expose them to alkylating agents. The lack of a stable intermolecular disulfide is also consistent with the EII concentration-dependent association data presented in this paper.

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Registry No. EII, 37278-09-4; DMPC, 13699-48-4; DMPG, 61361-72-6; DOPC, 10015-85-7; DOPG, 62700-69-0; PPPC, 95864-17-8; mannitol, 69-65-8.

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A Deuterium and Phosphorus-31 Nuclear Magnetic Resonance Study of the Interaction of Melittin with Dimyristoylphosphatidylcholine Bilayers and the Effects of Contaminating Phospholipase A_2

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ABSTRACT: The interaction of bee venom melittin with dimyristoylphosphatidylcholine (DMPC) selectively deuteriated in the choline head group has been studied by deuterium and phosphorus-31 nuclear magnetic resonance (NMR) spectroscopy. The action of residual phospholipase A_2 in melittin samples resulted in mixtures of DMPC and its hydrolytic products that underwent reversible transitions at temperatures between 30 and 35 °C from extended bilayers to micellar particles which gave narrow single-line deuterium and phosphorus-31 NMR spectra. Similar transitions were observed in DMPC–myristoyllysophosphatidylcholine (lysoPC)–myristic acid mixtures containing melittin but not in melittin-free mixtures, indicating that melittin is able to stabilize extended bilayers containing DMPC and its hydrolytic products in the liquid-crystalline phase. Melittin, free of phospholipase A_2 activity, and at 3–5 mol % relative to DMPC, induced reversible transitions between extended bilayers and micellar particles on passing through the liquid-crystalline to gel phase transition temperature of the lipid, effects similar to those observed in melittin–acyl chain deuteriated dipalmitoylphosphatidylcholine (DPPC) mixtures [Dufourc, E. J., Smith, I. C. P., & Dufourcq, J. (1986) *Biochemistry 25*, 6448–6455]. LysoPC at concentrations of 20 mol % or greater relative to DMPC induced transitions between extended bilayers and micellar particles with characteristics similar to those induced by melittin. It is proposed that these melittin- and lysoPC-induced transitions share similar mechanisms. The effects of melittin on the quadrupole splittings and T_1 relaxation times of head-group-deuteriated DMPC in the liquid-crystalline phase share features similar to the effects of metal ions on DPPC head groups [Akutsu, H., & Seelig, J. (1981) *Biochemistry 20*, 7366–7373], indicating that the conformational properties of the choline head group in PC bilayers may be affected by melittin and by metal ions in a similar manner.

Many studies on the interaction of bee venom melittin (Habermann & Jentsch, 1967) with model membranes composed of synthetic lipids have been carried out with the aim of defining the structural basis for the lytic effects of the peptide (Bernheimer & Rudy, 1986). It is still not clear, however, whether melittin acts by perturbing the structural organization of bilayer membrane lipids or by inducing discrete pores through which ions may diffuse, producing osmotic lysis.

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Membranes composed of the saturated phospholipid dimyristoylphosphatidylcholine (DMPC)[1] have been used in several studies of melittin–lipid interactions (Verma & Wallach, 1976; Dufourcq & Faucon, 1977; Lavialle et al., 1980; Prendergast et al., 1982; Jahnig et al., 1982; Vogel et al., 1983), and in a number of cases, melittin-induced increases in the main gel–liquid-crystalline phase transition temperature of the lipid have been reported (Verma & Wallach, 1976; Lavialle et al., 1980; Prendergast et al., 1982), indicative of marked perturbation of the organizational properties of the lipid. Studies by Dasseux et al. (1984) have shown, however, that these elevated phase transition temperatures are not due to the effects of melittin alone but are a consequence of the presence of residual bee venom phospholipase A_2 (Shipolini et al., 1971) in the melittin preparation.

Phosphorus-31 NMR and deuterium NMR of bilayer membranes composed of selectively deuteriated lipids are sensitive and nonperturbing methods for studying the conformational properties of bilayer lipids and their response to the presence of membrane binding proteins (Seelig & Seelig, 1980; Bloom & Smith, 1985; Watts et al., 1985). In this paper, we present a study of the interaction of melittin with bilayers composed of head-group-deuteriated DMPC using deuterium and ^{31}P NMR spectroscopy. We have found raised lipid phase transition temperatures similar to those previously reported when using melittin contaminated with residual phospholipase A_2 and have characterized the nature of these transitions using mixtures of DMPC and its hydrolytic products (myristoyllysoPC and myristic acid) to simulate the effects of phospholipase A_2. The interaction of phospholipase-free melittin with DMPC as studied by deuterium and ^{31}P NMR spectroscopy is found to share features similar to the interaction of myristoyllysoPC with DMPC bilayers. These results are discussed in relation to the ability of both lysolipids (Inoue et al., 1977) and melittin (Dufourcq et al., 1986; Dufourc et al., 1986a) to induce bilayer to micellar disk transitions in saturated phosphatidylcholines.

Materials and Methods

Materials. Melittin was purified from whole bee venom (Bulgarcoop, Sofia, Bulgaria) by forced dialysis (to isolate low molecular weight venom components including melittin) and gel filtration on tandem columns of Sephadex G-25 and G-50 (Gauldie et al., 1976) followed by heparin–Sepharose affinity chromatography to remove other low molecular weight peptides and phospholipase A_2 (Banks et al., 1981). Although peaks for melittin and phospholipase A_2 are well resolved by heparin–Sepharose chromatography, traces of residual phospholipase A_2 remained in the melittin samples after this step as described under Results. Purification of melittin by cation-exchange chromatography on SP-Sephadex G-25 (Gauldie et al., 1976) also failed to remove phospholipase activity below detectable levels.

Dimyristoylphosphatidylcholine (DMPC) was purchased from Fluka and gave a single spot on silica gel TLC. DMPC-d_9 (deuteriated in the terminal methyls of the choline head group) and DMPC-d_4 (deuteriated in the head group α- and β-methylenes) were synthesized as previously described (Sixl & Watts, 1982). In the synthesis of DMPC-d_4, incorporation of deuterium into the α-position of ethanolamine by catalytic exchange using Raney nickel was greater than for the β-methylenes as determine by high-resolution proton NMR, and this allowed unambiguous assignment of the α- and β-methylenes in DMPC-d_4 from the relative peak areas in the deuterium NMR spectra.

Determination of Residual Phospholipase Activity in Melittin Samples. Following NMR spectroscopy, a small amount of lipid sample was extracted into $CHCl_3$/MeOH (2:1) and examined by TLC on silica plates using hexane/ether (1:1) to resolve DMPC from myristic acid and $CHCl_3$/MeOH/NH_4OH (65:35:5) to resolve DMPC and lysoPC. Sufficient lipid was loaded on the plates that 1% of lysolytic products were detectable.

