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EFFECT OF *N*-ALKYL-*N*,*N*,*N*-TRIMETHYLAMMONIUM IONS ON PHOSPHATIDYLCHOLINE MODEL MEMBRANE STRUCTURE AS STUDIED BY ^{31}P-NMR

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The interaction of $[C_nH_{2n+1}N^+(CH_3)_3]\cdot I^-$ ($n = 3, 6, 9, 12, 14, 16$ or 18) with egg-yolk phosphatidylcholine-water dispersions has been studied by ^{31}P-NMR spectroscopy. It is shown that the effective anisotropy of ^{31}P chemical shift ($-\Delta\sigma_{eff}$) of the lamellar phospholipid liquid-crystalline phase L_α increases with increasing concentration and alkyl chain length of the drug. Addition of $[C_6H_{13}N^+(CH_3)_3]\cdot I^-$ or $[C_9H_{19}N^+(CH_3)_3]\cdot I^-$ to the phospholipid-water dispersion at a molar ratio ammonium salt: phospholipid > 0.8 induces in the dispersion a structure with an effective isotropic phospholipid motion. This structure is unstable and slowly transforms into the hexagonal phase. These effects have not been observed in phospholipid-water dispersions mixed with the ammonium derivatives with the longer alkyl chains $n = 12, 14, 16$ or 18. It is proposed that these results might explain the effects of the investigated drugs on the nerve, muscle and bacterial cells.

Introduction

The biological activity of *N*-alkyl-*N*,*N*,*N*-trimethylammonium ions (C_nTMA) and *N*-alkyl-*N*,*N*,*N*-triethylammonium ions (C_nTEA) depends strongly on the length of the alkyl substituent. For example, C_3TMA, C_4TMA, C_5TMA, and C_8TMA accelerate the multiple-drug-resistance (R) transfer from resistant bacterial cells of *Shigella flexneri* to sensitive bacterial cells of *Escherichia coli*, while the ions with the longer alkyl substituent C_{12}TMA, C_{14}TMA and C_{18}TMA have an inhibitory effect on the R transfer [1]. The effect of C_nTMA on muscle preparations is also different at $n > 10$ in comparison to $n < 10$. The C_2TMA–C_8TMA ions have a cholinolytic effect, while the C_{10}TMA–C_{20}TMA ions are cholinomimetics [2,3]. Experiments with squid axon have indicated that after depolarization, intracellular quaternary ammonium ions and their derivatives diffuse into open K^+ channels, producing a time-dependent block of the outward potassium current [4]. Swenson [5] has noted that the maximal effect of C_nTEA ions on the K^+ channel is observed for C_8TEA–C_{10}TEA derivatives, and has hypothesized that the interaction of the alkyl chain of C_nTEA with the lipid region surrounding the channel in the membrane is important for the channel inactivation. A similar hypothesis has been proposed by Heyer et al. [6]. They have found that C_3TMA, C_9TMA, and C_{12}TMA ions inactivate the voltage-dependent conductance induced in thin (black) lipid membranes by monazomycin. They have also

† Deceased.

Abbreviations: TMA, trimethylammonium; TEA, triethylammonium; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

proposed that, aside from their specific interaction with the K^+ channels, these ions bind to the lipid bilayer.

Also, interesting biological effects have been demonstrated and there have been various indications of the association of these molecules with the lipid part of the biological membrane; the interaction of C_nTMA and C_nTEA with model lipid membranes has been studied only rather fragmentarily. However, we have found in the literature remarks indicating structural effects of C_nTMA on the bilayer lipid membrane. Heyer et al. [6] have observed the increase of fragility of black lipid membranes with monazomycin in the presence of C_{12}TMA. De Smedt et al. [7,8] have found that the interaction of dimyristoylphosphatidylcholine vesicles with C_{16}TMA micelles results in the lysis of bilayer vesicles into 'fragments' which can reaggregate into 'large structures' under critical conditions of temperature, ionic strength and concentration of C_{16}TMA. Jain and Wu [9], using differential scanning calorimetry, observed that C_{18}TMA induces the formation of a new modified phase in the dipalmitoylphosphatidylcholine bilayer phase with higher gel-liquid crystal phase transition temperature.

