polarity profile) across the membrane using A_z as a convenient experimental observable. To obtain rigid limit value of A_z, the membrane was frozen and the ESR spectrum was measured at −196°C (to suppress the motional effect).

We employed this method to assess water accessibility to the membrane surface by using cholestane spin label. Cholestane spin label is intercalated in the membrane in such a way that the oxazolidine ring which contains the nitroxide group is placed in the hydrophilic domain of the membrane [34]. We obtained all principal values of g- and A-tensor by performing computer simulation of the Q-band second derivative spectrum taken at −120°C (Fig. 7 and Table IV). We assessed water accessibility to the nitroxide moiety using A_{iso}

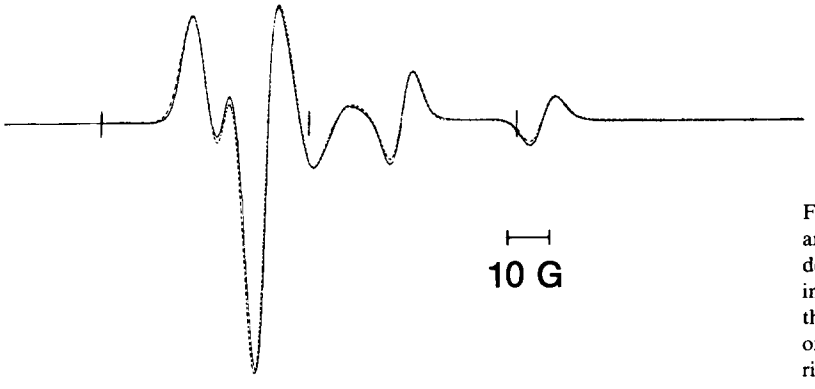

Fig. 7. Superimposed experimental (solid line) and simulated (broken line) Q-band second derivative ESR spectra of cholestane spin label in DPPC membranes at −120°C. Ticks on the baseline indicate magnetic field intensity of 12350, 12400 and12450 gauss from left to right.

$(=(A_x+A_y+A_z)\times 1/3)$ as well as A_z to increase the reliability of evaluation. A_{iso} values obtained in frozen solution were shown to be nearly the same as A_0 measured in liquid solution [25]. Thus A_{iso} is a simpler and more sound parameter to observe the polarity of the environment of nitroxide radicals than A_z. A_{iso} directly reflects the spin density on the nitrogen nucleus of the nitroxide because anisotropic electron-nuclear dipolar interaction is cancelled in A_{iso}. Addition of cholesterol to the membrane increases both A_z and A_{iso} in all phosphatidylcholine (DLPC, DPPC, and DOPC) membranes examined in this study (Table IV). It is concluded that the inclusion of cholesterol in phosphatidylcholine membranes increases water accessibility to the nitroxide radical group of cholestane spin label.

Increase of A_z and decrease of A_x are characteristic effects of cholesterol (Table IV). If we assume the increase of water accessibility to the nitroxide radical, this observation is expected from the molecular structure of the nitroxide ring, because a hydrogen atom in a water molecule interacts with the oxygen atom of the nitroxide in the direction of N-O bond (x axis).

We note the decrease of g_x and g_{iso} with addition of cholesterol in Table IV. This observation is also consistent with the increased water accessibility to the nitorxide group on cholestane spin label, because g_x and g_{iso} values decrease as the solvent polarity increases [25].

It is somewhat difficult to assess the range of error in estimation of magnetic parameters listed in Table IV because of the complex interplay of many parameters. If a single parameter is varied, deterioration of the fit is easily detected with the change of ± 0.00003 in g values and ± 0.05 gauss in A values. Difference of magnetic constants in DLPC and DPPC membranes appears small. However, if the rigid-limit values in DLPC membranes are used to simulate the rapid-motion ESR spectrum of CSL in DPPC membranes above the phase transition temperature, the quality of fit is much less satisfactory compared with that obtained by using rigid-limit values in DPPC membranes. This can be taken as an indication that the accuracy of these values is high.

