Cholesterol dynamics in membranes

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ABSTRACT Time-resolved fluorescence anisotropy of the sterol analogue, cholestatrienol, and ^{13}C nuclear magnetic resonance (NMR) spin lattice relaxation time (T_{1c}) measurements of [$^{13}C_4$] labeled cholesterol were exploited to determine the correlation times characterizing the major modes of motion of cholesterol in unsonicated phospholipid multilamellar liposomes. Two modes of motion were found to be important: (*a*) rotational diffusion and (*b*) time dependence of the orientation of the director for axial diffusion, or "wobble." From the time-resolved fluorescence anisotropy decays of cholestatrienol in egg phosphatidylcholine (PC) bilayers, a value for $\tau_\perp$, the correlation time for wobble, of 0.9×10^{-9} s and a value for $S_\perp$, the order parameter characterizing the same motion, of 0.45 s were calculated. Both $\tau_\perp$ and $S_\perp$ were relatively insensitive to temperature and cholesterol content of the membranes. The T_{1c} measurements of [$^{13}C_4$] labeled cholesterol did not provide a quantitative determination of $\tau_\parallel$, the correlation time for axial diffusion. T_{1c} from the lipid hydrocarbon chains suggested a value for $\tau_\perp$ similar to that for cholesterol. Steady-state anisotropy measurements and time-resolved anisotropy measurements of cholestatrienol were used to probe sterol behavior in a variety of pure and mixed lipid multilamellar liposomes. Both the lipid headgroups and the lipid hydrocarbons chains contributed to the determination of the sterol environment in the membrane, as revealed by these fluorescence measurements. In particular, effects of the phosphatidylethanolamine (PE) headgroup and of multiple unsaturation in the lipid hydrocarbon chains were observed. However, while the steady-state anisotropy was sensitive to these factors, the time-resolved fluorescence analysis indicated that $\tau_\perp$ was not strongly affected by the lipid composition of the membrane. $S_\perp$ may be increased by the presence of PE. Both steady-state anisotropy measurements and time-resolved anisotropy measurements of cholestatrienol were used to probe sterol behavior in three biological membranes: bovine rod outer segment (ROS) disk membranes, human erythrocyte plasma membranes, and light rabbit muscle sarcoplasmic reticulum membranes. In the ROS disk membranes the value for $S_\perp$ was marginally higher than in the PC membranes, perhaps reflecting the influence of PE. The dramatic difference noted was in the value for $\tau_\perp$. In both the ROS disk membranes and the erythrocyte membranes, $\tau_\perp$ was one-third to one-fifth of $\tau_\perp$ in the phospholipid bilayers. This result may reveal an influence of membrane proteins on sterol behavior.

INTRODUCTION

Membrane dynamics is as important to understand as membrane structure in modeling molecular features of membrane function. Accurate determination and assignment of correlation times to the modes of motion of lipids in membranes is essential for a complete description of the dynamics of membrane structure (Yeagle, 1987).

For phospholipid hydrocarbon chains, an elegant picture of their motion has been described through extensive studies of deuterium relaxation parameters (Meier et al., 1986). For cholesterol, however, the situation has been less clear, in part because of a lack of adequate methods to directly probe sterol behavior. ^{13}C nuclear magnetic resonance (NMR) studies of [$^{13}C_4$] labeled cholesterol suggested a correlation time for axial diffusion of ~0.1 ns in sonicated egg phosphatidylcholine (PC) vesicles (Yeagle, 1981; Brainard and Szabo, 1981). However, ^2H NMR studies suggested a correlation time substantially longer (~3 ns) for this same motional mode (Dufourc and Smith, 1986). Both of these analyses were model dependent; the first in terms of the calculation of the correlation time and the second in terms of the assignment of the correlation time derived from the temperature dependence of T_{1z}. Another study reported a correlation time of 0.42 ns for cholesterol in *N*-palmitoylgalactosylsphingosine at 69°C using ^2H NMR relaxation data (Siminovitch et al., 1988). Therefore, resolution of the details of cholesterol motion required an independent approach that would not be as model dependent.

The ability to more clearly describe cholesterol behavior derives from the introduction of a minimally-perturbing probe of cholesterol behavior, cholestatrienol (Fig. 1).

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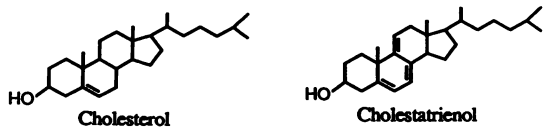

FIGURE 1 Chemical structure of cholestatrienol.

This is different from cholesterol only to the extent of the introduction of two additional double bonds into the ring system. Cholestatrienol can be incorporated into the plasma membranes of LM fibroblasts up to a level equivalent to 90% of the total sterol without effects on cell growth or on the activity of the Na^+K^+ATPase (Schroeder et al., 1985). The fundamental optical properties of this probe have been described previously (Smutzer et al., 1986). The use of this probe to study sterol behavior in membranes has been reviewed (Yeagle, 1989).

The transition dipole for this probe is nearly parallel to the long axis of the molecule (Smutzer et al., 1986). As a consequence, fluorescence depolarization is determined by wobble of the director, and is independent of rotational diffusion about the long axis of the sterol. Therefore, time-resolved analysis of fluorescence anisotropy decays provides unambiguous information about the wobble of the sterol; in particular, we have obtained the order parameter, $S_\perp$, and the correlation time, $\tau_\perp$, relevant to sterol wobble in the lipid bilayer.

MATERIALS AND METHODS

[$^{13}C_4$]-labeled cholesterol (90% enriched in ^{13}C) was obtained from E. Merck (Darmstadt, FRG). Phospholipids were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Cholestatrienol was synthesized and purified by HPLC as described previously (Smutzer et al., 1986). Retinas was obtained from J. Lawson, Inc. (Lincoln, NE). Digitonin was obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of phospholipid liposomes

Phospholipids dissolved in organic solvent (chloroform or chloroform/methanol) was mixed with ^{13}C labeled cholesterol as the only cholesterol, or cholestatrienol, with cholesterol added to the desired mole percent. The fluorescence probes were included at 0.5 mol %. The mixture was dried to a film under a stream of nitrogen and subsequently under vacuum for 1 h. The lipid was suspended in 1 mM EDTA, 10 mM histidine, pH 7. The samples for the fluorescence measurements contained in addition 100 mM NaCl. For the fluorescence measurements, the lipid was suspended at 0.5 mg/ml by vortexing. As a fluorescence blank identical samples were prepared without fluorescence probe. For the NMR measurements, the lipid was suspended at 100 mg/ml.

