Biochimica et Biophysica Acta, 470 (1977) 141—151

BBA 77812

^{13}C NMR MEASUREMENTS OF UNSONICATED PHOSPHATIDYLCHOLINE BILAYERS OF DIFFERENT FATTY ACID AND STEROL COMPOSITION

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(Received March 23rd, 1977)

Summary

(1) ^{13}C NMR linewidths were measured for various ^{13}C resonances in unsonicated dispersions of synthetic and natural phosphatidylcholines both in the absence and presence of cholesterol at temperatures where the acyl chains are in the liquid-crystalline state.

(2) In the absence of cholesterol the linewidths of the various resolved chain resonances were decreased with increasing unsaturation and decreasing chain length. The motion of the Δ9-*cis* olefinic carbon atoms in dioleoylphosphatidylcholine was more restricted than the motion of the Δ9-*cis* olefinic carbon atoms in 1-stearoyl-2-oleoyl-phosphatidylcholine despite the higher overall fluidity of dioleoylphosphatidylcholine. The polar head-group motion was not dependent upon the fatty acid composition.

(3) Incorporation of cholesterol broadens all observed chain resonances of all phosphatidylcholines, thus demonstrating a reduction in chain motion by cholesterol. For both the saturated and unsaturated phosphatidylcholines the reduction of the chain motion is decreased with increasing chain length.

(4) The chemical shift of the carbonyl resonances of sonicated dipalmitoyl phosphatidylcholine vesicles labelled with ^{13}C in both chains in the 1-position was slightly decreased by the incorporation of 50 mol% of cholesterol. In contrast, 50 mol% of epicholesterol, the 3α-OH isomer, produced a large downfield shift and a splitting of the carbonyl resonance.

Introduction

The dynamical behaviour of lipids in membranes is intimately linked to the structure and function of biomembranes. In understanding the basic principles

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of the molecular motion of lipids, NMR has proved to be a valuable technique. Most of our present knowledge on the motional characteristics of the membrane lipids comes from ^{1}H, ^{13}C, ^{31}P and ^{2}H NMR studies on model membrane systems. ^{1}H and ^{31}P NMR are of limited use in the investigation of the motions at different positions within the lipid molecule. In the ^{1}H spectrum of membrane systems only a few protons are resolved. ^{31}P NMR can, of course, only probe the phosphate region of the lipid molecule. In deuterium NMR studies using specifically deuterated lipids the mobility gradient along the dipalmitoylphosphatidylcholine molecule in lipid bilayers has been elucidated [1]. Although this technique has great potential for the future the special instrumentation required and the need for elaborate lipid synthesis are major limitations.

The advantages of ^{13}C NMR include a wide range of chemical shifts and limited resonance broadening in structures with restricted molecular motion. In the ^{13}C NMR spectrum of lipids in model membrane systems (even unsonicated ones) resonances of many of the ^{13}C nuclei in the lipid molecule are resolved. Thus, this technique has given information about the motions of egg phosphatidylcholine and dipalmitoyl phosphatidylcholine in sonicated and non-sonicated liposomes [2–7]. Although the exact mechanisms determining the relaxation of the ^{13}C nuclei in membrane systems are not yet fully understood both spin-lattice relaxation times (T_1) and spin-spin relaxation times (T_2) (derived from the linewidth) can be used to investigate the various motions of lipids in membrane systems [2–8]. The T_2 relaxation times have been shown to be particularly sensitive to changes in membrane motion [6].

In this paper, ^{13}C NMR linewidth measurements are reported for non-sonicated liposomes of various synthetic and natural phosphatidylcholine species at temperatures where the fatty acid chains are in the liquid-crystalline state. It will be shown that there is a significant effect of chain length and kind and degree of unsaturation of the fatty acid chains on the molecular motion of the various parts of the phosphatidylcholine molecule.

The interaction between cholesterol and membrane phospholipids is still not fully understood (for recent review see ref. 9). Only recently new data have been presented on this topic. In particular ^{2}H NMR studies of deuterated fatty acids incorporated in egg phosphatidylcholine-cholesterol bilayers [10] and of mixed bilayers of specifically deuterium labeled dipalmitoyl phosphatidylcholine and cholesterol [11] have given a clearer picture of the so-called condensing effect of cholesterol.

