Structural and Dynamic Effects of Oxidatively Modified Phospholipids in Unsaturated Lipid Membranes†

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ABSTRACT: Phospholipid hydroperoxides and phospholipid alcohols are two of the major forms of oxidatively modified phospholipids produced during oxidant stress and lipid peroxidation. The process of lipid peroxidation is known to affect the physiological function of membranes. We, therefore, investigated the effects of lipid peroxidation products on the molecular interactions in membranes. Our study was specifically focused on the effects of lipid peroxidation products on static membrane structure (molecular orientational order) and on the reorientational dynamics of the probe molecules in lipid bilayers. The study was done by performing angle-resolved fluorescence depolarization measurements (AFD) on the fluorescent probe diphenylhexatriene (DPH) and by performing angle-resolved electron spin resonance (A-ESR) measurements on cholestane (CSL) nitroxide spin probes embedded in macroscopically oriented planar bilayers consisting of 2–10% 1-palmitoyl-2-(9/13-hydroperoxylinoleoyl)phosphatidylcholine (PLPC-OOH) or 1-palmitoyl-2-(9/13-hydroxylinoleoyl)phosphatidylcholine (PLPC-OH) in 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC) or dilinoleoylphosphatidylcholine (DLPC). Both probe molecules have rigid cylindrical geometries and report on the overall molecular order and dynamics. However, being more polar, the nitroxide spin probe CSL is preferentially located near the surface of the membrane, while the less polar fluorescent probe DPH reports preferentially near the central hydrophobic region of the lipid bilayers. The results show that the presence of relatively small amounts of oxidatively modified phospholipids within the PLPC or DLPC membranes causes pronounced structural effects as the molecular orientational order of the probe molecules is strongly decreased. In contrast, the effect on membrane reorientational dynamics is minimal.

Lipid peroxidation in membranes occurs during many pathological as well as some physiological processes. Examples include inflammation, ischemia/reperfusion injury, cytochrome P-450 metabolism, and cellular aging. Exposure of the membrane to oxidant species may lead to radical propagation reactions that result in accumulation of oxidized phospholipids. Indeed, lipid hydroperoxides are known to be primary products of the peroxidation process (Porter, 1990) and can accumulate under conditions favoring their stability to represent the initial derived free-radical products in membranes. Although there is a vast array of literature describing lipid peroxidation, very little is known as to how these lipid peroxidation products affect membrane function. The introduction of hydroperoxy or alcohol groups into the unsaturated *sn*-2 fatty acyl chain is characteristic of many oxidized biological phospholipids and may have a significant impact on either or both the static order of the membrane and the dynamic reorientational and diffusional rates of the phospholipid molecules. It has been proposed that, due to the hydrophilic nature of the hydroperoxy/alcohol groups, the fatty acyl chain may locate closer to the head group interface than a corresponding unoxidized chain (van Kuijk et al., 1988). It has also been shown that phospholipase A_2 hydrolytic activity is increased in membrane preparations containing oxidatively modified phospholipids and that fatty acyl chains with an oxidative modification are hydrolyzed to a much greater extent than control (nonoxidized) fatty acyl chains (Sevanian et al., 1988).

To obtain detailed information on the effects of lipid peroxidation products on the orientational order and reorientational dynamics of the membrane molecules, it is necessary to use planar oriented lipid bilayers and to employ techniques with a proper time window (van Ginkel et al., 1989a; van Gurp et al., 1988). Angle-resolved fluorescence depolarization (AFD)[1] measurements and angle-resolved electron spin resonance (A-ESR) measurements on oriented planar multibilayers with embedded reporter molecules satisfy this requirement and are highly informative in identifying changes in the structural and dynamic properties of membranes, as has been shown in a number of papers (Koole et al., 1984; Mulders et al., 1986; Korstanje et al., 1989, 1990; Deinum et al., 1988; van Langen et al. 1989; van Ginkel et al., 1986, 1989a,b).

The advantage of using planar multibilayers lies in the optimal resolution for structural details afforded by a membrane which is macroscopically ordered with respect to the bilayer normal. When the angle of the membrane in the electromagnetic field is varied (polarized light with AFD, the

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[1] Abbreviations: AFD, angle-resolved fluorescence depolarization; A-ESR, angle-resolved electron spin resonance; CSL, 3-doxyl-5-α-cholestane: DLPC, dilinoleoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; PLPC, 1-palmitoyl-2-linoleoylphosphatidylcholine; PLPC-OH, 1-palmitoyl-2-(9/13-hydroxylinoleoyl)phosphatidylcholine; PLPC-OOH, 1-palmitoyl-2-(9/13-hydroperoxylinoleoyl)phosphatidylcholine; SLE, stochastic Liouville equation.

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magnetic field with A-ESR), structural information is obtained in the form of the equilibrium orientational distribution function of the probe molecules. Dynamic information is extracted by studying the fluorescence depolarization in AFD or the details of the absorption line shape in ESR.