In our work we have investigated the effects of C_nTMA on the phase behaviour of the phosphatidylcholine model membrane system. The method we used was ^{31}P-NMR spectroscopy, which provides a possibility for studies of phospholipid headgroup conformation and dynamics, and for the identification of phospholipid phases [10,11].

Material and Methods

C_nTMA iodides prepared by Dr. A. Paul, Humboldt University, Berlin, and by Dr. D. Devínsky, Comenius University, Bratislava, were analytically pure. Egg-yolk phosphatidylcholine was isolated and purified according to the method of Singleton et al. [12]. The purity of egg-yolk PC before and after ^{31}P-NMR experiments was checked by thin-layer chromatography.

C_nTMA and egg-yolk PC were mixed at the proper molar ratio, dissolved in methanol and evaporated to dryness in vacuum. From this mixture, the dispersion was prepared with ^2H_2O at the weight ratio PC : ^2H_2O = 1 : 1. The samples were inclosed in glass tubes and equilibrated at room temperature for 12–14 h before the first ^{31}P-NMR measurement. Before the next ^{31}P-NMR experiments the samples were stored at 4°C, and immediately before the measurement they were briefly heated to 50°C and equilibrated as described above. In several experiments the samples were frozen in liquid nitrogen and thawed at room temperature before NMR experiments. The freeze-thaw cycle was repeated several times.

^{31}P-NMR spectra were recorded on a Bruker HX 90 spectrometer operating at 36.4 MHz, which had facilities for temperature control, a deuterium lock and a home-build proton decoupling unit. All spectra were obtained in the presence of high-power (600 W) proton decoupling. For the Fourier transform spectra, up to 2000 free induction decays were accumulated, employing 6–8 μs radio-frequency pulse, 25 μs dwell time and 0.7 s delay time.

Results and Discussion

As indicated in Fig. 1a, PC + C_nTMA dispersions at 25°C exhibit broad asymmetric ^{31}P-NMR spectra, characteristic of the lamellar liquid-crystalline phospholipid phase L_α [10,11]. This type of

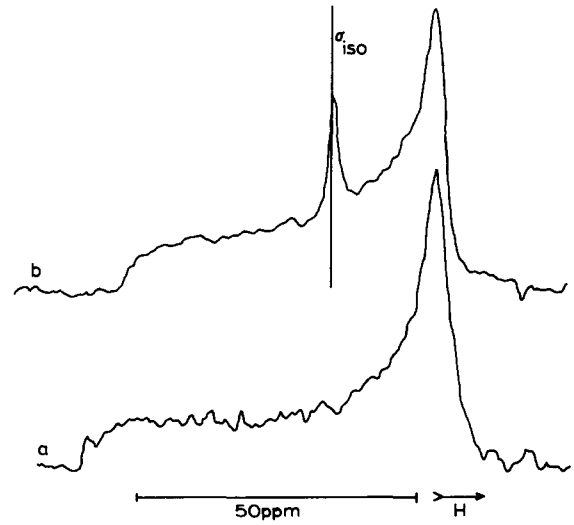

Fig. 1. 36.4 MHz ^{31}P-NMR proton-decoupled spectra of (a) egg-yolk PC: *N*-octadecyl-*N*,*N*,*N*-trimethylammonium iodide (1 : 1.2 molar ratio) dispersion in ^2H_2O (1 : 1 weight ratio), (b) egg-yolk PC : *N*-nonyl-*N*,*N*,*N*-trimethylammonium iodide (1.1 : 1 molar ratio) dispersion in ^2H_2O (1 : 1 weight ratio).

