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# THE INTERACTION OF CHOLESTEROL WITH BILAYERS OF PHOSPHATIDYLETHANOLAMINE

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**The interaction of cholesterol with the glycerol backbone segments of phospholipids was studied in bilayers of phosphatidylethanolamine containing equimolar amounts of cholesterol. Glycerol selectively deuterated at various positions was supplied to the growth medium of *Escherichia coli* strain 131 GP which is defective in endogeneous glycerol synthesis. The procedure enables the stereospecific labeling of the three glycerol backbone segments of the membrane phospholipids. Phosphatidylethanolamine with wild-type fatty acid composition was purified from *E. coli* cells and deuterium magnetic resonance spectra were obtained either from dispersions of pure phosphatidylethanolamine or from equimolar mixtures of phosphatidylethanolamine with cholesterol. For comparative purposes 1.2-di[9,10-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphoethanolamine and [3-$\alpha$-$^2$H]cholesterol were synthesized in order to monitor the behavior of the fatty acyl chains and of the cholesterol molecule itself. For all deuterated segments the deuterium quadrupole splittings as well as the deuterium spin-lattice ($T_1$) relaxation times were measured as a function of temperature. The glycerol backbone was found to be a remarkably stable structural element of the phospholipid molecule. The quadrupole splittings of the backbone segments changed only by at most 2 kHz upon incorporation of 50 mol % cholesterol. This was in contrast to the fatty acyl chains where the same amount of cholesterol increased the quadrupole splitting by more than 20 kHz. The glycerol segments exhibited the shortest $T_1$ relaxation times of all $CH_2$ segments indicating that the glycerol backbone is the slowest motional moiety of the lipid molecule. Addition of cholesterol has no effect on the backbone motion but the fast reorientation rate of the *trans*-double bonds in 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine is increased dramatically.**

## Introduction

Cholesterol can be dissolved in phospholipid bilayers to the level of about one molecule of cholesterol per molecule phospholipid. Neutron diffraction data indicate that the cholesterol OH group is located in the vicinity of the ester linkages by which the fatty acyl chains are attached to the phospholipid glycerol backbone [1]. The bulk of the cholesterol molecule is thus immersed deeply in the hydrophobic bilayer interior. The influence of cholesterol on the thermodynamic and structural properties of phospholipid bilayers has been investigated by numerous techniques (for a review see Ref. 2). Probably the best documented change at the structural level is the stiffening of the hydrocarbon chains upon addition of cholesterol. The rigid steroid ring restricts the *trans-gauche* isomerisations of the flexible fatty acyl chains leading

Abbreviations: PE, phosphatidylethanolamine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulphonic acid).

to a more extended chain configuration. At high cholesterol levels and low temperatures the fatty acyl chains may approach the completely extended all-*trans* conformation [3–9]. In contrast to the extensive and by now well-understood studies on the hydrocarbon layer, there is a paucity of data on the interaction of cholesterol with the glycerol backbone and the phospholipid polar groups, apparently because these segments are not easily resolved by most spectroscopic techniques. The purpose of the present study was therefore to investigate more systematically the interaction of cholesterol with the three glycerol segments of a naturally occurring phosphatidylethanolamine with *cis*-usaturated fatty acid chains. In particular, we were interested whether the cholesterol-induced stiffening of the fatty acyl chains was also felt at the attachment sites of the fatty acids, i.e. the glycerol segments C-1 and C-2.

We have recently shown that selective deuteration of the glycerol backbone can be achieved either by chemical synthesis [10] or by using glycerol-auxotrophs of the bacterium *E. coli* [11]. The three glycerol segments can be distinguished readily by deuterium nuclear magnetic resonance ($^2$H-NMR) and are characterized by distinct sets of quadrupole splittings $\Delta\nu_Q$. We have employed this approach to study the interaction of cholesterol with synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) where the deuterium label was at the *sn*-3 position of the glycerol backbone [12]. Here we report results of equimolar mixtures of cholesterol with backbone-deuterated phosphatidylethanolamine. The phospholipid was selectively deuterated at each of the three glycerol segments and the deuterium quadrupole splittings as well as the spin lattice ($T_1$) relaxation times were measured with and without cholesterol. For comparative purposes we have also added deuterated cholesterol to the same phosphatidylethanolamine bilayers and have studied the ordering and dynamics of the cholesterol molecule itself. Finally, cholesterol was added to bilayers of 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) carrying the deuterium label at the *trans*-double bond of the fatty acyl chains. This experiment allowed us to monitor the influence of cholesterol on the hydrocarbon region of an unsaturated phosphatidylethanolamine bilayer.

## Materials and Methods

*Preparation of selectively deuterated lipids.* A cardiolipin-deficient glycerol-auxotroph of *Escherichia coli* (mutant T131 GP; [11]) was grown on either *sn*-[1,1-$^2H_2$]-, *sn*-[2-$^2$H]-, or *sn*-[3,3-$^2H_2$]glycerol. The preparation of the selectively deuterated glycerols has been described previously [11,13] and followed the synthesis suggested by Lok et al. [14]. A total phospholipid fraction was prepared from the cells by chloroform/methanol extraction [15] and purified by silicic acid chromatography. Phosphatidylethanolamine (PE) and phosphatidylglycerol were separated on a DEAE-cellulose column [16]. The fatty acid composition is that of wild-type *E. coli* with palmitic acid predominantly in the *sn*-1 position and vaccenic and palmitoleic acid in the *sn*-2 position. The fatty acid composition was determined by gas chromatography for two different batches (Borle, F., private communication) and was found to be independent of the type of glycerol employed. Cholesterol selectively deuterated at the 3-position was prepared according to Rosenfeld et al. [17]. 1,2-Di[9,10-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphatidylethanolamine (DEPE) was synthesized by the base exchange reaction of phospholipase D from the corresponding 1,2-di[9,10-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphocholine (DEPC). The latter compound was prepared as described before [18].