The purpose of the present investigation was to determine how phospholipid hydroperoxides and alcohols affect membrane order and dynamics. We used the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) for the AFD measurements and the membrane spin label 3-doxyl-5-α-cholestane (CSL) for the A-ESR measurements. Both probes can be described as rigid cylindrically shaped molecules that report on the overall molecular order and dynamics of the phospholipids. Recognizing that the two probes may be localized differently in the membrane, it may be possible to distinguish localized vs general effects of the oxidized lipids. DPH is proposed to report within the central hydrophobic region of the bilayer whereas CSL is proposed to report closer to the polar head group interface (Korstanje et al., 1989).

EXPERIMENTAL PROCEDURES

1-Palmitoyl-2-linoleoylphosphatidylcholine (PLPC) (Avanti Polar Lipids, Alabaster, AL) was purified by HPLC in order to remove peroxidation products and stored under nitrogen at −20 °C until use. 1-Palmitoyl-2-(9/13-hydroperoxylinoleoyl)phosphatidylcholine (PLPC-OOH) was prepared from PLPC using soybean lipoxidase IV as described previously (Eskola & Laahso, 1983). 1-Palmitoyl-2(9/13-hydroxylinoleoylphosphatidylcholine (PLPC-OH) was prepared from PLPC-OOH by reduction with $NaBH_4$ and subsequent purification by HPLC (Sevanian & Kim, 1985). Dilinoleoylphosphatidylcholine (DLPC) was purchased from Lipid Products (Nutfield, U.K.) and checked for purity by HPTLC (Koole et al., 1984). Macroscopically aligned planar bilayer systems were prepared as described previously (Kooyman et al., 1983; Van de Ven & Levine, 1984; Korstanje et al., 1989), and the alignment was monitored under a polarizing microscope equipped with a first-order red plate. The samples contained approximately 25% water. The molecular probe to lipid ratio was 1:250 for AFD experiments with DPH and 1:100 for A-ESR experiments with CSL. Experiments using CSL to lipid ratios of 1:500 were also performed for comparison with the 1:100 ratios. There were no observable differences in spectral shape or line width between the 1:500 and 1:100 probe to lipid ratios.

All sample preparations were done under an inert atmosphere with low light intensity to prevent background oxidation. Steady-state angle-resolved depolarization (AFD) measurements and angle-resolved electron spin resonance (A-ESR) measurements were performed under similar conditions, as described in previous papers (Mulders et al., 1986; Korstanje et al., 1989). The AFD measurements were carried out with a custom-built fluorometer; the A-ESR experiments were carried out using a Varian E-9 X-band spectrometer equipped with a TM 110 cavity. The experimental details of these techniques have been described previously by Mulders et al. (1986) and Korstanje et al. (1989).

AFD measurements were obtained at 56 different combinations of excitation–emission angles, whereas 4 different angles (0°, 45°, 60°, and 90° with respect to the magnetic field) were adequate for A-ESR. Interpretation of the AFD data required knowledge of the fluorescence decay times of DPH, which are dependent on the molecular environment of the fluorescent probe. These quantities were, therefore, independently obtained from time-resolved fluorescence experiments on planar-oriented multibilayers containing PLPC, PLPC + oxidized lipids, DLPC, and DLPC + oxidized lipids, using the synchrotron facility in Daresbury (U.K.) as a source of flash light (van Langen et al., 1988). Dynamic information from the AFD data is derived in the form of rotational diffusion coefficients, which are calculated by using the weighted-average DPH lifetimes (Mulders et al., 1986; van Langen et al., 1988).

Dynamic information from the ESR data is derived in the form of rotational correlation times. The ESR data were analyzed using an analytical solution of the stochastic Liouville equation (SLE) based on a separable model for the molecular tumbling dynamics (Van Faassen, 1990, 1991).