spectrum we observed at molar ratios C_nTMA : PC < 0.7 for ammonium derivatives with an alkyl length of $n = 6.9$, and up to a molar ratio C_nTMA : PC = 1.2 for derivatives with $n = 3$, 12, 16 or 18, and up to C_nTMA : PC = 4.0 for C_{14}TMA. From the spectra we determined the effective anisotropy of the chemical shift ($-\Delta\sigma_{eff}$). The plot of $-\Delta\sigma_{eff}$ as a function of C_nTMA : PC molar ratio and as a function of length of C_nTMA alkyl substituents is shown in Fig. 2. It is evident that the $-\Delta\sigma_{eff}$ increases with the increase in C_nTMA concentration, as well as with increasing the alkyl-chain length. This increase in $-\Delta\sigma_{eff}$ in dependence on C_nTMA concentration can be caused not only by the changes of the conformation of the phospholipid polar region, but also by changes in the type of the whole phospholipid molecule motion. A similar increase in $-\Delta\sigma_{eff}$ was observed in several binary systems consisting of phospholipids and drugs with quaternary nitrogen atoms – for example DPPC + diethazine hydrochloride and DPPC + chlorpromazine hydrochloride [13], and phosphatidylserine + dibucaine [14]. It has been concluded from these results that the motion of the phosphate is hindered in the presence of diethazine and chlorpromazine [13], or that the dibucaine induces appreciably more order in the phosphate region [14]. We have found that it is possible to obtain some information on the lipid conformation from the undecoupled ^{31}P-NMR spectra. In the lamellar phase L_α, the addition of C_nTMA induces broadening of undecoupled spectra, and a shift of the maximum to a higher field (Fig. 3). We have simulated undecoupled spectra as described in the Appendix and have found that the simultaneous upfield shift of the maximum and broadening of the signal can be ascribed to the substantial decrease of proton-phosphorus dipole-dipole coupling constants $\bar{\alpha}_A$ and $\bar{\alpha}_B$. As an example, some simulated spectra are shown in Fig. 4. It is noteworthy that the similarity between measured (Fig. 3a and b) and calculated (4a, b and c) spectra indicates changes of the polar headgroup conformation under the influence of C_nTMA ions.

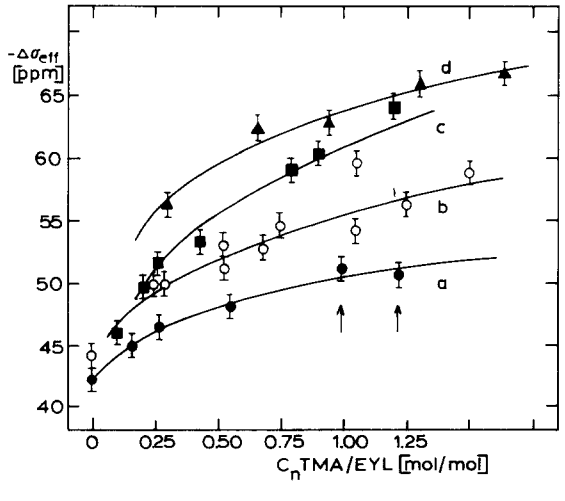

Fig. 2. Effect of *N*-alkyl-*N*,*N*,*N*-trimethylammonium iodides on the ^{31}P-NMR chemical-shift anisotropy of egg-yolk PC dispersion in ^2H_2O. (a) *N*-hexyl-*N*,*N*,*N*-trimethylammonium iodide; (b) *N*-nonyl-*N*,*N*,*N*-trimethylammonium iodide; (c) *N*-octadecyl-*N*,*N*,*N*-trimethylammonium iodide; (d) *N*-hexadecyl-*N*,*N*,*N*-trimethylammonium iodide.

