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The actions of melittin on membranes

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I. Introduction

The venom of the European honey bee *Apis mellifera* contains as its major protein component a 26 amino

Abbreviations. CD, circular dichroism, CL, cardiolipin, DAPC, di-arachidoylphosphatidylcholine, DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine, DPPC, dipalmitoylphosphatidylcholine, DSC, differential scanning calorimetry, DSPC, distearoylphosphatidylcholine, DTPC, ditetradecylphosphatidylcholine, EDTA, ethylenediaminetetraacetic acid, ESR, electron spin resonance, HPLC, high-performance liquid chromatography, NMR, nuclear magnetic resonance, PC, phosphatidylcholine, PE, phosphatidylethanolamine, PG, phosphatidylglycerol; PS, phosphatidylserine, NOE, nuclear Overhauser enhancements, PBP, *p*-bromophenacylbromide.

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acid peptide, called melittin, which has a powerful hemolytic activity. Numerous studies have been undertaken to determine the nature of the interaction of melittin with membranes, both with the aim of understanding the molecular mechanisms of melittin-induced hemolysis and as a model for studying the general features of the structures of membrane proteins and their interactions with lipids in membranes. It is probably uncontroversial to state that the mechanism of membrane lysis has not been proven and that there is no consensus on the nature of the interaction of melittin with membrane lipids. One reason relates to the problems inherent in the study of proteins in membranes, namely that many of the biophysical techniques used to study protein structures and interactions are difficult to apply to membrane systems. A second reason, more specific to melittin, is that the peptide has several actions on membranes and that these activities are not necessarily related; melittin may interact with mem-

branes in different ways, depending on the lipid composition of the bilayer, peptide concentration, bulk solution pH, the presence or absence of a membrane potential, and the hydration level of the bilayer

In addition to its hemolytic activity, melittin induces voltage-dependent ion-conductance across planar lipid bilayers and causes selective micellization of bilayers as well as membrane fusion. In this review the membrane activities of melittin are discussed and evidence relating to the molecular mechanisms of these activities and their relationship to one another are assessed. A number of effects of melittin on cells and enzymes that may result either from the binding of melittin to membranes or to proteins have been described but will not be discussed here. The reader is referred to several earlier reviews of melittin [1–3].

II. The structure of melittin

II-A Structure and conformational properties of melittin in water

In common with other membrane-binding peptides and membrane proteins, melittin is predominantly hydrophobic (Table I) [4]. The peptide has a net charge of +6, four of these charges are in the highly basic C-terminal tetrapeptide sequence Lys-Arg-Lys-Arg, with only the N-terminal amino group and Lys-7 in the N-terminal sequence. There are no acidic groups in melittin. A further skewed distribution of polar and non-polar amino acids is apparent if the peptide is placed in an α -helical configuration (Fig. 1) [5]. The polar amino acids in the first 21 residues lie on one face of the helix with the non-polar amino acids segregated almost completely on an opposite side of the helix. This so-called amphipathic nature is a characteristic of many membrane-binding peptides and of putative transmembrane helices of many membrane proteins [6–8]. A number of peptides that share some of the properties of melittin in membranes are shown in Table I and their amphipathic nature is illustrated in Fig. 1.

Despite its high proportion of hydrophobic side-chains, melittin is very soluble in water (> 250 mg/ml) and moderately soluble in methanol (up to 20 mg/ml at pH values below the pK_a of the N-terminal amino

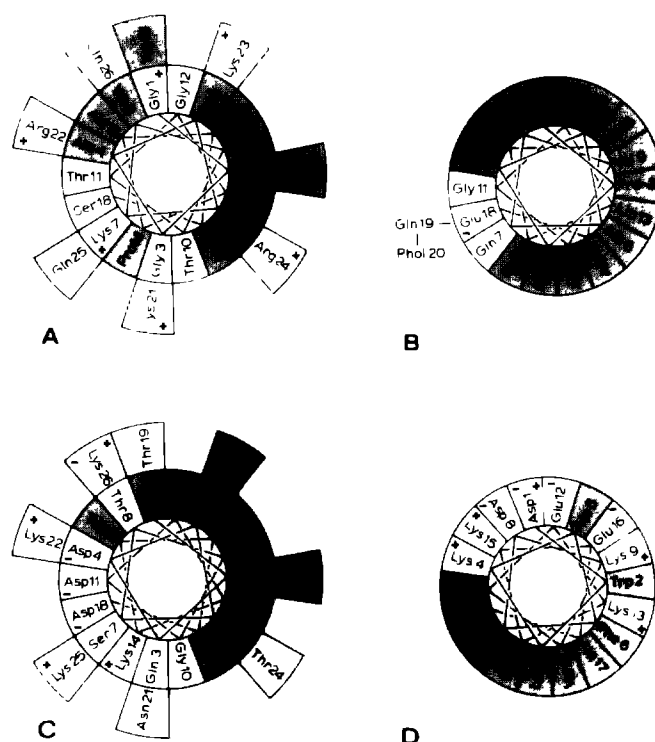


Fig. 1 α -Helical wheel representation of melittin and some related peptides. (A) Melittin, (B) alamethicin, (C) δ -hemolysin, (D) apolipoprotein analogue. Amino acids with hydrophobic side-chains are shaded. The sequences of melittin and δ -hemolysin are shown as helices extending to the C-terminus although there is evidence for non-helical conformations at the C-terminus for these peptides under some conditions (subsections II-B and IV-A).

group). Melittin reaches membranes through the aqueous phase, so that a consideration of the properties of the peptide in water is relevant to its effects on membranes.

In aqueous solution melittin adopts different conformations and aggregation states depending on several factors including peptide concentration, pH, ionic strength and the nature of the negative counterion. The results of a large number of studies may be summarized as follows:

Depending on the factors listed above, melittin is either monomeric in water or associated as a tetrameric aggregate [9–14]; stable dimers are not formed in water [15]. The aqueous monomer has no detectable secondary structure, as determined by circular dichroism

TABLE I

Amino acid sequences of melittin and some structurally and functionally related peptides

Melittin	Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH ₂
δ -Hemolysin	for-Met-Ala-Gln-Asp-Ile-Ile-Ser-Thr-Ile-Gly-Asp-Leu-Val-Lys-Trp-Ile-Ile-Asp-Thr-Val-Asn-Lys-Phe-Thr-Lys-Lys
Alamethicin	ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol
Apolipoprotein analogues	Asp-Trp-Leu-Lys-Ala-Phe-Tyr-Asp-Lys-Val-Ala-Glu-Lys-Leu-Lys-Glu-Ala-Phe

for, formyl, ac, acetyl, Aib, α -aminobutyric acid, Phol, phenylalaninol

(CD) [11,16,17] and high-resolution ^1H -NMR which shows chemical shifts typical of 'random-coil' polypeptides for the assigned resonances of the NMR spectrum [12]. In common with other Pro-containing peptides, the Leu-13-Pro-14 peptide bond undergoes *cis-trans* isomerism with a rate sufficiently slow ($< 10/\text{s}$) for separate signals to be observable in the proton NMR spectrum of the aqueous melittin monomer [12]. The tetramer, on the other hand, consists of melittin monomers predominantly in a helical conformation [11,16-18]. The conformational and aggregational properties of melittin in water result from two opposing forces; promoting self-association is the hydrophobic effect that acts to sequester non-polar amino acids in the interior of proteins (or molecular aggregates) and opposing self-association are the high positive-charge density of melittin and the entropic term associated with the formation of secondary structure in the self-associated tetramer. Factors that suppress charge strongly promote self-association. High ionic strength leads to self association, and the marked effect of phosphate in inducing tetramerization [19] may be related to the favorable binding of this counterion to the guanidino group of arginine. A natural derivative of melittin formylated on the N-terminal amino group [20] self-associates at lower peptide concentrations and ionic strengths than melittin at pH values below the pK_a of the N-terminal amino group of melittin [12]. Details of the conditions under which melittin is either monomeric or tetrameric may be found in the NMR studies of references [12] and [13], and the thermodynamics of the melittin monomer-tetramer equilibrium are discussed in Ref. 14. The structure of the aqueous tetramer is described below (subsection II-B).

