Schwyzer, M., & Hill, R. L. (1977) *J. Biol. Chem. 252*, 2338–2345.
Stollery, J. G., Boggs, J. M., & Moscarello, M. A. (1980) *Biochemistry 19*, 1219–1226.
Surewicz, W., Moscarello, M. A., & Mantsch, H. (1987) *Biochemistry 26*, 3881–3886.
Tressel, P. S., & Kosman, D. (1982) *Methods Enzymol. 89*, 163–171.

Interaction of Melittin with Phosphatidylcholine Membranes. Binding Isotherm and Lipid Head-Group Conformation†

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ABSTRACT: The binding of melittin to nonsonicated bilayer membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was studied with an ultracentrifugation assay and with ^2H and ^{31}P nuclear magnetic resonance. Melittin binding could best be described by a partition equilibrium with $K_p = (2.1 \pm 0.2) \times 10^3$ M^{-1}, measuring the binding isotherm in the concentration range of 0–100 μM melittin and taking into account electrostatic effects by means of the Gouy–Chapman theory. This partition coefficient is smaller than that deduced for small sonicated vesicles and attests to the tighter lipid packing in the nonsonicated bilayers. Deuterium magnetic resonance revealed a conformational change of the phosphocholine head group upon melittin binding. The quadrupole splittings of the α and β segments of the choline head group varied linearly with the amount of bound melittin but in opposite directions; i.e., the α splitting decreased, and the β splitting increased. This conformational change is not specific to melittin but is a response of the phosphocholine head group to positive membrane surface charges in general. Quantitatively, melittin is one of the most efficient head-group modulators, the efficiency per unit charge comparable to that of charged local anesthetics or hydrophobic ions.

Melittin, the main component of bee venom, is a cationic peptide composed of essentially hydrophobic (positions 1–20) and hydrophilic (positions 21–26) amino acids (Haberman & Jentsch, 1967): Gly-Ile-Gly-Ala-Val[5]-Leu-Lys(+)-Val-Leu-Thr[10]-Thr-Gly-Leu-Pro-Ala[15]-Leu-Ile-Ser-Trp-Ile[20]-Lys-(+)-Arg(+)-Lys(+)-Arg(+)-Gln[25]-Gln-$CONH_2$. Because of the uneven distribution of polar and hydrophobic residues, melittin shows amphiphilic properties, interacting strongly with micelles, pure lipid membranes, and biological membranes. Conflicting views about the aggregational state of melittin and about its membrane orientation may be found in the literature, but the data seem to converge to the following picture (Stanislawski & Rüterjans, 1987; Batenburg et al., 1987; Altenbach & Hubbell, 1988): (I) Mellitin is bound in a monomeric form to the lipid membrane. (II) The N-terminus with its 20 mainly hydrophobic amino acids adopts an α-helical conformation in the presence of lipid, with the helix axis more or less parallel to the membrane surface. (III) When added to sonicated phosphatidylcholine vesicles, melittin will not permeate the lipid membrane, but the N- and C-termini will remain on the same side of the membrane.

Melittin induces structural perturbations of the lipid bilayer which have been visualized by a number of different techniques [see Dufourc et al. (1986a,b) and Dempsey and Watts (1987) and references cited therein]. At high concentrations, the peptide disrupts the bilayer, leading to the formation of nonbilayer phases or micelles. In the present study, we were interested mainly in the effect of melittin on the phospholipid head groups, and the concentration of the peptide was kept low enough to leave the bilayer intact.

Phospholipid head groups are sensitive to electric charges at the membrane surface (Seelig et al., 1987). The binding of quite different chemical compounds such as metal ions (Akutsu & Seelig, 1981; Altenbach & Seelig, 1984; Macdonald & Seelig, 1987a,b), hydrophobic ions (Altenbach & Seelig, 1985), or charged local anesthetics (Boulanger et al., 1981; Seelig et al., 1988) induces a unique conformational change of the lipid head group which is quantitatively similar for all positively charged agents and opposite that induced by negatively charged molecules (Seelig et al., 1987; Macdonald & Seelig, 1988). Hence, melittin with its six positive charges should exert a relatively large effect on the phospholipid head groups when bound to the membrane. Deuterium nuclear magnetic resonance (NMR)[1] experiments with head-group-deuteriated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) have indeed provided first indications of such an effect but have been inconclusive concerning a quantitative interpretation (Dempsey & Watts, 1987).

A reliable and sensitive method for monitoring conformational changes of lipids in membranes is NMR spectroscopy and, in particular, deuterium NMR spectroscopy (Seelig, 1977). Combined with a selective deuteriation of the phospholipid head groups, the measurement of the so-called deuterium quadrupole splitting, $\Delta\nu_Q$, provides a quantitative handle on changes of the head-group conformation and lipid

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[1] Abbreviations: NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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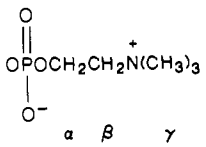
ordering. In the present study, we have selectively deuteriated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) at the two methylene segments of the choline moiety and at the cis-double bound of the oleic acyl chain and have measured the variation of the deuterium quadrupole splittings as a function of melittin concentration. POPC is a common, naturally occurring lipid; it is found, for example, in egg yolk lecithin where it accounts for about 70% of the total lipid (Tattrie et al., 1968). Membranes composed of POPC therefore most closely mimic the packing density of biological membranes which also contain predominantly cis-unsaturated phospholipids. The phase properties of the melittin–POPC mixtures were monitored with ^{31}P NMR (Seelig, 1978). The chemical shielding anisotropy, $\Delta\sigma$, provided information on the orientation and fluctuations of the phosphate segment.