Inactivation of Phospholipase Activity in Melittin Samples. Samples of melittin containing residual phospholipase A_2 activity were treated with *p*-bromophenacyl bromide (PBP) as described by Volwerk et al. (1974) for inactivation of the pancreatic enzyme and by Abe et al. (1977) for snake venom phospholipases A_2. An aliquot of PBP (68 mM in acetone) was added to the melittin–phospholipase A_2 sample in buffer (either 0.1 M sodium cacodylate, pH 6.0, or 0.1 M sodium acetate, pH 6.0, or 0.1 M Tris-HCl, pH 8.0) to give 0.68 mM PBP. The solutions were incubated at 30 °C. Aliquots were removed after various time and assayed semiquantitatively for phospholipase A_2 activity using DMPC as substrate as described above.

Preparation of Melittin–DMPC Complexes for NMR. Complexes were prepared by hydrating the lipid in buffer (50 mM Tris-HCl, pH 7.5, in deuterium-depleted water with EDTA at the concentrations indicated) containing melittin to give the appropriate melittin:lipid ratio. The final lipid concentration was 50 100 mg mL^{-1}. The samples were hydrated at temperatures above the gel–liquid-crystalline phase transition temperature (T_c = 24 °C for DMPC) and incubated for at least 2 h at 30–35 °C. In some experiments, melittin and lipid were cosolubilized in methanol and then suspended in buffer after removing the methanol in a stream of dry N_2 and pumping under reduced pressure overnight. Samples prepared by either method gave similar results.

Our criterion for the attainment of equilibrium within the samples was the reproducibility of deuterium NMR spectra as a function of temperature in consecutive heating runs. In some cases, long-term variations in the deuterium NMR spectra were observed, but these could usually be correlated with the production of lysophosphatidylcholine as described under Results.

Samples of DMPC containing myristoyllysoPC and myristic acid (the latter compounds in equimolar amounts) were prepared by cosolubilization of the lipids in methanol at the appropriate ratios followed by removal of the solvent by vacuum evaporation and suspending in deuterium-depleted buffer.

Deuterium and ^{31}P NMR Spectroscopy. NMR spectra were recorded on a Bruker WH-300 spectrometer operating at frequencies of 46.1 MHz for deuterium and 120.9 MHz for phosphorus using single 60–90° pulses (20–29 μs) for ^2H and 90° pulses (18 μs) for ^{31}P. After beginning the study, upgrading of our deterium NMR facility by using a dedicated deuterium probe and a high-power pulse amplifier improved the 90° pulse for deuterium to 11 μs. Spectra taken under both conditions are presented here. Deuterium spin–lattice relaxation rates (T_1) were measured by using an inversion–recovery pulse sequence with single pulses. Phosphorus-31

[1] Abbreviations: CSA, chemical shift anisotropy; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; lysoPC, lysophosphatidylcholine; myristoyllysoPC, 1-myristoyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; PBP, *p*-bromophenacyl bromide; TLC, thin-layer chromatography; $\Delta\nu_Q$, quadrupole splitting; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

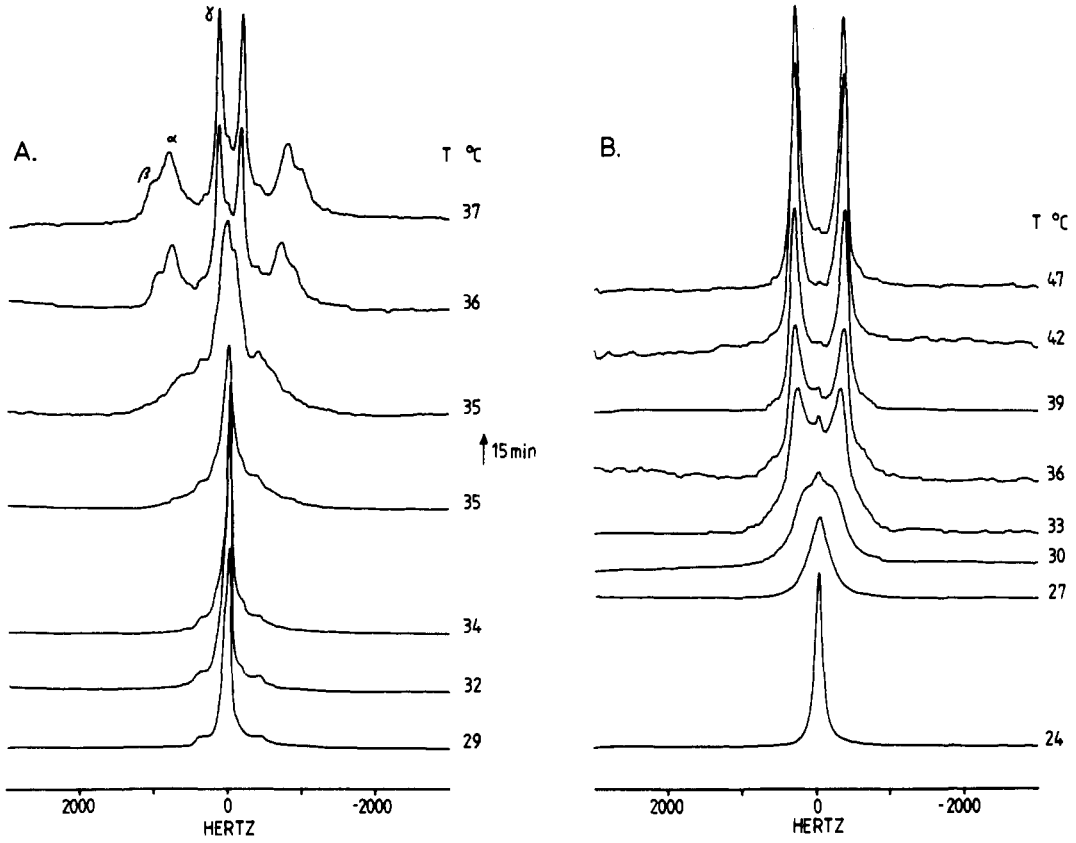

FIGURE 1: Deuterium NMR spectra as a function of temperature for samples of DMPC-d_9/DMPC-d_4, 10:1 (w/w), containing 4 mol % melittin (A) and of DMPC-d_9 containing 10 mol % melittin (B). The melittin preparation was contaminated with phospholipase A_2 activity, and between 20% and 30% of the DMPC was hydrolyzed to hydrolytic products in the samples. In (A), the upper 35 °C spectrum was taken 15 min after the lower spectrum as indicated.

spectra were accumulated with continuous broad-band proton decoupling at 7–10 W. Temperatures were controlled with a thermostated gas flow unit and were accurate to ±1 °C as determined by calibration against the DMPC phase transition temperature.