Preparation of human erythrocyte ghosts

Fresh human red blood cells were used. White or slightly pink erythrocyte ghosts were obtained by established procedures (Dodge et al., 1963).

Preparation of light sarcoplasmic reticulum

Rabbit muscle light sarcoplasmic reticulum was prepared as described previously (Selinsky and Yeagle, 1984).

Preparation of bovine rod outer segment (ROS) disk membranes

Retinal ROS disk membranes were prepared from frozen bovine retinas (J. Lawson, Inc.) as described by Smith et al. (1975). All manipulations of the ROS and disk membranes were performed under a Kodak 1A red filter. The isolated disks were washed and resuspended in 10 mM Hepes, 1 mM EDTA, 0.1 mM DTT pH 7.0 (buffer A) to a final rhodopsin concentration of 4–6 mg/ml. The buffers used were made 1 mM in EDTA, 0.5 mM in DTT, and perfused with nitrogen or argon to reduce lipid oxidation (Stone et al., 1979).

Digitonin-treated ROS disk membranes

Digitonin was purchased from Sigma Chemical Co. and recrystallized according to the method of Bridges (1977). Stock solutions of digitonin were made 50–100 mg/ml in 90% EtOH. To completely solubilize the digitonin, this solution was gently heated. The stock digitonin solution was stored at 10°C. The digitonin was added (1:1 mole ratio with cholesterol) from the stock solution to freshly prepared disks. Control disks were treated with an equal volume of 90% EtOH. Both disk preparations were incubated at 5°C for 1 h.

Modulation of the cholesterol content of ROS disk membranes

The procedure used is essentially that described previously (Yeagle, 1983). Equimolar phospholipid concentrations of small, sonicated unilamellar vesicle (SUV) (PC/

cholesterol or PC SUV) and membranes were incubated together in a shaking water bath at 37°C for varying lengths of time. The biological membranes were then separated from the SUV by centrifugation at 30,000 *g* for 20 min. The pellet was washed twice with 10 mM Hepes pH 7.0. The phospholipid/protein ratio was determined in the cholesterol-modified membranes as a control for sticking or fusion of the PC SUV to the biological membranes. In most cases, no sticking or fusion was observed. In the rare case of an increase in this ratio, the material was discarded. After isolation of the biological membranes, the cholesterol/phospholipid ratio was also determined.

Preparation of ROS disk lipids

Disk lipids were obtained using ConA Sepharose 4B chromatography as described previously by Albert et al. (1984). The isolated disk lipids were dialyzed against a 1,000-fold excess of 10 mM Hepes, 1 mM EDTA, 0.5 mM DTT pH 7.0 for 24–36 h at 4°C to remove the octylglucoside. The disk lipids were Folch extracted (Folch et al., 1957), dried down under a stream of nitrogen, and cosolubilized with cholestatrienol in acetonitrile/methanol (95:5). This suspension was dried down and place under a vacuum for 2–6 h. The lipids containing the cholestatrienol were resuspended in 10 mM Hepes pH 7.0, forming multilamellar vesicles. The amount of cholestatrienol added was 0.1–0.3 mol %.

Introduction of cholestatrienol into biological membranes

Small unilamellar PC (PC-SUVs) and PC-cholesterol vesicles were formed by sonication. Two different types of cholestatrienol donor vesicles were used, PC/cholestatrienol, and PC/cholesterol/cholestatrienol vesicles. In the cholesterol-containing vesicles, the cholesterol content of the donor vesicles matched the cholesterol content of the acceptor membranes. The lipids were cosolubilized in acetonitrile/methanol (95:5), dried under a stream of nitrogen, and lyophilized for 2–6 h. The lipids were then hydrated with 10 mM Hepes pH 7.0 to an aqueous suspension. The suspension was sonicated four times (5 min each) using a 350 Sonifier (Branson Sonic Power Co., Danbury, CT). The large phospholipid vesicles and the multilamellar species were removed by centrifugation at 45,000 *g* for 90 min in a rotor (Ti-50; Beckman Instruments, Inc.). The biological membranes were then incubated with these cholestatrienol-containing vesicles for 24 h under nitrogen at 22°C and the fluorescent sterol was incorporated by exchange into the biological membranes. Control membrane preparations were incubated with donor vesicles containing no cholestatrienol. In the case of the erythrocytes only, the donor vesicles containing the fluorescent sterol were incubated with whole, fresh cells. After incubation, ghosts were made of the whole cells. The ghosts retained the fluorescent sterol.

Labeling of lipid extracts of the biological membranes with cholestatrienol

Incorporation of cholestatrienol into bilayers of lipid extracts of the biological membranes was achieved by incubation of the biological membranes (or red cells) with the donor vesicles containing cholestatrienol, as described above. The lipid extracts of the light sarcoplasmic reticulum (LSR) and erythrocyte ghost membranes were then obtained using the procedure of Folch et al. (1957). The extraction procedure for the disk membranes was described above. These lipid extracts retained the fluorescent sterol.

Steady-state fluorescence measurements

Steady-state fluorescence measurements were obtained on a spectrofluorimeter (L55; Perkin-Elmer Corp., Norwalk, CT) equipped with polarizers on both the excitation and emission sides. Some samples were also checked on a Specs spectrofluorimeter equipped with polarizers to ensure accurate, repoducible values for r_{ss}. Fluorescence anisotropy was calculated according to the time-independent version of Eq. 1 below (Lakowicz, 1983). Multiple measurements were made on each sample and more than one independent sample for each condition reported was analyzed.

Time-resolved fluorescence measurements

Time-resolved fluorescence anisotropy measurements were carried out on a flashlamp-based system from PRA (London, Ontario, Canada). Excitation was provided by a thyraton-gated flashlamp at an output of 6 kV and wavelength was selected using a monochrometer. Emission was selected with a 3-mm KV 370 Schott filter (high pass, 50% transmittance at 370 nm). A lamp profile was obtained before and after each anisotropy decay with glycogen solution. Usually 20,000 or more counts were collected in the maximum emission channel. Temperature was regulated by means of a water bath.

