undecanoic acid nor oleic acid has much effect on the ATPase activity of (Ca^{2+}-Mg^{2+})-ATPase, or of (Ca^{2+}-Mg^{2+})-ATPase reconstituted with dioleoylphosphatidylcholine, although oleic acid does cause a large stimulation of (Ca^{2+}-Mg^{2+})-ATPase reconstituted with the shorter chain phospholipid dimyristoleoylphosphatidylcholine (A. C. Simmonds et al., unpublished experiments).

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Interactions between Phospholipid Head Groups at Membrane Interfaces: A Deuterium and Phosphorus Nuclear Magnetic Resonance and Spin-Label Electron Spin Resonance Study†

Frank Sixl and Anthony Watts*

ABSTRACT: The head group interactions in fully hydrated, mixed bilayers of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, specifically deuterated in the head groups at the α- and β-methylene and $N(CD_3)_3$ positions, have been investigated by deuterium and phosphorus-31 nuclear magnetic resonance (NMR) and 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) spin-label electron spin resonance (ESR) studies at pH 7.5. In binary lipid bilayers, the two lipids were found to be completely miscible by spin-label partitioning and phosphorus-31 NMR experiments. Also the phosphorus-31 NMR studies showed no evidence that the mean orientation of the phosphate group of either lipid was significantly altered in the binary system. In contrast, large changes in the deuterium residual quadrupole splittings for the deuterated head group segments were observed by deuterium NMR for both phosphatidylcholine and phosphatidylglycerol when one lipid was titrated against the other. The quadrupole splittings for the choline methyls decreased from 1.4 kHz in pure phosphatidylcholine bilayers to 0.4 kHz for bilayers containing 75 mol % of phosphatidylglycerol at 28 °C. A similar behavior was found for the head group β-CD_2 segment; the quadrupole splittings were reduced from 5.8 to 1.1 kHz in the same range of concentration and temperature. A converse observation was made for the α-CD_2 group in that the quadrupole splittings increased from 6.3 to about 10 kHz, and in mixed bilayers containing less than 50 mol % of phosphatidylcholine; magnetic inequivalence of the two deuterons gave two very well resolved NMR lines. In bilayers containing head group deuterated dimyristoylphosphatidylglycerol, the two α-CD_2 deuterons showed quadrupole splittings of 9.0 and 10.8 kHz, which increased to 10.2 and 11.3 kHz, respectively, on adding 75 mol % of phosphatidylcholine at 28 °C; for the β-CD the quadrupole splittings increased from 1.8 to 6.6 kHz for the same mixture ratio and temperature. The deuterium spin-lattice relaxation times T_1 remained essentially unchanged for the α-CD_2 and β-CD_2 groups of dimyristoylphosphatidylcholine but increased for the choline methyls by about 20% when dimyristoylphosphatidylglycerol and dimyristoylphosphatidylcholine were mixed in a 1:1 mole ratio. The results indicate that some reorientations in the lipid head groups and changes in their amplitudes of motion are induced in the two-component bilayers by the presence of one lipid on the other but their rates of motion remain rather similar.

The properties of biological and model membranes depend to a large extent on the head group structure of their phospholipid molecules. The binding of ions (Akutsu & Seelig, 1981) and antibiotics (Sixl & Galla, 1979, 1980), as well as the function of membrane-bound enzymes (Sanderman, 1978), is believed to be determined by the presence of structurally different phospholipid head groups and may be triggered by the surface charge of the lipid matrix (Watts et al., 1978). In particular, the choline head group of synthetic lecithins has been studied in detail by a variety of techniques, such as X-ray (Pearson & Pascher, 1979), proton NMR[1] (Hauser, 1981),

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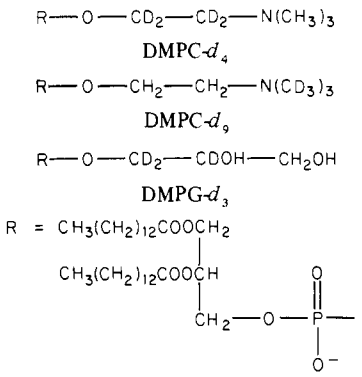
laser Raman scattering (Akutsu, 1981), and phosphorus-31 and deuterium NMR spectroscopy (Seelig et al., 1977; Gally et al., 1975; Skarjune & Oldfield, 1979). Also, phosphorus and deuterium NMR have been used to study the influence of cholesterol (Brown & Seelig, 1978), anesthetics (Boulanger et al., 1981), and metal ions (Akutsu & Seelig, 1981) on the structure and motion of the choline residue in lecithin bilayers.