RESULTS

Effect of Melittin Containing Residual Phospholipase on DMPC Bilayers. Figure 1 shows deuterium NMR spectra as a function of temperature for a sample of DMPC-d_4/DMPC-d_9 (10:1) containing 4 mol % melittin and for DMPC-d_9 containing 10 mol % melittin. These samples were prepared in buffers containing 1 mM EDTA and were kept for 1 week at 4 °C before the spectra were recorded. Examination of lipid from both samples by TLC showed that a considerable proportion of the DMPC was hydrolyzed to lysoPC and myristic acid. Although the proportions of lysolipid to DMPC were not accurately quantitated, we estimate from the relative densities of TLC spots that 20–30% of the DMPC was hydrolyzed.

The dispersion for which deuterium NMR spectra are illustrated in Figure 1A underwent a rather sharp phase transition at 35 °C. Visually, the transition was from a clear solution (low temperature) to a thick, cloudy dispersion. The transition is quite different from the gel to liquid-crystal phase transition in melittin-free DMPC dispersions, and the gross rearrangement of the components in the mixture is reflected in the slow formation of the high-temperature phase on holding the sample at just above the phase transition temperature of 35 °C (Figure 1A). Similar behavior was observed with melittin to DMPC ratios of between 1 and 4 mol % whenever significant hydrolytic products were present (see, for example, Figure 3B).

The complex with a higher melittin:lipid ratio of 10 mol % underwent a broader phase transition between 24 and 33 °C (Figure 1B). Although only one transition was observed by deuterium NMR, the sample underwent a low-temperature transition from a fluid transparent solution (present at least between 4 and 12 °C) to a transparent gel which was sufficiently solid that the sample could be inverted without falling from the NMR tube. This gel-like complex existed over a temperature range between about 13 and 24 °C above which temperature the dispersion became cloudy and gave deuterium (Figure 1B) and ^{31}P NMR spectra characteristic of lipid in a bilayer arrangement. The solid gel phase existing between 13 and 24 °C gave single lines in both deuterium and ^{31}P spectra (not shown) characteristic of isotropic motions on the NMR time scale.

Phase Behavior of DMPC Containing MyristoyllysoPC and Myristic Acid. Figure 2 illustrates the phase behavior of a sample of DMPC containing myristoyllysoPC (35 mol %). This sample underwent a macroscopic phase change from a configuration giving isotropic deuterium NMR spectra to a bilayer phase with a T_c around 23–24 °C. Below the transition temperature, the isotropic phase coexisted with a small amount of a lipid phase having a characteristic bilayer deuterium NMR spectrum. At the transition, spectral components from the two phases coalesced to give a spectrum characteristic of a single bilayer phase with a varying amount of isotropic component. The phases observed in this sample were rather unstable, and the nature of the deuterium NMR spectrum was markedly dependent on the thermal history of the sample. For example, on heating too rapidly, the isotropic phase was maintained up to at least 40 °C. On warming the sample slowly through the transition temperature and incubating for 24 h at 24 °C, the isotropic component disappeared to give

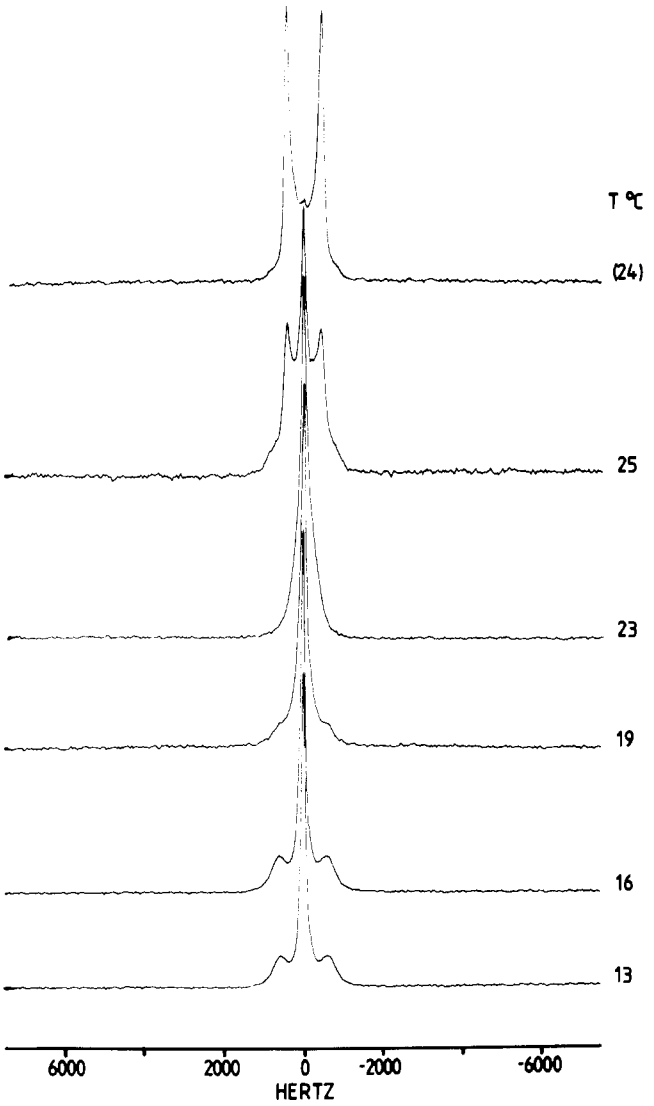

FIGURE 2: Deuterium NMR spectra as a function of temperature for a sample of DMPC-d_9 containing 35 mol % myristoyllysophosphatidylcholine. The temperature was lowered from 25 °C for the lower series of spectra, and the top spectrum was obtained after warming the lipid slowly through the phase transition and incubating for 24 h at 24 °C.

a single-component bilayer-type spectrum above T_c (Figure 2; top spectrum).