DESCRIPTION OF THE SPECTROSCOPIC METHODS

Information on both structural organization and molecular dynamics within a lipid bilayer is accessible by angle-resolved fluorescence depolarization as well as electron spin resonance spectroscopy. Both techniques provide only indirect information in that they study the response of fluorescent or paramagnetic probe molecules which have been added in low concentration to the lipid system under study. In AFD, the sample is continuously irradiated with incoherent polarized light, which preferentially excites those fluorescent probe molecules whose absorption moment is aligned more parallel to the electric field component of the incoming light. The excited molecules relax to their ground state with a characteristic fluorescence decay time τ_F by emitting a photon polarized in the plane containing the emission moment. Structural information on the average orientations of the probe in the lipid bilayer is obtained by studying the selectivity of the absorption process under various orientations of the sample. Dynamic information is obtained by measuring the extent of depolarization of the emitted radiation. The latter is caused by reorientation of the emission moment due to molecular motions in the short time interval between the absorption and emission processes. Obviously, this method is sensitive for dynamic processes which induce loss of orientational correlation and for which the characteristic correlation time is of the order of the fluorescence decay time τ_F. The fluorescence decay time of DPH in multiplanar bilayers is approximately 7 ns. The theoretical description of AFD experiments has been elaborated by Mulders et al. (1986) and Deinum et al. (1988). The process of molecular tumbling is described by the so-called Brownian rotational diffusion model. This model incorporates two types of anisotropy which characterize the tumbling motion of the fluorescent probe. The first is motional anisotropy, as expected for a particle with nonspherical shape, where the rotational diffusion around the long molecular axis should be faster than the tumbling of the axis itself. This implies a description with two diffusion rates $D_\parallel > D_\perp$. Second, it incorporates the anisotropy due to the surrounding lipid matrix, which is a reflection of the microscopic order inside the lipid bilayer. This implies a microscopic equilibrium orientation distribution function P_{eq} for the fluorescent probes, which is conveniently parametrized by an ordering potential U:

$$P_{eq}(\Omega) = e^{-U(\Omega)} / \int e^{-U(\Omega)}\, d\Omega \qquad (1)$$

which is normalized to unity under integration over all molecular orientations with respect to the bilayer normal.

This distribution is the unique stationary mode of the Brownian diffusion process. For the average potential U we employ

$$U(\Omega) = U(\alpha,\beta,\gamma) = \lambda_2 P_2(\cos\beta) + \lambda_4 P_4(\cos\beta) \quad (2)$$

which depends only on the Euler angle β between the bilayer normal and the long axis of the molecule. In the Brownian diffusion framework, therefore, the molecular tumbling process is characterized by four parameters λ_2, λ_4, $D_\perp$, and $D_\parallel$. Dependence on the last of these is negligible for situations (as arise with our probe here) where the emission moment is nearly aligned with the long axis of the fluorescent probe. The analysis of the experimental data proceeds with a fitting algorithm for the three remaining parameters based on numerical solution of the Brownian diffusion problem in terms of angular eigenfunctions and their characteristic correlation times. Details of this numerical procedure are given by Freed (1976). Information on the average microscopic probe distribution is obtained in the form of optimal values for λ_2 and λ_4. It is customary, however, to characterize molecular orientational order not in terms of the these parameters or the distribution function itself but rather in the first even weighted averages of the Legendre polynomials:

$$P_2 = (^1/_2)(\cos^2\beta - 1)$$

$$P_4 = (^1/_8)(35\cos^4\beta - 30\cos^2\beta + 3) \quad (3)$$

$$\langle P_n \rangle = \int P_{eq}(\Omega) P_n(\cos\beta)\, d\Omega \quad (n = 2, 4, \ldots) \quad (4)$$

In ESR spectroscopy, one observes radiative transitions between energy levels, the splitting of which is not determined by internal molecular structure, as in AFD, but rather by application of a static external magnetic field. The probes employed are paramagnetic amphiphiles, i.e., stable radicals which embed themselves in the membrane. Under the magnetic field, the unpaired electron of the probe exhibits a Zeeman splitting typically corresponding to microwave radiation in the 10-GHz range (X-band). In contrast to the fluorescence process, the relaxation is nonradiative and leads to detectable absorption of the microwave radiation. For the relevant cases, this Zeeman coupling is anisotropic; i.e., the absorption frequency depends on the molecular orientation with respect to the magnetic field. A variety of orientations of the probes thus lead to broadening of the absorption line shape. Clearly, structural information on preferred orientations of the ESR probe may be obtained by inspecting the relative weights of the various frequencies in the total line shape, i.e., detailed line-shape analysis. Dynamic information may be obtained also. This follows from the observation that, under extremely fast reorientational motion, the Zeeman interaction may be replaced by its orientational average. The latter leads to a single averaged absorption frequency, implying a single, narrow absorption line. Closer inspection reveals that this line-width collapse will occur for reorientation correlation times τ near the inverse line width $\Delta\omega$: $\Delta\omega\tau \sim 1$. For the most common nitroxide spin probes as used in this work, the typical line width is about 5 ns.