Titration of the amino groups of melittin by increasing pH results in suppression of positive charge density and promotes self-association of melittin [12,13,18,21]. There is considerable interest in the pK_a values of the amino groups of membrane-bound melittin because of the expectation that their titration behavior may be representative of the location of the groups in the bilayer (subsection IV-B). Several studies of amino group titrations in aqueous melittin have yielded conflicting information. A value of pH 7.8 for the pK_a of the N-terminal amino group of monomeric melittin in salt-free D_2O was found by ^1H -NMR; under these conditions the lysine amino groups were assigned pK_a values above pH 9 [12]. Highly perturbed pK_a values of pH 6.5 and 8.6 were reported for the amino groups of Lys-21 and Lys-23, respectively, in monomeric melittin from the pH-dependence of reaction with trinitrobenzenesulfonic acid [21]. Using the same method, Lys-21 and Lys-23 were assigned pK_a values of 7.4 in tetrameric melittin. These highly suppressed pK_a values were attributed to the high positive charge density in the C-terminal sequence [21]. However, several NMR

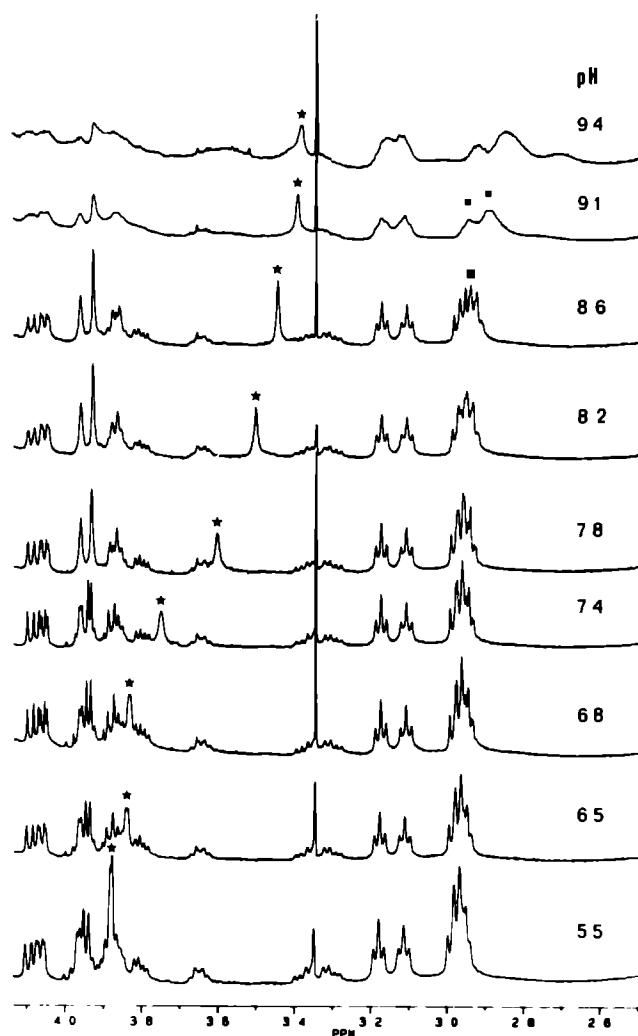


Fig. 2 pH-Dependence of part of the ^1H -NMR spectrum of melittin (0.8 mM) in salt-free D_2O . The signals corresponding to the Gly-1 $\alpha\text{-CH}_2$ protons (*) and Lys-7, -21 and -23 $\epsilon\text{-CH}_2$ protons (■) are indicated.

pH titration studies support the original finding that the C-terminal lysines of aqueous melittin titrate with pK_a values that are closer to the normal range expected for the ϵ -amino group of lysine of polypeptides. ^1H -NMR pH titrations similar to those reported previously [12] confirm the pK_a of 7.8 for the N-terminal amino group of monomeric melittin in salt free D_2O (Fig. 2) and show that the lysine $\epsilon\text{-CH}_2$ resonances (Fig. 2, boxes) begin to titrate only around pH 9 upon which self-association occurs as indicated by marked broadening of spectral lines; a broad multiplet corresponding to one lysine $\epsilon\text{-CH}_2$ (possibly Lys-7) titrates with a higher pK_a than the other two lysine $\epsilon\text{-CH}_2$ groups (unpublished results). ^{13}C -NMR pH titrations of tetrameric melittin amino-methylated with $[^{13}\text{C}]$ methyl groups indicate pK_a values of pH 9.65 for one lysine (proposed to be Lys-7) and pH 8.84 for two lysine amino groups (Lys-21 and Lys-23) in the tetramer [22]. Dimethylamino groups have pK_a values lowered by around 0.6 pH units com-

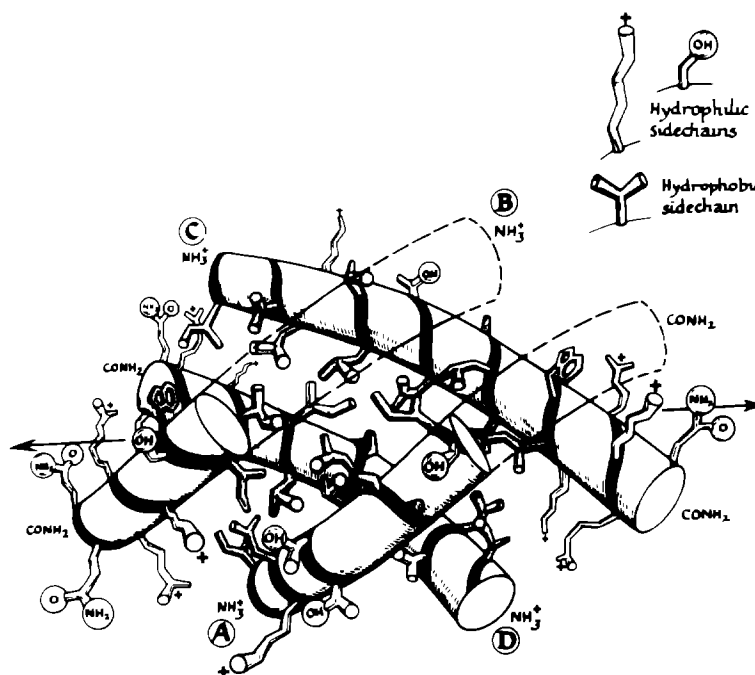


Fig 3 Illustration of the arrangement of melittin α -helical monomers in the crystalline tetramer (From Ref. 25, with permission.)

pared with unmethylated amino groups [23] so that the pK_a values correspond to around pH 10.2 and 9.4, respectively. While the high positive charge density in the C-terminal sequence of melittin causes a lowering of the pK_a values of the amino groups of lysines-21 and -23, the perturbation in aqueous melittin in either the monomeric or tetrameric states is only around 1 pH unit or less.

II-B High-resolution structures of melittin

The structure of the aqueous melittin tetramer is known to atomic resolution from the analysis of X-ray diffraction data from melittin crystals grown from solutions of high ionic strength [24–26]. The crystal structure of melittin has had considerable influence on subsequent ideas about the interaction of melittin with membranes and the structure will be described here in some detail. High-resolution structures of monomeric melittin in methanol, and in dodecylphosphocholine micelles have recently been determined using high-resolution proton NMR and amide exchange analysis [27–29]. The conformations of short polypeptides lacking disulphide bonds are expected to be heavily dependent on their environment and for this reason it is instructive to describe the X-ray derived crystal structure and the two NMR-derived structures together.