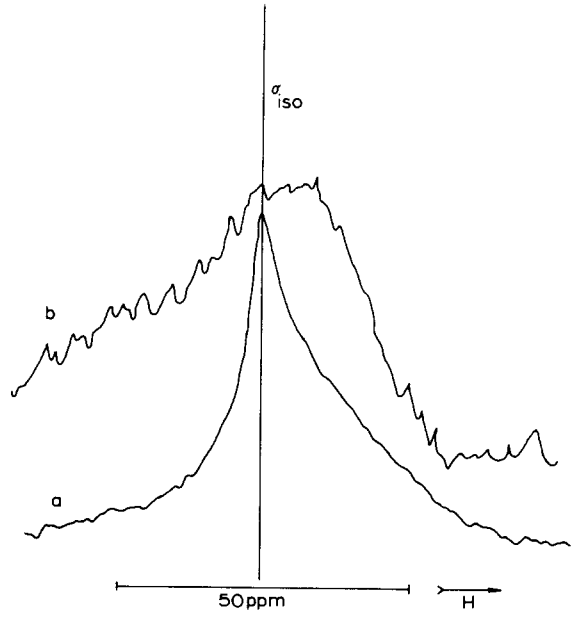

Fig. 3. 36.4 MHz ^{31}P-NMR proton-undecoupled spectra of (a) egg-yolk PC dispersion in ^2H_2O (1 : 1 weight ratio), (b) egg-yolk PC : *N*-hexadecyl-*N*,*N*,*N*-trimethylammonium iodide (1.1 : 1 molar ratio) dispersion in ^2H_2O (1 : 1 weight ratio).

The increase in $-\Delta\sigma_{eff}$ with increasing alkyl chain length of C_nTMA can be explained by two effects. The first one relates to the increased partition of derivatives with longer alkyl chains in the lipid bilayer. It was demonstrated that a dissociation constant describing the dissociation of C_nTMA from the DPPC bilayer is $2.6 \cdot 10^{-4}$ mol/l for C_{10}TMA, but only $2.1 \cdot 10^{-5}$ mol/l for C_{12}TMA

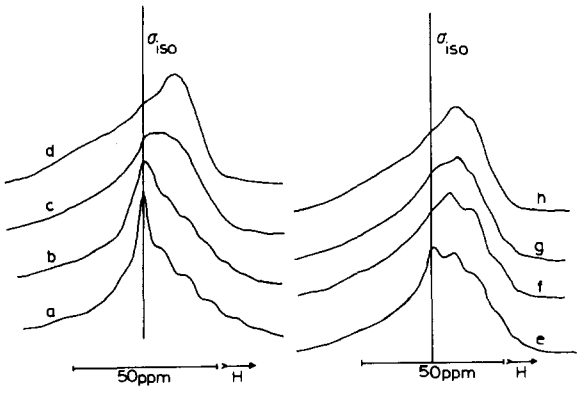

Fig. 4. 36.4 MHz ^{31}P-NMR proton-undecoupled spectra calculated as described in the Appendix.
(a) $\bar{\alpha}_A = 1214$ Hz $\bar{\alpha}_B = 607$ Hz, (b) $\bar{\alpha}_A = 970$ Hz $\bar{\alpha}_B = 485$ Hz,
(c) $\bar{\alpha}_A = 606$ Hz $\bar{\alpha}_B = 303$ Hz, (d) $\bar{\alpha}_A = 364$ Hz $\bar{\alpha}_B = 182$ Hz,
(e) $\bar{\alpha}_A = 606$ Hz $\bar{\alpha}_B = 485$ Hz, (f) $\bar{\alpha}_A = 606$ Hz $\bar{\alpha}_B = 121$ Hz,
(g) $\bar{\alpha}_A = 485$ Hz $\bar{\alpha}_B = 364$ Hz, (h) $\bar{\alpha}_A = 485$ Hz $\bar{\alpha}_B = 121$ Hz.
In all the calculated spectra $\Delta\nu_{1/2} = 60$ Hz, $\Delta\nu_{1/2}^{(DD)} = 120$ Hz.