In contrast to the previous case of AFD, the theoretical description not only incorporates the stochastic tumbling motion but deterministic time development of the Zeeman Hamiltonian as well. A suitable mathematical framework has been developed in the form of the so-called stochastic Liouville equation (SLE) (Kubo, 1969; Freed, 1976). Its numerical solution has conventionally proceeded via eigenfunction expansions. This method has been seriously compromised by three factors: though a linear equation of motion, its mathematical properties are such that the possibility of an eigenfunction expansion is not guaranteed. Worse, physically plausible counterexamples may be constructed where the eigenfunction method may be shown to be unfeasible. Moreover, even in the case where it may be applied, the eigenvalue algorithm is numerically very involved and time consuming and will become numerically unstable in the limit of very slow molecular motions where the eigenvalues become dense. The experimental ESR spectra were, therefore, analyzed by solving the SLE with a recently developed separable model for stochastic motion rather than the Brownian diffusion mentioned above. Due to its separable character, the ESR line shape may be found in closed analytical form by algebraic means (Van Faassen, 1990). In contrast to the case of AFD, which employs Brownian rotational diffusion rates D, the separable tumbling operator is parametrized in terms of correlation times $\tau_\parallel$ and $\tau_\perp$ for motions around the long molecular axis and of the long axis, respectively. As discussed below, a qualitative comparison with the diffusion model used for AFD may be made by comparing $\tau_\parallel$ and $\tau_\perp$ with the longest correlation times arising in the restricted Brownian diffusion problem.

Aside from this motional anisotropy, the separable operator also accounts for anisotropy of the surrounding lipid matrix. The formulation in terms of an equilibrium orientation distribution function in the bilayer was taken to be identical to the previous case of the AFD experiments. (see eqs 1 and 2).

The magnetic couplings of the nitroxide probes were taken to be a standard Zeeman coupling with hyperfine splitting due to the nitrogen nuclear spin. The diagonal elements of the coupling tensors were taken as g = diag (2.0081, 2.0024, 2.0061) and A = diag (5.3, 33.8, 5.1). The line-shape simulations were simplified by treating the hyperfine coupling in the pseudosecular approximation (van Faassen, 1991). As it turned out, the line-shape dependence of the ratio $\tau_\perp/\tau_\parallel$ was mild, provided the latter did not exceed 10. It was, therefore, kept at 7, leaving three free parameters for fitting: λ_2, λ_4, and $\tau_\perp$. Estimated uncertainties were 10%, 20%, and 20% respectively.

RESULTS

The analysis of the AFD measurements show that with increasing amounts (2–5 mol %) of oxidatively modified phospholipids in the PLPC membranes there is an overall decrease in molecular orientational order as defined by the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ of DPH in the bilayers. In contrast, the reorientational dynamics defined by the diffusion coefficient $D_\perp$ are not affected (see Table I).

This is consistent with the ESR data. Simulation of the ESR spectra, using the algebraic solution of the SLE with the dynamical model of Van Faassen (1990), shows that the presence of 10% of oxidatively modified phospholipids in the PLPC bilayers strongly decreases the molecular orientational order, but does not affect the reorientational dynamics as expressed by the values of the rotational correlation times $\tau_\perp$ in Table II.

The effects of oxidatively modified phospholipids on the structural characteristics of the membrane are clearly demonstrated by the graphic display of the orientational distribution functions described by eqs 1 and 2. Figure 1 illustrates the effects of 10% PLPC-OH or PLPC-OOH in planar PLPC membranes containing CSL (using A-ESR), and Figures 2 and 3 illustrate the effects of 2–5% PLPC-OOH or PLPC-OH in planar membranes containing DPH (using AFD) at

Table I: Structural and Dynamic Parameters Obtained from AFD Measurements on DPH in PLPC and DLPC Bilayers without and with PLPC-OH or PLPC-OOH at 25 °C.[a]

lipid	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$D_\perp$ (ns^{-1})	$(8D_\perp)^{-1}$ (ns)	$\langle \tau_F \rangle$ (ns)
PLPC	0.56 ± 0.03	0.34 ± 0.03	0.04	3.0	7.42
PLPC + 2% PLPC-OH[b]	0.47	0.27	0.04	3.0	7.56
PLPC + 5% PLPC-OH	0.40 ± 0.6	0.22 ± 0.01	0.04	3.0	7.66
PLPC + 2% PLPC-OOH	0.50 ± 0.03	0.28 ± 0.02	0.04	3.0	7.67
PLPC + 5% PLPC-OOH	0.39 ± 0.02	0.19 ± 0.01	0.05	2.5	7.54
DLPC	0.45 ± 0.03	0.25 ± 0.02	0.03	4.2	6.90
DLPC + 2% PLPC-OH	0.23 ± 0.02	0.08 ± 0.02	0.06	2.1	
DLPC + 5% PLPC-OH	0.20 ± 0.01	0.07 ± 0.01	0.06	2.1	
DLPC + 2% PLPC-OOH	0.17 ± 0.01	0.04 ± 0.01	0.05	2.5	
DLPC + 5% PLPC-OOH	0.17 ± 0.01	0.05 ± 0.02	0.04	3.0	6.84

[a] The average fluorescence lifetimes $\langle \tau_F \rangle$ of DPH in the PLPC bilayers are listed as well. [b] Only one measurement.