In addition to recording ^{2}H and ^{31}P NMR spectra of melittin–POPC dispersions, we have also measured the amount of bound melittin by a centrifugation assay. This approach differs from previous studies (Vogel, 1981; Batenburg et al., 1987; Schwarz & Beschiaschvili, 1989) in that we need not rely on a conformational change of melittin when binding to the membrane but can measure the free melittin concentration before and after addition of lipid. More important, the binding isotherm refers to nonsonicated phospholipid dispersions, i.e., to essentially planar bilayers with little or no significant curvatures. Such bilayers have a higher lipid packing density than sonicated unilamellar vesicles and correspond more closely to the situation in biological membranes.

Melittin, as available commercially, usually contains traces of phospholipase A_2 which cause rapid hydrolysis of phospholipids and can lead to incorrect results in the interpretation of melittin–membrane interactions. Following the procedure of Batenburg et al. (1987a,b), special care was taken to remove all impurities of phospholipase A_2. Repeated measurements of the same sample in intervals of several hours or days showed no change in the quadrupole splittings.

Materials and Methods

Chemicals. To simplify the discussion, the following notation for the phosphocholine head-group segments is introduced:



1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was selectively deuteriated at the α or β segment of the choline moiety as described previously (Tamm & Seelig, 1983). 1-Palmitoyl-2-[9′,10′-$^{2}H_2$]oleoyl-*sn*-glycero-3-phosphocholine was prepared as described by Seelig and Waespe-Sarcevic (1978).

Unlabeled POPC was purchased from Avanti Polar-Lipids Inc. (Birmingham, AL) and was used without further purification. Sephadex G-25 and thiopropyl-Sepharose 6B were from Pharmacia (Upsalla, Sweden).

Melittin was purchased from Sigma (St. Louis, MO) (grade II, phospholipase free) and Serva (Heidelberg, FRG) (reinst, 51560) and extensively purified, similar to the procedure described by Batenburg et al. (1987a). Fifty milligrams of melittin was dissolved in 1 mL of buffer (0.3 M $NaHCO_3$/1 mM EDTA, pH 8.4) in the presence of 1% (w/v) dithiothreitol. After centrifugation and gel filtration (3 × 90 cm G-25 column, 0.01 M sodium acetate buffer, pH 4), the product was lyophilized and twice incubated with 500 mg of thiopropyl-Sepharose 6B for 20 min (20 mL of 0.1 M Tris-HCl/1 mM EDTA, pH 7.5). The solution was filtered (glass filter and Millipore 0.5-μm filter) and loaded via multiple injection on an HPLC ET 250-1/2″-10 mm column (Macherey-Nagel, Düren, FRG) packed with Nucleosil 100-5 C18 and equipped with two Beckman 112 pumps and a variable-wavelength detector (Kontron Uvicon 725) set at 280 nm. The column was eluted at a flow rate of 2 mL/min with a linear gradient of 45–95% methanol in water (0.1% trifluoroacetic acid). Melittin was collected at 70–73% methanol, lyophilized, and reinjected; the N-formylated derivative was collected at 75–77%. The final product showed a single peak on an HPLC analytical column (packed with the same material as described above) and a single band on electrophoresis. Incubation of POPC with the purified melittin at 37 °C for 24 h gave only one band for the lipid on TLC. NMR measurements of lipid–melittin mixtures were stable over days. We therefore concluded that the final product was free of phospholipase A_2 and contained only melittin which was not formylated at the amino terminus.

Membrane Preparation and Melittin Binding Assay. Standard solutions of lipid in methylene chloride and of melittin in methanol were prepared. Aliquots of the solutions (containing 5–14 μmol of lipid and 1–500 nmol of melittin) were mixed in a glass tube and were subsequently dried under high vacuum. The mixture was hydrated in a well-defined amount of buffer (3–4 mL), vortexed for a short time, and subsequently freeze–thawed. After several freeze–thaw cycles, the samples were equilibrated at 25 °C for at least 4 h and vortexed from time to time. This was sufficient to reach equilibrium as judged by the ^{2}H NMR spectra which showed only one averaged spectrum. For equilibrated samples, we never observed two different signals, i.e., signals due to free lipid and to melittin-bound lipid. Next, the lipid dispersion was centrifuged at 300000*g* at 25 °C for 90–120 min using polyallomer vials, resulting in a clear and a lipid-free supernatant.

All experiments were performed in 40 mM phosphate buffer, pH 6.9, or 40 mM citrate buffer, pH 6.8. The latter buffer was chosen to allow simultaneous ^{31}P NMR experiments and lipid phosphorus analysis.