The decay of the polarized components, $I_{vv}(t)$ and $I_{vh}(t)$ were collected along with the excitation profiles for each sample. The anisotropy decay was calculated

according to:

$$r(t) = \frac{I_{vv}(t) - QI_{vh}(t)}{I_{vv}(t) + 2QI_{vh}(t)}, \quad (1)$$

where I is the intensity, v represents the excitation or emission polarizer in the vertical position, respectively, h represents the excitation or emission polarizer in the horizontal position, respectively, and Q is the correction factor ($Q = I_{hv}/I_{hh}$).

The anisotropy decay data were fit to a simple exponential decay expression (Lakowicz, 1983):

$$r(t) = (r_0 - r_\infty)e^{-t/\tau_\perp} + r_\infty. \quad (2)$$

In practice this analysis proved satisfactory. No more complicated exponential expression was necessary to adequately describe the experimental data. The correlation time obtained from this analysis, $\tau_\perp$, corresponds to the wobble.

The order parameter, $S_\perp$, characterizing the wobble is readily calculated from the residual anisotropy, r_∞, of the anisotropy decay. In ordered systems, the anisotropy does not decay to zero, but to a finite value. The larger the value of r_∞, the more ordered the sterol. Quantitatively:

$$S = \left\{\frac{r_\infty}{r_0}\right\}^{1/2}, \quad (3)$$

where S is the order parameter. r_0 is the limiting anisotropy, determined to be 0.385 (Fischer et al., 1985).

^{13}C NMR

The ^{13}C NMR measurements were obtained on a spectrometer (NT-150; Nicolet Instrument Corp., Madison, WI) at a carbon frequency of 37.735 MHz with a homebuilt magic angle spinning (MAS) unit. The decoupling field was 45 kHz. The spinner system is a modified version of Wind et al. (1983), with a sample volume of 0.3 ml. The samples were spun at 3,200 rps. 4 K data points were collected with a spectral width of 20 kHz and an acquisition time of 104 ms. Chemical shifts are relative to external tetramethylsilane, using the signal from the silicone rubber plug as an internal intermediate standard. The lipid was introduced into a Teflon sleeve and a silicone rubber plug was inserted into the sleeve, which was then pressed into the Kel-F rotor body. Nonviscous, air-sensitive liquids have been successfully spun with this arrangement with no liquid leaking out and no air leaking in. The rotor was spun with dry nitrogen gas which had passed through a heat-exchange coil immersed in an ice bath. The sample was maintained at 20°C.

Longitudinal relaxation data were collected with a modified inversion-recovery pulse sequence (Freeman and Hill, 1971) that results in peak intensities decaying exponentially to zero with increasing delay time. The experiment was modified to incorporate a composite π pluse (Levitt and Freeman, 1979) and proton decoupling was gated on during acquisition. The cross-polarization version of this approach was used because it suppresses contributions from the Teflon and Kel-F rotor materials. Delay times (12–20 values) were sequenced randomly and varied from 1 ms to 5 s. The peak height vs. delay time data were fitted with a nonlinear, least squares routine in the Nicolet 1180 NMR program (DEXPN), and selected sets were also treated graphically to verify the fit. The T_1 values are reliable to ~10%, estimated from replicate experiments and scatter within each experiment. The first and last delay times were both 1 ms to check for drift in spectrometer performance or change in the sample. No significant changes were detected.

THEORY

Motions important to fluorescence anisotropy decay of cholestatrienol in lipid bilayers

To derive meaningful information from the fluorescence anisotropy decay of cholestatrienol in phospholipid bilayers, the most likely contributions to that decay must be known. Motions of the fluorophore that were to be important to the anisotropy decay must be characterized by correlation times near to, or shorter than, the lifetime of the excited state of the fluorophore. Motions with much longer correlation times would not contribute because the excited state would have already decayed. Because the fluorescence lifetime of cholestatrienol was in the nanosecond range, only relatively rapid motions needed to be considered.

For a lipid in a bilayer, three types of motion were most likely to contribute to rapid reorientation of the molecule or portion of the molecule (Brainard and Szabo, 1981). They were rotational diffusion about an axis perpendicular to the membrane surface, time-dependent reorientation of the director for axial diffusion (wobble), and segmental motion within the molecule due to *trans-gauche* isomerizations about molecular bonds.

For the fluorescent sterols (and cholesterol), the number of likely motions could be reduced. The fluorophore was in the *B* and *C* rings of the fused ring system of the sterol. This fused ring system was essentially incapable of the rapid *trans-gauche* isomerizations that characterize the carbon–carbon single bonds along the hydrocarbon chains of phospholipids. Therefore segmental motions, consisting of these rapid *trans-gauche* isomerizations, could be ruled out as significant motional modes for cholestatrienol.

Recently the orientation of the transition dipole for cholestatrienol was discussed in the context of the work of Hudson et al. (1982). It was concluded that the transition dipole was oriented nearly parallel to the long axis of the sterol (Smutzer et al., 1986). Therefore axial diffusion, with the director oriented perpendicular to the membrane surface, was incapable of significantly contributing to the decay of the fluorescence anisotropy because axial diffusion involved no change in the orientation of the transition dipole.

The above considerations suggested that only one motion of cholestatrienol is capable of significantly contributing to the decay of the fluorescence anisotropy in lipid bilayers. Time-dependent reorientation of the director for axial diffusion, or wobble as it will be referred to in the remainder of this presentation, should be the major contributor to the decay of fluorescence anisotropy in the systems to be described here. Energy transfer was not expected to be a major contributor to the anisotropy decay because the probe was used at the 0.5 mol % level or less.

Motions important to spin lattice relaxation of carbon-13 labeled cholesterol

^{13}C labeled cholesterol used in these studies was enriched at position 4, in the A ring of cholesterol. Therefore, since the labeled carbon was part of the fused ring system of the sterol, segmental motion was not likely to be important to spin lattice relaxation of the carbon at position 4.