Table II: A-ESR Measurements on CSL in PLPC Bilayers at Different Temperatures[a]

$\langle P_2 \rangle$	$\langle P_4 \rangle$	$\tau_\perp$ (ns)	temp (°C)	α
		PLPC		
0.62	0.39	30	10	0.05
0.62	0.39	12	25	0.10
0.56	0.32	8–9	35	0.20
0.50	0.28	5	45	0.55
		PLPC + 10% PLPC-OOH		
0.54	0.31	30	10	0.20
0.52	0.29	8	25	0.40
0.52	0.29	8	38	0.45
0.39	0.23	6	45	0.50
		PLPC + 10% PLPC-OH		
na[b]	na	na	10	na
0.45	0.23	10	25	0.40
0.45	0.23	7	35	0.50
0.41	0.20	7	45	0.60

[a] α is the relative weight of the planar contribution to the total absorption spectrum (cf. eq 5). $\tau_\perp$ was taken as $7\tau_\parallel$ throughout the analysis. [b] na, not available.

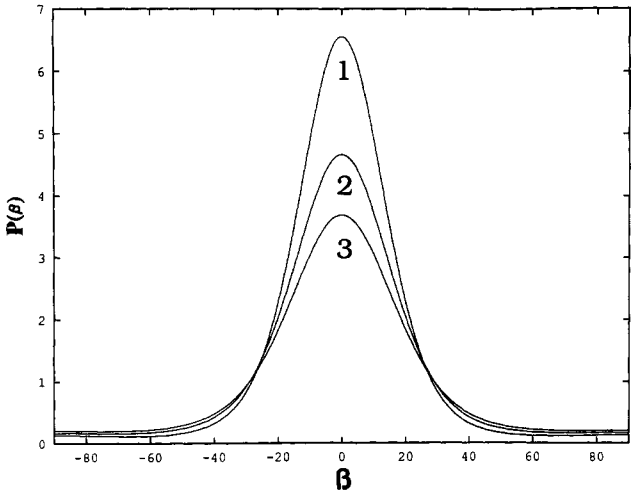

FIGURE 1: Normalized orientational distribution functions $P(\beta)$ of cholestane spin label molecules in planar bilayers of PLPC (1), PLPC + 10% PLPC-OH (2), and PLPC + 10% PLPC-OOH (3) obtained from simulation of ESR spectra measured at 25 °C. The orientational distribution functions were obtained from the calculated $\langle P_2 \rangle$ and $\langle P_4 \rangle$ values using the maximum entropy formalism [see Mulders et al. (1986)].

25 °C. Both techniques showed that addition of oxidatively modified phospholipids caused a significant decrease in the microscopic orientational order of the probes in PLPC bilayers.

Turning to the effects of oxidatively modified phospholipids on molecular dynamics, we recall that the AFD experiments

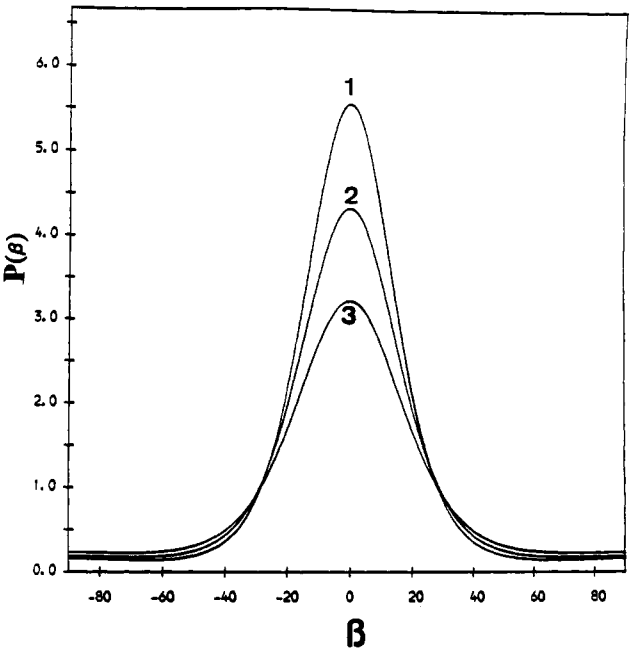

FIGURE 2: Normalized orientational distribution functions $P(\beta)$ of DPH in planar bilayers of PLPC (1), PLPC + 2% PLPC-OOH (2), and PLPC + 5% PLPC-OOH (3) obtained from AFD measurements at 25 °C.

were analyzed using the Brownian diffusion model, whereas the ESR employed the separable dynamics.

The use of two different dynamic models complicates a direct comparison of the dynamic parameters (diffusion rate D for Brownian diffusion vs correlation time $\tau_\perp$ in the separable operator approach). A rough comparison may be made from the observation that $\tau_\perp$ should be comparable to the longest decay time arising in the Brownian diffusion problem. For free diffusion without any external ordering potential (U = 0 in eq 2), this correlation time is analytically known (Nordio, 1976) to be $\tau = 1/(6D_\perp)$. In the presence of an ordering potential, this correlation time is shortened as the reduction of admissible orientations speeds up the decay of the correlation at a given, fixed diffusion rate $D_\perp$. For potentials typically found in phospholipid bilayers, the longest correlation time is approximately $1/(8D_\perp)$. When these quantities are compared with the correlation times obtained from ESR, it should be kept in mind that these experiments employed probes of a different geometry in a different location in the bilayers, for which differences in the dynamical parameters are to be expected.