In spite of the fact that a wide variety of different phospholipid head groups is usually present in natural membranes, very little is known about the mutual perturbation of one lipid head group by the others and any concomitant changes in the acyl chain motions. Only recently has information on head group interactions in binary lipid mixtures been reported (Browning, 1981a–c).

In the present study, mixtures of two naturally occurring lipid types, phosphatidylcholine and phosphatidylglycerol, have been investigated by ESR and deuterium and phosphorus-31 NMR spectroscopy. Both lipids are completely miscible (Findlay & Barton, 1978; Van Dijck et al., 1978), and basic structural information is available not only about the phosphatidylcholine (Gally et al., 1975) but also about the phosphatidylglycerol head group (Wohlgemuth et al., 1980) in single component bilayers. In addition, when the two lipids have identical acyl chains, the bilayer solid–fluid melting behavior is very similar, with pretransition and main transition temperatures for the 1,2-dimyristoyl derivatives of 12 and 24 °C, respectively (Marsh et al., 1977; Watts et al., 1978).

Materials and Methods

Lipid Synthesis. The two lipids, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol, were specifically deuterated in their head groups.



DMPC-d_9 was synthesized by methylation of DMPE with CD_3I as described by Eibl (1980). DMPC-d_4 was prepared in the same way with DMPE-d_4 and CH_3I. DMPE-d_4 was synthesized from dimyristoylglycerol (Eibl, 1978) with perdeuterated ethanolamine, which was produced by repeated catalytic exchange of protonated ethanolamine against D_2O (Taylor & Smith, 1981).

The synthesis of DMPG is described elsewhere (Harlos & Eibl, 1980). For the preparation of the head group deuterated derivative, 1,2-*O*-isopropylidene-*sn*-glycerol-*3*-d_2 was used. This compound was prepared by oxidation of 1,2:5,6-diisopropylidene-D-mannitol with $NaIO_4$ and reduction of the isopropylidene-D-glyceraldehyde with $NaBD_4$ in D_2O. A limited fraction of the C(2) H atom was found to be exchanged against deuterium, yielding a mixture of DMPG-d_2 and DMPG-d_3, which will be called DMPG-d_3 in the following. All lipids were purified by silica acid chromatography in basic solvents, crystalized from acetone, and stored at −20 °C until required.

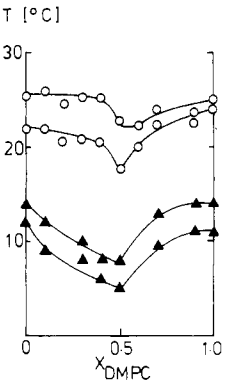


FIGURE 1: Phase diagram for DMPC/DMPG liposomes obtained from Tempo partitioning experiments. The onset and completion points of the pretransition (▲) and main transition (○) are derived from the heating curves according to Shimshick & McConnell (1973). Buffer 0.1 M Tris-HCl, pH 7.5; lipid concentration 50–100 mg/mL; Tempo concentration 10^{-4} M; heating rate 20 °C/h.

Sample Preparation. Samples for NMR experiments were prepared from stock solutions of each lipid in $CHCl_3/CH_3OH$ (50 mg/mL) and by mixing suitable amounts to give the desired composition. The solvent was removed with nitrogen at 40 °C. The lipid mixture was dried under high vacuum over night and then fully hydrated at 40 °C with an excess of 0.1 M Tris-HCl, pH 7.5 (Watts et al., 1981). The final lipid concentration was 50–100 mg/mL. Buffer solutions were made from deuterium-depleted water, and pH values were checked after each preparation by spotting onto pH paper. Thin-layer chromatography revealed no evidence for lipid decomposition after the NMR or ESR measurements.

Samples for Tempo spin-label partitioning studies were prepared as described by Recktenwald & McConnell (1981) for DMPC/cholesterol mixtures, with 0.1 M Tris-HCl, pH 7.5, instead of phosphate-buffered saline. As in the NMR experiments, lipid concentrations of 50–100 mg/mL were used, and a TEMPO concentration of 10^{-4} M was employed.

Measurements. NMR spectra were recorded on a Bruker WH-300 spectrometer at 46.1 MHz (D) and 121.4 MHz (^{31}P). Single-pulse modes were used for the D NMR spectra with a 90° pulse width of 29 μs. The low radio frequency power of the spectrometer resulted in spectral distortions, particularly for the broad spectra from DMPG-d_3 samples. As reported by Gally et al. (1981), such distortions have no effect on the observed quadrupole splittings. Deuterium spin–lattice relaxation times T_1 were measured by the inversion–recovery technique, 180°–τ–90° pulse sequences being employed. ^{31}P NMR spectra were recorded under broad-band proton decoupling at a power of 7–10 W. ESR experiments were carried out on a Varian E109 ESR spectrometer. Both NMR and ESR spectrometers were equipped with nitrogen gas flow variable temperature units. The temperature was measured to an accuracy of ±0.5 °C.