These results point toward the necessity to determine rigid-limit values in each membrane when motion of nitroxide spin radicals in membranes is studied. In most studies published so far, magnetic constants determined in crystals were used to simulate rapid-motion ESR spectra observed with various membranes. To the best of our knowledge, magnetic constants listed in Table IV are most accurate estimates of these parameters of spin labels in membranes. Such evaluation was made possible by simulating Q-band second-derivative ESR spectra.

To confirm CSL data on the cholesterol effect of increasing water accessibility to the hydrophilic loci, we utilized Tempo-PC as another probe. Only A_z data directly obtained from X-band ESR spectra are listed in Table V. It is clear that water accessibility to the nitroxide radical of Tempo-PC greatly increases in the presence of 50% cholesterol.

TABLE IV

MAGNETIC PARAMETERS OF CHOLESTANE SPIN LABEL IN VARIOUS MEMBRANES

Lipid	g_x	g_y	g_z	g_{iso} [a]	A_x	A_y	A_z	A_{iso} [a]
DLPC	2.00854	2.00600	2.00231	2.00561	5.65	5.0	33.8	14.8
DLPC/cholesterol (1:1)	2.00835	2.00590	2.00226	2.00550	5.2	4.9	34.8	15.0
DPPC	2.00849	2.00598	2.00229	2.00559	6.1	5.15	34.0	15.1
DPPC/cholesterol (1:1)	2.00835	2.00585	2.00224	2.00548	5.65	5.15	35.05	15.3
DOPC	2.00880	2.00619	2.00229	2.00576	5.85	4.8	33.05	14.6
DOPC/cholesterol (1:1)	2.00845	2.00593	2.00226	2.00555	5.1	4.9	35.0	15.0

[a] g_{iso} and A_{iso} is $1/3\times$ trace of g- and A-tensor, respectively. A values are presented in gauss.

TABLE V

PRINCIPAL A_z VALUES OF TEMPO-PC IN VARIOUS PHOSPHATIDYLCHOLINE-CHOLESTEROL MEMBRANES

The measurement error is estimated to be ±0.3 gauss.

Phospholipid	A_z (gauss)	
	0% cholesterol	50% cholesterol
DLPC	33.50	37.00
DPPC	33.75	37.50
DOPC	33.13	37.25

The very large A_z values reported here for 50% cholesterol membranes are indicative of hydrogen bondings between water molecules and the nitroxide radical [35].

Effects of cholesterol on water accessibility to the hydrophobic loci

We also studied the effect of cholesterol on the water accessibility to the hydrophobic loci of the membrane. We employed a fluorescently-labelled stearic acid, 12-AS, because its fluorescence emission spectrum is sensitive to polarity of the environment of anthroyloxy group [36,37]. The fluorescence emission spectra obtained in DPPC-cholesterol membranes at various mole fractions of cholesterol are shown in Fig. 8. The emission peak shifts to shorter wavelengths as the cholesterol mole fraction increases, indicating that the environment around the anthroyloxy group becomes more hydrophobic as cholesterol concentration increases. Decrease of water accessibility to hydrophobic loci of membranes in the presence of cholesterol observed here is consistent with previous studies that showed that cholesterol incorporation decreases the water permeation through liposomal membranes [14].

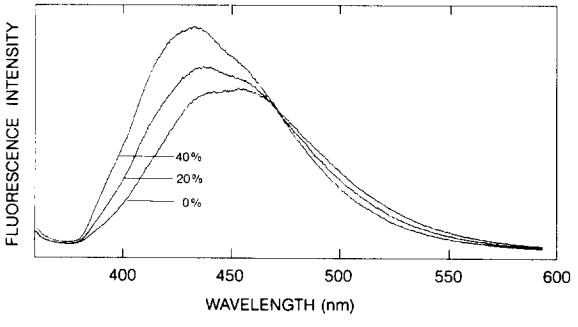

Fig. 8. Fluorescence emission spectra of 12-AS in DPPC cholesterol membranes at 50°C. The spectrum shifts hypochromically as cholesterol mole fraction increases at 0, 20, 40 mol%.