Two crystal forms of tetrameric melittin have been solved to a resolution of 2 to 2.5 Å and in each crystal form the conformation of the peptide is essentially the same [25,26]. Within each tetramer the structures of the monomers are also very similar. In the crystalline tetra-

mer (Fig. 3) melittin adopts a helical conformation (Fig. 4). The presence of proline at position 14 results in loss of the X-14 amide to Thr-10 carbonyl helical hydrogen bond and the peptide helix is bent so that lines drawn through the helix axes of residues 1–10 and 16–26 intersect with an angle of about 120° . The large helix bend allows optimal packing of hydrophobic side-chains within the melittin tetramer. As predicted from the helical wheel representation (Fig. 1), the non-polar amino acids are almost completely sequestered on the inside of the helix bend, while the polar and charged side-chains lie largely on the convex face of the helix bend. The Leu-13–Pro-14 peptide bond is in the *trans* configuration required for an α -helix. An anomalous feature of the crystal monomer is that the peptide amide bond between residues 11 and 12 is rotated by 180° from the orientation expected in a regular α -helix. Despite this feature and the large bend around Pro-14, the crystal monomer has an essentially helical conformation throughout. The degree of helicity of crystalline tetrameric melittin is greater than expected from the values

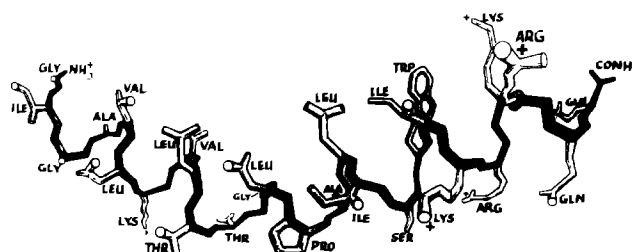


Fig 4 The conformation of monomeric subunits of melittin from the crystalline tetramer (From Ref. 25, with permission.)

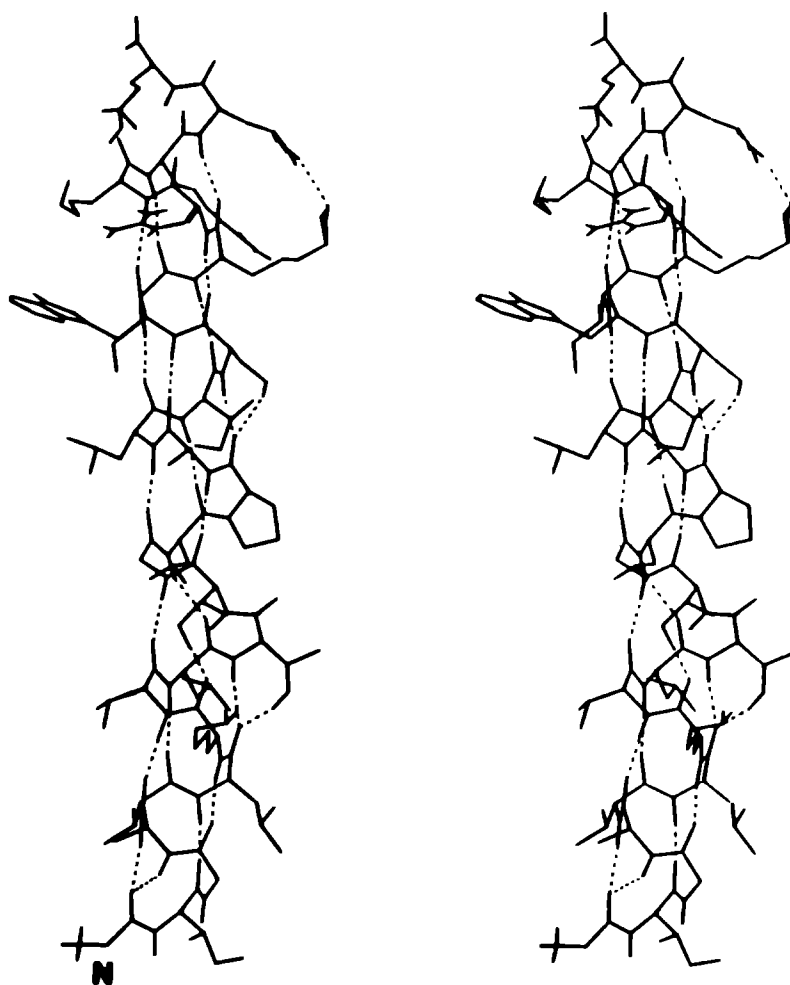


Fig. 5. Stereo view of the structure of monomeric melittin in methanol determined from ^1H -NMR and molecular dynamics simulations (From Ref 27, with permission.)

determined by CD for tetrameric melittin in solution (around 50–60% [17,18]) probably because of the very high ionic strength and peptide concentration used in the crystallizations [25]. A helical content approaching that of the crystalline peptide was determined by Raman spectroscopy for an aqueous solution of melittin at very high concentration (230 mg/ml) [30].

Melittin is monomeric and α -helical in methanol [27,28]. Helical structure is apparent from characteristic $^3J_{\text{NHCH}\alpha}$ coupling constants for amide protons, nuclear Overhauser enhancements (NOEs) that define distance constraints among backbone and side-chain protons and the pattern of slowly exchanging hydrogen-bonded backbone amide protons. The absence of NOEs characteristic of an α -helix through residues 10–12 and the large coupling constants for Thr-10 and Thr-11 amide protons support the interpretation of a relaxation of helical conformation around the Pro-14 residue. The Leu-13–Pro-14 peptide bond adopts the *trans* configuration. In the ^1H -NMR study [27], no inter-residue NOEs were obtained that would define the nature of the bend between the helical segments; molecular dy-

namics simulations of the structure encompassing the NOE-derived distance constraints indicate that in the monomeric state the bend in the helical structure may be considerably smaller than the value of 60° found in the crystal structure. A value of around 20° was obtained by averaging structures obtained in the final 30 ps of a molecular dynamics calculation. The averaged structure is shown in Fig. 5 [27]. While this structure does not define a unique solution to the NMR data, it indicates the proline residue need not induce a large bend in the melittin structure and this should be considered in models of the membrane bound conformation in the absence of direct determination of the perturbation induced by the proline.

Direct information on intramolecular hydrogen-bonding and conformational dynamics involving hydrogen-bond-breaking backbone fluctuations has been obtained from a complementary study of amide exchange from the melittin monomer in methanol [28] and this supports similar conclusions about the structure of the peptide. Backbone amide hydrogens in the N-terminal sequence (amides of residues 5–11) and in the C-termi-

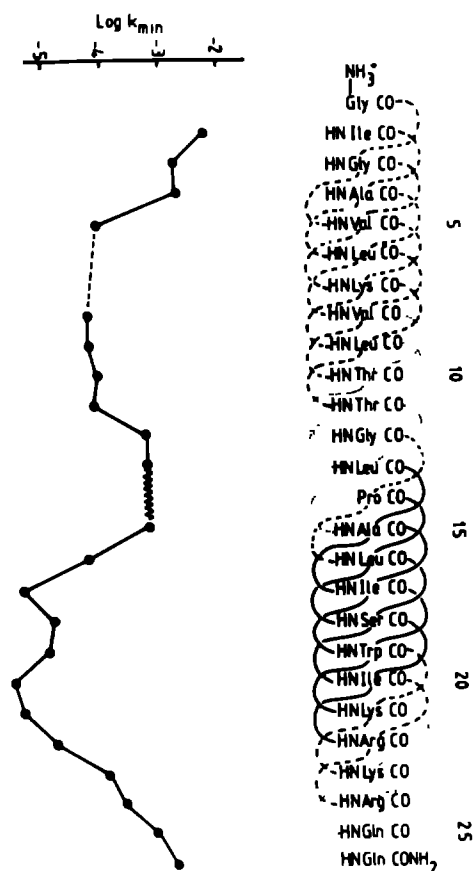


Fig. 6 Variation in the stability of backbone amides of monomeric melittin in methanol to exchange with solvent deuterons, demonstrating rapid exchange in a central turn of helix around the Pro-14 residue [Large negative log $k_{\min}$ values indicate stable hydrogen bonds] The relative stability of helical hydrogen bonds is illustrated schematically above (From Ref. 28, with permission)

nal sequence (amides of residues 16–23) are stable to exchange with solvent, indicating protection by hydrogen bonding in a stable helical structure (Fig. 6). The amides in a central turn of helix (residues 12–15) are weakly protected from exchange indicating that the N- and C-terminal helical segments are connected by a flexible turn of helix with unstable helical hydrogen bonds. The progressive decrease in amide stability towards the C-terminus indicates a loosening of the helix structure in the last few residues.