Phosphate and Peptide Analysis. The amount of lipid in the supernatant was determined by a phosphorus assay after destruction with perchloric acid (Fiske et al., 1925; Dittmer & Wells, 1969). The maximum content of lipid in the supernatant was 3% of the total lipid, but generally less than 1%.

The concentration of free melittin, C_{eq}, in the supernatant was determined by means of three methods: (i) For the upper concentration range, the melittin concentration was determined with ultraviolet absorption spectroscopy using a molar optical absorption coefficient ϵ = 5570 M^{-1} cm^{-1} at 280 nm (Quay & Condie, 1983). (ii) Low concentrations were determined by measuring the tryptophan fluorescence. The emission spectrum in the range of 300–400 nm was recorded with an SLM PR-8000 fluorometer, the excitation wavelength being 280 nm. (iii) For all concentration ranges, the adapted Bio-Rad protein assay (Bio-Rad, München, FRG) was used. Lipid controls were tested in all three methods. In the absence of lipid, no significant sedimentation of melittin after 120 min of 300000*g* centrifugation could be detected.

From the difference between the melittin content of the supernatant and that of the starting solution, it was possible to calculate the amount of melittin bound per mole of POPC. This parameter is denoted X_b (moles of melittin per mole of POPC) in the following.

Table I: Binding of Melittin to POPC Membranes (25 °C, 40 mM NaH_2PO_4/Na_2HPO_4 Buffer, pH 7.2)

C_{eq}[a] (μM)	X_b[b] (mmol/mol)	$\Delta\nu_\alpha$[c] (kHz)	$\Delta\nu_\beta$[c] (kHz)	σ[d] (mC/m²)	ψ_0[e] (mV)	C_M[f] (μM)	X_b/C_M (M^{-1})
0	0	6.3	5.0				
1.60 ± 0.08	4.4 ± 0.3	6.2	5.34	2.25	3.4	1.2	3670
2.2 ± 0.3	3.6 ± 1.8	5.76	5.29 ± 0.03	1.8	2.8	1.7	2080
3.8 ± 0.7	5.5₄ ± 1.0	5.75 ± 0.26	5.49 ± 0.10	2.8	4.2	2.7	2090
6.2 ± 1.0	7.54 ± 1.5	5.47 ± 0.25	5.44	3.8	5.7	3.8	1980
7.3 ± 0.2	8.18 ± 1.0	5.44 ± 0.17	5.41	4.1	6.1	4.3	1900
8.4 ± 0.8	9.78 ± 2.0	5.08 ± 0.16	5.47	4.9	7.2	4.5	2170
11.2 ± 1.4	12.08 ± 1.6	5.17 ± 0.18	5.63	6.1	8.8	5.3	2290
20.5 ± 1.0	16.4 ± 5.4	4.67 ± 0.41		8.1	11.5	7.6	2150
26.8 ± 2.0	18.9 ± 3.7	4.59 ± 0.07		9.3	13.0	8.8	2150
40.2 ± 2.8	23.2 ± 3.1	4.13 ± 0.11		11.3	15.4	10.7	2170
66.9 ± 0.7	24.8 ± 1.5	3.95		11.9	16.2	16.6	1490

[a] Data points represent the average of three to five measurements. [b] Moles of bound melittin per mole of POPC. [c] Quadrupole splittings of α-CD_2- and β-CD_2-POPC as measured by ^2H NMR. [d] Calculated according to eq 3 with lipid area A_L = 68 Å, protein area A_p = 200 Å², and protein charge Z_p = 2.2. [e] Calculated by means of Gouy–Chapman theory. [f] Calculated according to eq 5 with z_p = 2.2.

The melittin concentration before and after equilibration with lipid differed by about 50–90% (high and low concentration range). The variation of the melittin concentration in a binding assay without lipid was only 1.5–2.5%. This latter effect can mainly be attributed to melittin adsorption at the tube walls. The error of the evaluation of the melittin concentration therefore is large (about 20%) for the low concentration range and becomes smaller (about 4%) for higher melittin concentrations.

Nuclear Magnetic Resonance. All ^2H NMR and ^{31}P NMR experiments were performed with coarse lipid dispersions. The lipid pellets obtained from the centrifugations were used without further manipulation. The observed quadrupole splitting and the chemical shift anisotropy could then be related to the amount of bound melittin, X_b, and to the equilibrium melittin concentration of the bulk solution, C_{eq}.

^2H NMR measurements were recorded on a Bruker CXP-300 spectrometer, operating at 46.1 MHz and using a quadrupole echo sequence (Davis, 1976). The pulse width for a 90° pulse was 3.5–4.0 μs, the spectral width was 50–100 kHz, and the recycle time was 0.25 s. Typical measurement times were on the order of 1 h/spectrum.

^{31}P NMR measurements were performed at 121.4 MHz. A gated decoupling technique was used, with a recycle time of 7 s, a pulse width of 3.5–4.5 μs, and a 50-kHz sweep width. A total of 500–2000 free induction decays were accumulated. The chemical shift anisotropy was determined from the distance between the edges of the spectrum measured at half-height of the low-field shoulder.