However, both axial diffusion and wobble were expected to contribute to spin lattice relaxation of the carbon at position 4. Axial diffusion and wobble could be simultaneously modeled (Brainard and Szabo, 1981), but it is difficult theoretically to uniquely deconvolute correlation times for axial diffusion and wobble from spin lattice relaxation measurements.

RESULTS

Steady-state fluorescence studies of cholestatrienol in membranes

The steady-state fluorescence anisotropy, r_{ss}, of cholestatrienol in a variety of lipid bilayers was determined at 22°C and the results of those experiments were summarized in Table 1. The temperature dependence of r_{ss} in all these systems was measured. Fig. 2 shows the temperature dependence of cholestatrienol r_{ss} in several of these systems. The data in Fig. 2 showed either no dependence of r_{ss} upon temperature or a modest decrease with increasing temperature. The same result was obtained for all the other systems listed in Table 1.

TABLE 1 Values of steady-state anisotropy for cholestatrienol in lipid membrane systems

System	r_{ss}
By phospholipid class:	
66% TPE/Egg PC	0.13 ± 0.01
Egg PC	0.19 ± 0.01
Bovine phosphatidylserine	0.20 ± 0.02
Egg phosphatidylglycerol	0.21 ± 0.02
Egg PC/Bovine phosphatidylinositol (PI)	0.21 ± 0.02
16:0,18:1 phosphatidate (PA)	0.22 ± 0.02
Egg sphingomyelin	0.23 ± 0.02
Egg PA	0.25 ± 0.02
Bovine PI	0.26 ± 0.02
By hydrocarbon chains on PC:	
Dimyristoylphosphatidylcholine	0.25 ± 0.02
Egg PC	0.19 ± 0.01
16:0,18:1 PC	0.18 ± 0.02
16:0,20:4 PC	0.22 ± 0.02
20:4,20:4 PC	0.23 ± 0.02

However, the data in Table 1 did show a dependence of r_{ss} upon lipid composition, both with respect to lipid headgroup structure and with respect to hydrocarbon chain composition. For example, the value of r_{ss} appeared to be reduced by the presence of phosphatidylethanolamine (PE). Therefore, a series of experiments were performed at increasing transphosphatidylated phosphatidylethanolamine (TPE) concentrations in egg PC. The TPE was transphosphatidylated from egg PC so that the hydrocarbon chain composition was the same for both the PC and the TPE. Fig. 3 shows the dependence of r_{ss} on TPE content of these bilayers. A monotonic decrease in r_{ss} was observed with increasing PE.

The sensitivity of the fluorescence technique allows the extension of the approach using r_{ss} from cholestatrienol to the study of sterols in biological membranes. In Table 2, we report r_{ss} data from cholestatrienol in ROS disk

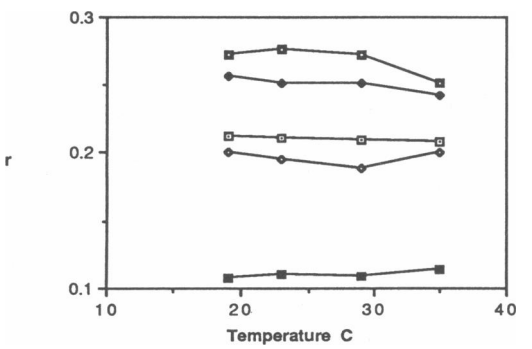

FIGURE 2 Steady-state anisotropy, r_{ss}, for cholestatrienol at 0.5 mol % in phospholipid bilayers as a function of temperature (°C): □, egg PA; ♦, egg sphingomyelin; ⊡, egg PG; ◊, bovine PS; ■, TPE/egg PC (2:1).

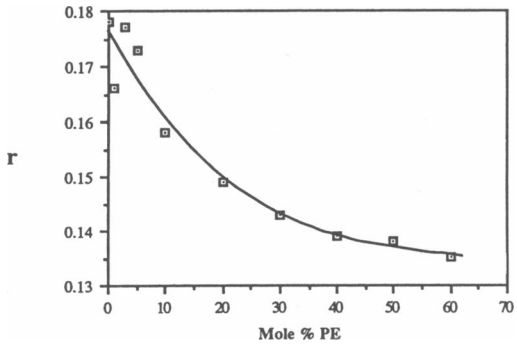

FIGURE 3 Steady-state anisotropy, r_{ss}, for cholestatrienol at 0.5 mol % in TPE/egg PC as a function of TPE content.

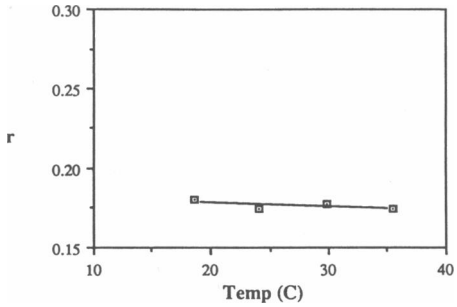

FIGURE 4 Steady-state anisotropy, r_{ss}, for cholestatrienol at 0.2 mol % in LSR membranes as a function of temperature.

membranes, human erythrocyte membranes, and light sarcoplasmic reticulum membranes. Values for r_{ss} in both the biological membranes and the lipid extracts of those membranes are listed. The values for r_{ss} are distinctly different from one membrane to the next. However, the range observed for r_{ss} from the biological membranes was contained within the range observed for r_{ss} from the lipid bilayers listed in Table 1.

As shown in Fig. 4, r_{ss} was essentially independent of temperature for cholestatrienol in LSR. The same result was obtained for the erythrocyte ghosts and the ROS disks (data not shown).

r_{ss} was also determined in the ROS disk membrane as a function of cholesterol content. As in the model systems, no strong dependence of r_{ss} upon cholesterol content was observed.

Steady-state anisotropy measurements were not readily interpretable in terms of molecular motion because r_{ss} was constituted of a complex formulation of molecular dynamic parameters and fluorescent lifetime. It has been proposed (Heyn, 1979, van Blitterswijk et al., 1981) that r_{ss} can be described by the following equation:

$$r_{ss} = r_\infty + \frac{r_0 - r_\infty}{1 + \frac{\tau}{\tau_\perp}}.$$

TABLE 2 Values of steady-state anisotropy for biological membrane systems

	System	r_{ss}
Light sarcoplasmic reticulum	Biological membrane	0.19 ± 0.01
	Lipid extract	0.21 ± 0.02
Human erythrocyte ghosts	Biological membrane	0.25 ± 0.01
	Lipid extract	0.29 ± 0.02
Bovine ROS disks	Biological membrane	0.15 ± 0.01
	Extract	0.18 ± 0.01
	Digitonin-treated	0.25 ± 0.01

Thus r_{ss} was not a direct measure of $S_\perp$. Changes in either of those parameters or in the fluorescent lifetime could have led to a change in r_{ss}.