The values of the rotational diffusion coefficient $D_\perp$ in Table I, obtained from the AFD analyses, were calculated using the weighted-average lifetimes $\langle \tau_F \rangle$ of DPH in the different planar

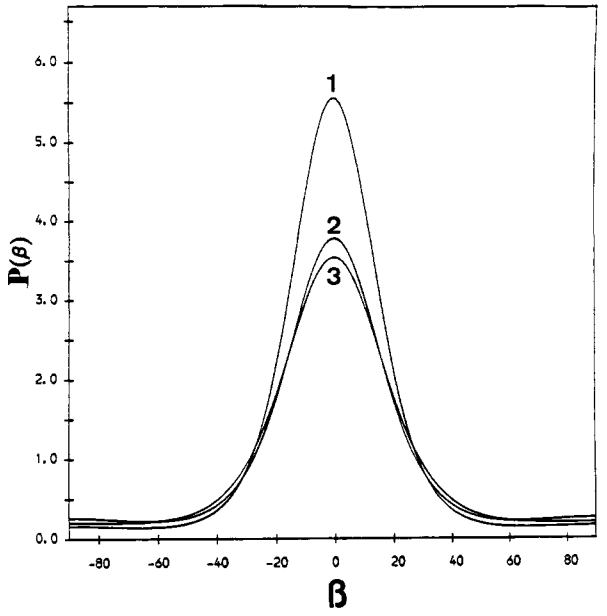

FIGURE 3: Normalized orientational distribution functions $P(\beta)$ of DPH in planar bilayers of PLPC (1), PLPC + 2% PLPC-OH (2), and PLPC + 5% PLPC-OH (3) obtained from AFD measurements at 25 °C.

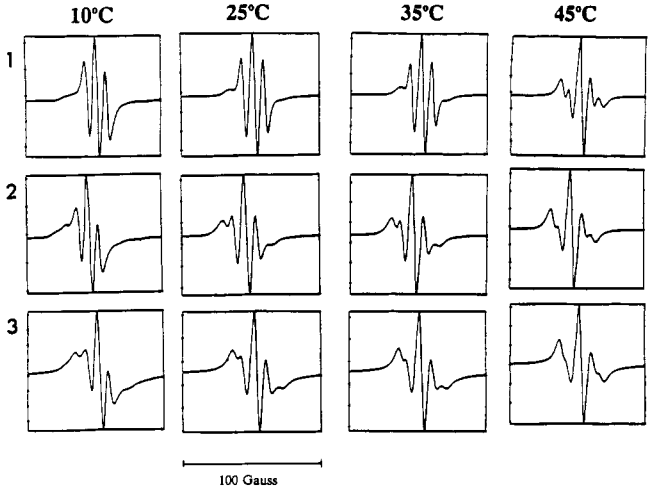

FIGURE 4: X-band ESR spectra of cholestane spin label in planar bilayers of PLPC (1), PLPC + 10% PLPC-OH (2), and PLPC + 10% PLPC-OOH (3) at different temperatures and at 0° sample orientation. The spectra show a field sweep of 100 G.

bilayers shown in Table I. In DLPC bilayers the presence of PLPC-OH or PLPC-OOH causes a significant increase in $D_\perp$ in contrast to PLPC bilayers where $D_\perp$ is not affected.

The A-ESR spectra were measured for the PLPC system in the temperature range of 10–45 °C (Figure 4). All samples were visually inspected with a polarization microscope and found perfectly and homogeneously aligned. At higher temperatures (above 35 °C) all preparations yielded five-line spectra at 0° sample orientation and three-line spectra at all other measured sample orientations. At lower temperatures (10–35 °C) a small extra absorption band outside the three-line spectra was obtained from PLPC membranes at 0° sample orientation. This band showed a marked increase with temperature (Figure 4) and upon addition of PLPC-OH or PLPC-OOH. The changes we observed appear to be related to a physical property of either the oxidized or nonoxidized lipids, rather than a breakdown of the model or poor alignment, as the changes we observed were reversible with temperature. This reversibility also suggests that the appearance of the isotropic component in the PLPC bilayers was not due to oxidation of PLPC during the time course of the experiment, as the isotropic component was not apparent when the sample was cooled from 45 °C to lower temperatures. Upon repeated temperature changes, no traces of temperature hysteresis were detected in any of the measured spectra by using concentrations of 1–10% oxidized phospholipids or by varying the probe ratios of 1:500–1:100 (data not shown). At first we attempted to simulate the ESR spectra by assuming that the lipid bilayers are oriented in a perfectly planar configuration. This approach failed to reproduce the characteristic five-line spectra found at 0° sample orientation.