Results

Tempo Partitioning. In order to investigate the phase behavior or DMPC/DMPG mixtures, it is necessary to study the partitioning of the spin probe Tempo between liposomes

[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; *X*, molar fraction; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; NMR, nuclear magnetic resonance; ESR, electron spin resonance; Tris, tris(hydroxymethyl)-aminomethane.

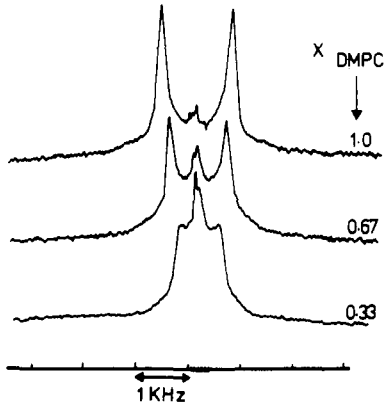


FIGURE 2: D NMR spectra (46.1 MHz) of DMPC-d_9/DMPG mixtures for different molar fractions ($X_{DMPC\text{-}d_9}$): temperature 28 °C; buffer 0.1 M Tris-HCl, pH 7.5. The samples contained 20–30 mg of DMPC-d_9. 10-μs (30°) pulses were applied. The number of scans was 300.

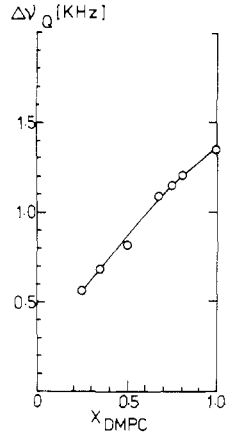


FIGURE 3: Quadrupole splittings $\Delta\nu_Q$ of DMPC-d_9 in mixtures with DMPG as a function of molar fraction ($X_{DMPC\text{-}d_9}$). Conditions were as for Figure 2.

and the aqueous phase as a function of temperature. A phase diagram (Figure 1) was constructed from the phase-transition–temperature curves, according to Shimshick & McConnell (1973). Both lipids were found to be completely miscible with each other in every proportion, as indicated by the occurrence of a single main transition. A pretransition was also detected for all DMPG/DMPG ratios.

The phase diagram shows that the temperatures and widths of the phase transitions in DMPC/DMPG mixtures are quite similar to those of the pure components. Only a small broadening of the main transition was observed for molar fractions of DMPC of about 0.5. For this composition only, a minimum was found in the midpoints of pretransition and main transition, which was 4 °C for the main transition and 6 °C for the pretransition below the values of 12 and 24 °C in both pure DMPC and pure DMPG liposomes. These observations agree very closely with some calorimetric studies (Van Dijck et al., 1978) but are in contrast to other similar studies (Findlay & Barton, 1978), possibly due to different pH values and ion concentrations used by these latter workers.

Head Group Deuterated DMPC. Deuterium NMR spectra of DMPC-d_9 in mixtures with DMPG are shown in Figure 2. The line shapes are typical for randomly oriented bilayers of deuterated lipids (Seelig, 1977). Increasing amounts of DMPG reduce the observed quadrupole splitting (Figure 3) such that in pure DMPC-d_9 bilayers at 28 °C the quadrupole splitting was 1.38 kHz and decreased to 0.55 kHz at a DMPC-d_9/DMPG molar ratio of 1:3. A similar decrease of

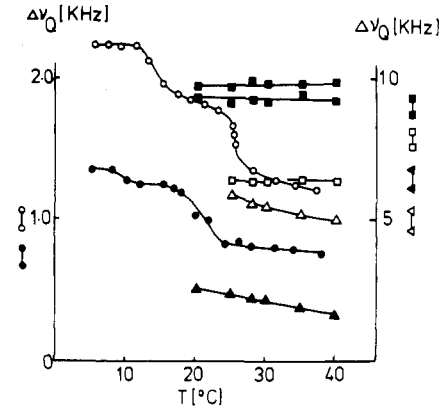


FIGURE 4: Quadrupole splittings $\Delta\nu_Q$ of DMPC-d_9 (○, ●) and DMPC-d_4 [(□, ■) α-CD_2; (△, ▲) β-CD_2] as a function of temperature: (open symbols) DMPC bilayers; (filled symbols) bilayers of DMPC/DMPG in a 1:1 molar proportion. Conditions were as for Figure 2.