Time-resolved analysis of the anisotropy decay of cholestatrienol offered a means to obtain specific information about molecular dynamics, as described in Theory. Therefore, a study of the time-resolved fluorescence anisotropy decay for cholestatrienol in the membranes investigated above was initiated.

The multilamellar membrane system consisting of egg PC, cholesterol, and cholestatrienol was studied as a function of temperature and as a function of cholesterol content. The following parameters were measured or calculated: r_{ss}; fluorescence lifetime (τ); time constant for the anisotropy decay, Φ (which in this case is a measure of $\tau_\perp$); and order parameter $S_\perp$. These data are presented in Figs. 5 and 6. These data are quantitatively similar to values for 1 mol % cholestatrienol in 1-palmitoyl-2-oleoyl-PC vesicles (Schroeder et al., 1988).

To better understand the changes induced by PE seen in Fig. 3, time-resolved anisotropy measurements of cholestatrienol in TPE/PC (2:1 mol ratio) multilamellar liposomes were obtained. The fluorescence lifetime was determined to be monoexponential with a value of 0.9 ns. $\tau_\perp$, as determined from the value of Φ derived from the anisotropy decay, was 1.9 ns. $S_\perp$ for the TPE-rich membranes was 0.6.

Dynamic measurements were also obtained from cholestatrienol in biological membranes used in this study. The results are shown in Table 3.

The order parameter, $S_\perp$, for cholestatrienol in the ROS disk membranes, the erythrocyte ghosts, and in the LSR membranes, may have been on the high side of the range of $S_\perp$ in the model systems with PC, but similar to $S_\perp$ for the TPE-rich lipid bilayers just described.

The highest value for the order parameter, $S_\perp$, was found for digitonin-treated ROS disk membranes. This result was expected because digitonin complexes with cholesterol. Formation of a cholesterol-digitonin complex

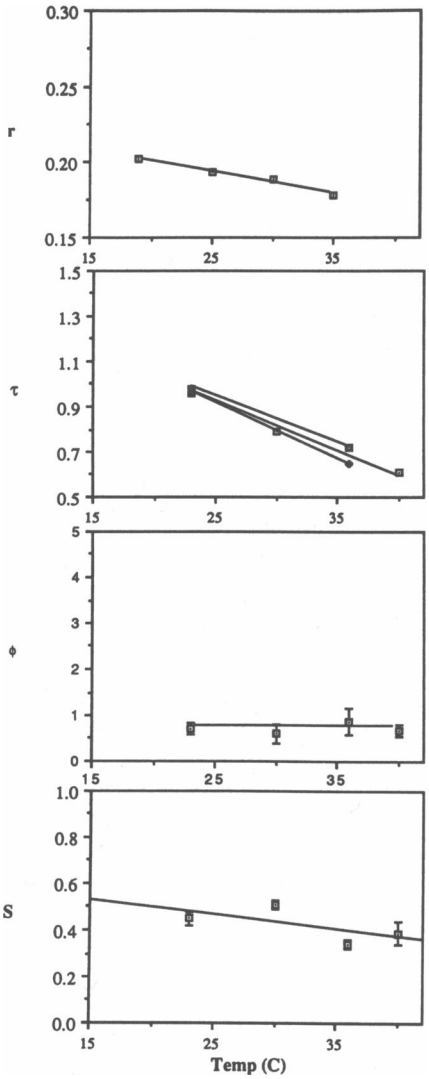

FIGURE 5 Steady-state and time-resolved fluorescence data for cholestatrienol in egg PC multilamellar liposomes at 15% cholesterol as a function of temperature. The solid lines represent a linear least squares best fit to the data and do not have theoretical significance.

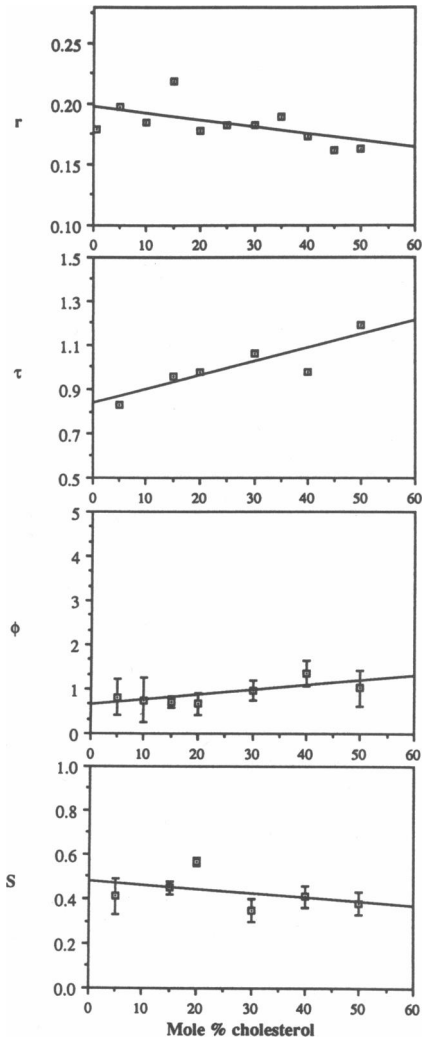

FIGURE 6 Steady-state and time-resolved fluorescence data for cholestatrienol in egg PC multilamellar liposomes at 23°C as a function of cholesterol content. The solid lines represent a linear least squares best fit to the data and do not have theoretical significance.

would be expected to damp the wobble of the sterol, leading to an increase in $S_\perp$ characterizing the wobble.