[15]. Furthermore, the activation energy of the C_nTMA interaction with DPPC monolayers is a linear function of n for $n = 6$, 8, 12 or 16 [16]. The second possible explanation supposes that the C_nTMA ions have different effects on $-\Delta\sigma_{eff}$ also at equal bilayer concentrations, due to the different deformations of the bilayer structure. At the low water concentrations used in our experiments, the effect of different partition coefficients play probably a minor role.

Noteworthy were the properties of samples prepared with the $C_{18}TMA$ ion. We have observed that these samples differ macroscopically from samples with other C_nTMA ions. Samples with $C_{18}TMA$ were powder-like, water added to these samples after evaporation of organic solvent separated as an additional phase, which indicates reduced swelling in comparison to samples with ions of another C_nTMA. It is possible that addition of $C_{18}TMA$ ions induces a new lamellar phase in egg yolk PC, which probably differs in its hydration properties. Some indication for the formation of this new phase in DPPC bilayers under the influence of $C_{18}TMA$ has also been obtained by Jain and Wu [9] in their calorimetric studies.

At molar ratios $C_nTMA : PC > 0.8$ and alkyl

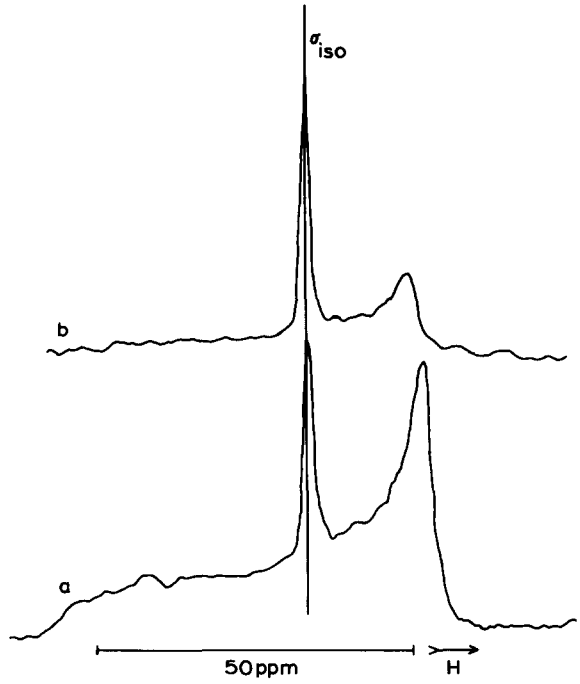

Fig. 5. Effect of temperature on the 36.4 MHz ^{31}P-NMR proton-decoupled spectrum of egg-yolk PC: *N*-nonyl-*N*,*N*,*N*-trimethylammonium iodide (0.8:1 molar ratio) dispersion in ^2H_2O (1:1 weight ratio). (a) 25°C; (b) 85°C.

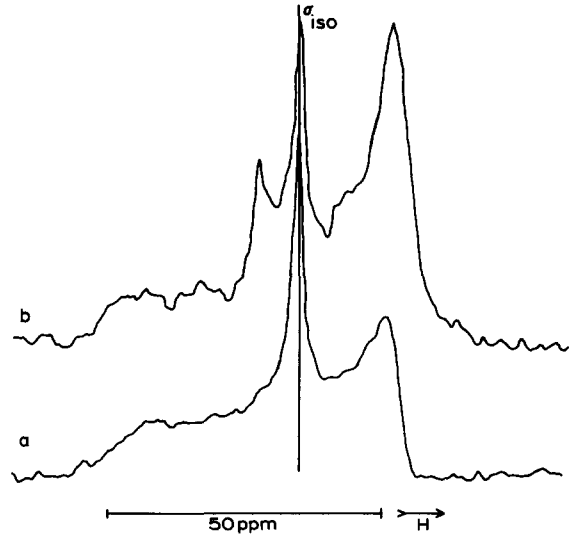

Fig. 6. 36.4 MHz ^{31}P-NMR proton-decoupled spectra of *N*-hexyl-*N*,*N*,*N*-trimethylammonium iodide: egg-yolk PC (0.99:1 molar ratio) dispersion in ^2H_2O (1:1 weight ratio). Spectra were measured immediately after preparation (a), or after 1 week incubation at 4°C (b).