The ^1H -NMR structure of melittin bound to perdeuterated phosphatidylcholine micelles [29,31,32] shares most of the features of the NMR structure in methanol. Micelle-bound melittin is α -helical with a loss of NOE connectivity in the central region, indicating the relaxation of helical structure around residues 11–12 [29]. Like the structure in methanol, the angle of the bend between the N- and C-terminal helices was not uniquely defined from the NMR distance constraints. Amides on the hydrophobic side of the amphipathic helix show the largest chemical-shift changes compared with the amides of melittin in methanol and the slowest amide exchange

rates, supporting a surface location for the peptide with the non-polar side of the helix facing the micelle interior, protecting the amides on this helix face from exchange with solvent.

Melittin provides an interesting example of a membrane binding polypeptide whose structure has been determined to high resolution in the crystalline state, in solution of low dielectric constant and in a detergent micelle. The similarities in the three structures lends confidence to the idea that the structures are good models for the membrane-bound conformation, particularly in view of the strong evidence for a helical structure of the membrane-bound peptide determined by CD spectroscopy [19,33] (subsection IV-A). The NMR and amide-exchange studies emphasize the effect of the central proline residue in enhancing flexibility at the centre of the helical molecule [34] which seems to act as a 'hinge' allowing the helix to bend to accommodate conformational constraints induced by intermolecular interactions. This flexibility seems to be important for some of the actions of melittin in membranes (subsection III-E). The relationship between the high resolution structures of melittin and the effects of the peptide in membranes are discussed in the following sections. The structure of membrane-bound melittin is discussed in subsection IV-A.

III. The actions of melittin on membranes

III-A A note about phospholipase A_2 contamination

Bee venom contains an endogenous phospholipase A_2 [35] and this presents the potential for artifacts in experiments with melittin involving phospholipid membranes unless the peptide is purified free of the enzyme. Although baseline resolution of melittin and phospholipase A_2 may be achieved in several chromatographic systems, the melittin frequently retains phospholipase activity, probably as a result of binding of the peptide to the enzyme [36]. Moreover, the phospholipase A_2 activity is not inhibited by low concentrations of EDTA (1 mM), indicating very tight complexing of Ca^{2+} with the enzyme [35,37,38]. Unlike other venom phospholipases, bee venom phospholipase A_2 is not readily inhibited by the histidine-modifying agent *p*-bromophenacylbromide (PBP) at pH 6, reaction with PBP is more effective at pH 8 [38]. A number of reports of melittin-induced raised thermal transition temperatures in melittin-DMPC complexes have been shown to result from contaminating phospholipase A_2 activity [37]. The raised transition temperatures are a property of the mixed system of melittin, DMPC and myristic acid and lysophosphatidylcholine produced by the action of phospholipase A_2 [38].

There are a number of methods reported for the purification of melittin free of endogenous phospho-

lipase A₂ activity [37,39–41]. Standard reverse-phase HPLC in acetonitrile gradients yields melittin preparations with very low phospholipase activity. Where practical, it is useful to include EDTA at concentrations of 5 mM in experiments with melittin employing phospholipids to ensure the suppression of residual phospholipase activity, and to assay the lipid for the formation of hydrolytic products following the experiment.

III-B Hemolytic activity

The characteristic effect of melittin on cell membranes is its hemolytic activity [1,42]. At concentrations of 1 $\mu\text{g/ml}$ ($3 \cdot 10^{-7}$ M) and higher melittin binds rapidly to erythrocytes and induces the release of hemoglobin into the extracellular medium. It has been shown that the binding of melittin to erythrocytes as a monomer is necessary for expression of hemolytic activity [43]. Phosphate suppresses hemolysis to an extent that correlates with its effect on inducing tetramerization of melittin; complete suppression of hemolysis occurs at 0.5 M phosphate. Linear aggregates of melittin (obtained by cross-linking with dimethylsuberimidate) retain lytic activity in high phosphate concentrations and this is consistent with the observation that cross-linking suppresses phosphate-induced self-association in solution [43]. Whether lysis requires reassociation of monomeric membrane-bound melittin remains a controversial question.

Because of the difficulties in determining direct structural properties of erythrocyte-bound melittin, interpretations about the molecular mechanism of lysis have come from kinetic studies [43–45] and from structure–activity studies with peptide analogues [44,46]. The relevant experiments and conclusions may be summarized as follows:

On addition of melittin to a stirred population of erythrocytes under conditions where melittin is expected to be monomeric, all the peptide binds within seconds (there is a lag of about 15 s in the onset of hemoglobin release [44]; Fig. 7). There are about $1.8 \cdot 10^7$ binding sites for melittin per erythrocyte [44,45], indicating that the primary site of interaction is the membrane lipid rather than specific 'receptors'. The dissociation constant of 10^{-7} M [44] to $3 \cdot 10^{-8}$ M [45], depending on the details of the binding conditions, is similar to the dissociation constant for binding of melittin to membrane lipids. Melittin-induced hemolysis follows reproducible biphasic kinetics (Fig. 7) with characteristic temperature dependencies [43,44]. A fast phase of hemoglobin release seems to be triggered by the binding of melittin to the membrane, and decays to a slow steady-state release of hemoglobin. At 4°C the fast phase dominates lysis, whereas at 37°C the fast phase rapidly decays to a slow phase that accounts for most of the release of hemoglobin over a period of 30 min [44].

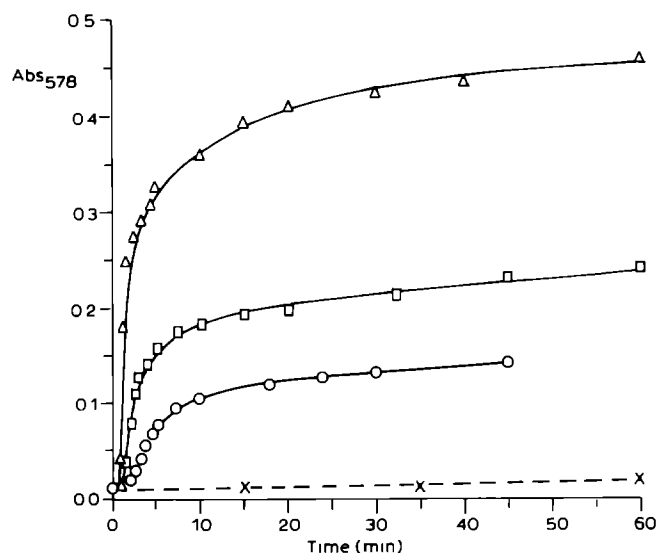
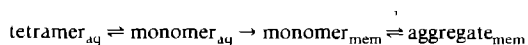


Fig. 7 Time-dependence of release of hemoglobin from washed human erythrocytes incubated at 4°C in 0.15 M NaCl, 20 mM Tris buffer (pH 7.6). Melittin concentrations are 0.3 μM ($\circ$), 0.6 μM ($\square$), and 1.2 μM ($\triangle$). The broken line is hemoglobin release from melittin-free erythrocytes.