On hydrolysis of DMPC by the action of phospholipase A_2, myristic acid and myristoyllysoPC are produced in equimolar amounts. To simulate such dispersions, samples of DMPC containing between 15 and 40 mol % of equimolar myristoyllysoPC and myristic acid were hydrated in buffer. These mixtures gave clear solutions and single, narrow-line deuterium and ^{31}P NMR spectra over the whole temperature range measured (5–40 °C). Deuterium NMR spectra for a mixture containing 20 mg of DMPC-d_9, 4 mg of myristoyllysoPC, and 2 mg of myristic acid are shown in Figure 3A. On addition of 1 mol % melittin relative to total lipid, the sample became opaque (over 30–60 s) and had an appearance similar to lipid bilayer dispersions within 2–3 min. Deuterium NMR spectra for the melittin-containing sample at increasing temperatures are shown in Figure 3B and indicate a transition at 30 °C between small rapidly tumbling particles, giving rise to isotropic reorientations on the NMR time scale, and an extended bilayer phase. The behavior of this sample including the transition temperature, the nature of the phases above and below the transition temperature, and the slow formation of

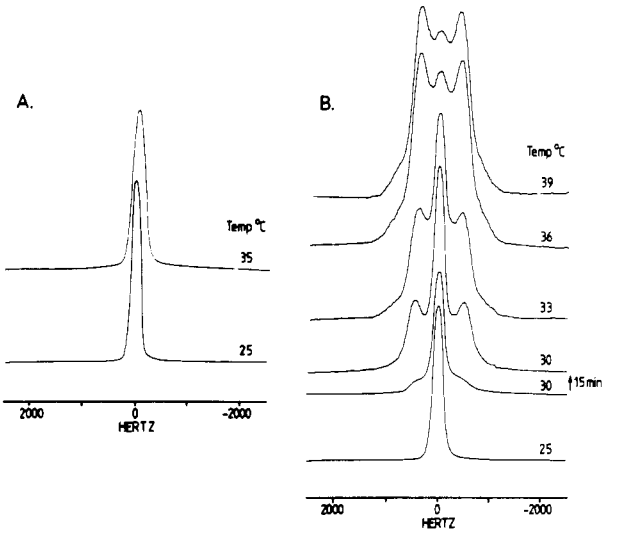

FIGURE 3: Deuterium NMR spectra as a function of temperature for a mixture of DMPC-d_9/myristoyllysoPC/myristic acid (60:20:20, mol %) (A) and (B) after the addition of melittin to give 1 mol % relative to total lipid. In (B), the upper 30 °C spectrum was taken 15 min after the lower 30 °C spectrum as indicated.

Table I: Semiquantitative Measure of the Stability to Various Inactivation Treatments of Contaminating Phospholipase A_2 in Melittin Samples[a]

sample	time		
	90 min	20 h	48 h
DMPC	N[b]	N	N
DMPC + 3 mol % mel	1–2%	10–15%	35–40%
DMPC + 3 mol % mel, from methanol[c]	1–2%	10–15%	35–40%
DMPC + 3 mol % mel, butanol extracted[c]	N	5–10%	35–40%
DMPC + 3 mol % mel + 1 mM EDTA	N	1–2%	3–5%
DMPC + 3 mol % mel + 2 mM EDTA	N	N	N
DMPC + 3 mol % PBP (pH 6.0)-treated mel[d]	1–2%	5–10%	15–20%
DMPC + 3 mol % PBP (pH 8.0)-treated mel[d]	N	N	N
DMPC + 3 mol % mel, pH 4.0[e]	N	N	N

[a] Phospholipase A_2 activity was determined by TLC analysis for hydrolytic products of DMPC after incubating the lipid dispersion at 5 mg mL^{-1} in 50 mM Tris-HCl, pH 7.5, at 30 °C with melittin (mel) for the times indicated. [b] No hydrolytic products of DMPC were detectable by TLC. [c] The melittin samples were used after extraction into methanol or butanol as described under Results. [d] Phospholipase-contaminated melittin was pretreated with the PBP for 24 h as described under Materials and Methods. [e] Assay for phospholipase A_2 activity was done at pH 4 (50 mM sodium acetate).

the bilayer phase under isothermal conditions is very similar to the samples whose NMR spectra are illustrated in Figure 1. The ^{31}P NMR spectra at temperatures above the transition temperature are characteristic of phospholipid in a bilayer configuration with a ^{31}P CSA of 44 ± 2 ppm (not shown), confirming that the high-temperature phase is bilayer. The low-temperature phase is assigned as micellar on the basis of the single narrow-line ^{31}P and deuterium NMR spectra and the complete clarification of the solution, indicating that small vesicles which give turbid suspensions in concentrated solutions were not present. As with the complexes whose spectra are illustrated in Figure 1, the deuterium NMR spectra and their temperature dependence were completely reversible and reproducible over subsequent temperature runs.

Inactivation of Residual Phospholipase Activity in Melittin Samples. Table I illustrates a qualitative assessment of residual phospholipase A_2 activity in melittin preparations after various inhibitory treatments. As previously described (Shipolini et al., 1971; Dasseux et al., 1984), bee venom

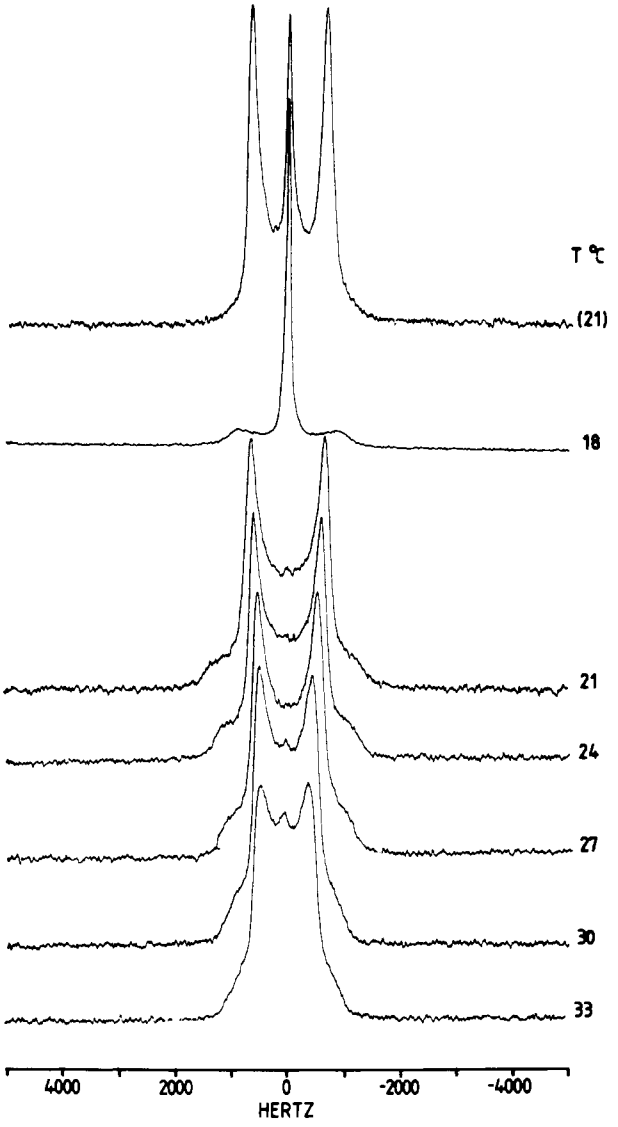

FIGURE 4: Deuterium NMR spectra as a function of temperature for DMPC-d_9 containing 3 mol % melittin. Spectra were obtained in a cooling run from 33 to 18 °C, and the top spectrum was taken after warming the sample slowly back through the phase transition to 21 °C.