Results

Binding Isotherm. The extent of melittin binding to POPC bilayers (25 °C, 40 mM phosphate buffer, pH 6.9) as measured by the centrifugation assay is summarized in Table I. The molar amount of melittin bound per mole of POPC, X_b, is given as a function of the equilibrium concentration, C_{eq}, of melittin in the buffer phase. The cutoff of the binding isotherm at high concentrations ($C_{eq} > 70$ μM) was due to the onset of bilayer disruption and micellization. Compared to published binding isotherms for sonicated lipid vesicles (Vogel, 1981; Schwarz & Beschiaschvili, 1989), the binding affinity of melittin to nonsonicated membranes appears to be distinctly reduced. This may be explained by the higher packing density of the lipids in the latter case (cf. below).

^2H and ^{31}P NMR Measurements. Selectively deuteriated lipids were employed in the centrifugation assay. It was therefore possible to measure simultaneously the ^2H and, in part, the ^{31}P NMR spectra and to correlate the observed spectral changes with the binding isotherm. Figure 1 displays ^2H NMR spectra of α- and β-deuteriated POPC membranes

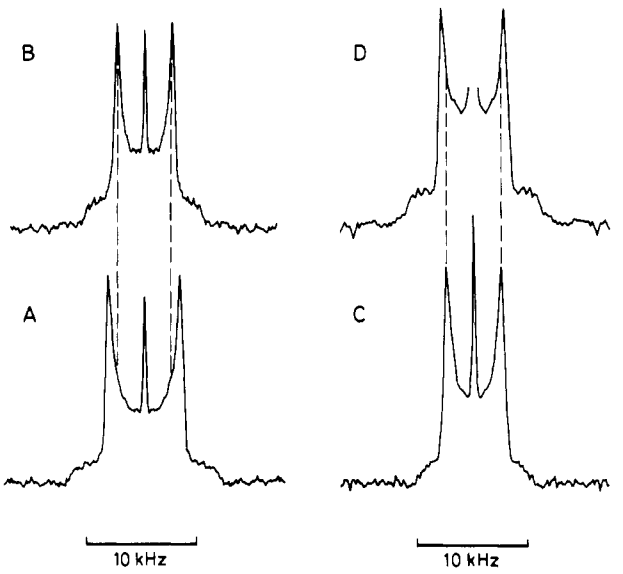


FIGURE 1: Deuterium NMR spectra (46.1 MHz) of multilamellar dispersions of POPC with and without melittin. (A) [α-CD_2]POPC without melittin: $\Delta\nu_\alpha$ = 6.3 kHz. (B) [α-CD_2]POPC with melittin: X_b = 13.0 mmol/mol; $\Delta\nu_\alpha$ = 4.88 kHz. (C) [β-CD_2]POPC without melittin: $\Delta\nu_\beta$ = 5.0 kHz. (D) [β-CD_2]POPC with melittin: X_b = 11.9 mmol/mol; $\Delta\nu_\beta$ = 5.63 kHz.

with and without melittin. Addition of melittin affects the two head-group segments in opposite direction: the α splitting decreases whereas the β splitting increases. For well-equilibrated samples, all ^2H NMR spectra were characterized by a single quadrupole splitting, $\Delta\nu_Q$, indicating a time-averaged head-group conformation at all melittin concentrations. The average residence time of a melittin molecule at a particular phospholipid head group was therefore short on the time scale of deuterium NMR ($\approx 10^{-5}$ s). The variation of the α- and β-head-group splittings with the amount of bound melittin is shown in Figure 2. As with other charged ligands [cf. Altenbach and Seelig (1984) and Macdonald and Seelig (1987a,b, 1988)], a linear dependence of $\Delta\nu_Q$ on X_b was found. Linear regression analysis yielded[2]

$$\Delta\nu_\alpha \text{ (kHz)} = 6.25 - 93.3X_b \text{ (mol/mol)} \quad (R = 0.98) \quad (1)$$

$$\Delta\nu_\beta \text{ (kHz)} = 5.10 + 44.4X_b \text{ (mol/mol)} \quad (R = 0.91) \quad (2)$$

In order to verify the bilayer structure, some lipid pellets were

[2] It would be more appropriate to correlate the quadrupole splittings with the surface charge density σ as given by eq 3 [cf. Seelig et al. (1988)]. However, at low loading ($X_b < 0.05$), eq 1 and 2 are sufficiently good approximations.

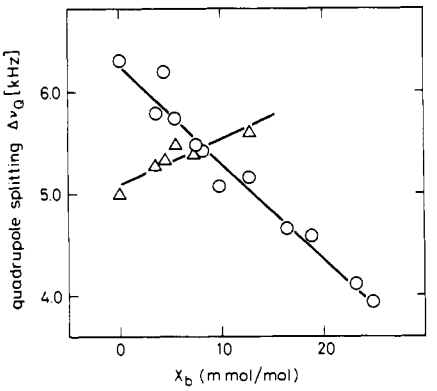


FIGURE 2: Conformational change of the phosphocholine head group upon binding and intercalation of melittin. POPC was deuteriated at the α segment (O) and at the β segment (Δ) of the choline moiety. Variation of the deuterium quadrupole splitting with mole fraction of bound melittin, X_b (millimoles of melittin bound per mole of POPC).