We therefore simulated these orientation-dependent absorption line shapes $P(\omega|\theta)$ as a geometrical superposition P_g of two components

$$P_g(\omega|\theta) = \alpha P_p(\omega|\theta) + (1-\alpha)P_{iso}(\omega) \quad (5)$$

where P_p is the spectrum of a planar bilayer sample with perfect macroscopic geometrical order of the bilayer. The second contribution P_{iso} is taken as the absorption spectrum resulting from an isotropic superposition of planar spectra

$$P_{iso}(\omega) = \int_{-1}^{1} \frac{d\cos\theta}{2} P_p(\omega|\theta) \quad (6)$$

As $P_{iso}(\omega)$ is independent of the orientation θ of the sample with respect to the magnetic field, all angular dependence enters through the planar contribution P_p in eq 5. In principle, the two contributions P_p and P_{iso} may be computed with different parameters τ for molecular motion and λ for microscopic order. For simplicity we have assumed common values for these, implying that the expression (eq 5) for the absorption line shape introduces a single new parameter α. It is important to stress that a similar superposition was required for a good fit to line shapes from a pure PLPC sample at higher temperatures. The relative weight α of the two contributions was consistently reproducible from sample to sample within a few percent and did not depend on the concentration of the CSL probe. The physical and mathematical treatment of the simulation of such spectra has been described in more detail elsewhere (Van Faassen, 1990, 1991; Eviatar et al., 1992). The above expression for ESR absorption line shapes calls into question whether AFD experiments could similarly identify a component with isotropic geometrical distribution. In this respect it is necessary to clearly distinguish between anisotropy on a microscopic, molecular, level and anisotropy on a macroscopic, geometrical, scale. Letting $P_m(\Omega_m/\Omega_n)$ be the microscopic distribution probability of a molecular orientation Ω_m with respect to a bilayer normal direction Ω_n, and $P_g(\Omega_n)$ be the geometrical distribution of bilayer normals within the sample, an AFD experiment will be able to identify only the total orientation distribution $P_{tot}(\Omega_m/\theta)$ of probe molecules given by

$$P_{tot}(\Omega_m|\theta) \equiv \int P_m(\Omega_m|\Omega_n)P_g(\Omega_n|\theta)\frac{d\Omega_n}{4\Pi} \quad (7)$$

In AFD, the macroscopic and geometrical distributions are inseparably intertwined. In contrast, in an ESR experiment, molecular motions induce partial averaging over P_m but not over P_g. In ESR, therefore, a natural separation of these two distribution functions occurs. Simulation of the ESR spectra with this approach showed that addition of PLPC-OH and PLPC-OOH causes a significant disordering of the lipid bilayers on a microscopic scale (i.e., reduction of $\langle P_2 \rangle$, with minimal or no effect on the reorientational dynamics) (Table

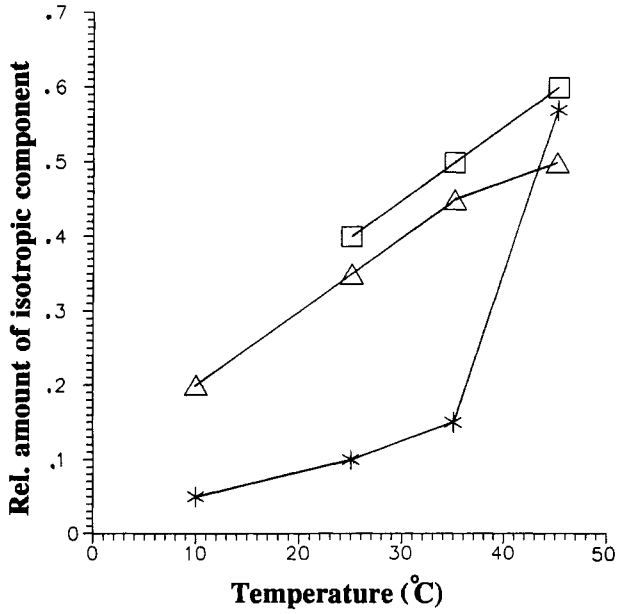

FIGURE 5: Relative amount of isotropic component contributing to the ESR spectra shown in Figure 4 at different temperatures: (*) PLPC, (△) PLPC + 10% PLPC-OH, and (□) PLPC + 10% PLPC-OOH.

II). These findings are in full agreement with the AFD results described above.