Interestingly, in two of the biological membranes (the erythrocyte ghosts and ROS disks), $\tau_\perp$ was significantly shorter than in the LSR membrane or in the model membrane systems. To determine whether this difference in $\tau_\perp$ was due to the lipid content of the erythrocyte ghosts and the ROS disk membranes, lipid extracts containing cholestatrienol (prepared as described in Methods) were examined. The values for $\tau_\perp$ determined from cholestatrienol in bilayers made from lipid extracts of these biological membranes appear in Table 3. These values for $\tau_\perp$ in the lipid extracts were in the same range as $\tau_\perp$ for the LSR membrane and the model membrane systems (near 1 ns). These data from Table 3 suggested that the dynamics of sterols in the ROS disk membrane and the erythrocyte membrane were significantly different than in the phospholipid membrane systems or in the LSR membrane.

Cross polarization magic angle spinning (CPMAS) ^{13}CNMR of labeled [$^{13}C_4$] cholesterol

The spin lattice relaxation time, T_{1c}, was determined for the $^{13}C_4$ resonance from cholesterol in PC multilamellar liposomes using MAS. High resolution resonances were

TABLE 3 Parameters from time-resolved fluorescence anisotropy for biological membrane systems

	System	ø	r_∞	$S_\perp$
		ns		
Bovine ROS disks	Biological membrane	0.3 ± 0.1	0.12 ± 0.02	0.57
	Digitonin-treated	0.3 ± 0.1	0.17 ± 0.02	0.65
	Lipid extract in bilayers	1.4 ± 0.2	0.15 ± 0.02	0.61
Human erythrocyte ghosts	Biological membrane	0.3 ± 0.1	0.10 ± 0.01	0.50
	Lipid extract in bilayers	1.0 ± 0.3	0.15 ± 0.03	0.60
Light sarcoplasmic reticulum	Biological membrane	1.3 ± 0.3	0.09 ± 0.02	0.49
	Lipid extract in bilayers	0.9 ± 0.3	0.13 ± 0.03	0.58

seen from this fully hydrated multilamellar suspension, using the CPMAS technique, similar to previously reported data (Sefcik et al., 1983; Frye and Yeagle, 1987). The C_4 T_{1c} as well as T_{1c} for some of the natural abundance carbon resonances from the lipids appear in Table 4. These values are similar to the values reported previously for the same resonances in sonicated egg PC vesicles (Yeagle, 1981). In particular, the values for T_{1c} of C_4 cholesterol, midchain methylenes of the lipid hydrocarbon chains, choline β methylene, and choline α methylene are identical in the sonicated unilamellar and unsonicated multilamellar systems. The comparison is feasible because the frequencies of the measurement (37.7 MHz here and 50.3 MHz [Yeagle, 1981]) are similar and are relatively low field, reducing possible contributions from a chemical shift anisotropy mechanism to the spin lattice relaxation. The lack of significant differences between the T_{1c} for lipid resonances in sonicated and unsonicated phospholipid membranes mirrored the ^{31}P T_1 results for phospholipid phosphates, where the values in both systems were similar (Yeagle et al., 1977). Therefore, despite potential packing differences in the small unilamellar vesicles and the large multilamellar liposomes, the molecular dynamics on the MHz timescale were similar (as sensed by ^{13}C NMR spin lattice relaxation) in the two membrane systems.

Fig. 7 shows a decrease of the T_1 for the $^{13}C_4$ of cholesterol with increase in cholesterol content of the unsonicated, multilamellar PC liposomes. The same results were obtained previously in sonicated vesicles (Yeagle, 1981). Data were not obtained as a function of temperature in the present study because of the difficulty in accurately regulating the sample temperature in the MAS probe. T_1 for C_4 of cholesterol was observed previously in sonicated vesicles to increase modestly with increase in temperature (Yeagle, 1981).

The phospholipid carbons with long T_{1c}'s showed a significant decrease with increasing cholesterol content (Fig. 8). This may reflect an increase in the correlation times for intramolecular rotations: because a positive NOE was observed for these carbons in the sonicated vesicles (Yeagle, 1981), the relevant (to spin lattice relaxation) correlation times must be in the "fast motion" regime ($\omega_o\tau_c < 1$). Therefore an increase in the relevant correlation time would be expected to lead to a decrease in the observed T_{1c}.

The T_{1c} for the lipid chain methylenes was substantially shorter at low cholesterol (than T_{1c} for the resonances just discussed), and as cholesterol is increased, this T_{1c} appeared to decrease modestly and then perhaps even to increase again. The correlation time for the midchain methylenes may be longer at low cholesterol content than for the terminal methylenes and the choline *N*-methyls. As that correlation time increased with cholesterol content, the spin lattice relaxation, T_{1c}, passed through a

TABLE 4 T_{1c} for C_4 of cholesterol and for lipid carbons at 20°C and 5 mol percent cholesterol

Carbon	T_1 (*ms*)*	NT_1 (*ms*)
Cholesterol C_4	130	260
Lipid chain $-CH_2-CH_3$	2,000	4,000
Lipid chain $-CH_2-$	635	1,270
Choline $-{}^+N-(CH_3)_3$	927	2,781
Choline $-CH_2-O-P-$	443	886
Choline $-CH_2-N^+$	523	1,046
Glycerol 2'	158	158

*±10%.

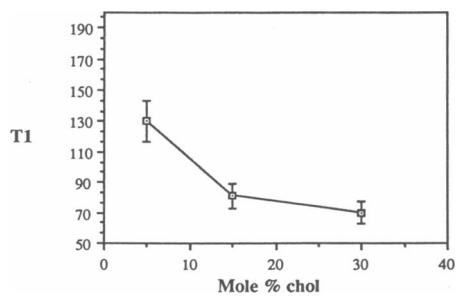

FIGURE 7 T_{1c} for [$^{13}C_4$] labeled cholesterol at 19°C in multilamellar liposomes of egg PC as a function of cholesterol content.