Monolayer and permeability studies have shown that the interaction between cholesterol and phosphatidylcholine is strongly dependent upon the fatty acids present in the phosphatidylcholine molecule [12]. Therefore the effect of cholesterol incorporation on the linewidth of the ^{13}C NMR resonances of non-sonicated liposomes of different phosphatidylcholines has also been investiated. 3α-OH sterols like epicholesterol have a much weaker interaction with membrane phospholipids than the naturally occurring 3β-OH sterols [4]. It has been hypothesized that differences in hydrogen bond formation between the sterol hydroxyl group and the ester carbonyl oxygens of the phospholipid molecule might be the cause of this difference in interaction [13,14]. Since the chemical shift of the carbonyl resonance is sensitive towards hydrogen bonding [15–17] we thought it of interest to study in more detail the behaviour of the carbonyl

resonances in mixed phosphatidylcholine-cholesterol and phosphatidylcholine-epicholesterol bilayers.

Experimental

Lipids. 1,2-Dilauroyl-*sn*-glycerol-3-phosphorylcholine (12 : 0/12 : 0-phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycerol-3-phosphorylcholine (14 : 0/14 : 0-phosphatidylcholine), 1,2-dipalmitoleoyl-*sn*-glycerol-3-phosphorylcholine (16 : 1_c/16 : 1_c-phosphatidylcholine), 1,2-dioleoyl-*sn*-glycerol-3-phosphorylcholine (18 : 1_c/18 : 1_c-phosphatidylcholine), 1,2-didocosenoyl-*sn*-glycerol-3-phosphorylcholine (22 : 1_c/22 : 1_c-phosphatidylcholine), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine (18 : 0/18 : 1_c-phosphatidylcholine) and 1,2-dielaidoyl-*sn*-glycerol-3-phosphorylcholine (18 : 1_t/18 : 1_t-phosphatidylcholine) were synthesized and purified as described before [18]. 1,2(1-[^{13}C]palmitoyl)-*sn*-glycerol-3-phosphorylcholine (1,2(1-[^{13}C]-16 : 0)-phosphatidylcholine) was prepared similarly using 1-[^{13}C]palmitic acid (90% ^{13}C) obtained from I.R.E. Fleurus (Belgium). Egg phosphatidylcholine was isolated from hen eggs according to established procedures. Soya phosphatidylcholine, which is a highly unsaturated phosphatidylcholine, was the kind gift of Dr. H. Eikermann from Natterman and Cie., Köln, G.F.R. Cholesterol was obtained from Fluka (Buchs, Switzerland).

Preparation of unsonicated liposomes and sonicated vesicles. Liposomes were prepared as described before [19] by dispersing 100—150 μmol of phosphatidylcholine with or without cholesterol in 1.5 ml ^2H_2O containing 25 mM Tris · HCl, p^{2}H, 7.0, and 0.2 mM EDTA by agitation on a vortex mixer. Lipid vesicles were obtained by subsequent ultra-sonication of the liposome dispersion as described before [20].

Nuclear magnetic resonance. Two ^{13}C Fourier transform NMR spectrometers were used: a Varian CFT 20 and a Bruker WS 360 which operate on ^{13}C at 20 and 90.5 MHz respectively. Both machines were equipped with broad band proton noise decoupling. The high field spectrometer was equipped with a variable temperature unit, and quadrature detection. The low field spectrometer operated at 30 ± 2°C. Typically a spectral width of 20 KHz was used using 4000 or 8000 data points. 10 000—100 000 transients were accumulated with an interpulse time of 1 s using 90° pulses. A decoupling power of 8 W was used in all experiments on both spectrometers. Care was taken to position the 10 mm NMR tube in such a manner in the probe that the liposomes which float in the ^2H_2O buffer, were always between the receiver coils.