phospholipase A_2 is not completely inhibited by EDTA at 1 mM. Treatment of bee venom phospholipase A_2 with *p*-bromophenacyl bromide at pH 6 as described by Abe et al. (1977) does not completely inhibit the enzyme. Phospholipase A_2 activity was completely inhibited by carrying out the *p*-bromophenacyl bromide inactivation at pH 8. Phospholipase A_2 activity in melittin samples was retained after dissolving the peptide in methanol followed by pumping under high vacuum at room temperature and after extracting phospholipase-contaminated melittin from alkaline solution (pH 10.5) into butanol followed by freeze-drying. This indicates that bee venom phospholipase A_2 is not irreversibly denatured in pure methanol or water-saturated butanol at least in the presence of melittin. No phospholipase activity was detected at pH 4 even in the absence of EDTA.

Effect of Phospholipase-Free Melittin on DMPC Dispersions. Phospholipase A_2 activity in the samples of melittin described in this section was inhibited by pretreatment of the melittin with PBP at pH 8 or by the inclusion of EDTA at a concentration of 4 mM in the lipid dispersions. Preparation of melittin–DMPC complexes by suspending the lipid in melittin-containing buffer above the lipid phase transition

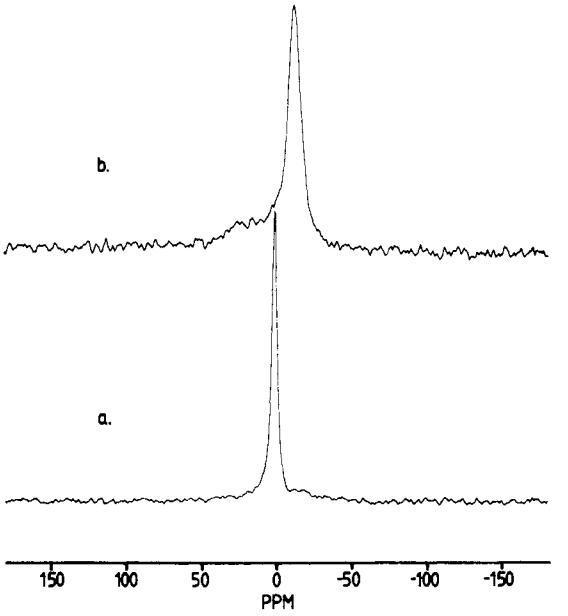

FIGURE 5: Phosphorus-31 NMR spectra for DMPC-d_9 containing 3 mol % melittin at temperatures above and below the gel to liquid-crystalline phase transition temperature (see Figure 4): (a) 18 °C; (b) 27 °C.

temperature resulted in rapid attainment of equilibrium so that reproducible temperature runs could be obtained within 30 min of sample preparation. Similarly, all of the melittin was associated with the lipid at concentrations up to at least 4 mol % as determined by deuterium NMR experiments using selectively deuteriated melittin (Dempsey et al., 1987). Each of these findings is in contrast to previous reports either that equilibrium is attained only under specific conditions or after long incubation periods (Prendergast et al., 1982) or that DMPC is able to support only up to 2 mol % of melittin (Vogel, 1981). These differences may be due to the lack of phospholipase activity in the samples described here (phospholipase-contaminated melittin–DMPC complexes attain equilibrium very slowly and only after phospholipid hydrolysis reaches a limiting state) and to the method of sample preparation which, in the present case, allows rapid access of melittin to all the lipid within the sample.

Figures 4–7 show the effects of melittin on the properties of DMPC in bilayers under conditions where phospholipase activity was completely suppressed. The results are illustrated for melittin at 2, 3, and 4 mol % relative to DMPC. At a concentration of 10 mol %, melittin caused complete micellization of the DMPC bilayers, resulting in clear solutions from which isotropic deuterium and ^{31}P NMR spectra were obtained at all temperatures (not shown). At melittin:DMPC ratios between 3 and 6 mol %, the bilayer phase was stable above the gel–liquid-crystalline phase transition temperature, but collapse of deuterium and ^{31}P spectral anisotropy occurred at lower temperatures (Figures 4 and 5).

At 2–4 mol % melittin:DMPC ratios, there was no increase in the gel–liquid-crystalline phase transition temperature compared to pure lipid dispersions, and the samples gave deuterium (Figures 4 and 6) and ^{31}P NMR spectra (Figures 5 and 7) characteristic of lipids in an extended bilayer configuration at all temperatures examined *above* T_c. Melittin caused a marked decrease in the quadrupole splitting of the α-deuterons of the head group (adjacent to the phosphate) without significantly affecting the spectral line shape. At a melittin:DMPC ratio of only 4 mol %, the quadrupole splitting was reduced by 65% from 6360 Hz for pure DMPC-d_4 bilayers to 2650 Hz (Figure 6). The quadrupole splitting of the β-

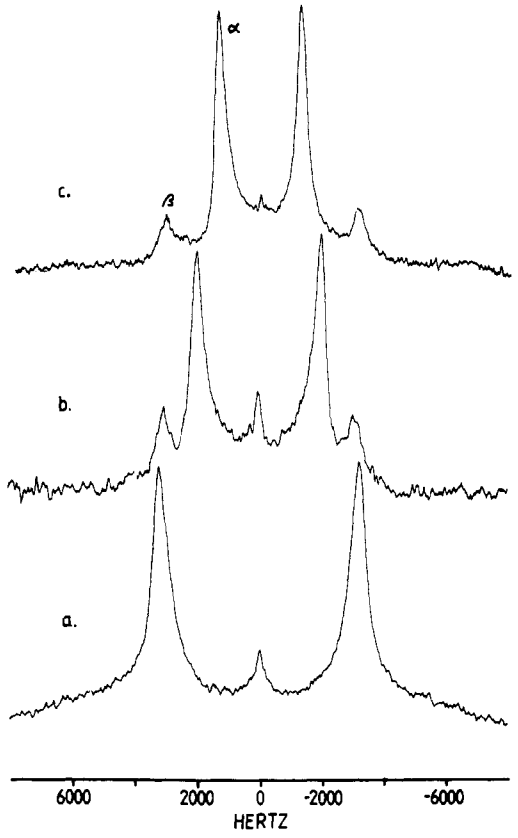

FIGURE 6: Deuterium NMR spectra at 27 °C for DMPC-d_4 (a) and for DMPC-d_4 containing melittin at 2 mol % (b) and 4 mol % (c).