*Sample preparations.* Samples without cholesterol: dried phosphatidylethanolamine (50–100 mg) was dispersed at 50°C in 0.01 M Pipes buffer, pH 7.0, containing 0.1 M NaCl and 0.001 M EDTA. The lipid concentration was 30 weight-%. Samples containing cholesterol: dried phosphatidylethanolamine and cholesterol (in the molar ratio of 1 : 1) were dissolved in chloroform and this solution was evaporated under a stream of $N_2$. An average molecular weight of 800 was assumed for the *E. coli* phosphatidylethanolamine. The sample was then dried thoroughly under high vacuum for 24 h and finally dispersed in buffer as described above. Deuterium depleted water was used in all sample preparations to eliminate isotropic contributions from the natural abundance of deuterium in water.

*NMR measurements.* Deuterium NMR spectra were recorded at 46.1 MHz with a Bruker-Spectro-

spin CXP-300 pulse spectrometer using the quadrupole echo technique [19]. 90° pulses of 4.5 μs were used with an echo pulse separation of 30 μs. All spectra were recorded with quadrature phase detection and a phase alternating sequence was employed to reduce coherent noise and phase errors. The spectral width was normally 100 kHz, the recycle time 250 ms. Deuterium spin-lattice ($T_1$) relaxation times were measured by the conventional 180°-$\tau$-90° sequence modified to include the quadrupole echo. Since the deuterium $T_1$ relaxation times were found to be 3–15 ms, a relaxation delay of 250 ms was sufficient for a full recovery of the magnetization.

*Differential scanning calorimetry.* The thermodynamic properties of the lipid-water dispersions were measured with a MC-1 differential scanning calorimeter (MICROCAL Inc., Amherst, MA).

## Results

*Thermodynamic properties of phosphatidylethanolamine bilayers*

Differential scanning calorimetry of dispersions of purified *E. coli* phosphatidylethanolamine (strain T 131 GP) reveals a very broad endothermic transition. The midpoint of the transition is at $T_c \simeq 25°C$, the width $\Delta T \simeq 30°C$, and the transition enthalpy $\Delta H \simeq 8$–$9$ kcal/mol. In contrast, dispersions of synthetic phosphatidylethanolamines exhibit very sharp gel-to-liquid crystal transitions, characterized by well-defined transition temperatures. Relevant comparisons for *E. coli* phosphatidylethanolamine are: 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) with $T_c = 37°C$ [20,21], 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) with $T_c = 27°C$ (Ganz, P. and Seelig, J., unpublished data), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine with $T_c = -16°C$ [20]. The predominant single species in natural *E. coli* phosphatidylethanolamine is 1-palmitoyl-2-vaccenic-*sn*-glycero-3-phosphoethanolamine which is similar in structure to POPE. This explains why the midpoint of the transition is found at $T_c \simeq 25°C$. On the other hand, the heterogeneity of the fatty acid composition in wild-type *E. coli* accounts for the width of the phase transition.

Differential scanning calorimetry also detects a second phase transition at about 65°C. This phase change has a much smaller transition enthalpy and corresponds to a transition from a bilayer to a hexagonal mesophase as has been noted previously for similar *E. coli* PE [22,23]. Addition of cholesterol at a 1:1 molar ratio eliminates the gel-to-liquid crystal transition and the mid-point of the lamellar → hexagonal transition is lowered to 60°C.

*Deuterium magnetic resonance*

*sn-[1,1-$^2H_2$]Phosphatidylethanolamine.* Fig. 1 shows the deuterium NMR spectra of *sn*-[1,1-$^2H_2$]phosphatidylethanolamine in the absence and presence of 50 mol% cholesterol measured at 37°C. As observed previously [11] the spectrum exhibits two components of quite different quadrupole splittings $\Delta\nu_Q$, that is, a strong central signal with $\Delta\nu_Q \simeq 0$ kHz and outer signal with $\Delta\nu_Q \simeq 17$ kHz. The central peak was not due to a non-bilayer component since the $^{31}$P-NMR spectra were characteristic of a pure bilayer phase. Instead, it was shown previously by stereospecific monolabeling that the two signals arise from the motional inequivalence of the two deuterons. The central signal was assigned to the 1($S$) deuteron, the outer splitting to the 1($R$) deuteron [11]. Additional support for this earlier interpretation comes from the measurement of the $^2$H $T_1$ relaxation times as shown in Fig. 2. Both signals exhibit identical $T_1$

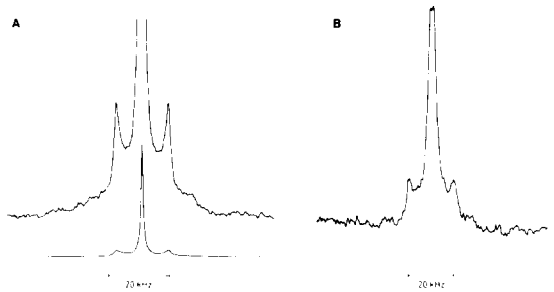



Fig. 1. $^2$H-NMR spectra at 46.1 MHz of coarse liposomes of [1,1-$^2$H]PE from *E. coli* in the absence (A) and presence (B) of 50% (mol/mol) cholesterol. Samples were made up in deuterium-depleted water and the quadrupole echo was used. Spectral width 100 kHz; all samples contain ~100 mg lipid.

relaxation times at all temperatures, indicating that both signals arise from deuterons attached to the same methylene group. Upon introduction of 50 mol% cholesterol the outer splitting increased from 17.2 kHz to 18.5 kHz and the inner central line was split into a doublet with a separation of 1.1 kHz. The latter could only be resolved at lower temperatures. At higher temperatures the splitting decreased and at 45°C was no longer resolvable.