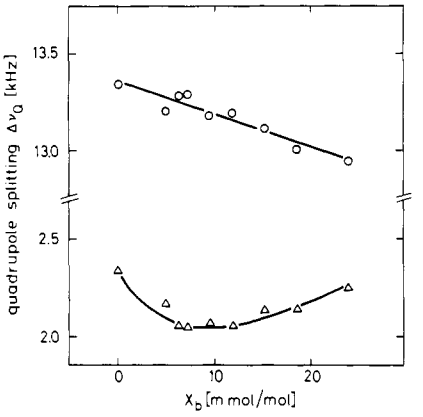


FIGURE 3: Influence of melittin on the hydrocarbon chain ordering of multilamellar dispersions composed of 1-palmitoyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine. The variation of the quadrupole splittings of the two deuterons of the cis-double bond is shown as a function of the amount of bound melittin, X_b. (O) C-9 carbon segment; (Δ) C-10 carbon segment.

further characterized by ^{31}P NMR using 40 mM citrate buffer. All ^{31}P NMR spectra were characteristic of bilayer spectra (Seelig, 1978) with no contributions from isotropic phases. The chemical shift anisotropy, $\Delta\sigma$, remained approximately constant ($\Delta\sigma \approx -48.5$ ppm) in the concentration range investigated.

The influence of melittin on the hydrocarbon chain ordering was studied with membranes composed of chain-deuteriated POPC [1-palmitoyl-2-(9′,10′-$^2H_2$)oleoyl-*sn*-glycero-3-phosphocholine]. The two deuterons at the cis-double bond give rise to different quadrupole splittings (Seelig & Waespe-Sarcevic, 1978) of about 13 and 2 kHz for the C-9′ and C-10′ positions, respectively. Binding of melittin induced the changes shown in Figure 3. The quadrupole splittings of the C-9′ deuteron decreases smoothly with increasing melittin, but the variation of $\Delta\nu_Q$(C-9′) was about a factor of 5 smaller than that observed for the α-head-group segment. For the C-10′ deuteron, a parabolic curve was obtained. $\Delta\nu_Q$ decreased up to $X_b \approx 0.01$ by about 0.5 kHz and returned to the original value at $X_b \approx 0.03$, indicating a shift in the average orientation for the cis-double bond [cf. Seelig and Waespe-Sarcevic (1978)].

Discussion

Analysis of the Binding Isotherm. Binding of melittin to an electrically neutral POPC membrane generates a positive electric charge at the membrane surface. From the extent of binding, X_b, the surface charge density, σ, can be calculated according to (Seelig et al., 1988)

$$\sigma = (z_p e_0 X_b/A_L)/[1 + X_b(A_p/A_L)] \quad (3)$$

z_p is the effective valency of melittin, e_0 is the elementary electric charge, and $A_L \approx 68$ Å^2 is the average surface area of a POPC molecule. A_p/A_L denotes a correction factor which takes into account the possible penetration of melittin into the lipid membrane, thereby increasing the surface area by the effective protein area A_p (per molecule). A_p can vary between $A_p = 0$ (no penetration) to $A_p \approx 200$ Å^2 (approximate surface requirement of the melittin α-helix). However, since X_b is small in the present study, the influence of the A_p/A_L ratio on the surface charge density is also small. The surface charge density, in turn, generates a surface potential, ψ_0, which can be calculated by using the Gouy–Chapman theory (Aveyard & Haydon, 1973; McLaughlin, 1977):

$$\sigma^2 = 2000\epsilon_0\epsilon_R RT\sum_i C_{i,eq}(e^{-z_i F_0\psi_0/RT} - 1) \quad (4)$$

where $\epsilon_r = 78$ is the dielectric constant of water, ϵ_0 the permittivity of free space, R the gas constant, $C_{i,eq}$ the concentration of the ith electrolyte in the bulk aqueous phase (in moles per liter), and z_i the signed charge of the ith species. The effect of the surface potential is to repel ions of like charge, making the binding of melittin increasingly more difficult. The concentration of melittin, C_M, at the plane of melittin binding, i.e., in the solution immediately adjacent to the membrane surface, is thus smaller than the equilibrium concentration and can be calculated according to

$$C_M = C_{eq} \exp(-z_p\psi_0 F_0/RT) \quad (5)$$

The unknown parameter in this approach is z_p, the effective charge of melittin. McLaughlin and co-workers have pointed out that when the distance of the charges on a multivalent cation is comparable with the Debye length, the effective charge as seen by the membrane surface can be smaller than the true electric charge and the screening effect of such ions on the electrostatic potential is smaller than predicted (Carnie & McLaughlin, 1983; Alvarez et al., 1983). A second factor could be the discreteness-of-charge effect [cf. Schoch and Sargent (1980)] since one of the major assumptions of the Gouy–Chapman theory is the replacement of discrete charges by a surface of uniform charge density. However, the discreteness-of-charge effect has recently been tested experimentally and was found not to be significant in determining the potential-sensitive partitioning of membrane probes (Winiski et al., 1986; Hartsel & Cafiso, 1986). In the present analysis, a consistent picture using the Guoy–Chapman theory was obtained with $z_p = 2.2$ which is considerably smaller than the true electric charge of 6+ of melittin (cf. below). Numerical data for σ, ψ_0, and C_M calculated with $z_p = 2.2$ are summarized in Table I.