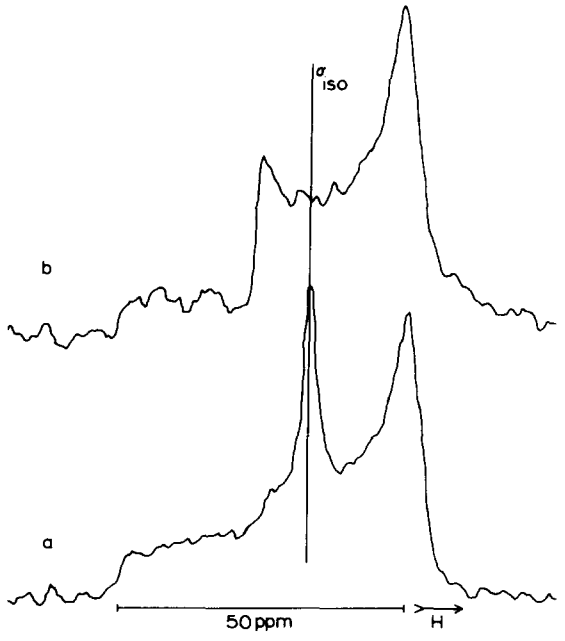

Fig. 7. 36.4 MHz ^{31}P-NMR proton-decoupled spectra of *N*-hexyl-*N*,*N*,*N*-trimethylammonium iodide: egg-yolk PC (1.22 : 1 molar ratio) dispersion in $^{2}H_2O$ (1 : 1 weight ratio). Spectra were measured immediately after preparation (a), or after 1 month incubation at 4°C (b).

chain lengths $n = 6$ or 9, we have again observed broad asymmetric L_α type ^{31}P-NMR spectra but with a superposed narrow spectral component indicating a fraction of the phospholipid in structures allowing effectively isotropic motion of the phosphate group (Fig. 1b, Fig. 6a, Fig. 7a). There are several factors which are responsible for the changes of the relative phospholipid concentration in the structure allowing isotropic motion, because at the same C_6TMA : PC molar ratio we observed spectra with different amplitudes of the isotropic signal, even in the case when the samples were prepared from the same stock solutions of PC and C_nTMA (compare spectra in Fig. 6a and Fig. 7a). The equilibration of sample seems to be very important, because we have observed different isotropic signal amplitudes after the different sample preparation procedures described in Materials and Methods. The second important factor is the temperature. We observed that the amplitude of isotropic signal increases with the increase of temperature; this temperature effect was fully reversible (Fig. 5). When studying the effect of sample equilibration, we have accidentally found that, in samples stored at 4°C for 1 to 4 weeks, the amplitude of the isotropic signal slowly decreases and a new peak appears upfield to the isotropic signal (Fig. 6b, Fig. 7b). This new spectral component can be ascribed to the liquid-crystalline phospholipid phase in which the phosphatidylcholine molecules undergo rapid diffusion around cylindrical structures, such as in hexagonal phases. The effective anisotropy of the ^{31}P chemical shift for such a type of motion is $-1/2$ of the effective anisotropy of chemical shift of the phospholipid ^{31}P nucleus in the lamellar L_α phase [17]. We analyzed the prepared samples 1 month after preparation, and found that the concentration of impurities (mainly lysophosphatidylcholine) was about 5% of the concentration of egg-yolk PC. We cannot exclude some effects of the lysophosphatidylcholine formation in the sample on the transition to the hexagonal phase, but we think the more probable cause for the transition is that the structure with the isotropic signal is an intermediate metastable phase between the lamellar phase, L_α, and the hexagonal phase. Intermediate phases with an isotropic ^{31}P-NMR peak have been found to exist in several phospholipid dispersions, for example in the binary systems phosphatidylethanolamine-phosphatidylcholine [17] and cardiolipin-Ca^{2+} [18], and in the ternary system phosphatidylserine-phosphatidylethanolamine-Ca^{2+} [14]. The narrow spectral component has a chemical shift characteristic of sonicated phospholipid vesicles. However, beside the vesicles, effective isotropic motion should occur also in the cubic, rhombohedral, micellar and inverted micellar phases, as well as in the vesicles [17]. Therefore, it is impossible to escribe the isotropic signal to some definite phase.