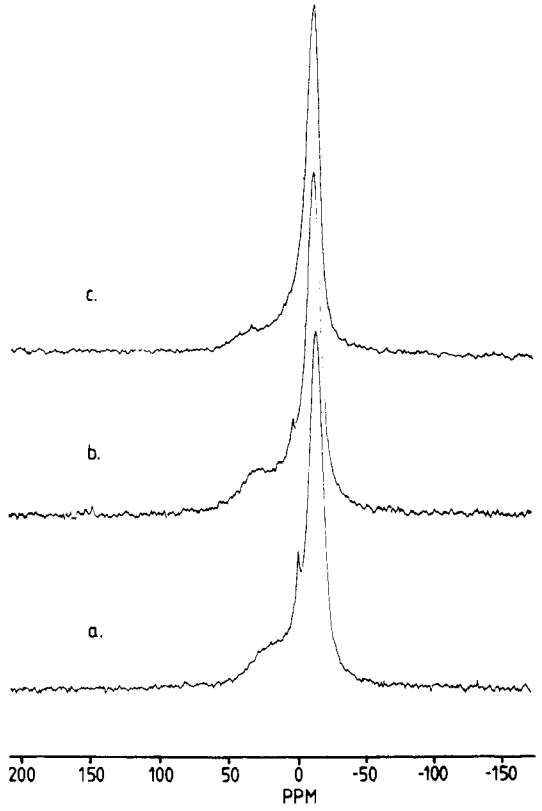

FIGURE 7: Phosphorus-31 NMR spectra at 27 °C for the samples whose deuterium NMR spectra are shown in Figure 7. (a) DMPC-d_4; (b) DMPC-d_4 containing 2 mol % melittin; (c) DMPC-d_4 containing 4 mol % melittin.

deuterons was unaffected by melittin at concentrations up to 4 mol % (Figure 6), and the γ-deuterons underwent a small concentration-dependent decrease in quadrupole splittings (from 1280 Hz in pure lipid, 27 °C, to 1100 Hz at 4 mol % melittin). Melittin decreased the ^{31}P chemical shift anisotropy from 54 ± 2 ppm in pure DMPC bilayers to 46 ± 2 ppm at a melittin:DMPC ratio of 4 mol % (Figure 7).

The effects of melittin on the spin–lattice relaxation times (T_1) of head-group-deuteriated DMPC were small. The T_1 values for the α- and β-deuterons were measured at 42 °C to resolve the α and β splittings in pure lipid. Melittin at 4 mol % caused a small decrease in the T_1 values of the α- and β-deuteriomethylenes (from 27 to 23 ms for the α-CD_2 group and from 35 to 27 ms for the β-CD_2 group). The T_1 for the γ-methyl deuterons measured at 32 °C (50 ms) was unaffected by melittin at concentrations up to 4 mol % relative to DMPC-d_9.

Discussion

Phospholipase-Contaminated Melittin. The presence of residual phospholipase A_2 activity in samples of melittin incubated with DMPC results in the generation of lipid mixtures which undergo phase transitions at temperatures that are raised to 30–35 °C compared with the transition temperature of pure DMPC of 23 °C (Dasseux et al., 1984; our Figure 1). The results presented here show that these elevated thermal transitions are associated with the coalescence of a micellar organization of DMPC–lysoPC–myristic acid–melittin dispersions into a melittin-stabilized extended bilayer phase. The gross rearrangement of the lipid during the transition is apparent from the slow isothermal formation of the bilayer phase as determined from the NMR spectra (Figures 1 and 3) and by the change in appearance of the sample from a clear solution to a thick milky white dispersion. The assignment of the high-temperature phase to bilayers is made on the basis of the deuterium and ^{31}P NMR spectra which are characteristic of lipids arranged in a bilayer configuration (Seelig & Seelig, 1980). In the low-temperature micellar phase, no residual anisotropy is retained either in the deuterium or in the ^{31}P NMR spectra, indicating that the labeled groups are undergoing rapid isotropic motions on the respective NMR time scales, i.e., with rates greater than the deuterium quadrupolar splitting or the phosphorus chemical shift anisotropy in hertz, respectively (Burnell et al., 1980). The collapse of the NMR spectra to single narrow lines, together with the clarification of the solution, indicates that the low-temperature phase is micellar. The narrow-line NMR spectra are uninformative about the conformational or organizational properties of lipids within the micellar particles existing at low temperature, and we cannot say whether these particles are spherical micelles or are disk-shaped regions of bilayer as shown for the interaction of melittin (Dufourcq et al., 1986) and other peptides forming amphipathic helices (Forte et al., 1974; Segrest et al., 1976) with lipids.

DMPC samples containing myristoyllysoPC also undergo transitions between a phase giving single, narrow-line deuterium and ^{31}P NMR spectra and an extended bilayer phase (Figure 2), but the transition temperatures either are similar to that of pure DMPC or are suppressed, and the phases are rather unstable and dependent on the thermal history of the sample. The presence of myristic acid in equimolar amounts relative to lysoPC (to simulate the effects of phospholipase A_2) and at mole ratios of 0.2 or greater relative to DMPC yields micellar solutions at all temperatures tested (Figure 3A), indicating that the high-temperature fluid bilayer phase in DMPC–myristoyllysoPC mixtures is destabilized relative to a mixed micellar phase on introducing myristic acid equimolar with lysolipid. The addition of melittin to a level of 1 mol % to these DMPC–lysoPC–myristic acid dispersions rapidly

(0.5–2 min) induces the formation of a bilayer phase which undergoes a reversible transition at 30–35 °C to a phase giving deuterium and ^{31}P NMR spectra having a single narrow line (Figure 3B). This melittin-induced transition is similar to the transitions illustrated in Figure 1 in the slow formation of the bilayer phase under isothermal conditions, the spectral shape above and below T_c, and the transition temperature. These results show that the macroscopic changes in lipid morphology associated with the elevated phase transitions induced by phospholipase A_2 contaminated melittin in DMPC bilayers are not only due to the formation of lysolipid and myristic acid but also require the presence of melittin to stabilize the high-temperature extended bilayer phase.