The contribution of the field inhomogeneity to the linewidth was estimated to be a maximum of 2 Hz which was the linewidth of a resonance of Tris present in the solution. The ^{13}C linewidths were measured for at least two different samples, with similar results. For linewidth measurements in spectra containing overlapping resonances first the most probable baseline was chosen whereafter the individual peaks were reconstructed using the peak heights and shape of the peak as obtained from the non-overlapping site assuming symmetrical peaks.

Results and Discussion

Phosphatidylcholine liposomes. In the 20 MHz ^{13}C NMR spectrum of all phosphatidylcholine liposomes tested several resonances are resolved. Two

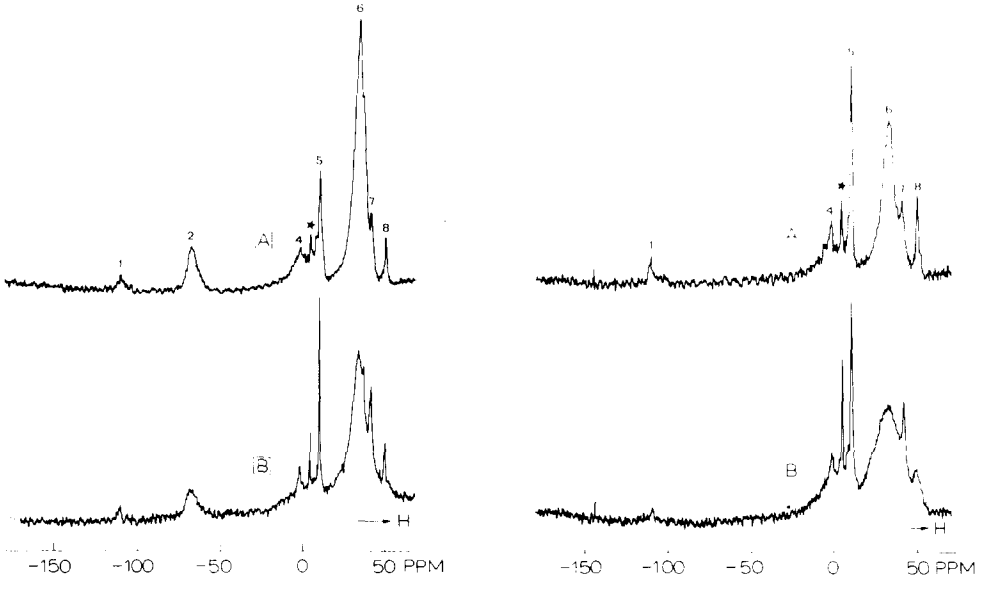

Fig. 1. 20 mHz ^{13}C spectra of unsonicated 18 : 1_c/18 : 1_c-phosphatidylcholine liposomes at 30°C (A) in the absence and (B) in the presence of 50 mol% cholesterol. ★, a resonance of Tris. Shifts are from external 1,4-dioxane. Peaks are numbered according to Table I.

Fig. 2. 20 MHz ^{13}C spectra of unsonicated 12 : 0/12 : 0-phosphatidylcholine liposomes at 30°C (A) in the absence and (B) in the presence of 50 mol% cholesterol. ★, a resonance of Tris. Shifts are from external 1,4-dioxane. Peaks are numbered according to Table I.

examples are presented in Figs. 1A and 2A in which the spectra of 18 : 1_c/18 : 1_c-phosphatidylcholine and 12 : 0/12 : 0-phosphatidylcholine liposomes are shown. The various resonances were assigned according to previously published ^{13}C NMR spectra of egg phosphatidylcholine, 16 : 0/16 : 0-phosphatidylcholine and 18 : 1_c/18 : 1_c-phosphatidylcholine [2—7,21] (Table I).

In the polar head-group region the resonances of the carbonyl atoms and two of the three resonances of the choline group are resolved. The resonances of the remainder of the polar head-group carbon atoms are not visible because these atoms undergo restricted motion which broadens the resonances beyond detection. Of the acyl chains the resonances of the double bond carbons, the terminal methyl group and the adjacent methylene group are present in the spectrum. The bulk of the chain methylene groups give rise to one signal where part of the linewidth is determined by differences in chemical shift of the various methylene carbons.