*sn-[2-$^2H$]Phosphatidylethanolamine.* Fig. 3 shows $^2$H-NMR spectra for PE labelled at the 2-position of the glycerol backbone in the absence (A) and presence of cholesterol (B), at 37°C. The addition of cholesterol (50 mol%) causes the quadrupole splitting to increase from 24.4 kHz to 27.5 kHz. The $T_1$-relaxation of the 2-deuteron appears to be only weakly dependent upon the presence of cholesterol and falls exactly within the range of values exhibited by the 1,1-deuterons (cf. Fig. 5)

*sn-[3.3-$^2H_2$]Phosphatidylethanolamine.* Fig. 3 also contains $^2$H-NMR spectra of PE labelled at the 3,3-position of the glycerol backbone in the presence and absence of cholesterol at 37°C. As noted previously [11] in the absence of cholesterol two splittings are observed due to the motional inequivalence of the two deuterons. However, the difference between the inner and outer splitting is only 1.1 kHz, somewhat smaller than that observed by Gally et al. [11]. This difference was observed to be reproducible for each batch of *E. coli* PE prepared. Interestingly, the addition of cholesterol causes the doublet to disappear, suggesting that the two deuterons become motionally equivalent, probably due to a small conformational change of the backbone. At the same time the total splitting is reduced by about 2 kHz.

In comparison to the other positions of the backbone the $T_1$ relaxation time for the 3,3-deuterons are somewhat larger and also somewhat more temperature-dependent. Nevertheless, this temperature dependence appears to be unchanged by the presence of cholesterol.

The quadrupole splittings $\Delta\nu_Q$ and $T_1$-relaxation data of the three backbone segments are summarized in Figs. 4 and 5, respectively. Below

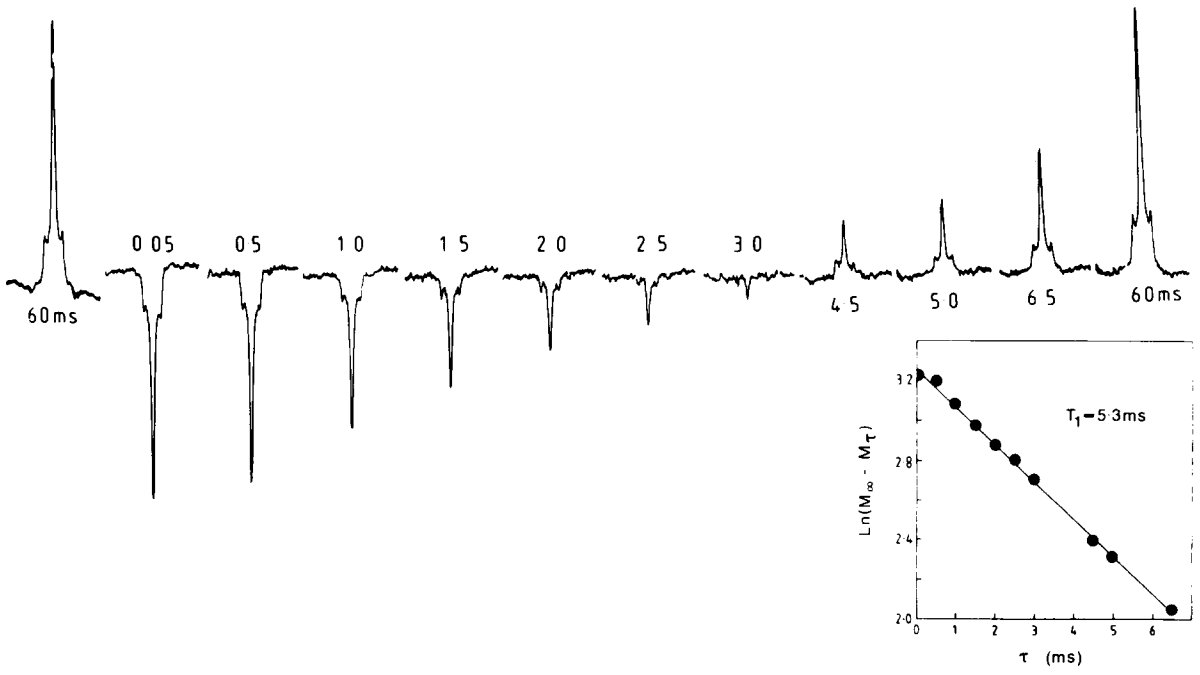



Fig. 2. Measurement of the $^2$H $T_1$ relaxation time (at 46.1 MHz) using the inversion recovery technique for [1.1-$^2H_2$]PE from *E. coli*. Inset: semilogarithmic plot of the intensity of the central peak vs. time. The outer splitting relaxed identically (data not shown) at all temperatures. Measuring temperature: 35°C.