Phospholipase-Free Melittin. The purification of melittin free from contamination by phospholipase A_2 is difficult, and even where base-line resolution of the two proteins is achieved by chromatography, residual phospholipase activity can be present in the melittin preparation. This seems to reflect the ability of melittin to complex with other proteins (Banks et al., 1981; Compte et al., 1983). We have found that the standard conditions for the inhibition of phospholipases (*p*-bromophenacyl bromide at pH 6; Abe et al., 1977) are rather ineffective in the case of the bee venom enzyme but that complete inhibition occurs readily at pH 8. Simiarly, the ability of EDTA to inhibit bee venom phospholipase A_2 activity is variable, and EDTA at a concentration of 1 mM was generally insufficient to inhibit the enzyme when samples were incubated for long periods. Similar effects have previously been observed (Shipolini et al., 1971; Dassieux et al., 1984). The most valiid criterion for the lack of phospholipase A_2 activity in melittin samples was the inability to detect hydrolytic products in the membrane preparations after the NMR experiments. By this criterion, all the samples described in the following discussion were free of residual phospholipase activity.

The effects of melittin were studied only at mole ratios up to 4 mol % because higher concentrations of the peptide in the bilayer (around 8–10 mol % and greater) resulted in micellization of all lipid phases. At melittin concentrations of 3 mol % or greater, the deuterium and ^{31}P NMR spectra of melittin-deuteriated DMPC complexes collapsed to single narrow lines on reducing the temperature through the gel–liquid-crystal phase transition (e.g., Figure 4 for a 3 mol % melittin:DMPC ratio). The lipid in the low-temperature phase is now undergoing rapid reorientations averaging the CSA and quadrupolar anisotropy, indicating the collapse of the extended bilayers to smaller particles on cooling. Recently, Dufourcq et al. (1986) have shown that melittin induces a reversible transition between bilayers and disk micelles in DPPC bilayers on passing through the phase transition of the lipid, and similar effects have been observed by deuterium NMR of acyl chain deuteriated DPPC dispersions containing melittin (Dufourc et al., 1986b). These morphological changes are likely to be responsible for the reversible transition from extended bilayers to micelles in DMPC–melittin dispersions observed here by deuterium NMR.

The reversible transition from extended bilayers to micelles induced by phospholipase-free melittin is very similar to the transitions induced by lysoPC as determined by NMR (compare Figures 2 and 4). In each case, the transition is reversible and is triggered by the gel to liquid-crystalline phase transition of the lipid. Electron microscopic studies have shown that lysophosphatidylcholine can induce the formation of micellar disks of phospholipids and that disk formation depends on the fluidity of the bilayer lipid (Inoue et al., 1977). Disk formation was observed in DPPC–palmitoyllysoPC mixtures when the lipids were fixed at 20 °C (below the phase transition temperature of DPPC) but not from membrane preparations fixed above T_c (Inoue et al., 1977). We assume that the reversible bilayer to micelle transitions in DMPC–lysoPC mixed dispersions observed by deuterium and ^{31}P NMR arise from this bilayer to micellar disk transition. Disk formation in gel phase lipid induced by lysoPC is a consequence of the wedge shape of the lysolipid. The increase in bilayer surface volume relative to the bilayer interior in membranes containing lysoPC can be accommodated in fluid phase lipid (so that fluid phase membranes containing lysoPC contract in bilayer thickness proportionally to the concentration of lysolipid; Mandersloot et al., 1975). Gel phase lipid is unable to accommodate high concentrations of lysoPC, and the retention of strong intralipid interactions requires the expulsion of lysolipid from the gel phase. Disk formation is presumably a consequence of the high local concentration of lysoPC resulting from phase separation and the high micelle-forming potential of the lysolipid. The similarity in these effects of melittin and lysoPC on lipid organization in DMPC bilayers as determined by NMR supports a similar mechanism for disk formation in melittin–DMPC complexes. The positioning of melittin toward the polar–apolar interface of the bilayer (Dawson et al., 1978; Terwillinger et al., 1982) will similarly cause a volume expansion of the head-group region of DMPC bilayers relative to the region occupied by the acyl chains that can be accommodated only in fluid phase lipid. The surface volume expansion may have a further contribution from the high positive charge density in neutral bilayers containing melittin.

A further similarity in the effects of melittin and lysoPC on the deuterium NMR spectra of head-group-deuteriated DMPC is the overrepresentation of the 90° orientational component of the deuterium NMR powder pattern on passing through the phase transition into the extended bilayer phase (see the top spectra of Figures 2 and 4). Although the receiver dead time (30 μs) following the nonselective excitation pulse in the single pulse excitation mode required with our spectrometer can result in distortions of the deuterium powder pattern, such distortions are small for deuterium atoms having small quadrupole splittings and long relaxation times, such as the DMPC choline methyl deuterons which are recorded in the lower sets of spectra of Figures 2 and 4, and show relatively undistorted powder patterns. The overrepresentation of the 90° component of the powder patterns in the top spectra of Figures 2 and 4 is not an artifact of spectral distortion but rather indicates that the membranes are macroscopically oriented, with the plane of the bilayer at 180° with respect to the magnetic field (Seelig et al., 1985). This orientational effect does not occur when DMPC bilayers containing melittin or lysoPC are formed above the phase transition temperature (lower sets of spectra in Figures 2 and 4) but only on warming into the fluid phase. We believe that this effect results from the nature of fusion of micellar disks of gel phase bilayers (Dufourcq et al., 1986) into fluid phase extended bilayers. Formation of extended bilayers requires the edge to edge fusion of disks in two dimensions. If the closing of these structures to give spherical liposomes is unfavorable, very large liposomes or membrane sheets may preferentially form. Large interacting lipid domains can be oriented by the interaction of the diamagnetically anisotropic lipid acyl chains with an external magnetic field (Seelig et al., 1985). This interpretation is supported by electron micrographs of lipid–apolipoprotein complexes undergoing disk to extended bilayer transitions [Figures 9 and 10 in Forte et al. (1974)] that show extended

regions of membrane apparently arising from edge to edge fusion of bilayer disks.

In the liquid-crystalline phase at melittin concentrations up to 4 mol %, the deuterium and ^{31}P NMR spectra show sensitivity to the orientational and dynamic properties of the choline head group. The effects of melittin on the deuterium NMR spectra of head-group-deuteriated DMPC are selective, with large changes in the quadrupole splitting of the α-deuteriomethylenes (adjacent to the lipid phosphate group) and small or insignificant changes of the γ- or β-deuteron splittings. A large decrease in the quadrupole splitting may be due to either a change in the orientation of the C–D bond relative to the main axis of molecular averaging (the director, which is perpendicular to the plane of the bilayer) or an increase in the amplitude of rapid reorientations, or both. The possibility that melittin causes the fragmentation of the extended DMPC bilayers in the liquid-crystalline phase, inducing a reduction in $\Delta\mu_Q$ through rapid lateral diffusion of lipids around small vesicles (Burnell et al., 1980), can be discounted because the quadrupole splittings of the β-deuteriomethylenes would, under these circumstances, also be reduced. The differential changes in quadrupole splittings of each deuteriated head-group segment also indicate that an increase in the amplitude of α-CD bond reorientations is unlikely to be the cause of the reduction of the α-CD_2 splitting because the quadrupole splittings of the β- and γ-CD bonds would be expected to be equally or more greatly affected. We conclude that melittin induces a selective and concentration-dependent change in the average orientation of the α-CD bonds of the choline head group.