Discussion

5-SASL mobilities plotted as a function of mole fraction of cholesterol at the same temperature in the fluid phases (Regions I and III) of DMPC-, DPPC-, and DSPC-cholesterol membranes are nearly the same in wide ranges of temperature (45–82°C) and cholesterol mole fraction (0–50%) (Fig. 4). This observation implies that outside of Region II interactions of cholesterol with saturated phospholipid in the membrane near the surface do not depend on acyl chain length between 14 and 18 carbons. The positions of the maxima in outermost splitting of 5-SASL in these membranes are 26% cholesterol at 27°C, 32% at 45°C and 40% * at 60°C. These values have been plotted on Fig. 1 and they coincide with the boundary between Regions I and III by the Tempo partitioning data. Since 5-SASL is presumed to mimic the motion of phospholipid alkyl chins, it follows that the phospholipid alkyl chains are more immobilized along the interface between Regions I and III and become more mobile away from the interface. It is notable that, in Region III, 5-SASL becomes progressively less immobilized (although the change is small) with the increase of cholesterol mole fraction.

No sensitivity of CSL to passage across the Region I/Region III interface is detected. The spectra indicate progressive monotonic increase in mobility as the amount of cholesterol increases. The motional modes and orientations of nitroxide principal axes of CSL and 5-SASL are completely different. Thus we hesitate to speculate on dynamics at a molecular level.

One of the most significant findings in this study is the effect of unsaturation on the membrane alkyl chain flexibility as observed with 5-SASL and 16-SASL. Unsaturated alkyl chains greatly decreases the ordering effect of cholesterol, while the order parameter at C-5 position in unsaturated phosphatidylcholine membranes is the same as that in saturated phosphatidylcholine

* The peak at 60°C is very broad and 35–45% of cholesterol gives nearly the same $2T'_{\parallel}$ value. However this range of error does not affect the later discussion.

membranes. Motional freedom of 16-SASL and lateral diffusion of NBD-PE also showed that alkyl chain unsaturation greatly moderates cholesterol effects while unsaturation alone (without cholesterol) gives only modest effects. The 'fluidizing' effect of unsaturated chains observed in biological membranes seems to manifest itself by moderating the 'solidifying' effect of cholesterol.

We have shown that cholesterol strongly influences hydrophilic loci as well as hydrophobic loci of the membrane. Water accessibility to the hydrophilic loci increases whereas the accessibility to the hydrophobic loci decreases as the cholesterol mole fraction in the membrane increases. It is important to take into account these effects of cholesterol on membrane-water interaction as well as the direct effects on hydrocarbon chains when cholesterol-phospholipid interaction is studied, because membrane properties may be fundamentally altered by increased water penetration into the hydrophilic domains of membranes.