Regardless of the actual structures of the non-lamellar phases, it is clear from our results that the length of alkyl substituent is important for their formation. A similar observation with mixtures of K^+ soaps of different chain length was published by Charvolin and Mely [19]. For small differences in the chain lengths they observed a lamellar phase, but an increase in the difference resulted in a phase transition to the cubic and hexagonal phases. Derzhanski and Bivas [20] have shown theoretically that formation of defects due to different chain lengths in the hydrophobic region of the

bilayer results in an elastic deformation, and the phase which needs the minimum elastic energy will be stabilised. From our experiments it thus follows that the deformation of the bilayer in the presence of C_{12}TMA, C_{14}TMA, C_{16}TMA and C_{18}TMA is smaller than in the presence of C_6TMA and C_9TMA. This is in agreement with the results of Frischleder and Gleichmann [21], according to which C_6TMA (and C_9TMA) decreases the transition temperature between gel and liquid-crystalline phases of DPPC, while in the case of C_{18}TMA a small increase in the transition temperature was observed.

We hope that our results may explain some biological effects of C_nTMA reviewed in the Introduction. Because of the different deformation effects of C_nTMA at $n < 10$ and $n > 10$ on the lipid model membranes, there should also be a different deformation of the lipidic part of biological membranes. Therefore, sharp changes in the character of action of alkylammonium ions may occur at a given alkyl chain length, such as in the case of muscles (cholinolytic-cholinomimetic effect), or the biological effect may show sigmoidal dependence on the alkyl length such as in the case of K^+-current inactivation in squid axon.

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Appendix

Simulations of ^{31}P-NMR spectra have been carried out as described earlier [22,23]. The Hamiltonian of ^{31}P nucleus is divided into time-dependent and time-independent parts. The time-independent part consists of the time-averaged chemical shift anisotropy and the dipolar interaction between phosphorus and protons of the neighbouring CH_2 groups. The assumptions of a fast rotation of the phospholipid molecule about the bilayer normal (director) and a fast lateral diffusion in the bilayer plane are used in the derivation of both parts of the Hamiltonian. Using the formalism of irreducible tensors and Wigner rotational matrices, the time-averaged chemical shift anisotropy part of the time-independent Hamiltonian yields for ^{31}P nucleus the resonance frequences:

$$\nu_L(\beta_{DL}) = \nu_{iso} + \tfrac{1}{3}\Delta\nu_{eff}\left(3\cos^2\beta_{DL} - 1\right) \quad (1)$$

with: ν_{iso}, resonance frequency in the case of rapid isotropic phosphate group reorientation; $\Delta\nu_{eff}$, effective anisotropy of chemical shift in frequency units; β_{DL}, angle between the Z axes of the laboratory coordinate system and the director coordinate system.