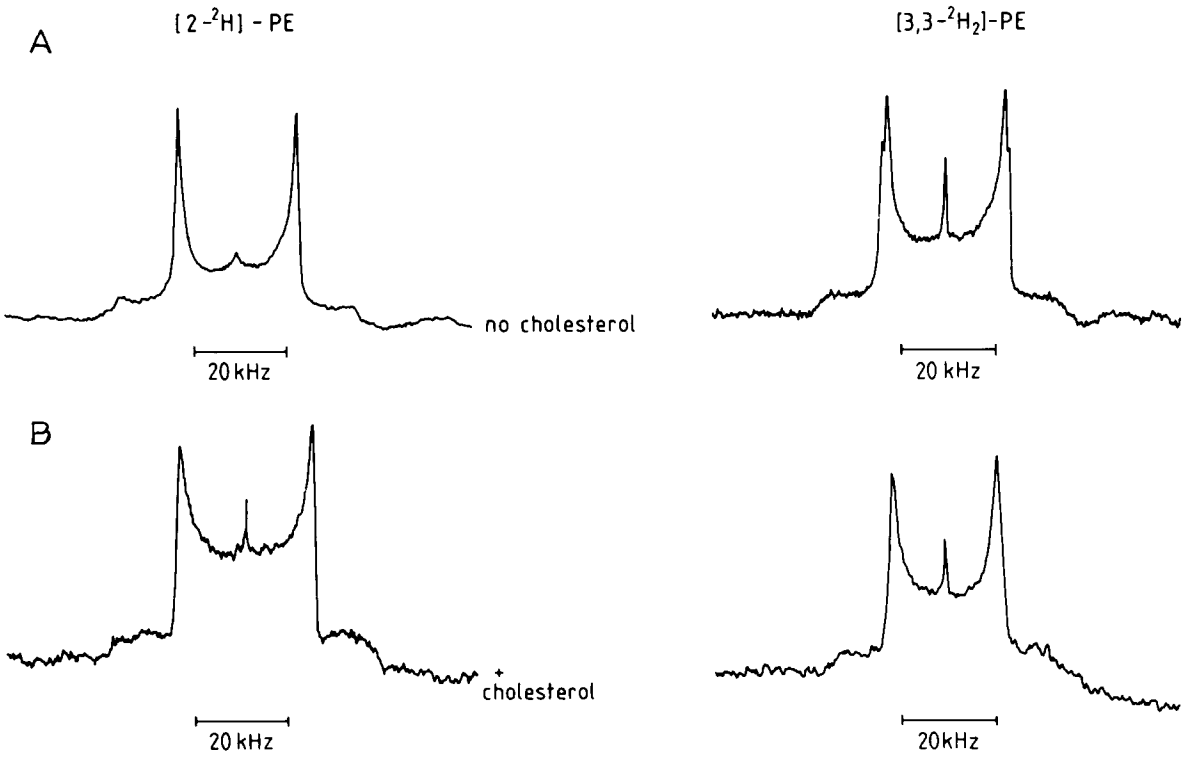
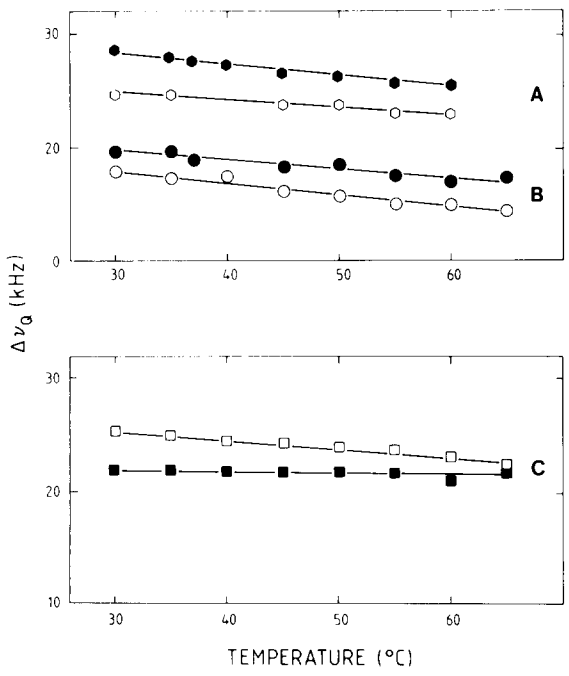



Fig. 3. $^2$H-NMR spectra of [2-$^2$H]- and [3,3-$^2H_2$]PE, respectively, in the absence (A) and presence (B) of 50 mol% cholesterol. Spectral width 100 kHz. In all cases the small central peak is due to the natural abundance of deuterium in deuterium-depleted water.




30°C it became increasingly more difficult to record $^2$H-NMR spectra due to the increasing amount of gel state lipids.

*[3-$^2$H]Cholesterol.* As an interesting comparison, cholesterol was deuterated at the 3-position and incorporated into a bilayer of [2-$^2$H]PE. The quadrupole splitting for [3-$^2$H]cholesterol is considerably larger ($\simeq$ 45 kHz) than that of the [2-$^2$H]PE, allowing both signals to be observed simultaneously. Both the quadrupole splitting and the $T_1$-relaxation times of [3-$^2$H]cholesterol are only weakly dependent upon temperature (Fig. 6) and the numerical values for the $T_1$-relaxation data coincide (accidentally) almost exactly with those obtained for [2-$^2$H]PE throughout the temperature range investigated.

Fig. 4. Variation of the quadrupole splittings with temperature for the three segments of the glycerol backbone of PE from *E. coli*, respectively, in the absence (open symbols) and presence (closed symbols) of 50 mol% cholesterol. (A) [2-$^2$H]PE (hexagonals), (B) [1,1-$^2H_2$]PE (circles) and [3,3-$^2H_2$]PE (squares).