The linewidth is directly related to the spin-spin relaxation time and is sensitive to the rate and/or mode of motion of the nucleus. Because the liposomes are large (in the order of microns) the vesicle tumbling is very slow. The only effective line-narrowing motions are the local anisotropic motions of the lipid molecule in the bilayer. The theory of the spin-spin relaxation in these systems is not fully understood in terms of the rate and/or modes of the various anisotropic motions involved. The measured linewidth therefore can provide only a qualitative picture of the motions of the various nuclei of the lipid molecule in the bilayer.

TABLE I

LINEWIDTHS OF THE ^{13}C NMR RESONANCES AT 20 MHz OF UNSONICATED PHOSPHATIDYLCHOLINE LIPOSOMES AT 30°C

Resonance	Phosphatidylcholine species (linewidth in Hz)								
	12 : 0/12 : 0	14 : 0/14 : 0	16 : 1_c/16 : 1_c	18 : 1_c/18 : 1_c	22 : 1_c/22 : 1_c	18 : 0/18 : 1_c	18 : 1_t/18 : 1_t	egg	soya
(1) *C = O	45	67	> 80	90	61	60	75	76	32
(2)-C = C—	—	—	84	130	110	80	76	59	33 [a]
(3)-C = C—C—C = C—	—	—	—	—	—	—	—	45	
(4) *CH_2—$N(CH_3)_3$	60	54	45	69	53	37	68	60	52
(5)-$N(*CH_3)_3$	19	18	16	29	26	17	19	16	9
(6)-$(*CH_2)_n$—	165	190	142	141	142	170	154	160	90
(7)-*CH_2—CH_3	25	— [b]	14	25	22	25	> 20	16	9
(8)-*CH_3	31	41	13	25	23	31	29	12	7

[a] Linewidth of both resonances which were only partially resolved.
[b] Too broad to be detected.
The error in the determination of the linewidth for resonances (1) and (4) is estimated to be 20% and for the other resonances 5%.

TABLE II

EFFECT OF THE INCORPORATION OF 50 MOL% CHOLESTEROL ON THE LINEWIDTHS OF THE ^{13}C NMR RESONANCES AT 20 MHz OF UNSONICATED PHOSPHATIDYLCHOLINE LIPOSOMES AT 30°C.

Resonance	Phosphatidylcholine species (linewidth in Hz)								
	12 : 0/12 : 0	14 : 0/14 : 0	16 : 1_c/16 : 1_c	18 : 1_c/18 : 1_c	22 : 1_c/22 : 1_c	18 : 0/18 : 1_c	18 : 1_t/18 : 1_t	egg	soya
(1) *C = O	53	47	49	55	107	> 55	60	44	62
(2)-*C = C—	—	—	170	170	195	200	110	94 [a]	33 [a]
(3)-*C = C—C—C— = C	—	—	—	—	—	—	—		
(4) *CH_2—$N(CH_3)_3$	26	37	33	36	37	36	25	26	39
(5)-$N(*CH_3)_3$	18	17	16	16	25	23	16	11	9
(6)-$(*CH_2)_n$—	400	346	327	200	190	265	284	300	220
(7)-*CH_2-CH_3	29	42	29	29	23	37	30	32	21
(8)-*CH_3	69	50	30	27	25	32	28	32	18

[a] Linewidth of both resonances which were only partially resolved.
The error in the determination of the linewidth for resonances (1) and (4) is estimated to be 20% and for the other resonances 5%.