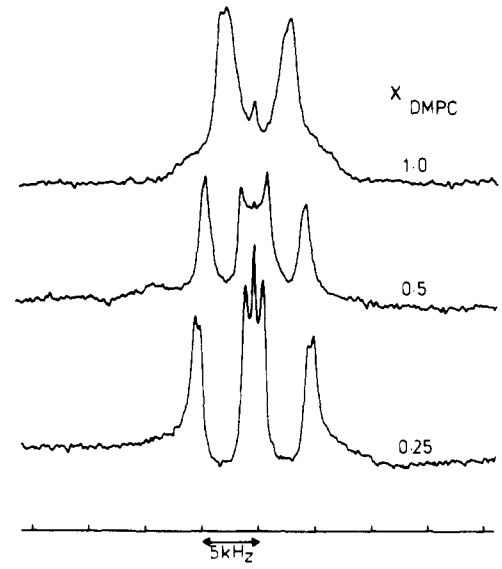


FIGURE 5: D NMR spectra (46.1 MHz) of DMPC-d_4/DMPG mixtures of different composition at 28 °C. The smaller quadrupole splittings are due to the β-CD_2 segment; the outer signals arise from the α-CD_2 group. In pure DMPC-d_4, the β-CD_2 component appears as a shoulder in the inner part of the spectrum. Samples contained 50–70 mg of DMPC-d_4. 5000 free-induction decays were accumulated, with 7-μs (20°) pulses.

the quadrupole splitting was found in the gel phase, below and above the pretransition. The temperature dependence of the $N(CD_3)_3$ quadrupole splittings is plotted in Figure 4 for DMPC-d_9 and a 1:1 mixture with DMPG. The phase transition curves are in accordance with the Tempo partitioning results for mixed bilayers (Figure 1) and for the individual pure lipid bilayers.

The temperature dependence of the α-CD_2 and β-CD_2 quadrupole splittings is essentially the same for pure DMPC-d_4 and for a 1:1 mixture with DMPG (Figure 4). For both bilayer compositions the α-CD_2 quadrupole splitting is almost independent of temperature, which is in agreement with results reported for DPPC (Gally et al., 1975), whereas for the β-CD_2 segment the splitting decreased significantly with temperature.

The effect of DMPG on the α-CD_2 and β-CD_2 segments in DMPC-d_4 is demonstrated in Figures 5 and 6; again the results are given for a temperature of 28 °C. At this temperature the quadrupole splittings for α-CD_2 and β-CD_2 segments in pure DMPC-d_4 bilayers are very close together with values of 5.8 kHz (β-CD_2) and 6.3 kHz (α-CD_2).

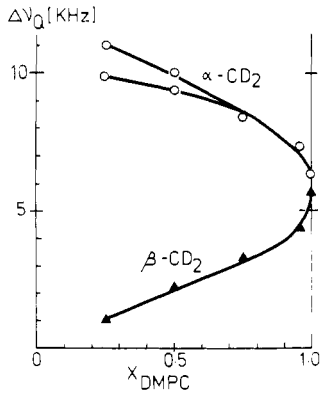


FIGURE 6: Quadrupole splittings $\Delta\nu_Q$ for the α-CD_2 and β-CD_2 segments of DMPC-d_4 in mixtures with DMPG as a function of molar fraction of DMPC ($X_{DMPC\text{-}d_4}$). Other conditions were as for figure 2.

Table I: Deuterium Spin-Lattice Relaxation Times (T_1) for DMPC-d_4 and DMPC-d_9 in Fully Hydrated Bilayers at 38 °C with and without DMPG at pH 7.5

	T_1 (ms)	
	pure DMPC	DMPC/DMPG (1:1)
DMPC-d_4		
α-CD_2	24 ± 4	26 ± 3
β-CD_2	25 ± 2	27 ± 2
DMPC-d_9	53 ± 3	62 ± 4

Therefore, the β-CD_2 powder pattern only appears as a shoulder at the inner edges of the spectrum. At temperatures above 35 °C both spectral components become very well resolved. The assignment of the two quadrupole splittings had been carried out by Gally et al. (1975) for DPPC.

The presence of DMPG in DMPC-d_4 bilayers causes a reduction in the quadrupole splittings of the β-CD_2 segment (Figure 6). At 28 °C, the quadrupole splitting for β-CD_2 was found to be 1.1 kHz in a mixture with a molar ratio of DMPC-d_4/DMPG of 1:3, compared to a 5-fold larger value of 5.8 kHz in pure DMPC-d_4. In contrast, the quadrupole splittings increase for the α-CD_2 group when DMPG is added, from 6.3 kHz in pure DMPC-d_4 to 9.8 and 11.1 kHz for the resolved doublet in DMPC-d_4/DMPG bilayers with a mole ratio of 1:3. A similar resolution of the two C(α) deuterons was previously observed in DPPC bilayers containing cholesterol (Brown & Seelig, 1978), and in the presence of Cd^{2+} (Akutsu & Seelig, 1981), and presumably arises from the magnetic nonequivalence of both deuterons (Büldt & Wohlgemuth, 1981).