The concentration dependence in the rates of the fast and slow kinetic phases have been interpreted in terms of the molecularity of the melittin species involved in each phase [44]. At 4°C in iso-osmotic sucrose (10 mM phosphate) the rate of the fast phase increases linearly with increasing peptide concentration while the rate of the slow phase increases as the square of the melittin concentration. The fast phase is interpreted as resulting from the perturbation of membrane structure and organization due to the rapid accumulation of melittin in the outer leaflet of the bilayer membrane and its decay into a slow phase a result of the reorganization of peptide and membrane lipid to recover favourable packing geometry. DeGrado et al. [44] propose that the internalization of a melittin dimer underlies the second, slow phase of hemoglobin release because of the second-order dependence of the rate on peptide concentration under the conditions of their experiment. Care must be taken in interpreting molecularity of kinetic events in terms of a single molecular mechanism for melittin-induced hemolysis because of the variation in concentration dependencies of the fast and slow kinetic phases obtainable under different experimental conditions. Thus, in iso-osmotic glucose at 22°C the rate of the initial, the fast phase varies as the fourth power of the melittin concentration [44], and in isotonic saline at 4°C the fast phase varies as the square of the peptide concentration with a slow phase almost independent of melittin concentration (unpublished results), whereas at 37°C the initial rate varies as the square of the peptide concentration and the slow rate varies linearly with melittin concentration (unpublished results). These variations are due at least in part to the rather complex

association behaviour of melittin in solution (and possibly in the membrane, subsection IV-C) and the multi-event processes involved in hemolysis incorporating



The hemolytic assays are relatively easy to perform [43] and careful systematic studies may yield a more consistent picture of the molecularity of the rate processes involved

Tosteson et al. [45] have concluded that melittin-induced hemolysis occurs by a colloid osmotic mechanism, the release of hemoglobin being secondary to the formation of ion-permeable (hemoglobin-impermeable) lesions or 'pores'. Evidence for such a mechanism includes (i) the observation that the permeability for Na^+ and K^+ of melittin-treated, unlysed cells increases 200–2000-fold over the first minute, before the bulk of hemoglobin release has occurred; (ii) that the cells viewed under the microscope swell before appreciable hemoglobin release occurs when treated with lytic concentrations of melittin (in contradiction to observations of DeGrado et al. [44]), and (iii) that replacing salts in the extracellular medium with high molecular mass osmolytes (raffinose, molecular mass 600 kDa; poly(ethylene glycol), 1000 kDa; or dextran, 10 kDa) inhibits lysis. From these experiments the size of the melittin-induced 'pore' was established to be smaller than the hydrodynamic radius of raffinose. This evidence for an osmotic mechanism for lysis is strong but leaves unanswered the molecular nature of the primary melittin-induced lesion

III-C Voltage-gated channel formation

The ability of melittin to induce enhanced ion permeability in planar lipid membranes under the influence of a trans-negative membrane potential provides a plausible mechanism for hemolysis by the colloid osmotic mechanism indicated by the results of Tosteson et al [45]. In black lipid membranes, melittin induces conductance changes in response to an applied potential when the potential is negative on the opposite side of the membrane to which the melittin is added [47–49]. No conductance is observed in the absence of an applied potential at sub-lytic melittin concentrations. The voltage-dependent increase in conductance is consistent with the formation of channels allowing ion migration, in response to a voltage-dependent reorientation of melittin in the membrane. Measurements on the accessibility of the melittin tryptophan residue (Trp-19) to acrylamide (a fluorescence quencher) and to proteolytic enzymes located on either side of the membrane show that the peptide can change its membrane location under the influence of the membrane potential and that

parts of the peptide become accessible to proteolysis on the trans-side of the membrane when a trans-negative potential is applied [48]

The 'pores' induced by melittin exhibit discrete levels of conductance [49] (suggestive of defined molecular structure), indicating that enhanced voltage-dependent conductance occurs by formation of discrete channels rather than through a voltage dependent change in the disposition of membrane-bound melittin resulting in a generalized effect on lipid organization. The voltage-gated 'pore' exhibits selectivity for anions over cations, probably as a result of electrostatic attraction of anions to the C-terminal cationic segment of melittin [47]. Amino-acetylated melittin also induces voltage-dependent conductance, indicating that the putative passage of the N-terminal helical section across the membrane under the influence of a trans-negative potential is not driven by interaction of the N-terminal, or Lys-7 amino groups with the membrane potential [49]. This conclusion has been reinforced by the observation that synthetic melittins having a blocked N-terminal amino group or a Lys-7 $\rightarrow$ Asn-7 substitution show voltage-dependent ion permeability [50]. As in the case of voltage-dependent conductance in alamethicin-containing black lipid membranes, the effective partial positive charge at the N-terminal end of the α -helix may act as the 'gating' charge in inducing transbilayer orientations [51]. A picture of the voltage-dependent channel forming properties of melittin is apparently completed by the report that the magnitude of the melittin-induced conductance changes in asolectin bilayers under the influence of a constant voltage increases with the fourth power of the melittin concentration, indicating that a tetrameric association of melittin monomers is required for channel formation [47]. However, the observations that melittin exhibits multiple conductance levels in POPC [49] and asolectin [52] bilayers, indicating heterogeneity in the 'pore' structure, and that structurally modified analogues show different concentration dependencies in their ion permeability [50], indicate that strictly tetrameric models for the melittin voltage-dependent 'pore' are probably unjustified. As with alamethicin [51] the size of the molecular aggregate that constitutes the voltage-dependent channel may vary depending on the lipid composition of the bilayer and changes in the structural properties of the peptide.

III-D Melittin-induced bilayer micellization and fusion

The permeabilization of membranes by melittin indicates an alteration in the organizational properties of membrane lipids and at high concentrations melittin is known to cause the breakdown of membranes into micelles in the manner of detergent solubilization [1]. Recent studies have demonstrated rather selective micellization of saturated phosphatidylcholine mem-

branes by melittin with, at moderate concentrations (3–6%, peptide/lipid; mol/mol), an interesting dependence on the lipid phase transition temperature [53–56,38]. While the effective concentrations of melittin, and the specificity for saturated phosphatidylcholines [57,58] make the relationship of the phenomenon to hemolysis uncertain (subsection III-E), it is instructive about the nature of interaction of melittin with membranes. The observations may be summarized as follows

At temperatures above T_m , the liquid crystalline to gel phase transition temperature of the lipid, bilayers composed of saturated phosphatidylcholines are stable as extended liposomes at melittin concentrations of up to 5 mol% (melittin to lipid). As the temperature is lowered into the gel phase the liposomes fragment into small particles (micelles). On raising the temperature back above T_m , extended bilayers re-form. This phase-dependent fragmentation is readily observable by spectroscopic methods such as phosphorus (^{31}P) [54] or deuterium (^2H) NMR [53,54,38] where the transition from bilayers to micelles is observed in a non-perturbing manner as a collapse of characteristic bilayer powder spectra into narrow lines characteristic of small particles like micelles (Fig 8). Gel-phase bilayer fragmentation is also observable by the collapse of the ^2H -NMR powder pattern arising from membrane-bound deuterated melittin on lowering the temperature below T_m (Fig. 9). Deuterium T_1 relaxation studies indicate that the peptide remains bound to the lipid micelles in the gel phase (Lemmon, M.A and Dempsey, C E., unpublished results). Freeze-fracture electron micrographs indicate that the micellar particles are discs of gel phase lipids organized in a bilayer with the melittin molecules