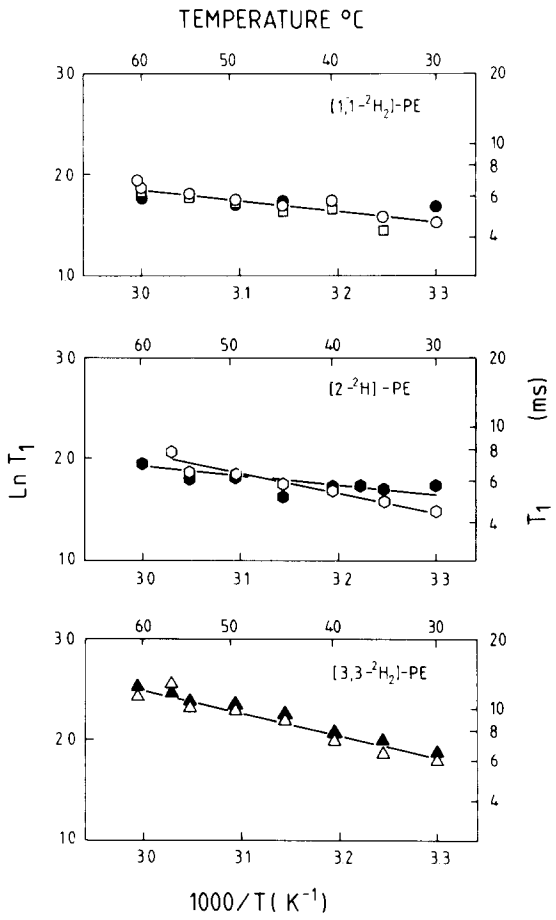



Fig. 5. Arrhenius plots of the $^2H$ $T_1$ relaxation times for each of the three $^2H$-labelled carbon atoms of the glygerol backbone, in the absence (open symbols) and presence of 50 mol% cholesterol (closed symbols), respectively. The fit of the solid lines to the data were determined by linear regression. The symbols ○ and □ refer to the outer splitting and the central line, respectively (cf. Fig. 2).

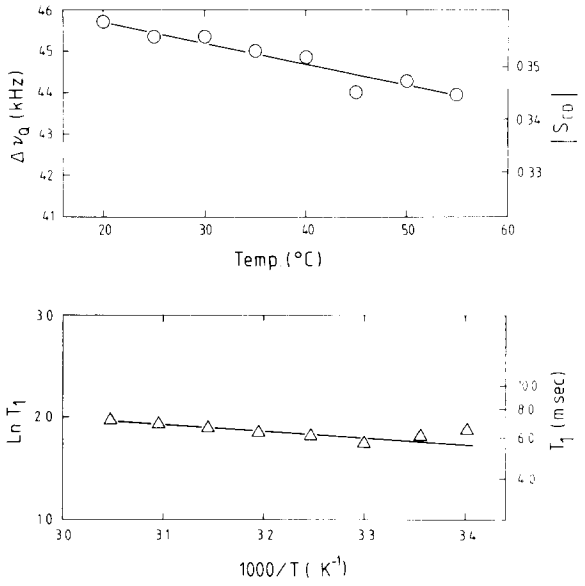



Fig. 6. Variation of the quadrupole splittings (upper figure) and $^2H$-relaxation times (lower figure) for [3-$^2H$]cholesterol incorporated into liposomes of *E. coli* PE at a mol ratio of 1 : 1.

*[9,10-$^2H_2$]DEPE.* As another comparison we have also examined the effect of cholesterol (50 mol%) upon the structure and motion of the [9,10-$^2H_2$]-*trans* double bond of DEPE. A rather simple $^2H$-NMR spectrum is obtained which can be characterized by just one quadrupole splitting. This means that at the level of the *trans* double bond both fatty acyl chains are motionally equivalent despite the fact that they have different initial orientations [18]. Fig. 7 shows the temperature dependence of the quadrupole splitting. For pure phosphatidylethanolamine bilayer the quadrupole splitting changes sharply from 55 kHz to 25 kHz at the phase transition temperature ($T_c = 37°C$). Below $T_c$ the hydrocarbon chains are ordered, giving rise to a large quadrupole splitting, above $T_c$ the chains are melted and disordered, and the quadrupole splitting decreases. Addition of equimolar amounts of cholesterol is seen to have two different effects. First, the phase transition of the pure DEPE bilayer is eliminated as indicated by the smooth variation of the quadrupole splitting over the whole temperature range. Second, the incorporation of cholesterol leads to an increase in the quadrupole splitting of the fatty acyl chains by about 20 kHz. This is comparable to the increase observed for bilayers of phosphatidylcholine [3–9]. Taken together these data demonstrate that the response of the hydrophobic part of an unsaturated phosphatidylethanolamine bilayer to cholesterol is much the same as that observed for bilayers of phosphatidylcholine. In particular, cholesterol exerts a quite distinct stiffening effect on the fatty acyl chains of the phosphatidylethanolamine bilayer.

Figure 7 also contains the $^2H$- $T_1$-relaxation times for DEPE with and without cholesterol. For