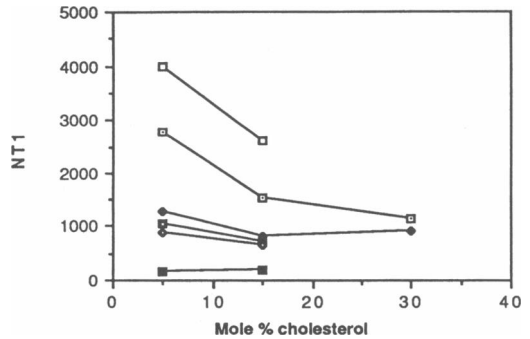

FIGURE 8 NT_{1c} for some of the phospholipid resonances in egg PC multilamellar liposomes as a function of cholesterol content. □, penultimate methylene of hydrocarbon chains; ⊡, N-methyl; ♦, hydrocarbon chain methylene envelope; ◘, choline -CH_2-N^+; ◊, choline -CH_2-O-P-; ■, glycerol 2′.

minimum ($\omega_0\tau_c = 1$). If this analysis is correct, a correlation time in the nanosecond range may be important to the T_{1c} relaxation for the hydrocarbon chains. This correlation time is similar to $\tau_\perp$ recently calculated for lipid hydrocarbon chains by Pastor et al. (1988).

Those carbons with very rapid spin lattice relaxation, such as the glycerol carbons, were not adequately resolved in these experiments at high cholesterol, even with the MAS, and therefore T_1 values were not reported from membranes with a high cholesterol content for some of those carbons.

DISCUSSION

Initially the data obtained from the egg PC/cholesterol/cholestatrienol system will be examined. As discussed in Theory, the only motion for the fluorescent cholesterol analogue in a phospholipid membrane that is capable of contributing significantly to the decay of the anisotropy of the fluorescence emission is wobble. Therefore, the time-resolved fluorescence anisotropy data provide an unambiguous evaluation of the correlation time of wobble ($\tau_\perp$) for a sterol in a phospholipid bilayer. The value for $\tau_\perp$ in PC bilayers is ~0.9 ns.

These data also provide an evaluation of the motional order associated with the wobble, $S_\perp$. The order parameter is labeled as $S_\perp$ because the order parameter obtained pertains only to the wobble. In the case of order parameters derived from ^{2}H NMR data, for example, S_{obs} is the product of all the order parameters corresponding to motions that contribute to averaging of the observed quadrupole splittings:

$$S_{obs} = \prod_{i=1}^{n} S_i. \qquad (4)$$

Because the fluorescence data reflects only the wobble, only one order parameter, $S_\perp$, can be derived from these data.

Table 5 shows an interesting comparison between the data obtained for $\tau_\perp$ and $S_\perp$ in this study and results from a recent ^{2}H NMR study using specifically deuterated cholesterol (Dufourc and Smith, 1986). The observation in that study of a minimum in a plot of T_{1z} vs. temperature clearly revealed the presence of a correlation time of ~3 ns that described motion important to T_{1z} relaxation. Those authors assigned the 3-ns correlation time to axial diffusion, and suggested that wobble would occur with $\tau_\perp \ll 10^{-9}$s. The data reported in this study were inconsistent with that assignment of Dufourc and Smith (1986).

However, Table 5 shows a strong similarity between the characteristics of wobble described in this study and the "long" correlation time identified in the study of Dufourc and Smith (1986). If one interchanged the assignments of the motions detected in the ^{2}H NMR study, one would have good agreement between the studies: $\tau_\perp$ = 1–3 ns and $S_\perp \approx 0.5$. The study of Siminovitch et al. (1988) cannot be compared with the present study because the system studied was very different and because the only temperature measured was much higher than used here. Pastor et al. (1988) recently calculated similar values for $\tau_\perp$ and $S_\perp$ for lipid hydrocarbon chains from ^{31}C T_1.

These data showed little dependence of $\tau_\perp$ on cholesterol content or on temperature. These results were not in contradiction to the data of Dufourc and Smith (1986) because the T_{1z} is experimentally more sensitive to small changes in correlation time than is Φ in this time regime. Such small changes in Φ would be lost in the intrinsic experimental uncertainty of the measurement.

Likewise there is little dependence of $S_\perp$ on cholesterol content or temperature. These results were in marked contrast to the increase in ordering of membrane lipids observed upon introduction of cholesterol into the lipid bilayer (Yeagle, 1985). However, the ordering of the C—D bond on the lipid hydrocarbon chains due to the introduction of cholesterol into the membrane, S_{CD}, was at least in part due to the reduction in *gauche-trans* isomerizations of the carbon–carbon single bonds in those

TABLE 5 Comparison of dynamics from fluorescence and NMR data

NMR analysis from Dufourc and Smith (1986):		
Motion	Order parameter	τ
Axial diffusion	$S_\parallel = \pm 0.51$	$\tau_\parallel = 3 \times 10^{-9}$ s
Wobble	$S_\perp = 0.79$	$\tau_\perp \ll 10^{-9}$ s
Analysis from fluorescence data:		
Wobble	$S_\perp = 0.45$	$\tau_\perp = 0.9 \times 10^{-9}$ s

hydrocarbon chains which control the fluctuations of the C—D bond. Because the steroid fluorophore was contained within a fused ring system, carbon–carbon rotations were not likely contributors to the observed $S_\perp$. Thus it was not surprising that $S_\perp$ did not change with cholesterol content of the membrane. In the case of ^2H NMR from the lipid hydrocarbon chains, $S_\perp$ contributed to S_{obs} as part of a product with S_{CD} according to Eq. 4 and without independent measurements of $S_\perp$, the extent to which the cholesterol content affects $S_\perp$ was not readily determined.

Likewise, the lack of temperature effects on $S_\perp$ and $\tau_\perp$ for the sterol could not be directly compared with the ^2H order parameter analysis of lipid hydrocarbon chains, which did exhibit a dependence on temperature. Once again, S_{obs} (from ^2H NMR) for the lipid hydrocarbon chains contained more terms than just $S_\perp$.

The question remained concerning the characteristics of axial diffusion. Previously we calculated a value of ~0.1 ns for $\tau_\parallel$ from a ^{13}C NMR spin lattice relaxation study in sonicated PC vesicles containing [$^{13}C_4$] labeled cholesterol (Yeagle, 1981). However, that study assumed a model for spin lattice relaxation with only one anisotropic motion contributing significantly to T_1. The single anisotropic correlation time model was too simple for an adequate description of the longitudinal relaxation of C_4 of cholesterol. Given the value for $\tau_\perp$ reported here, and assuming for the sake of argument that the value for $\tau_\parallel$ was 0.1 ns, then two highly anisotropic motions must be modeled to adequately describe ^{13}C relaxation for [$^{13}C_4$] labeled cholesterol. Present theory does not adequately treat this problem, especially because both a {^1H-^{13}C} dipolar term and a chemical shift anisotropy term contribute to the Hamiltonian. In the study of Brainard and Szabo (1981), the relationship $10\ \tau_\parallel = \tau_\perp$ was assumed. Therefore, $\tau_\parallel$ remains experimentally undetermined at this time, from the available NMR studies. However, spin label studies with a sterol analogue suggested that $\tau_\parallel$ = 0.1 ns (Schindler and Seelig, 1974).