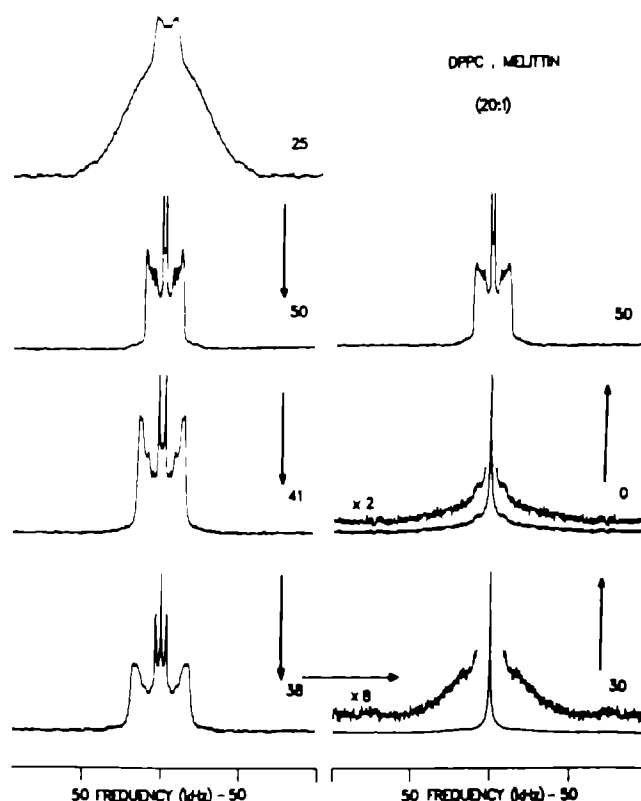


Fig 8 ^2H -NMR spectra of acyl-chain-deuterated DPPC bilayers containing melittin at 5 mol%. Deuterated lipids in extended bilayers exhibit broad deuterium NMR powder patterns. On lowering the temperature below the liquid-crystalline to gel phase transition temperature (41°C for DPPC), the powder patterns collapse into narrow lines characteristic of small particles undergoing rapid isotropic reorientations which averages orientation-dependent contributions to the NMR spectrum. Before warming melittin-containing samples above T_m , no collapse of the powder pattern is observed (top spectrum). The extended bilayers reform on rewarming above T_m (From Ref 55, with permission)

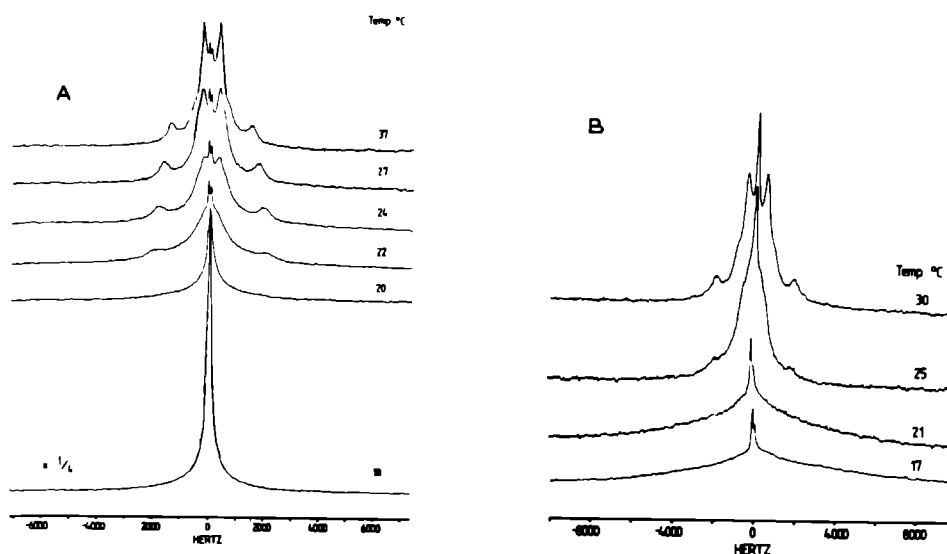


Fig 9 Temperature dependence of ^4H -NMR spectra of *N*-deuteromethyl melittin bound to DMPC bilayers at melittin/lipid ratios of 1:25 (A) and 1:100 (B). In (A) the NMR signal from the deuterated peptide collapses to a broad single line when the temperature is lowered below the lipid phase transition temperature (20°C for DMPC containing 4 mol% melittin). In (B) the spectrum of the bilayer-bound peptide becomes broadened beyond detection as the peptide becomes relatively immobilized in the gel phase bilayer (From Ref 56, with permission)

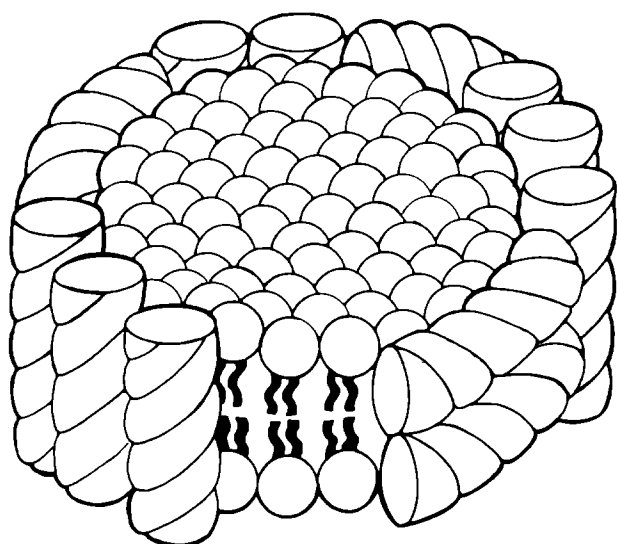


Fig 10 Schematic illustration of the structure of disc micelles formed by interaction of melittin (cylinders) with saturated phosphatidylcholine bilayers after cooling into the gel phase. The orientation of the melittin helix is uncertain. Modified from Refs 53 and 64

probably lining the disc edges protecting the acyl chains from interaction with aqueous solution [54] (Fig. 10). These structures are reminiscent of similar 'bicycle tyre' structures observed by electron microscopy with other amphipathic proteins or peptides, including the apolipoproteins [59], synthetic apolipoprotein analogues [60], glucagon [61] and detergent such as lysophosphatidylcholine [62]. Curious features shared both by melittin and lysophosphatidylcholine are the dependence of bilayer morphology on the lipid phase transition temperature and its reversibility as the gel phase micellar discs re-fuse to form extended bilayers when the temperature is taken back above the lipid phase transition temperature [38]. The observation that two widely disparate structure induce similar effects in DMPC bilayers indicates that the structural requirements for phase-transition-dependent reversible disc micellization in saturated phosphatidylcholine bilayers are non-specific. It has been argued [38] that the bilayer micellization results from the volume expansion of the head group region of the bilayer when these molecules partition into the membrane (in the case of melittin with the axis of the helix lying parallel to the membrane surface [26]). This volume expansion of the bilayer head group region can be accommodated in fluid phase lipid by an increase in disorder of the acyl chains which allows the bilayer interior to expand laterally in response to an increase in the head group volume (this acyl chain disorder has been observed in melittin-DPPC complexes in the fluid phase by ^2H -NMR [55]), and is expected to be associated with a compression of the bilayer combined with lateral expansion (as is observed in bilayers containing lysophosphatidylcholine [63]). Surface volume expansion cannot be accommodated in gel phase lipid and the

maintenance of strong cohesive forces between gel phase lipid acyl chains requires the redistribution of peptide (or lysoPC) and lipid in a manner (as yet undetermined) that results in disc micelle formation. There is some evidence from ^2H -HMR experiments that melittin moves deeper into the membranes as the bilayers enter the gel phase [55], as is ultimately required for disc micelle formation where the amphipathic helix must 'solvate' the lipid acyl chains.