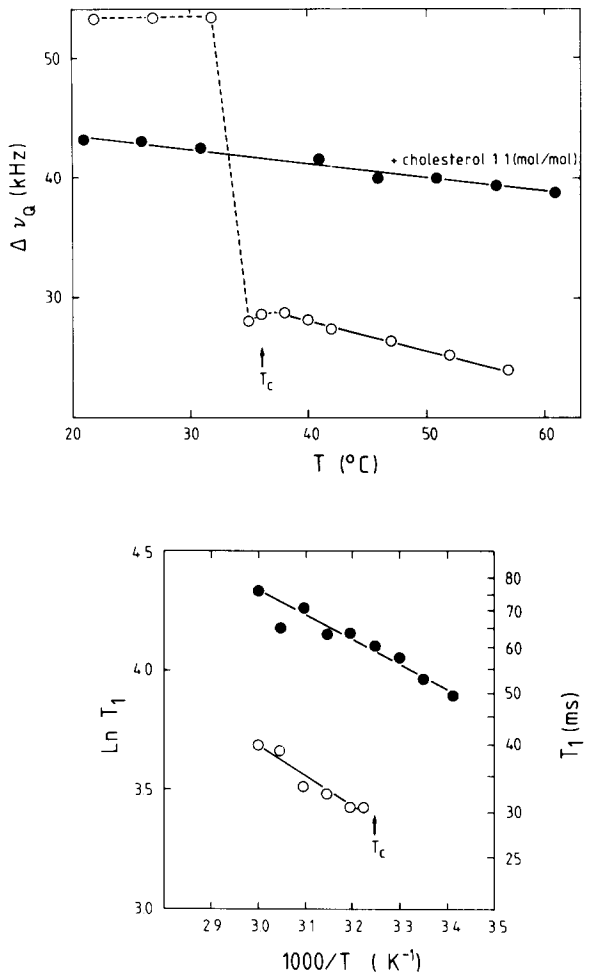



Fig. 7. Variation of the quadrupole splitting (upper figure) and $^2$H-relaxation times (lower figure) for liposomes of 1,2-[9,10-$^2H_2$]dielaidoyl-*sn*-glycero-3-phosphoethanolamine in the absence (open symbols) and presence (closed symbols) of 50 mol% cholesterol. The dotted line of the uppermost figure corresponds to the observation of gel-stat $^2$H-spectra, and reflects the width between inner edges of the powder pattern.

pure DEPE the observed $T_1$-relaxation times were of about the same magnitude as for DEPC [24]. In the presence of cholesterol, the relaxation time of the [9,10-$^2H_2$]-*trans* double bond was almost doubled in comparison to the pure lipid bilayer. The activation energy, however, was approximately the same both in the presence and absence of cholesterol.

## Discussion

### *Structural aspects of phosphatidylethanolamine-cholesterol interaction*

The addition of equimolar amounts of cholesterol to phosphatidylethanolamine bilayers has a dramatic effect on the fatty acyl chains (change in the quadrupole splitting from 25 kHz to 45 kHz) but only a small effect on the glycerol backbone. The quadrupole splittings at the C-1 and C-2 position of the glycerol backbone increase by not more than 2 kHz, those of the C-3 position even decrease by about the same amount (Fig. 4). These data suggest that the glycerol backbone of phosphatidylethanolamine undergoes a small conformational change upon incorporation of cholesterol but in contrast to the fatty acyl chains does not experience any restriction in its inherent motional freedom. This is also supported by the $T_1$-relaxation time measurements to be discussed below. Moreover, the numerical agreement between the quadrupole splittings of the pure phosphatidylethanolamine bilayers and intact cell membranes [11] provides a strong argument that the same backbone conformation is retained in biological membranes, even at the highest protein/lipid ratios.

A precise picture for the position of cholesterol in lipid membranes can be derived from neutron and X-ray diffraction studies on various model membranes. First using deuterated cholesterol ([3-α-$^2$H]cholesterol) Worcester and Franks [1] were able to determine the position of the deuterated segment in bilayers of egg lecithin with a precision of ±1.5 Å. The deuterated segment was found to be 18 Å away from the center of the bilayer, placing the OH-group at 19.5 Å from the center. Second, using X-ray diffraction [25] it was also possible to localize the phosphate segment at 23 ± 1 Å from the center. Third, neutron diffraction measurements yielded a separation between the phosphate segment and the glycerol C-3 segment of 3.6 Å, placing this glycerol backbone segment at 19.4 Å from the center (in egg lecithin) [26]. Taken together these data demonstrate that the cholesterol hydroxyl group is located approximately at the level of the glycerol backbone C-3 segment. Since the glycerol backbone of phosphatidylethanola-

mine is oriented perpendicular to the surface of the membrane [27,28] the glycerol segments C-1 and C-2 are penetrating more deeply into the bilayer interior and hence are in direct contact with the steroid frame. Nevertheless, the effect on the quadrupole splitting remains small. On the other hand, it is well-established that cholesterol restricts the fluctuations in all fatty acyl chain segments [3–9], including the C-2 segment of the *sn*-2 chain [6,8]. However, since the *sn*-2 chain starts out parallel to the bilayer surface and is bent only after the C-2 segment [29], this segment is at the same height as the backbone C-2 segment. Addition of equimolar amounts of cholesterol increases the quadrupole splittings of this segment by more than 50%. Thus we encounter the paradox situation that two lipid segments are located at the same distance from the lipid interface, one of which is strongly affected by the neighboring cholesterol molecule while the other is not. We must conclude therefore that the predominant effect of cholesterol is to restrict the *trans-gauche* isomerisations within the fatty aycl chains but that this is a purely steric effect of the flat steroid ring which is not propagated into the glycerol backbone. This result also argues against extensive trans-gauche-isomerisations around the C1-C2 axis of the glycerol backbone. Finally, it should be mentioned that cholesterol has only a small effect on the quadrupole splittings of the ethanolamine headgroup segments of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine [30] which is consistent with the model proposed here for the average location of cholesterol.

*Dynamical properties of cholesterol and of the phospholipid segments*

Table I summarizes the spin-lattice ($T_1$) relaxation times and deuterium quadrupole splittings of the various phospholipid segments and of [3-$^2$H]cholesterol (at 45°C). Let us first focus on the motional properties of cholesterol since comparative data of other techniques are also available. Cholesterol is an approximately axially symmetric rod-like molecule and its motional behavior requires the specification of at least two different rates of motion. The rate of rotation around the $C_\infty$ symmetry axis (rotational diffusion constant $R_\parallel$) can be expected to be faster than the wobbling motion perpendicular to this axis ($R_\perp$). Using spin labelled steroids the following rotational diffusion constants for cholesterol incorporated into soap-like bilayers were obtained [31]

$$R_\parallel \simeq 8 \cdot 10^8 s^{-1} \quad R_\perp \simeq 1 \cdot 10^8 s^{-1}$$

The rotational diffusion coefficients are related to rotational correlation times according to

$$\tau_{2m}^{-1} = 6 R_\perp + m^2 (R_\parallel - R_\perp)$$

TABLE I

ORDER PARAMETERS, $^2$H $T_1$ RELAXATION TIMES, ROTATIONAL CORRELATION TIMES AND ACTIVATION ENERGIES FOR EACH LABELLED SEGMENT OF THE GLYCEROL BACKBONE AND [9,10-$^2H_2$]DEPE AT 45°C IN THE PRESENCE AND ABSENCE OF CHOLESTEROL.