The effects of cholesterol shown in Fig. 8 on the T_{1c} of the carbon atoms of the lipid hydrocarbon chains appeared to mimic the effects in Fig. 7 of cholesterol content on the T_{1c} of the cholesterol $^{13}C_4$. However, it was not possible to uniquely describe the correlation functions contributing to the lipid hydrocarbon chain T_{1c} which were more complicated than for the sterol. The former must have had contributions from axial diffusion, wobble, and carbon–carbon rotations, at the minimum. Therefore the effects of sterol on phospholipid T_{1c} could not be properly interpreted.

The data reported here showed effects on r_{ss} of cholestatrienol due to the phospholipid composition of the membranes. As an example, the effects of PE were investigated in detail. The data in Fig. 3 showed that the presence of TPE in a PC/PE mixture in a membrane produced a measurable reduction in r_{ss}. Time-resolved fluorescence anisotropy revealed that the correlation time, $\tau_\perp$, may be longer in PE-rich membranes than in PC membranes, while the order parameter, $S_\perp$, was similar in PE-rich membranes and PC membranes. In this case, the change in r_{ss} reflected a change in sterol dynamics, though not as dramatic a change as the r_{ss} values might suggest. In fact, one might be more impressed at the lack of strong effects on sterol dynamics due to phospholipid content, mimicking in that regard the lack of strong effects from temperature and cholesterol concentration in the membrane on sterol dynamics. These data may be explained by the concept advanced previously (Smutzer and Yeagle, 1985; Schroeder et al., 1988) that cholesterol preferentially partitioned into sterol-rich microdomains even at low sterol contents.

One of the significant advantages of this fluorescent approach to studying sterol behavior was the relatively high sensitivity of the fluorescent technique (compared with techniques such as NMR). The sensitivity of the fluorescence technique allowed one to introduce the fluorescent sterol into biological membranes and study sterol behavior where nuclear magnetic resonance techniques were too insensitive. In this study, sterol behavior in three biological membranes, bovine ROS disk membranes, human erythrocyte ghosts, and light sarcoplasmic reticulum, was studied by exploiting the fluorescent properties of cholestatrienol.

Each of the biological membranes exhibited r_{ss} for cholestatrienol that was different from the others, with the lowest value for r_{ss} exhibited by the ROS disks and the highest value of r_{ss} exhibited by the erythrocyte ghosts. While the differences in r_{ss} among the biological membranes were significant, no dramatic differences were detected between the range of values for r_{ss} for phospholipid-cholesterol bilayers and range of r_{ss} for biological membranes. It was determined (see above) that interpretation of r_{ss} in terms of sterol dynamics was unwise. Therefore a time-resolved fluorescence study was performed with each membrane preparation.

$S_\perp$ for the biological membranes was on the high end of the range of values of $S_\perp$ observed in the phospholipid bilayer systems. These values for $S_\perp$ suggested that the ordering of the wobble in the erythrocyte ghosts and LSR membranes was similar to the ordering of the wobble of the sterol in PC bilayers. The wobble of the sterol in ROS disks may have been marginally more ordered than in the PC bilayers.

However, $S_\perp$ for cholestatrienol in a membrane treated with digitonin was distinctly greater than $S_\perp$ in any of the phospholipid bilayer systems. This increase in ordering was likely the result of complex formation between the digitonin and sterol which damps the wobble.

A curious observation was made concerning $\tau_\perp$ in the biological membranes. In the LSR, $\tau_\perp$ was similar to $\tau_\perp$ in the phospholipid bilayers. However, $\tau_\perp$ in the ROS disks and in the erythrocyte ghosts was three or fourfold shorter than $\tau_\perp$ observed in the phospholipid bilayers. Therefore, while sterol behavior was observed to be largely independent of environment in most systems, in the ROS disks and in the erythrocyte ghosts, a dramatic difference in $\tau_\perp$ was observed compared with other systems.

The source of this difference in $\tau_\perp$ could only be the subject of speculation. The biological membranes contained protein which the phospholipid bilayers did not. The anisotropy decay of cholestatrienol was therefore examined in bilayers made from lipid extracts of all three biological membranes studied here. In each case, the value for $\tau_\perp$ from the lipid extract bilayers was similar to $\tau_\perp$ in the other lipid bilayers reported in this study. Therefore, a possible role of protein in modulating sterol behavior could be postulated.

One must then understand the differences between LSR on the one hand and erythrocyte ghosts and ROS disks on the other. One difference in the biological membranes examined was the lipid/protein mole ratio. Although this number is difficult to determine in most biological membranes because of their relative complexity, both the ROS disk membrane and the LSR membrane contain a single dominant protein component: rhodopsin in the ROS disk membrane and calcium ATPase in the LSR membrane. With respect to this dominant protein component, the lipid/protein mole ratio is ~65 in the ROS disk membranes (Boesze-Battaglia et al., 1989) and 120 in the LSR membranes (Selinsky and Yeagle, 1984). This difference in lipid/protein ratio might play a role in the difference in values for $\tau_\perp$ for the two membranes.

Another possible difference is in cholesterol interactions with membrane proteins, which have been studied in two of these membranes. Cholesterol has been implicated in interactions with glycophorin and Na^+K^+ATPase of the human erythrocyte membrane (Yeagle, 1983; Yeagle, 1984; Yeagle et al., 1986). In the LSR, cholesterol may be excluded from direct interactions with the calcium pump (Warren et al., 1975). One could therefore suggest that interactions with membrane proteins in the human erythroctye membrane might modulate $\tau_\perp$, and that the absence of sterol interactions with the major protein of the LSR would lead to sterol behavior similar to that observed in phospholipid bilayers. This speculation should be subject to further testing.

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