The reverse process, the refusion of discs to give large membrane structures as the temperature is warmed above the lipid phase transition temperature is modeled schematically in Fig 11. In this model the increase in fluidity of gel phase lipid in the disc micelles as the temperature approaches T_m , results in a lateral expansion of the membrane associated with chain 'melting', and the exposure of acyl chains at a fixed melittin concentration. This effect, together with a tendency for melittin to adopt a bilayer surface location in fluid phase PC, will be a strong driving force for the lateral fusion of discs to yield large membrane sheets that may eventually fold into liposomes. Such a mechanism has been postulated to explain the ability of melittin and

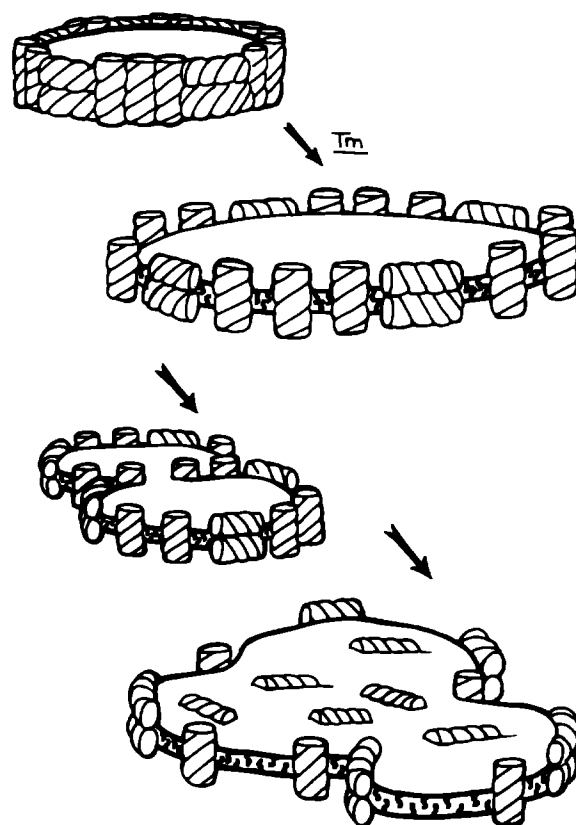


Fig 11 Highly schematic model for the re-fusion of melittin/PC disc micelles on warming above the lipid phase transition temperature. The vertical compression and lateral expansion of discs expected on warming the lipid into the fluid phase, together with a preference for a bilayer surface location for melittin in the fluid phase, promotes the edge to edge fusion of discs.

TABLE II

Effects of melittin and some related peptides on membranes

Peptide	Hemolytic (ED ₅₀ , μ M)	Voltage-gated ion conductance	Disc micelle formation	Refs.
Melittin	0.27 10	yes	yes (phase transition dependent)	see text
Peptide I ^a	0.5 $\times$ mol ^b	n.d. ^d	n.d.	[44]
[Ala-14]Melittin	0.5 $\times$ mel ^b	unstable channels	yes (rate of micellization = 0.01 $\times$ melittin)	[70]
δ -Hemolysin	28–100 ^c	yes	no (bilayer micellization at very high concentration)	[71,75]
Alamethucin	145	yes	n.d.	[49,51,74]
Apolipoproteins and analogues	no (lyse vesicles)	n.d.	yes (independent of phase transition)	[91]

^a The amino acid sequence of peptide I is: L-L-Q-S-L-L-S-L-L-Q-S-L-L-S-L-L-L-Q-W-L-K-R-K-R-Q-NH₂^b The hemolytic activity is twice that of melittin^c The hemolytic activity varies with the erythrocyte species^d n.d., not determined.

lysophosphatidylcholine to induce magnetic orientation of the membranes obtained upon refusion of micellar discs [38]. The resistance of saturated phosphatidylcholine bilayers to melittin-induced gel phase micellization increases with increasing acyl chain length supporting the idea that bilayer disruption is maximized when cohesion between lipid acyl chains is minimized in gel phase membranes [39]. Similarly, the stability of gel phase micellar discs is inversely related to the acyl chain length, with long-chain phosphatidylcholine-melittin micelles reforming bilayers sheets or large vesicles over extended periods by fusion without warming above the phase transition temperature [39,58].

Apolipoproteins and their synthetic analogues induce disc micelle formation in DMPC bilayers but these micellar discs form without cooling into the gel phase, are more stable than melittin-PC disc complexes and do not re-fuse on warming into the fluid phase. The apolipoproteins seem to interact more strongly with the exposed lipid surface and prevent a proportion of lipid (presumably the lipid at the disc edges in contact with the protein) from participating in the main gel to liquid crystalline phase transition with the interior lipid [60]. There is also some evidence that the apolipoproteins are able to accommodate size variation in the disc micelles (that may occur on chain melting above T_m) due to structural flexibility within the protein [64]. The overall charge compensation in apolipoproteins and their analogues (Fig. 1) may contribute to enhanced interaction of these proteins with lipids in a disc micelle. However, δ -hemolysin, which has a similar amphipathic structure to melittin and a low net charge like the apolipoproteins

(Fig. 1), does not induce the formation of disc micelles in saturated phosphatidylcholine membranes [58]. The structural requirements for amphiphile-induced disc micelle formation remain to be defined.

In common with a number of basic amphipathic peptides [65], melittin exhibits fusogenic activity. At concentrations of around 0.5 mol% melittin fuses a variety of phosphatidylcholine membranes when the temperature is cycled through the lipid phase transition temperature [66]. In negatively charged lipids, fusion occurs under isothermal conditions [67]. The mechanism of melittin-induced fusion of vesicles is probably not the same as for the fusion of bilayer disc micelles as depicted in Fig. 10, but may require a local disruption of bilayer structure and the apposition of vesicular membranes [66–68]. The kinetics of fusion of DPPC vesicles depends on the association state of melittin in solution [92,93].

III-E Relationship between the membrane actions of melittin

There is considerable interest in the mechanism of melittin-induced hemolysis and the evidence and interpretations fall into three main categories. Hemolysis results from the perturbation of bilayer lipid organization due to the presence of the amphipathic helix in the head group region of the bilayer [16,26,44], or due to the formation of ion-permeable channels in the manner of the voltage-gated 'pore' [45], or as a result of the ability of the peptide to micellize discs of membranes

leaving holes through which the cell contents may diffuse [53–55]. Each of these mechanisms is consistent with an osmotic lysis mechanism [45] as long as the initial perturbation results in enhanced permeability to ions (and water) before release of intracellular hemoglobin. While there is little direct evidence on the molecular mechanism of hemolysis, structure-activity studies with synthetic analogues shed some light on the relationships of the three main membrane activities of the peptide. It was shown early on using chemically modified or synthetic analogues of melittin that the hemolytic activity of melittin is not directly related to its surface activity [46,1]. Studies with a synthetic peptide analogue, peptide I (Table II) containing minimal sequence homology to melittin but retaining the overall amphipathic character of the first 20 residues [69] showed that neither a defined sequence nor the presence of a proline in the center of the helix are required for hemolytic activity [44]. The lack of importance for hemolytic activity of a helix bend has been confirmed using a synthetic analogue ([Ala-14]melittin) containing a Pro → Ala substitution at residue 14. NMR and amide exchange studies indicate that this peptide forms a very stable helix in methanol without the flexibility in the centre of the helix observed in native melittin (subsection II-B). Like the synthetic peptide I, [Ala-14]melittin is about twice as effective as melittin as a hemolytic agent, showing the same biphasic release of hemoglobin as melittin and peptide I. [Ala-14]melittin has very poor voltage-gated channel activity in planar bilayers and voltage-dependent ion conductance is supported only at very high ionic strengths (Dempsey, C.E., Bazzo, R., Boheim, G. and Campbell, I.D. (1989) unpublished data). The enhanced hemolytic activity of straight helical analogues of melittin having low structural flexibility is consistent with models for melittin-induced bilayer perturbation in which the location of the peptide in the head group region of the bilayer causes volume expansion near the membrane surface [26,44]. The low channel forming activity of [Ala-14]melittin indicates that the flexibility at the centre of the melittin helix is important for the stability of voltage-gated channels, possibly allowing the highly charged C-terminal sequence to bend away from the pore axis, minimizing channel-destabilizing charge repulsion. δ -Hemolysin, which lacks a central proline residue, nevertheless forms stable voltage-gated channels with characteristics similar to those of melittin [71]. The enhanced stability of δ -hemolysin channels compared with those formed by [Ala-14]melittin may be due to the lower net positive charge in the former peptide (Table I, Fig. 1). The dissociation of the hemolytic activity (enhanced) and voltage gated channel activity (suppressed) in the [Ala-14] analogue of melittin supports the idea that melittin-induced hemolysis does not result directly from 'pores' formed under the influence of the membrane potential.