Deuterium Spin–Lattice Relaxation Times (T_1). Deuterium spin–lattice relaxation times T_1 give information about the dynamic behavior of a deuterated segment. The T_1 values for DMPC-d_4 were measured at 38 °C, a temperature at which the quadrupole splittings of the α-CD_2 and β-CD_2 groups are well resolved. Results for pure DMPC-d_4, DMPC-d_9, and their 1:1 mixtures with DMPG are given in Table I. No difference in T_1 was observed between pure DMPC and the binary system with DMPG for either CD_2 groups. For the $N(CD_3)_3$ deuterons, T_1 increased slightly by 20% from 53 ms in DMPC-d_9 to 62 ms in an equimolar mixture with DMPG.

Head Group Deuterated DMPG. Deuterium NMR spectra were recorded for DMPG-d_3 in the presence of different amounts of unlabeled DMPC. Two typical spectra are shown in Figure 7, in which three quadrupole splittings were observed in each case. The outer two signals are assigned to the α-CD_2 group of the glycerol head group in 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol (Wohlgemuth et al., 1980). The narrow, low-intensity powder pattern is due to the partially deuterated β-CD segment (see Materials and Methods).

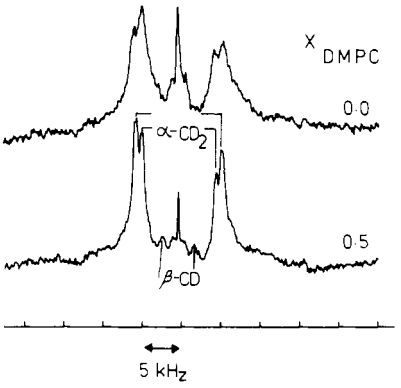


FIGURE 7: D NMR spectra (46.1 MHz) of DMPG-d_3 and 1:1 mixture with unlabeled DMPC. Samples contained 80–100 mg of DMPG-d_3. Spectra were recorded by accumulating 20 000 free-induction decays, with 7-μs (20°) pulses. Other conditions were as for Figure 2.

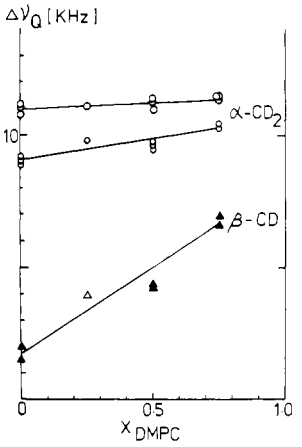


FIGURE 8: Quadrupole splittings of DMPG-d_3 in mixtures with DMPC as a function of molar fraction (X_{DMPC}). Conditions were as for Figure 2.

For both segments, an increase in the quadrupole splittings was measured when increasing amounts of DMPC were present (Figure 8). In particular, the β-CD group was very sensitive to the composition of the bilayers. The residual quadrupole splitting was 2.0 kHz in pure DMPG-d_3 but increased over 3-fold to 6.8 kHz in a DMPG-d_3/DMPC mixture with a molar fraction of $X_{DMPC} = 0.75$. Over the same concentration range the quadrupole splitting for the α segment changed by about 5% only.

^{31}P NMR Spectra. For a number of DMPC/DMPG ratios, proton-decoupled ^{31}P NMR spectra were recorded at 28 °C. The line shapes were always identical with theoretically calculated powder patterns of random dispersions of fluid phospholipid bilayers (Seelig, 1978). Chemical shift anisotropies were found to be −47 (DMPC) and −42 (DMPG) ppm. In mixtures of both lipids, one-component spectra were always observed with chemical shift anisotropies intermediate between these two limits (a value of −43 ppm was measured for an equimolar DMPC/DMPG bilayer). It was never possible to resolve two spectral components, as reported for DPPC/DPPE bilayers (Arnold et al., 1981).

Discussion

It has been shown previously that bilayers of phospholipids with different head groups have almost identical structural features in the hydrocarbon region. On a reduced temperature

scale, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were found to have fairly similar order parameters for the C(2) segments of their fatty acyl chains, varying maximally by 35% between an unsatuated, mixed acyl chain phosphatidylcholine (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and a saturated homoacyl chain phosphatidylethanolamine (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine) at temperatures close to the phase-transition temperature (Seelig & Browning, 1978). This similarity in acyl chain behavior is further substantiated for the case of DMPC and DMPG, as well as the dipalmitoyl derivatives, in that these two lipid types have remarkably similar pretransition and main transition temperatures at pH >4 when the phosphoglycerol lipid is deprotonated (Watts et al., 1978). The similarity in conformation extends to the most rigid part of phospholipids in bilayers, the glycerol backbone. For a variety of phospholipids deuterated in the glycerol backbone, deuterium NMR showed no differences in the quadrupole splittings between different lipid types (Gally et al., 1981).