Values for [3-$^2$H]cholesterol are also included for comparison. The correlation times were derived using Equation 1.

| Lipid | $\Delta\nu_Q$ | $S_{C^2H}$ | $T_1$ (ms) | $\tau_c$ ($10^{-10}$ s) | $E_a$ (kJ/mol) |
|---|---|---|---|---|---|
| [1,1-$^2H_2$]PE from *E. coli* | 16.07 | 0.12 | 5.39 | 4.17 | 9.3 |
| [1,1-$^2H_2$]PE + cholesterol (50 mol%) | 18.21 | 0.14 | 5.39 | 4.16 | 6.2 |
| [2-$^2$H]PE from *E. coli* | 23.84 | 0.18 | 5.8 | 3.86 | 15.6 |
| [2-$^2$H]PE + cholesterol (50 mol%) | 26.42 | 0.21 | 5.09 | 4.42 | 7.2 |
| [3,3-$^2H_2$]PE from *E. coli* | 24.28 | 0.18 | 9.01 | 2.48 | 15.6 |
| [3,3-$^2H_2$]PE + cholesterol (50 mol%) | 21.78 | 0.17 | 9.35 | 2.39 | 16.0 |
| [3-$^2$H]Cholesterol | 44.0 | 0.35 | 6.60 | 3.56 | 3.2 |
| [9,10-$^2H_2$]DEPE | 26.17 | 0.21 | 33.1 | 0.68 | 7.1 |
| [9,10-$^2H_2$]DEPE + cholesterol (50 mol%) | 39.19 | 0.31 | 65.0 | 0.35 | 7.6 |

$\Delta\nu_Q = (3/4)(e^2qQ/h)S_{C^2H}$

Insertion of the above numbers yields

$$\tau_{20} \triangleq \tau_{\perp} = 1.7\,ns \quad \tau_{22} \triangleq \tau_{\parallel} = 0.3\,ns$$

Brainard and Szabo [32] have analyzed the carbon-13 relaxation time ($T_1$) and the linewidth of cholesterol incorporated into single-walled bilayer vesicles arriving at the following correlation times

$$\tau_{\perp} \simeq 2.5\,ns \quad \tau_{\parallel} \simeq 0.25\,ns$$

in agreement with the spin label EPR results. Next we may analyze the deuterium $T_1$ relaxation times of cholesterol. As demonstrated in Fig. 6 the $T_1$ relaxation time of [3-$^2$H]cholesterol increases continously with increasing temperature indicating that the motions fall into the fast-correlation time regime. This simplifies the quantitative analysis since under this conditions $T_1$ is given to first approximation by [33]

$$\frac{1}{T_1} = \frac{3\pi^2}{2}\left(e^2qQ/h\right)^2\left(1 + \tfrac{1}{2}\,S_{C^2H} - \tfrac{3}{2}\,S_{C^2H}^2\right)\tau_c \quad (1)$$

where $(e^2qQ/h) = 170$ kHz. The last term in parentheses contains the order parameter correction. For cholesterol in a 1:1 mole ratio mixture with phosphatidylethanolamine the experimental results are $\Delta\nu_Q = 45$ kHz ($S_{C^2H} = 0.35$) and $T_1 = 6.6$ msec, yielding $\tau_c = 0.4$ nsec. The above $T_1$ formula assumes a single correlation time to describe the motion of the C$^2$H bond vector and is thus a poor motional model for the cholesterol molecule as a whole. Nevertheless, the numerical comparison with the spin label and carbon-13 data demonstrates that $\tau_c$ must be identified with the fast rotation around the long molecular axis for which the two other methods yield $\tau_{\parallel} \simeq 0.3$ ns.

We may now proceed to a discussion of the motional properties of the glycerol backbone. As mentioned above the glycerol backbone is oriented perpendicular to the bilayer surface. The motional freedom of the C-1 and the C-2 segments can be expected to be severely restricted due to the covalent linkage of these segments to the fatty acyl chains. As for cholesterol one would thus predict that the rotational diffusion of the glycerol backbone is characterized by an anisotropic diffusion tensor. Again, the measurement of $T_1$ relaxation times does not allow a complete determination of the diffusion tensor but provides only the fastest correlation time. Nevertheless, the following quantitative conclusions can be derived from the experimental results.

First, it should be noted from the temperature dependence of the $T_1$ relaxation times (Fig. 5) that the motional rates of all three backbone segments fall into the fast correlation time regime. Thus the above $T_1$ formula is agian a good approximation to evaluate $\tau_c$. Secondly, the C(1) and C(2) backbone segments are characterized by the same $T_1$ relaxations times, which are furthermore the shortest $T_1$ relaxation times observed in the whole molecule. This suggests that the C(1)-C(2) axis is the slowest moving part of the phospholipid molecule, which is in agreement with $^{13}$C $T_1$-relaxation time studies on sonicated vesicles of phosphatidylcholine [34]. The correlation time of both segments is $\tau_c \simeq 0.4$ ns and by analogy with cholesterol we assume that this correlation time describes the fast motion ($\tau_{\parallel}$) around the C(1)-C(2) axis. The off-axis motion are probably characterized by a slower correlation time ($\tau_{\perp}$) which is not accessible in the present experiments. Third, the $T_1$ relaxation time of the C(3) backbone segment is about twice as long as that of the C(1) and C(2) segments. This means that the glycerol backbone is not a completely rigid entity but that additional rotational isomerisations are possible around the C(2)-C(3) axis. Finally, the motional properties of all glycerol segments are little affected by the presence of cholesterol. The absolute values of the $T_1$ relaxation times are remarkably similar in the presence or absence of cholesterol. This may be contrasted with the behavior of the fatty acyl chain segments as illustrated by 1,2-di[9′,10′-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphoethanolamine. Addition of 50 mol% of cholesterol to DEPE bilayers increases the quadrupole splitting of the *trans* double bond from 27 kHz to 39.5 kHz, in agreement with earlier studies on synthetic saturated phosphatidylcholines [3–9]. The unusual aspect, however, is the simultaneous increase in the $T_1$ relaxation time by almost a factor of two. Even though the amplitudes of the angular fluctuations are more restricted the rate of motion apparently increases, at least as far as the fastest