Table I summarizes the linewidth measurements at 20 MHz of the ^{13}C resonances of different phosphatidylcholine liposomes at 30°C. In agreement with other ^{13}C NMR studies on egg phosphatidylcholine [6,7] the linewidth of the resonances towards both the end of the polar head-group and the end of the acyl chains are narrower than the resonances from the central part of the molecule, demonstrating the mobility gradient along the molecule [1]. 14 : 0/14 : 0-phosphatidylcholine has a higher transition temperature than 12 : 0/12 : 0-phosphatidylcholine. The greater rigidity of the 14 : 0/14 : 0-phosphatidylcholine molecule is manifested by increased linewidth of all the ^{13}C resonances of the carbon chain atoms. The introduction of double bond decreases the chain-chain interactions in the bilayer and therefore can be expected to cause increased motion in the hydrocarbon region. This is manifested by the narrower linewidths of the various chain resonances of the unsaturated phosphatidylcholine liposomes (Table I). Most of the resonances of the chain carbon atoms which are resolved are narrower than the resonances of the corresponding atoms in the chains of the saturated phosphatidylcholines which have even shorter fatty acid chains. The greatest chain motion is found for the highly unsaturated soya phosphatidylcholine which contains predominantly linoleic acid. The linewidth of the resonances of the chain methylene groups of 18 : 1_t/18 : 1_t-phosphatidylcholine is broader than the corresponding resonances in 18 : 1_c/18 : 1_c-phosphatidylcholine, which is in line with the more saturated character of the trans unsaturated lipids. The linewidth of the resonances of the trans unsaturated carbon atoms is significantly smaller than the *cis* unsaturated carbon atoms. This suggests that the motion of the trans olefinic carbon atoms is higher than the *cis* olefinic carbon atoms. It has to be realised however that the linewidth depends also on the orientation of the C-H vector with respect to the axes of motional averaging, which might be different for both double bonds.

The overall fluidity of 18 : 0/18 : 1_c-phosphatidylcholine is less than 18 : 1_c/18 : 1_c-phosphatidylcholine, as demonstrated by the increased linewidths of the signal from the methylene groups. The olefinic carbon atoms in 18 : 0/18 : 1_cphosphatidylcholine however have a higher motion than in 18 : 1_c/18 : 1_c-phosphatidylcholine. The presence of the saturated stearic acid chains must therefore impose less hindrance to the motion of the Δ9-olefinic carbon atoms at the 2-position. It would appear that the motions of these atoms in 18 : 1_c/18 : 1_c-phosphatidylcholine are sterically hindered and can be increased only when either both chains in the same molecule or some chains in neighbouring molecules can undergo a cooperative movement. In 22 : 1_c/22 : 1_c-phosphatidylcholine the double bond is in the Δ13-position, thus further away from the polar head-group. The observed narrower linewidth of the resonance of this group shows that the motion at this position is higher than in the Δ9-position of 18 : 1_c/18 : 1_c-phosphatidylcholine despite the longer chain length.

The linewidths of the resonances from the choline group are less sensitive to the fatty acyl constituents of the phosphatidylcholine molecule. The motion of this part of the molecule is rather independent of the molecular packing of the acyl chains in the bilayer. Only for 18 : 1_c/18 : 1_c- and 22 : 1_c/22 : 1_c-phosphatidylcholine the motion of the *N*-methyl carbons is decreased, whereas for soya phosphatidylcholine the motion of this group is increased. A similar

conclusion was drawn from ^{31}P NMR studies for the phosphate region of the polar head-group [22]. With the exception of 12 : 0/12 : 0-phosphatidylcholine and soya phosphatidylcholine the same can be said for the ester bond region because no marked changes were noticed in the linewidth of the carbonyl resonance from the various phosphatidylcholine species.

Phosphatidylcholine-cholesterol liposomes. In general the cholesterol-phospholipid interaction results in an increased broadening of the hydrophobic part of the bilayer at temperatures where the fatty acid chains are melted (for references to the numerous studies on this topic see ref. 9). Decreased motion of the acyl chains will give an increased linewidth of the ^{13}C NMR resonances as was demonstrated for egg phosphatidylcholine-cholesterol (1 : 1) liposomes [4,7].

In Figs. 1B and 2B the 20 MHz ^{13}C NMR spectra of 18 : 1_c/18 : 1_c-phosphatidylcholine and 12 : 0/12 : 0-phosphatidylcholine liposomes containing 50 mol% cholesterol are compared with the spectra of the cholesterol-free liposomes. Cholesterol causes the chain resonances to become much broader. No resonances of cholesterol can be detected which must be due to the restricted motion of the sterol ring system in particular. In fact, only a very broad resonance is observed for mixed 4-[^{13}C]-cholesterol phosphatidylcholine liposomes (Ref. 21 and de Kruijff, B., unpublished observations).