The effects of melittin on the quadrupole splittings and T_1 relaxation times of head-group-deuteriated DMPC are similar to the changes in the deuterium NMR spectra of head-group-deuteriated phosphatidylcholines induced by the presence of metal ions (Akutsu & Seelig, 1981). In both cases, the effects are relatively specific for the α-deuteriomethylenes; for lanthanum chloride, for example, the quadrupole splitting of the α-CD_2 group is reduced to a value of −1.27 kHz at saturating concentrations of the metal ion. Whereas the β-deuteriomethylenes of the choline head group are relatively unaffected by melittin, metal ions produce small increases in the quadrupole splitting of this group. In both cases, the γ-deuteriomethyls have slightly reduced values of $\Delta\mu_Q$, and the α- and β-CD_2 groups have T_1 values reduced by 15–25%. These similarities suggest that the major effects of melittin on the motional properties of the head group of DMPC reflected in the deuterium NMR spectra and relaxation properties may arise from electrostatic interactions of the positively charged groups of melittin (N-terminal and lysine amino groups, and arginine guanidino groups) with the lipid phosphates, as is the case with metal ions (Akutsu & Seelig, 1981). These interactions must be weak and transient (with lifetimes of less than 0.1 ms) because there is no evidence for immobilization of subpopulations of DMPC by melittin giving rise to multiple spectral components. Thus, for both metal ions and melittin, the lipids are in rapid exchange throughout all sites on the time scale of the static quadrupolar constant (10^{-6} s). A partial immobilization of "boundary" lipids by melittin, suggested from Raman spectroscopic studies of melittin–DMPC mixtures (Lavialle et al., 1980), is not apparent in the present work.

The present study of the interaction of melittin with head-group-deuteriated DMPC complements a recent deuterium NMR study (Dufourc et al., 1986b) of the interaction of the peptide with acyl chain deuteriated DPPC. Together these studies provide a concerted picture of the effects of the peptide on both the macroscopic organization of the lipid and the microscopic order and motion in different regions of the bilayer. The reversible transition between extended bilayers and micellar particles at the lipid phase transition, characterized by the reversible collapse of the deuterium and ^{31}P powder patterns arising from membrane lipids, is the major observation of each study and can be seen to be a general effect of melittin on saturated lipids. Dufourc et al. (1986b) propose that this "direct-lysis" effect of melittin, i.e., its ability to removal small regions of bilayer from phospholipid membranes, may underlie the lytic activity of the peptide, and we propose that the mechanism of this process may share features of the lysolipid-induced lysis of saturated lipid bilayers. The two studies indicate that melittin may induce changes in local conformation and order in all regions of the membrane in the liquid-crystalline phase. It is difficult to draw conclusions about the localization of melittin within bilayers from these local effects on order and dynamics in deuteriated lipids because the perturbation of the organization of membrane lipids may give rise to changes throughout the whole lipid molecule irrespective of the position of the peptide in the bilayer. However, the similarities in the effects of melittin and metal ions on the spectral properties of the deuteriated head group of PC, and of melittin and lysoPC on the macroscopic organization of saturated lipids, support a position for melittin near the membrane surface in fluid phase lipid. This conclusion is consistent with the interpretations of Dufourc et al. (1986b), who suggest that in the fluid phase melittin lies near the bilayer surface (Dawson et al., 1978; Terwilliger et al., 1982) moving deeper into the membrane as the temperature is lowered through T_c into the gel phase.

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Characterization of the Chromatin Acceptor Sites for the Avian Oviduct Progesterone Receptor Using Monoclonal Antibodies†

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ABSTRACT: Monoclonal antibodies (MAb) against the chromatin acceptor sites for the avian oviduct progesterone receptor were prepared with highly purified hen oviduct acceptor proteins reconstituted to hen DNA. Addition of the MAbs to a cell-free assay blocked progesterone receptor from chick oviduct (PRov) binding to native-like acceptor sites on nucleoacidic protein (NAP) representing a partially deproteinized chromatin, which has been shown to be enriched in these binding sites. However, the antibodies do not block PRov binding to pure DNA, nor do they affect the receptor itself. Estrogen receptor binding to NAP was not inhibited, supporting a receptor specificity of the PRov acceptor sites as reported previously from direct competition studies. These data support earlier studies showing that (1) the reconstituted PRov acceptor sites resemble the native sites, (2) the acceptor sites are receptor specific, and (3) the PRov binding sites of NAP are different from those of pure DNA. While some animal-species specificity in the PRov binding inhibition was observed, no tissue specificity was seen. Direct binding of the antibodies to native acceptor sites was demonstrated in an enzyme-linked immunosorbent assay (ELISA) system. The antibodies showed little recognition of free acceptor protein or DNA alone, indicating specificity for the protein–DNA complex. A partial evolutionary conservation of the nuclear acceptor sites for PRov was shown by the fact that about 50% of the inhibition seen with hen NAP was obtained with NAPs from several other species, and this partial cross-reactivity of the MAbs with the same NAPs from other animal species was also seen in the ELISA.

It is widely accepted that steroids bind to specific receptor proteins which in turn bind nuclear "acceptor" sites to regulate gene expression (Jensen & DeSombre, 1972). Specific nuclear binding sites for the avian oviduct progesterone receptor (PRov),[1] which appear to be composed of a group of acceptor proteins bound to certain DNA species, have been identified in avian oviduct chromatin [reviewed in Spelsberg et al. (1979, 1983), Thrall et al. (1978), and Spelsberg, (1982)]. These sites bind PRov in a saturable, high-affinity manner and display receptor dependency (Pikler et al., 1976), receptor specificity (Kon & Spelsberg, 1982), and an in vivo like pattern

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[1] Abbreviations: NAP, nucleoacidic protein (a 4 M Gdn·HCl extract of chromatin); Gdn·HCl, guanidine hydrochloride; [^{3}H]PRov, [^{3}H]-progesterone receptor from chick oviduct; [^{3}H]ERov, [^{3}H]estrogen receptor from chick oviduct; MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBS, 0.15 M NaCl and 0.01 M NaH_2PO_4, pH 7.2; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Ig, immunoglobulin.

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