The phosphorus-31 NMR results measured here show that both DMPC and DMPG in bilayers at pH 7.5 are completely miscible, at least within the experimental limits and uncertainties of the method. Single, one-component phosphorus-31 NMR powder patterns are observed, with averaged chemical shift anisotropies for mixtures of the lipids between the values for the two pure lipid bilayers (−47 ppm for pure DMPC and −42 ppm for DMPG at 28 °C). This indicates that no gross reorientation of the phosphate group occurs for either lipid on interaction with the other lipid type. Similarly, the Tempo partitioning ESR results indicate lipid miscibility by virtue of single transition temperatures measured for mixed bilayers (Shimshick & McConnell, 1973), being identical with those measured for the pure lipids individually (Watts et al., 1978). Thus no long-lived lipid domains exist, with different acyl chain properties from the pure lipids. The present ESR and phosphorus-31 NMR experiments therefore show that at all DMPC/DMPG ratios, the two lipids are completely miscible both in the acyl chains and phosphate groups, at least on the time scales of the two magnetic resonance methods. Calorimetric experiments have also been used to demonstrate the complete miscibility of DMPC and DMPG in bilayers at alkaline pH (Findlay & Barton, 1978; Van Djick et al., 1978).

In contrast to the very similar behavior of the acyl chains and phosphate groups in mixed bilayers, the deuterium NMR experiments give different results regarding the conformation of the methylene and terminal choline methyls in the head groups of the two lipids when mixed. In particular, the quadrupole splittings of the $N(CD_3)_3$ and the β-CD_2 groups in DMPC are very sensitive to the presence of DMPG. A considerable decrease of the quadrupole splittings for these groups by 63% for the $N(CD_3)_3$ group and by 86% for the β-CD_2 segment of DMPC was measured on the addition of DMPG to a DMPC/DMPG molar ratio of 1:3 (see Figures 3 and 6).

The α-methylene segment of phosphatidylcholines show a characteristic feature, namely, that its spectral line may split into a doublet under certain conditions (Brown & Seelig, 1978; Akutsu & Seelig, 1981) A similar doublet is resolved on introducing DMPG into the DMPC-d_4 bilayers (Figure 5). The appearance of this doublet in the head group of DMPC may be conformational or dynamic in origin. In pure DMPC, only one quadrupole splitting is observed, as a result either of two quickly interconverting conformers or of one single α-CD_2 conformation. As DMPG is titrated into the DMPC bilayer, the appearance of the spectral doublet may have two explanations. First, the rate of interconversion of the two possible conformations experienced in pure DMPC bilayers decreases to a value that is now slow on the deuterium NMR time scale. Second, the DMPG may induce the formation of additional or even new conformational states. The exchange rate between these two conformers in both situations must be slow on the deuterium NMR time scale, with an interconversion time of $\geq 10^{-3}$ s, the frequency difference between the two NMR lines. Since the deuterium spin–lattice relaxation times T_1, a dynamic indicator of motion, are virtually unchanged for the methylene head group segments when DMPG is added to the DMPC bilayers (Table I), the stronger probability is that a conformational rather than a dynamic perturbation is induced by the charged lipid on the choline head group.

The time-averaged amplitude of segmental motion for a CD bond under examination is usually expressed as an order parameter, S_{CD} (Seelig & Seelig, 1980). An inherent problem in the interpretation of CD order parameters is the contribution of two different factors to S_{CD} (Akutsu & Seelig, 1981): one is the mean orientation of the CD bond with respect to the axis of motional averaging; the other depends on the amplitude of fluctuations around this average orientation. The distinction between both contributions is difficult to discern. However, the results imply that the smaller glycerol head group of DMPG acts as a spacer for the bulky choline residues of DMPC. For further substantiation of this idea, X-ray evidence has shown that bilayers in the solid phase of dipalmitoylphosphatidylcholine and -phosphatidylglycerol (at pH 8.0) have very similar molecular areas of ~48 Å^2 at 20 °C (Chapman et al., 1967; Watts et al., 1981), defined predominantly by the interchain van der Waals forces. The phase-transition properties of these two lipids are also identical, as with the dimyristoyl derivatives (Watts et al., 1978). If this domination of the acyl chain interactions in determining the molecular area persists to the fluid lipid bilayer phase, then the area available to the glycerol lipid head group will be relatively much greater than that available to the choline head group on simply steric grounds. This suggestion is borne out by the results of Figure 4, in which the quadrupole splittings are seen to be considerably lower for the choline methyls when in a equimolar mixture with DMPG than in the pure DMPC bilayer over the whole temperature range examined. A similar behavior is observed for the β-CD_2 group of DMPC when mixed with DMPG (Figures 4 and 6). The reason why the quadrupole splittings for the α-CD_2 of DMPC increase on the addition of DMPG is also most probably an orientational phenomenon, as discussed earlier. In addition, the observation that the quadrupole splittings for the α- and β-CD_2 segments for DMPC can be measured to lower temperatures in the presence of DMPG than in pure lipid bilayers again reinforces the suggestion that the charged lipid head group allows more membrane surface area in which the choline head group as a whole can move. This perturbation of both methylene segments is in contrast to the differential effect induced by cholesterol in dipalmitoylphosphatidylcholine bilayers, the α-CD_2 remaining unchanged on the addition of cholesterol to phosphatidylcholine bilayers (Brown & Seelig, 1978). It should also be pointed out that the changes of the two methylene quadrupole splittings of DMPC, caused by the introduction of DMPG, are opposite to those caused by di- and trivalent ions on dipalmitoylphosphatidylcholine bilayers (Brown & Seelig, 1977; Akutsu & Seelig, 1981), where a decrease of the α-CD_2 and an increase of the β-CD_2 quadrupole splittings were observed.