The ^{13}C NMR linewidths of the resonances of various cholesterol-phosphatidylcholine (1 : 1) liposomes are presented in Table II. There are some interesting quantitative differences between the various species (compare Table I and II). The linewidths of the $-(CH_2)_n$-resonances of the saturated phosphatidylcholines are most strongly increased by cholesterol. For both the saturated and unsaturated phosphatidylcholines the increase is correlated with the chain length. Thus, for 12 : 0/12 : 0- and 14 : 0/14 : 0-phosphatidylcholine the increase is 232% and 183% respectively, and for 16 : 1_c/16 : 1_c- and 18 : 1_c/18 : 1_c-phosphatidylcholine the increase is 230% and 142% respectively. The smallest increase (133%) was observed for 22 : 1_c/22 : 1_c-phosphatidylcholine. The strong inter-chain interactions in the bilayer composed of this long chain phosphatidylcholine molecule interferes with a proper cholesterol-phospholipid interaction. Recently, it was demonstrated with differential scanning calorimetry that the phase transition of this phosphatidylcholine species was not completely removed by the incorporation of 50 mol% cholesterol in the bilayer, which also suggests a poor interaction with cholesterol [23]. In this light it is intriguing that the lipids of the cholesterol-rich myelin membrane contain a high percentage of similar long chain fatty acids [24].

The total length of a cholesterol molecule is roughly the same as the length of an 18-carbon-atom-long fatty acid. The cholesterol-phospholipid interaction is thought to be the strongest along the rigid sterol nucleus [9] in agreement with the above data. The differential increase in linewidth of the terminal chain methyl resonance of 12 : 0/12 : 0- and 14 : 0/14 : 0-phosphatidylcholine by the incorporation of cholesterol also points to such an interaction. In the absence of cholesterol the terminal methyl groups in 12 : 0/12 : 0-phosphatidylcholine liposomes have more motion than in 14 : 0/14 : 0-phosphatidylcholine liposomes. The incorporation of cholesterol, however, restricts the motion of this group more in the shorter chain phosphatidylcholine where the terminal methyl group is closer to the rigid ring system, which has a length of about 9 methylene groups.

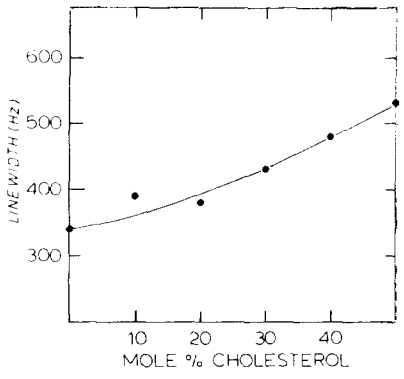

Fig. 3. Effect of cholesterol incorporation on the linewidth of the Δ9 *cis* olefinic resonance at 90.5 MHz of 18 : 1_c/18 : 1_c-phosphatidylcholine liposomes at 30°C.

The resonances of the Δ9 double bond carbon atoms of the unsaturated phosphatidylcholine species are broadened by cholesterol. By far the largest broadening of the Δ9 olefinic carbon atoms is observed for those contained in the oleic acid chain at the 2-position of 18 : 0/18 : 1_c-phosphatidylcholine (from 80 to 250 Hz).

To study the effect of cholesterol on the double bond carbon atoms in a more quantitative way the linewidth of the resonance of the Δ9 olefinic carbon atoms in 18 : 1_c/18 : 1_c-phosphatidylcholine was measured as a function of the bilayer cholesterol concentration. This was done at 90.5 MHz in order to increase sensitivity.

As shown in Fig. 3 there is a rather gradual increase in linewidth with increasing cholesterol concentration up to 50 mol% cholesterol.

Cholesterol does not influence the motion of the $-N(CH_3)_3$ part of the polar head-group of dipalmitoyl and egg phosphatidylcholine liposomes [4,7]. The linewidth of the $-N(CH_3)_3$ resonance of the various phosphatidylcholines was, in general, not affected by the incorporation of cholesterol. Cholesterol slightly increases the motion in the phosphate region [22]. The $\overset{*}{C}H_2$-$N(CH_3)_3$ resonance of all phosphatidylcholine species investigated was significantly narrower in the presence of cholesterol (Tables I and II) demonstrating that cholesterol does allow increased motion in this part of the polar head-group.