DISCUSSION

Both AFD and A-ESR experiments indicate that the accumulation or addition of phospholipid hydroperoxides and/or phospholipid alcohols causes significant structural aberrations, but does not appear to affect the bilayer reorientational dynamics. This is further supported by the fact that the two probes, used in the different experiments, respectively, DPH and CSL, although located differently within the membrane gave similar results with respect to membrane order and dynamics. While there were some differences in the reported order parameters between the two techniques, this probably simply reflects these different membrane locations. CSL is expected to report closer to the polar head groups while DPH is proposed to reside within the hydrophobic fatty acyl region of the bilayer, and the higher values of the order parameters in the former case would seem to be consistent with this.

A possible explanation to account for the structural changes caused by the oxidatively modified phospholipids may be based on the thermodynamic properties of these phospholipids. The addition of the hydroperoxy or the alcohol group to the linoleoyl chain would be expected to occur at the 9- or 13-carbon of the chain (Porter, 1990). The model proposed by van Kuijk et al. (1988) suggests that the hydroperoxy or alcohol moieties reside closer to the phospholipid polar head region due to the more hydrophilic nature of these groups. If the oxidative modification occurs fairly deep into the chain, this could have profound effects on the membrane organization.

Although both the hydroperoxy and the alcohol group of the oxidatively modified phospholipid would be expected to reside near the polar head group regions, there were significant differences produced by these two oxidatively modified phospholipids. Addition of phospholipid hydroperoxide caused the greatest decrease in order as measured by both AFD and A-ESR techniques. This can also be seen qualitatively from Figure 5, in that the contribution of the isotropic components was less for multibilayers containing PLPC-OH that it was for PLPC-OOH. The nature of this isotropic component has not been well characterized. It is not entirely caused by addition of the oxidized phospholipid, as it was also seen at high temperature in the control multibilayers of pure PLPC. In addition, this effect was reversible upon decreasing the temperature. This leads to the suggestion that the effects arise from an intrinsic physical property of the phospholipid that is dependent on temperature.

Several years ago it was proposed by Israelachvili that the physical arrangements of the phospholipids were dependent on the intrinsic "shape" of the molecule (Israelachvili, 1973). Thus if the phospholipid has a cylindrical shape in which the average head diameter is approximately equal to the average tail diameter, the phospholipids will tend to orient in a lamellar bilayer form. If the average tail group diameter is greater than the average acyl chain diameter, the phospholipid will tend to orient in an inverse micellar fashion, whereas if the average head diameter is less than the tail diameter, the phospholipid tends to form a hexagonal II structure. The "shapes" of the phospholipids are dependent on the length of the acyl chain, the type of head group, and the hydration of the tail and head groups. Addition of a hydroperoxy or alcohol moiety to a phospholipid may change the shape of the phospholipid in such a way that it no longer packs correctly in the bilayer. This is proposed for the oxidatively modified 18-carbon linoleoyl chain now residing closer to the polar head groups. The oxidatively modified phospholipid in this case would have one fairly long 16-carbon chain (with no bends from any trans–gauche isomerization) and one rather short bulky chain due to the location of the hydroperoxy or alcohol group. An increase in temperature can change the average acyl chain diameter, which may further augment this shape change. Shape changes due to the effects of temperature and/or the presence of lipid peroxidation products may also influence membrane permeability. In this respect, the observations by different investigators (Chatterjee & Agarwall, 1988; Kunimoto et al., 1981) that lipid peroxidation products in membranes cause increased permeability then become more understandable.

The differences that are seen between multibilayers containing phospholipid hydroperoxide vs multibilayers containing phospholipid alcohols may be due to slightly different location for the oxidized moieties. This may be one of the reasons for the preferential hydrolysis of phospholipid hydroperoxides over phospholipid alcohols or control phospholipids by phospholipase A_2.

There are numerous reports in the literature on the effects of oxidized phospholipids on membranes (Watanabe et al., 1990; Pradham et al., 1990; Gut et al., 1985; Szebeni & Toth, 1986; Galeotti et al., 1984; Ohyashiki et al., 1986; Bruch & Thayer, 1983; Eichenberger et al., 1982). However, direct comparison of the present data on membrane order and dynamics with data previously reported by other groups is difficult in that many of these groups used macroscopically isotropic vesicle systems. The analysis of membrane order and dynamics for such systems is fraught with difficulty, as discussed previously by van Ginkel et al. (1989a).

Studies in other laboratories have purported to show that accumulation of peroxidation products in the membrane caused an increase (Cavatorta et a., 1985; Gut et al., 1985; Eichenberger et al., 1982; Bruch & Thayer, 1983) or no change (Galeotti et al., 1984) in the order parameter, $\langle P_2 \rangle$. Our results do not agree with these findings. A possible explanation for this discrepancy might be that the cited authors used lipid preparations subjected to free-radical generating systems which give rise to a number of primary lipid peroxidations,

as well as secondary decomposition products. In general, these authors also used more extensive peroxidation than we employed.

Future research still must address the interesting question as to how the structural changes might affect the lipid peroxidation process.

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