type of motion ($\tau_{\|}$) is concerned. To our knowledge, this is the first example that such an effect has ever been observed in a lipid bilayer and it demonstrates that a clear distinction has to be made between structural and dynamic parameters. The insertion of cholesterol leads to a better ordered DEPE bilayer and, at the same time, also to a more 'fluid' bilayer, at least as judged from the increase in the rate of the fastest motions dominating the $T_1$ relaxation process.

In conclusion, the present studies demonstrate that the glycerol backbone is a remarkable stable and rigid structural element of phospholipid molecules. Neither the average conformation nor the dynamic properties are changed in the presence of cholesterol which must be contrasted with the quite dramatic effects exerted by cholesterol on the fatty acyl chains.

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## References

1 Worcester, D.L. and Franks, N.P. (1976) J. Mol. Biol. 100, 359–378
2 Demel, R.A. and De Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109–132
3 Oldfield, E., Chapman, D. and Derbyshire, W. (1971) FEBS Lett. 16, 102–104
4 Gally, H., Seelig, A. and Seelig, J. (1976) Hoppe Seyler's Z. Physiol. Chem. 357, 1447–1450
5 Stockton, G.W. and Smith, I.C.P. (1976) Chem. Phys. Lipids 17, 251–263
6 Haberkorn, R.A., Griffin, R.G., Meadows, M.D. and Oldfield, E. (1977) J. Am. Chem. Soc. 99, 7353–7355
7 Oldfield, E., Meadows, M., Rice, D. and Jacobs, R. (1978) Biochemistry 17, 2727–2740
8 Jacobs, R. and Oldfield, E. (1979) Biochemistry 18, 3280–3285
9 Rance, M., Jeffrey, K.R., Tulloch, A.P., Butler, K.W. and Smith, I.C.P. (1982) Biochim. Biophys. Acta 688, 191–200
10 Gally, H.U., Niederberger, W. and Seelig, J. (1975) Biochemistry 14, 3647–3652
11 Gally, H.U., Pluschke, G., Overath, P. and Seelig, J. (1981) Biochemistry 20, 1826–1831
12 Haberkorn, R.A., Griffin, R.G., Seelig, J. and Waespe-Šarčevič, N. (1982), in preparation
13 Wohlgemuth, R., Waespe-Šarčevič, N. and Seelig, J. (1980) Biochemistry 19, 3315–3321
14 Lok, C.M., Ward, J.P. and Van Dorp, D.A. (1976) Chem. Phys. Lipids 16, 115–122
15 Ames, G.F. (1968) J. Bacteriol. 95, 833–843
16 Rouser, G., Kritchevsky, G., Yamamoto, A., Simon, G., Galli, C. and Bauman, A.J. (1969) Methods Enzymol. 14, 272–317
17 Rosenfeld, R.S., Fukushima, D.K., Hellman, L. and Gallagher, T.F. (1954) J. Biol. Chem. 211, 301–311
18 Seelig, J. and Waespe-Šarčevič, N. (1978) Biochemistry 17, 3310–3315
19 Davis, J.H., Jeffrey, K.R., Bloom, M., Valic, M.I. and Higgs, T.P. (1976) Chem. Phys. Lett. 42, 390–394
20 Van Dijck, P.W.M., De Kruijff, B., Van Deenen, L.L.M., De Gier, J. and Demel, R.A. (1976) Biochim. Biophys. Acta 455, 576–587
21 Jackson, M.B. and Sturtevant, J.M. (1977) J. Biol. Chem. 252, 4749–4751
22 Cullis, P.R. and De Kruijff, B. (1978) Biochim. Biophys. Acta 513, 31–42
23 Gally, H.U., Pluschke, G., Overath, P. and Seelig, J. (1980) Biochemistry 19, 1638–1643
24 Seelig, J., Tamm. L., Hymel, L. and Fleischer, S. (1981) Biochemistry 20, 3922–3933
25 Franks, N.P. (1976) J. Mol. Biol. 100, 345–358
26 Büldt, G., Gally, H.U., Seelig, A., Seelig, J. and Zaccai, G. (1978) Nature 271, 182–184
27 Hitchcock, P.B., Mason, R., Thomas, K.M. and Shipley, G.G. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3036–3040
28 Büldt, G. and Seelig, J. (1980) Biochemistry 19, 6170–6175
29 Seelig, J. and Browning, J.L. (1978) FEBS Lett. 92, 41–44
30 Brown, M.F. and Seelig, J. (1978) Biochemistry 17, 381–384
31 Schindler, H. and Seelig, J. (1974) J. Chem. Phys. 61, 2946–2949
32 Brainard, J.R. and Szabo, A. (1981) Biochemistry 20, 4618–4628
33 Brown, M.F., Seelig, J. and Häberlen, U. (1979) J. Chem. Phys. 70, 5045–5053
34 Lee, A.G., Birdsall, N.J.M., Metcalfe, J.C., Warren, G.B. and Roberts, G.C.K. (1976) Proc. Roy. Soc. B. 193, 253–274