Having determined the amount of bound melittin as well as its concentrations immediately adjacent to the membrane interface, various binding models can be tested. A simple partition equilibrium would obey the relation:

$$X_b/C_M = K_p \quad (6)$$

and the last column of Table I demonstrates that such a model is indeed in agreement with the experimental data. The ratio X_b/C_M is approximately constant over the whole concentration range measured, yielding an average partition coefficient of $K_p = (2.1 \pm 0.2) \times 10^3$ M^{-1}.

Schwarz and Beschiaschvili (1989) have measured the binding of melittin to *sonicated* vesicles composed of 1,2-

dioleoyl-*sn*-glycero-3-phosphocholine with circular dichroism and fluorescence spectroscopy. They also arrived at a partition equilibrium but with a partition coefficient $K_p \approx 3 \times 10^4$ M^{-1} (0.11 M salt) which is 1 order of magnitude larger than our result. Electrostatic repulsions were taken into account with an effective charge of $z_p \approx 1.8$. An even higher binding constant of $K \approx 5 \times 10^5$ M^{-1} was derived by Vogel (1981) for DMPC vesicles. In the latter case, electrostatic repulsions were, however, neglected.

We believe that the different binding affinities are a consequence of not so much the different lipids employed but of differences in the lipid packing densities in planar bilayers and curved vesicles. Seelig (1987) has compared the binding of local anesthetics to POPC monolayers and planar POPC bilayers. Identical binding isotherms were only obtained if the monolayer was compressed at a lateral pressure of 32 mN/m which thus can be considered as the bilayer–monolayer equivalence pressure. Increased anesthetic binding was observed upon lowering the monolayer pressure. Schindler (1980) has measured the bilayer–monolayer equivalence pressure as a function of vesicle radius for 1,2-dioleoyl-*sn*-glycero-3-phosphocholine vesicles. Vesicles with a radius of 20 nm exhibited a bilayer–monolayer equivalence pressure of only 12 mN/m which increased asymptotically to a final value of 33 mN/m for vesicles with $r \geq 60$ nm. These results suggest that the packing difference between lipid vesicles of small curvature and planar lipid bilayers accounts for the enhanced melittin binding measured for lipid vesicles. Similar conclusions have been reached for the reactivity of apolipoprotein A-I with DPPC (Wetterau & Jonas, 1982) and for the association of apocytochrome *c* with phosphatidylserine (Berkhout et al., 1987). In both systems, the protein binding affinity decreased with decreasing vesicle curvature. For the apocytochrome *c*–phosphatidylserine, the binding constant of planar PS membranes was almost a factor of 300 smaller than that of unilamellar vesicles of small radius.

The Gouy–Chapman analysis suggested an effective charge of $z_p = 2.2$ for melittin bound to POPC membranes. This value was adopted for the following reasons. For small z_p values ($z_p < 2.2$), a representation of the data in terms of a Scatchard analysis resulted in *curved* Scatchard plots only. For $z_p > 2.2$, the Scatchard plots were characterized by *positive* slopes. Hence, $z_p = 2.2$, yielding constant X_b/C_M ratios over the whole concentration range, provided the simplest and physically most realistic model for interpreting the experimental data.

A reduced electric charge was also observed in previous melittin binding assays with negatively charged lipids. A stoichiometry of 1 melittin bound to 3–4 negative charges was found for melittin–phosphatidylserine (Dufourc & Faucon, 1977), melittin–cardiolipin (Batenburg et al., 1987a,b), melittin–phosphatidylglycerol, and melittin–phosphatidic acid (Batenburg, 1987c) complexes.

Melittin–Phosphocholine Head-Group Interaction. Analogous to the melittin–DMPC system (Dempsey & Watts, 1987), the binding of melittin to POPC bilayers induces a conformational change of the phosphocholine head group. We observe a counterdirectional change for the two choline quadrupole splittings, the latter varying linearly with the amount of bound melittin.[3] By eliminating X_b from eq 1 and 2, we obtain a linear relationship between the two quadrupole splittings (α–β plot):

$$\Delta\nu_\beta = -0.48\Delta\nu_\alpha + 8.1 \text{ (kHz)} \quad (7)$$

The significance of this result is 2-fold. First, the counterdirectional change of $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ rules out a disordering of the lipid head group since this would decrease both splittings simultaneously. Evidence for a constant lipid ordering is further provided by the almost constant chemical shift anisotropy of the phosphate group and by the rather small changes of the hydrocarbon chain ordering as reflected in the quadrupole splittings of the cis-double bond (cf. Figure 3). Second, eq 7 is consistent with α–β plots obtained for other positively charged compounds which associate with the membrane surface. We note the following systems and α–β relations: Ca^{2+}–, Mg^{2+}–, Cd^{2+}–, or La^{3+}–DPPC (Akutsu & Seelig, 1981)