Deuterium spin–lattice relaxation times T_1 can be used to give a measure of the correlation time (τ_c) for CD segmental motions (Brown et al., 1979). For small values of the order parameter for the CD bond, T_1 is inversely proportional to the rotational correlation time. In this study, the quadrupole splittings give small order parameters of $S_{CD} < 0.08$. The approximate correlation times for the α- and β-CD_2 segments of DMPC-d_4 are all around $(9–10) \times 10^{-11}$ s. These results indicate that the rate (τ_c^{-1}) of conformational change in the $-C(\alpha)D_2-C(\beta)D_2-$ segment is at least faster than 1×10^{10} s^{-1}, with less than a 10% decrease on the addition of 50 mol % of DMPG to the DMPC bilayer. A somewhat faster rate of $(CD_3)_3$ motion occurs at the membrane surface of phosphatidylcholine bilayers on the addition of DMPG, decreasing the correlation time by about 20% in the presence of 50 mol % DMPG. Again, as the quadrupole splitings indicated, the maximal perturbation incurred on the head group motion is at the choline moiety; this time the perturbation is dynamic in nature. The observed change is presumably due to organized surface water and hydrogen bonding, which would be more pronounced in the presence of the extra hydroxyl groups of the glycerol head group of DMPG (Browning, 1981b).

The perturbation of the glycerol head group in DMPG bilayers by DMPG was studied in DMPG-d_3/DMPC mixtures. The α-CD_2 segment was much less sensitive than the β position to mixing with the zwitterionic lipid, the α-CD_2 quadrupole splittings being only slightly larger in mixtures with DMPC than in pure DMPG-d_3 (Figure 8). Each of the α deuterons had its own NMR signal for every DMPG/DMPC ratio, although they converged as the DMPC concentration increased. These observations suggest that the effect of phosphatidylcholines on the glycerol head group is opposite to the perturbation of the choline residue under the influence of DMPG. The more bulky choline head group is therefore restricting the smaller glycerol head group, probably conformationally. Deuterium spin–lattice relaxation times for these segments were not measured and the distinction between dynamic and conformational perturbations cannot be made as readily as for the choline head group. However, proton NMR studies of DMPG bilayers sonicated into small unilamellar vesicles suggest that the glycerol hydroxyl groups are gauche to each other, which makes a favorable conformation for hydrogen-bond formation within the bilayer surface (Marsh & Watts, 1978). The presence of DMPC may disrupt this hydrogen-bonding stability at the bilayer surface and induce a tilt in the glycerol group from the averaged parallel orientation (Wohlgemuth et al., 1980) to an angular orientation with respect to the bilayer surface.

The present study of conformation and dynamics of lipid head group interactions at membrane surfaces demonstrates the high degree of sensitivity of the approach. Relatively large conformational sensitivity has been observed, distinct from dynamic perturbations, by mixing one lipid with another in hydrated bilayers. Although the angular changes in averaged head group conformation may be relatively small, the changes in the measured quadrupole splittings are dramatic. This is in sharp contrast to the acyl chain behavior, which appears to be rather insensitive to the presence of even proteins (Seelig & Seelig, 1980). Having demonstrated the viability of deuterium NMR and less sensitive phosphorus-31 NMR to study lipid head group interactions, it should now be feasible to use similar methods to investigate protein–lipid associations with various lipid types and their functional relationship, if any, as already demonstrated by spin-label ESR methods (Watts et al., 1979; Knowles et al., 1981; Marsh & Watts, 1982).