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References

1 Lentz, B.R., Barrow, D.A. and Hoechli, M. (1980) Biochemistry 19, 1943–1954
2 Recktenwald, D.J. and McConnell, H.M. (1981) Biochemistry 20, 4505–4510
3 Presti, F.T. and Chan, S.I. (1982) Biochemistry 21, 3821–3830
4 Presti, F.T., Pace, R.J. and Chan, S.I. (1982) Biochemistry 21, 3831–3835
5 Hui, S.W. and He, N.-B. (1983) Biochemistry 22, 1159–1164
6 Dufourc, E.J., Parish, E.J., Chitrakorn, S. and Smith, I.C.P. (1984) Biochemistry 23, 6062–6071
7 Tardieu, A., Luzzati, V. and Reman, F.C. (1973) J. Mol. Biol. 75, 711–733
8 Shimshick, E.J. and McConnell, H.M. (1973) Biochem. Biophys. Res. Commun. 53, 446–451
9 Haberkorn, R.A., Griffin, R.G., Meadow, M.D. and Oldfield, E. (1977) J. Am. Chem. Soc. 99, 7353–7355
10 Cadenhead, D.A. and Muller-Landau, F. (1979) Chem. Phys. Lipids 25, 329–343
11 Kusumi, A. and Hyde, J.S. (1982) Biochemistry, 21, 5978–5983
12 McIntosh, T.J. (1978) Biochim. Biophys. Acta 513, 43–58
13 Tanford, C. (1980) The Hydrophobic Effect. Formation of Micelles and Biological Membranes, Wiley-Interscience, New York
14 Blok, M.C., Van Deenen, L.L.M. and De Gier, J. (1977) Biochim. Biophys. Acta 464, 509–518
15 Oldfield, E., Meadows, M., Rice, D. and Jacobs, R. (1978) Biochemistry 17, 2727–2740
16 Rubenstein, J.L., Owicki, J.C. and McConnell, H.M. (1980) Biochemistry 19, 569–573
17 Sanson, A., Ptak, M., Rignaud, J.L. and Gary-Bobo, C.M. (1976) Chem. Phys. Lipids 17, 435–444
18 Egret-Charlier, M., Sanson, A., Ptak, M. and Bouloussa, O. (1978) FEBS Lett. 87, 313–316
19 Kusumi, A., Subczynski, W.K. and Hyde, J.S. (1982) Fed. Proc. 41, 1394, Abstr. 6571
20 Träuble, H. and Eibl, H. (1974) Proc. Natl. Acad. Sci. USA 71, 324–335
21 Papahadjopoulos, D. (1968) Biochim. Biophys. Acta 163, 240–254
22 Popp, C. and Hyde, J.S. (1981) J. Magn. Reson. 43, 249–258
23 Chang, C.-H., Takeuchi, H., Ito, T., Machida, K. and Ohnishi, S. (1981) J. Biochem. 90, 997–1004
24 Griffith, O.H., Dehlinger, P.J. and Van, S.P. (1974) J. Membrane Biol. 15, 159–192
25 Pasenkiewicz-Gierula, M., Hyde, J.S. and Pilbrow, J.R. (1983) J. Magn. Reson. 55, 255–265
26 Hubbell, W.L. and McConnell, H.M. (1971) J. Am. Chem. Soc. 93, 314–326
27 Gaffney, B.J. (1976) in Spin Labeling, Theory and Applications (Berliner, L.J., ed.), pp. 567–571, Academic Press, New York
28 Gaffney, B.J. and McConnell, H.M. (1974) J. Magn. Reson. 16, 1–28
29 McConnell, H.M. (1976) in Spin Labeling, Theory and Applications (Berliner, L.J., ed.), pp. 525–560, Academic Press, New York
30 Müller-Landau, F. and Cadenhead, D.A. (1979a) Chem. Phys. Lipids 25, 299–314
31 Müller-Landau, F. and Cadenhead, D.A. (1979b) Chem. Phys. Lipids 25, 315–328
32 Wu, S.H. and McConnell, H.M. (1975) Biochemistry 14, 847–854
33 Jain, M.K. and Wagner, R.F. (1980) Introduction to Biological Membranes, Wiley-Interscience, New York
34 Griffith, O.H. and Jost, P.C. (1976) Spin Labeling. Theory and Application (Berliner, L.J., ed.), Chap. 12, pp. 453–525, Academic Press, New York
35 Johnson, M.E. (1981) Biochemistry 20, 3319–3328
36 Waggoner, A.S. and Stryer, L. (1970) Proc. Natl. Acad. Sci. USA 67, 579–589
37 Lakowicz, J.R. (1983) Principles of Fluorescence Spectroscopy, Chap. 7, pp. 187–215, Plenum Press, New York