With respect to the influence of cholesterol on the motion in the ester bond region of phosphatidylcholine the present study does not provide a clear picture. Although the error in the determination of the linewidth of the natural abundant carbonyl resonances is considerable it appears that with 14 : 0/14 : 0, 16 : 1_c/16 : 1_c-, 18 : 1_c/18 : 1_c- and egg phosphatidylcholine cholesterol increases the motion of these atoms whereas for others no effect (12 : 0/12 : 0-, 18 : 0/18 : 1_c and 18 : 1_t/18 : 1_t-phosphatidylcholine) or a decreased motion (22 : 1_c/22 : 1_c- and soya phosphatidylcholine) was observed.

Differential interaction of cholesterol and epicholesterol with the ester carbonyl atoms in the bilayer. Sterols with the naturally occurring 3β-OH group show a much stronger interaction with membrane phospholipids than sterols with a 3α-OH group [25—27]. It has been suggested on theoretical grounds that

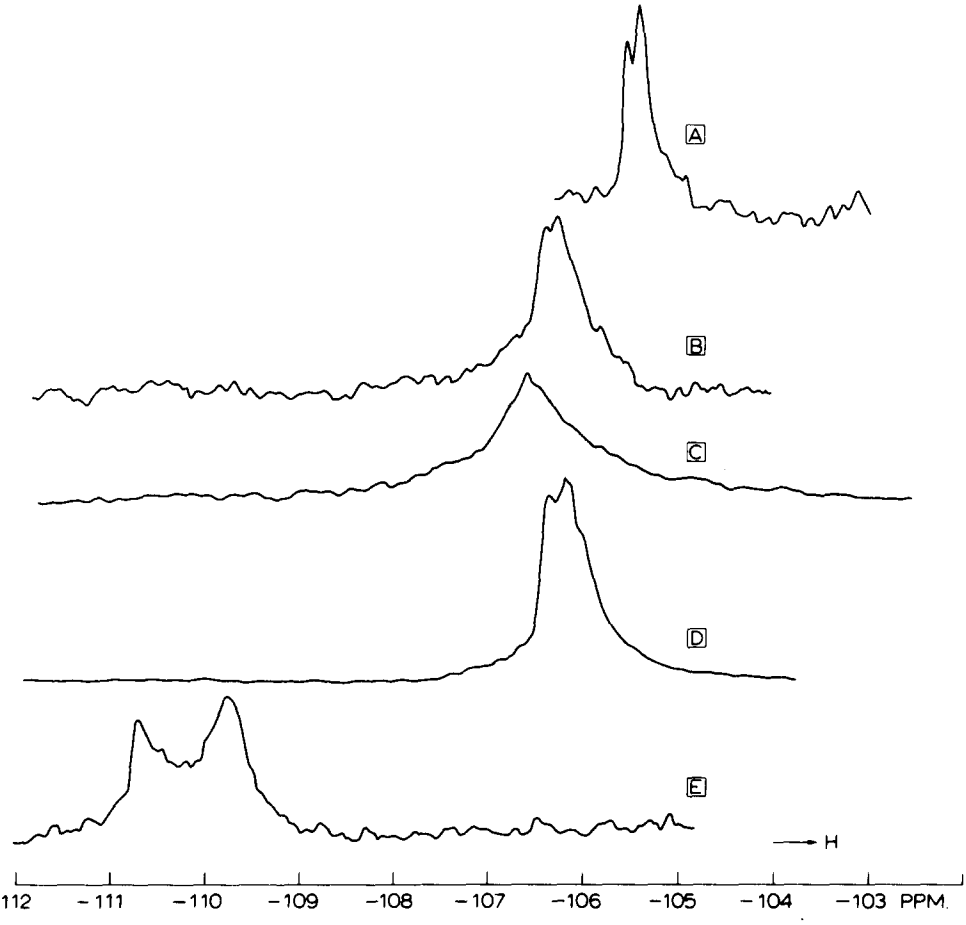

Fig. 4. 90.5 MHz ^{13}C NMR spectrum of 1,2-(1-[^{13}C]-16 : 0)-phosphatidylcholine at 45°C. (A), phosphatidylcholine in CCl_4; (B), sonicated phosphatidylcholine vesicles; (C), sonicated phosphatidylcholine-cholesterol (1 : 1) vesicles; (D), sonicated phosphatidylcholine-epicholesterol (7 : 3) vesicles; (E), sonicated phosphatidylcholine-epicholesterol (1 : 1) vesicles. Each sample contained 10—15 μmol phosphatidylcholine. Chemical shifts downfield from external 1,4-dioxane.

differences in the ability of these hydroxyl groups to participate in hydrogen bonds with water or the ester carbonyl atoms of the lipid molecule in the bilayer are the reason for this difference in interaction [13,14]. The chemical shift of the carbonyl groups is sensitive towards hydrogen bonding and conformational changes [15—17]. Therefore we investigated the effect of cholesterol and epicholesterol incorporation upon the chemical shift of the carbonyl resonance in 16 : 0/16 : 0-phosphatidylcholine in which both carbonyl atoms were 90% ^{13}C enriched. To obtain maximal sensitivity and resolution the measurements were done at 90.5 MHz using sonicated vesicles. The carbonyl resonances of sonicated 1,2-(1-[^{13}C]-16 : 0)-phosphatidylcholine vesicles are shifted about 1.4 ppm to lower field when compared to the resonances in CCl_4 (Fig. 4). This downfield shift of the resonance in the vesicles is most easily explained by a hydrogen bonding of both carbonyl atoms in the vesicle bilayer as was observed previously [17]. The doublet observed in CCl_4 arises from a slight difference in