It is supposed that the resonance lines are Lorentzians with the linewidth $\Delta\nu_{1/2}^{(0)}$. The proton decoupled ^{31}P-NMR spectrum can be described as:

$$J(\nu) = \frac{1}{2\pi}\int_0^{\pi} I_0 \Delta\nu_{1/2}^{(0)} \sin\beta_{DL}\left\{\Delta\nu_{1/2}^{(0)2} + 4\left[\nu_{iso} + \tfrac{1}{3}\Delta\nu_{eff}\left(3\cos^2\beta_{DL} - 1\right) - \nu\right]^2\right\}^{-1} d\beta_{DL} \quad (2)$$

The intermolecular dipolar interaction between phosphorus and protons in different phospholipid molecules is unimportant because of fast phospholipid rotation and lateral diffusion in the liquid-crystalline state. These motions average the angular dependences of the intermolecular dipolar interaction, so that there could be only some influence of these interactions on the homogeneous line broadening (i.e., relaxation processes).

The effects of intramolecular dipolar interaction are separated into two parts. The first part is an orientation-dependent splitting of the resonance lines. Using the same formalism as described above, the ^{31}P-NMR line is split into a set of lines occuring at frequences:

$$\nu_K(\beta_{DL}) = \tfrac{1}{2}\left(3\cos^2\beta_{DL} - 1\right)\sum_j m_j \alpha_j^{eff} + \nu_L(\beta_{DL}) \quad (3)$$

with: m_j, spin quantum number of the jth proton; and α_j^{eff}, effective dipolar coupling constant. It is supposed that the splitting is observed only in the case of phosphorus dipolar interaction with the protons of the nearest neighbouring CH_2 groups. Furthermore, it is supposed that the coupling constants of both protons in the same CH_2 group is

the same. There are thus obtained two coupling constants $\bar{\alpha}_A$ and $\bar{\alpha}_B$ and splitting of the resonance line into nine lines at frequencies (in respect to ν_L frequency):

$$\Delta\nu = \tfrac{1}{2}\left(3\cos^2\beta_{DL} - 1\right) A_K \tag{4}$$

with $A_K = -\bar{\alpha}_A - \bar{\alpha}_B$; $-\bar{\alpha}_A$; $-\bar{\alpha}_A + \bar{\alpha}_B$; $-\bar{\alpha}_B$; 0; $+\bar{\alpha}_B$; $+\bar{\alpha}_A - \bar{\alpha}_B$; $+\bar{\alpha}_A$; $+\bar{\alpha}_A + \bar{\alpha}_B$. The ratio of intensities, I_K, of the nine lines is 1 : 2 : 1 : 2 : 4 : 2 : 1 : 2 : 1.

The second part of the effects of intramolecular dipolar interaction is described as an orientation-dependent broadening of the resonance lines:

$$\Delta\nu'_{1/2} = \Delta\nu_{1/2} + \Delta\nu_{1/2}^{(DD)}\left(\frac{3\cos^2\beta_{DL} - 1}{2}\right) \tag{5}$$

where $\Delta\nu_{1/2}^{(DD)}$ is the angular dependent contribution caused by dipolar interaction of phosphorus with protons except CH_2 groups in the nearest neighbourhood, and $\Delta\nu_{1/2}$ is the angular-independent contribution. The angular-independent contribution includes also the effects of intermolecular dipolar interactions.

The ^{31}P-NMR undecoupled spectrum is described as

$$J(\nu) = \frac{1}{2\pi}\sum_{K=1}^{9}\int_0^{\pi} I_K \Delta\nu'_{1/2}\sin\beta_{DL}\left\{\Delta\nu'^2_{1/2} + 4\left[\nu_{iso} + \left(\tfrac{1}{3}\Delta\nu_{eff} + \tfrac{1}{2}A_K\right)\left(3\cos^2\beta_{DL} - 1\right) - \nu\right]^2\right\}^{-1} d\beta_{DL} \tag{6}$$

For calculation, the integration in Eqn. 6 was replaced by a summation with angular intervals of one degree or less. Calculations were carried out on EC 1040 and KRS 4200 computers.

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