$$\Delta\nu_\beta = -0.43\Delta\nu_\alpha + 6.7 \text{ (kHz)} \quad (8)$$

Ca^{2+}–POPC (Altenbach & Seelig, 1984)

$$\Delta\nu_\beta = -0.49\Delta\nu_\alpha + 7.6 \text{ (kHz)} \quad (9)$$

Etidocaine (cation)–POPC (Seelig et al., 1988)

$$\Delta\nu_\beta = -0.49\Delta\nu_\alpha + 8.4 \text{ (kHz)} \quad (10)$$

cationic integral membrane protein–DMPC [calculated from Roux et al. (1988)]

$$\Delta\nu_\beta = -0.52\Delta\nu_\alpha + 7.3 \text{ (kHz)} \quad (11)$$

The almost quantitative agreement between the α–β plot of melittin and those of other cationic substances suggests that the chemical nature of the adsorbed ion or bound substance is of secondary importance but that the driving force in perturbing the choline head-group conformation is the positive electric charge generated at the membrane surface. The larger the membrane surface charge, the larger are the changes in the quadrupole splittings. This conclusion is also supported by the observation that neutral, dipolar, and negatively charged molecules are characterized by different α–β plots (different for each class of substances) (Akutsu & Seelig, 1981; Scherer & Seelig, 1987; Seelig et al., 1987; Macdonald & Seelig, 1988).

A closer inspection of eq 1 and 2 further attests to the predominant role of membrane surface charges. Compared to other cations, melittin induces an exceptionally large *molar* change of the quadrupole splittings; e.g., the change in $\Delta\nu_\alpha$ upon melittin binding is 93 kHz/mol of bound melittin (eq 1). On the other hand, the melittin molecule is characterized by a large electric charge. If we choose an effective charge of $z_p = 2.2$, in order to remain consistent with the Gouy–Chapman analysis, the variation of the α-quadrupole splitting per unit charge corresponds to $93/2.2 \approx 42$ kHz per mole unit charge. Similar values have been obtained for cationic dibucaine (Seelig et al., 1988) and tetraphenylphosphonium (Altenbach & Seelig, 1985) both with a 1+ charge. Thus, on the basis of the number of electric charges, the melittin behavior is no longer so unusual.

The latter result also attests to the hydrophobic nature of melittin binding. The electric charges of melittin as sensed by the membrane surface are as effective in changing the lipid conformation as those of hydrophobic ions. This conclusion is consistent with fluorescence spectroscopy data which demonstrate that tryptophan-19 penetrates at least up to the glycerol backbone area of PC vesicles (Batenburg et al., 1987).

The exact nature of the conformational change induced by melittin and other cations is not known at present. However, it can be estimated that quite small changes of the bond rotation angles are sufficient to produce the observed variations

[3] Dempsey and Watts (1987) observe only an insignificant change of the β-deuteron splitting which is in contrast to the present results.

in the quadrupole splittings (Akutsu & Seelig, 1981; Roux et al., 1989). This problem is at present under investigation.

The molecular interactions between lipids and proteins have been extensively studied with NMR methods [for reviews, see Seelig and Seelig (1980), Davis (1983), Deveaux and Seigneuret (1985), Bloom and Smith (1987), and Watts (1987)]. In general, the hydrophobic part of the lipid bilayer appeared to be only little influenced by the presence of extrinsic or intrinsic proteins [cf. Tamm and Seelig (1983), Dufourc et al. (1986a,b), and Zidovetzki et al. (1988)]. On the other hand, a number of specific effects have been noted for the phospholipid head groups (Sixl & Watts, 1985; Sixl et al., 1984; Dempsey & Watts, 1987; Deveaux et al., 1986; Roux et al., 1989), and most head-group studies report distinct changes in the head-group conformation of the lipids involved. The present data on melittin as well as the study of Roux et al. (1989) on a synthetic membrane peptide then point to a common mechanism underlying these conformational changes. Independent of the mode of peptide binding (extrinsic or intrinsic), the overriding influence on the head-group conformation comes from the electric charge at the membrane surface.