shift between the resonances from the 1- and 2-position of the lipid molecule [17].

Incorporation of equimolar amounts of cholesterol in the vesicles caused a broadening and a small downfield shift of the carbonyl resonance. Yeagle et al. [17] observed a similar effect for the ^{13}C resonance of the natural abundance carbonyl atoms in egg phosphatidylcholine vesicles. The resonance broadening can either arise from a decreased local motion or from a reduced tumbling rate of the larger cholesterol containing vesicle [28].

The incorporation of 30 mol% of the 3α-OH isomer, epicholesterol, does not affect the chemical shift of the carbonyl resonance (Fig. 4). However, the incorporation of 50 mol% of epicholesterol caused a large downfield shift and a pronounced splitting of the carbonyl resonance (Fig. 4). This shows that at this sterol concentration there is a marked difference in the effect of the 3α-OH and the 3β-OH group of the sterol molecule on the state of the ester carbonyl atoms in the bilayer. The large downfield shift might suggest an increased hydrogen bonding although conformational changes cannot be excluded. The splitting of the carbonyl resonance by epicholesterol can either arise from a different interaction of epicholesterol in the outer and inner monolayer of the vesicle or is caused by a difference in interaction between the carbonyl atoms at the 1- and 2-position of the molecule. It is interesting that 50 mol% epicholesterol cannot be homogeneously incorporated in egg phosphatidylcholine bilayers [27,29]. Above 25 mol% a new phase of epicholesterol could be detected by X-ray [27]. No data are available on the mixing properties of epicholesterol and 16 : 0/16 : 0-phosphatidylcholine. We suggest that the downfield shift of the carbonyl resonance is associated with the formation of epicholesterol clusters in the bilayer of 16 : 0/16 : 0-phosphatidylcholine. Up to 30 mol%, the 3α-OH group can be accommodated in the ester bond region without effecting the state of the carbonyl atoms. Above this concentration epicholesterol clusters are formed which perturb the molecular organization of the ester bond region.

Acknowledgements

The 90.5 MHz ^{13}C NMR measurements were carried out at the SON NMR facility in Groningen. The 20 MHz ^{13}C NMR measurements were performed on the CFT-20 of the NMR centre of the Subfaculty of Chemistry in Utrecht. We thank all members of the Biomembranes Group for stimulating discussions and helpful suggestions.

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