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Characterization of Two Uterine Proteases and Their Actions on the Estrogen Receptor†

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ABSTRACT: We have characterized two previously undetected proteases from the calf uterine cytosol and measured their actions on the estrogen receptor. One is an exopeptidase, purified 60-fold, that hydrolyzed amino acid (lysine-, and alanine-, or leucine-) *p*-nitroanilide substrates and leucylglycylglycine, did not hydrolyze [^{14}C]methemoglobin, was completely inhibited by 1 mM bestatin or puromycin (specific inhibitors of leucine aminopeptidase like enzymes), and was unable to influence the sedimentation of the 8S form of the estrogen receptor in sucrose gradients containing dilute Tris buffer. A commercial porcine leucine aminopeptidase, like the calf uterine aminopeptidase, did not convert the 8S estrogen receptor to a 4S form. Evidently, removal of the N-terminal amino acid(s) from the estrogen receptor by exopeptidase action cannot alter the sedimentation of the 8S form of the receptor, or the N-terminal amino acid(s) of the receptor is (are) unaccessible or resistant to exopeptidase activity. The second, a receptor-active protease, is an endopeptidase that did not hydrolyze any of the synthetic amide or peptide substrates tested but did possess [^{14}C]methemoglobin-degrading activity and the ability to convert the 8S estrogen receptor to a modified 4S form in sucrose gradients containing dilute Tris buffer. The modified 4S receptor was separable from the native receptor by DEAE-cellulose chromatography. The endopeptidase did not require Ca^{2+} for activity, and its chromatographic properties were distinctly different from a previously isolated Ca^{2+}-activated protease. It was inhibited by leupeptin or dipyridyl disulfide, suggesting the presence of a thiol group that is essential for its activity. These data indicate that a decrease in the sedimentation rate of the estrogen receptor in sucrose gradients with low salt or a change in the receptor's elution on DEAE-cellulose chromatography is not related to receptor activation but is produced by the receptor-active protease or other proteases.

Resolution of the molecular characteristics of the estrogen receptor and other steroid hormone receptors is a key step in relating structure to function for genomic regulatory proteins. Multiple forms of the estrogen receptor have been reported (Notides, 1978; Stancel et al., 1973). Several laboratories including our own have demonstrated two major forms of the receptor. The cytoplasmic or nonactivated estrogen receptor sediments into sucrose gradients containing 0.15 or 0.4 M KCl as a 4S protein, while the nuclear or activated estrogen receptor sediments at 5S (Jensen & DeSombre, 1973; Notides & Nielsen, 1974). The activated 5S receptor is a dimer composed of two 4S estrogen receptor molecules (Notides et al., 1975, 1981). The cytoplasmic estrogen receptor can also be transformed into the activated 5S receptor by salting out during ammonium sulfate fractionation (Weichman & Notides, 1979). Furthermore, analysis of the estrogen receptor in sucrose gradients containing low salt concentrations (only dilute Tris buffer) aggregates the estrogen receptor to a third form—the 8S species. The 8S estrogen receptor aggregate is observed either before or after purification by ammonium sulfate precipitation.

In studying the molecular properties of the steroid hormone receptors, it is important to discern those properties of the receptor that are inherent in its molecular structure from those that are inadvertently produced by the action of extraneous factors such as endogenous proteases. The endogenous proteases are also of concern when isolation and purification of the native receptor are undertaken. Previous reports have indicated that the steroid hormone receptors are hydrolyzed by endogenous proteases to species having decreased sedimentation coefficients in sucrose gradients containing low salt concentrations (Notides et al., 1972, 1973; Puca et al., 1972, 1977; Sherman et al., 1978; Wilson & French, 1979). Proteolysis of the receptor frequently may lead to a loss of the DNA-binding domain of the receptor (Notides et al., 1976); however, a very limited proteolysis of the receptor may result in retention of part or the complete DNA-binding domain of the receptor (Wrange & Gustafsson, 1978).

In this report we characterize two calf uterine proteases, previously undetected, and describe their effects on the estrogen receptor.

Materials and Methods

Materials. 17β-[2,4,6,7-$^{3}H_4$]Estradiol (90 or 102 Ci/mmol) and [*methyl*-^{14}C]methylated methemoglobin (24.4 μCi/mg) were obtained from New England Nuclear. Leucine aminopeptidase was purchased from Worthington Biochemical Corp. Alkaline phosphatase (*Escherichia coli*), diisopropyl fluorophosphate (DFP), puromycin hydrochloride, 4,4′-dipyridyl disulfide, and lysine-, leucine-, and alanine-*p*-nitroanilide were obtained from Sigma Chemical Company. Leupeptin was purchased from Peninsula Laboratories. Sucrose and am-

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