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References

Akutsu, H., & Seelig, J. (1981) *Biochemistry 20*, 7366–7373.
Altenbach, Ch., & Seelig, J. (1984) *Biochemistry 23*, 3913–3920.
Altenbach, Ch., & Seelig, J. (1985) *Biochim. Biophys. Acta 818*, 410–415.
Altenbach, Ch., & Hubbell, W. L. (1988) *Proteins: Struct., Funct., Genet. 3*, 230–242.
Alvarez, O., Brodwick, M., Latorre, R., McLaughlin, A. C., McLaughlin, S., & Szabo, G. (1983) *Biophys. J. 44*, 333–342.
Aveyard, R., & Haydon, D. A. (1973) *An introduction to the principles of surface chemistry*, Cambridge University Press, London.
Batenburg, A. M., Hibbeln, J. C. L., & de Kruijff, B. (1987a) *Biochim. Biophys. Acta 903*, 155–165.
Batenburg, A. M., Hibbeln, J. C. L., Verkleij, A. J., & de Kruijff, B. (1987b) *Biochim. Biophys. Acta 903*, 142–154.
Batenburg, A. M., van Esch, J. H., Leunissen-Bijvelt, J., Verkleij, A. J., & de Kruijff, B. (1987c) *FEBS Lett. 223*, 148–154.
Berkhout, T. A., Rietveld, A., & de Kruijff, B. (1987) *Biochim. Biophys. Acta 897*, 1–4.
Bloom, M., & Smith, I. C. P. (1985) in *Progress in Lipid Protein Interactions* (Watts, A., & de Pont, J. J. H. H. M., Eds.) Vol. I, pp 68–88, Elsevier, Amsterdam.
Boulanger, Y., Schreier, S., & Smith, I. C. P. (1981) *Biochemistry 20*, 6824–6830.
Carnie, S., & McLaughlin, S. (1983) *Biophys. J. 44*, 325–332.
Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117–172.
Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390–394.
Dempsey, C. E., & Watts, A. (1987) *Biochemistry 26*, 5803–5811.
Deveaux, P. F., & Seigneuret, M. (1985) *Biochim. Biophys. Acta 822*, 63–125.
Deveaux, P. F., Hoatson, G. L., Favre, E., Fellmann, P., & Farren, B. (1986) *Biochemistry 25*, 3804–3812.
Dittmer, J. C., & Wells, A. M. (1969) *Methods Enzymol. 14*, 482–530.
Dufourc, E. J., Smith, I. C. P., & Dufourcq, J. (1986a) *Biochemistry 25*, 6448–6455.
Dufourc, E. J., Faucon, J. L., Fourche, G., Dufourcq, J., Gulik-Krzywicki, T., & Le Maire, M. (1986b) *FEBS Lett. 201*, 205–209.
Dufourcq, J., & Faucon, J. F. (1977) *Biochim. Biophys. Acta 467*, 1–11.
Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–389.
Habermann, E., & Jentsch, J. (1967) *Hoppe Seyler's Z. Physiol. Chem. 348*, 37–50.
Hartsel, S. C., & Cafiso, D. S. (1986) *Biochemistry 25*, 8214–8219.
Macdonald, P. M., & Seelig, J. (1987a) *Biochemistry 26*, 1231–1240.
Macdonald, P. M., & Seelig, J. (1987b) *Biochemistry 26*, 6292–6298.
Macdonald, P. M., & Seelig, J. (1988) *Biochemistry 27*, 6769–6775.
McLaughlin, S. A. (1977) *Curr. Top. Membr. Transp. 9*, 71–144.
Quay, S. C., & Condie, C. C. (1983) *Biochemistry 22*, 695–700.
Roux, M., Neumann, J. M., Hodges, R. S., Deveaux, P. F., & Bloom, M. (1989) *Biochemistry 28*, 2313–2321.
Scherer, P. G., & Seelig, J. (1987) *EMBO J. 6*, 2915–2922.
Schindler, H. (1980) *FEBS Lett. 122*, 77–79.
Schoch, P., & Sargent, D. F. (1980) *Biochim. Biophys. Acta 602*, 234–247.
Schwarz, G., & Beschiaschvili, G. (1989) *Biochim. Biophys. Acta 979*, 82–90.
Seelig, A. (1987) *Biochim. Biophys. Acta 899*, 196–204.
Seelig, A., Allegrini, P. R., & Seelig, J. (1988) *Biochim. Biophys. Acta 939*, 267–276.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.
Seelig, J. (1978) *Biochim. Biophys. Acta 505*, 105–141.
Seelig, J., & Waespe-Sarcevic, N. (1978) *Biochemistry 17*, 3310–3315.
Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.
Seelig, J., Macdonald, P. M., & Scherer, P. G. (1987) *Biochemistry 26*, 7535–7541.
Sixl, F., & Watts, A. (1985) *Biochemistry 24*, 7906–7910.
Sixl, F., Brophy, P. J., & Watts, A. (1984) *Biochemistry 23*, 2032–2039.
Stanilawski, B., & Rüterjans, H. (1987) *Eur. Biophys. J. 15*, 1–12.
Tamm, L. K., & Seelig, J. (1983) *Biochemistry 22*, 1474–1483.
Tattrie, N. H., Bennett, J. R., & Cyr, R. (1968) *Can. J. Biochem. 46*, 819–824.
Vogel, H. (1981) *FEBS Lett. 134*, 37–42.
Watts, A. (1987) *J. Bioenerg. Biomembr. 19*, 625–653.
Wetterau, J. R., & Jonas, A. (1982) *J. Biol. Chem. 257*, 10961–10966.
Winiski, A. P., McLaughlin, A. C., McDaniel, R. V., Eisenberg, M., & McLaughlin, S. (1986) *Biochemistry 25*, 8206–8214.
Zidovetzki, R., Banerjee, U., Harrington, D. W., & Chan, S. (1988) *Biochemistry 27*, 5686–5692.