^2H -NMR studies [57] indicate that there is no specific association of melittin with negatively charged lipids in reconstituted membranes that might minimize charge repulsion and stabilize 'pores', although it remains possible that some other form of pore stabilization might exist in erythrocyte membranes. Despite the evidence that hemolysis does not require voltage-dependent 'pore' formation, it has been shown that the hemolytic activity of melittin increases with an increase in the transmembrane potential of the erythrocyte [72].

There is so far little evidence to support the idea that hemolysis results from disc micellization of erythrocyte lipids. ^{31}P -NMR studies of erythrocytes containing melittin show no evidence of micellization below melittin/lipid ratios of 1 : 20, indicating that if some micellization occurs it is below levels detectable by ^{31}P -NMR [58]. While it is likely that hemolysis could result from the formation of only one or a few of these micelles involving only a small amount of the erythrocyte membrane lipids, these micelles have yet to be found. The incorporation of negatively charged lipids (DMPS) in DMPC bilayers greatly stabilizes the membranes to melittin-induced micellization [57], indicating that more complex lipid mixtures are more resistant to disc micellization. [Ala-14]Melittin causes disc micellization of DMPC bilayers at a rate 100-fold slower than melittin despite its enhanced hemolytic activity, further indicating a structural dissociation of the two activities.

Although each of the hemolytic peptides melittin, alamethicin [74] and δ -hemolysin [75] form voltage-gated channels in planar bilayers, there is no direct evidence that voltage-gated channel formation underlies the hemolytic activity of melittin. The results obtained with synthetic analogues support the conclusion that there is no necessary relationship between the various effect of melittin on membranes. At low concentrations, melittin forms voltage-dependent channels probably consisting of cylindrical aggregates of melittin helical monomers that do not greatly perturb the overall structural integrity of the lipid bilayer. At higher concentrations the peptide induces hemolysis by an osmotic lysis mechanism that need not require either discrete channel formation or micellization of regions of the membrane as bilayer discs. Bilayer disruption at high melittin concentrations occurs in a manner dependent on the lipid composition of the membrane, resulting in phase-transition-dependent micellization of phosphatidylcholine membranes, and other morphologies in different lipids (subsection IV-D). Whether some of the effects on bilayer organization in model membranes discussed in the following sections (or, indeed, the observation of melittin binding to integral membrane proteins [76]) are directly relevant to the structural effects on membranes occurring in the hemolytic process probably requires direct structural analysis of erythrocyte membranes containing melittin.

IV. Physical studies of the interaction of melittin with model membranes

A detailed understanding of the molecular mechanisms underlying the actions of melittin in membranes requires determination of the conformation of melittin in membranes, its location and orientation within the bilayer and its self-association properties. Because of the difficulties of studying these properties of melittin in erythrocyte membranes or in planar bilayers under the influence of a membrane potential, the large majority of physical studies of melittin and its interactions with lipids in membranes have been made using model membranes. Considering that the molecular mechanisms for the different activities of melittin may differ (subsection III-E) and that the peptide may adopt different orientations, self-association states and locations within the bilayer under different conditions (see following sections), the assignment of molecular mechanisms by extrapolation from model systems must be made with caution. It is clearly desirable to perform biophysical studies on membrane systems closely related to the activity of interest. These considerations indicate that the mechanisms of melittin-induced micellization of saturated phosphatidylcholine membranes is most amenable to physical study, and that the molecular mechanism underlying voltage-gated channel activity is least accessible to direct study requiring measurements in the presence of a transmembrane potential.

IV-A. The conformation of melittin in membranes

All of the studies of the structure of melittin in membranes support the conclusion that the peptide is helical. The ^1H -NMR studies of the peptide bound to dodecylphosphocholine micelles [29,32,33] confirms previous evidence [16,17] that melittin forms a helix on interaction with a membrane-like interface (subsection II-B). CD and Raman spectroscopic studies have shown that melittin adopts a helical conformation in egg PC [16,19], DMPC [77] and DTPC membranes [30,33]. The main uncertainty concerns the extent of the helix at the C-terminus. CD and Raman experiments indicate that the helix contains about 20 amino acids in DTPC membranes [30,33], suggesting that the C-terminal tetrapeptide sequence may adopt a non-helical conformation. Although the side-chains of Lys-23 and Arg-24 fall on the non-polar face of the amphipathic helix when the helix is extended through these residues, the long side-chains of these residues may allow the charged groups to fold back into the polar regions of the membrane as depicted in the model of a surface lying helix (Fig. 12; [26]). There is no direct information on the angle between the helical segments of membrane-bound melittin

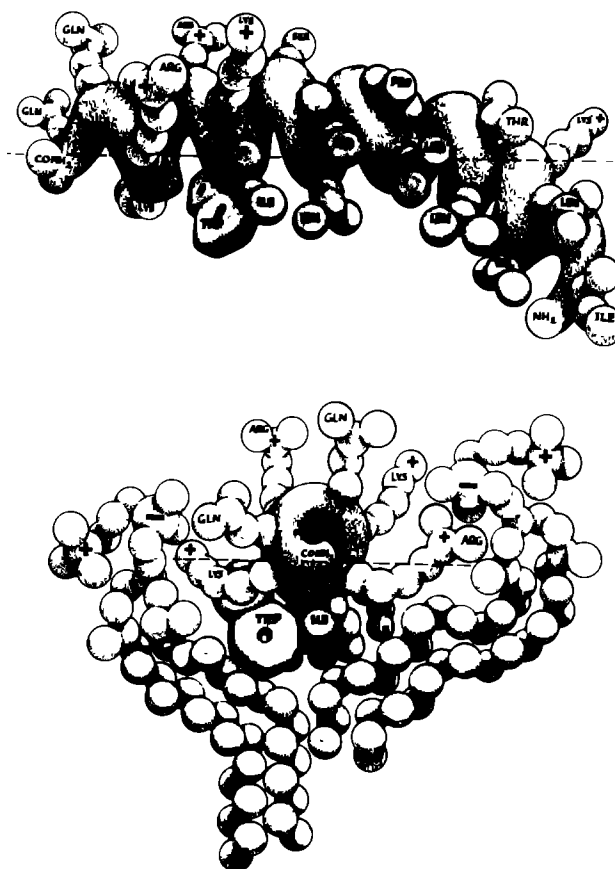


Fig. 12 A model for the interaction of melittin with phosphatidylcholine bilayers. The peptide is given the structure from the crystal tetramer and lies embedded in the head group region of the bilayer with the non-polar face of the helix towards the bilayer interior (From Ref. 26, with permission)

IV-B. The orientation of melittin in membranes

Under the influence of a transmembrane potential melittin becomes accessible to proteolytic enzymes on the trans side of the membrane, whereas no proteinase susceptibility is found in the absence of a membrane potential [48] (subsection III-B). This result is compatible with a voltage-dependent change in the orientation of the melittin helix from a bilayer surface orientation to a transbilayer one but is also consistent with the potential-catalysed movement of the whole peptide across the membrane. All other studies of the orientation of the melittin helix have been done in model systems in the absence of a transbilayer potential. Direct determination of helix orientation based on linear dichroism of the peptide amide vibrational bands (infrared spectroscopy), or from orientational order parameters measured by circular dichroism, have been made using oriented bilayers. In gel phase ditetradecylphosphatidylcholine (DTPC) or DMPC at low hydration levels (6% w/w corresponding to 2–3 water molecules per lipid) the α -helical segments of melittin are oriented roughly perpendicular to the plane of the mem-

brane [30]. This observation is compatible either with a transbilayer orientation or a model in which the peptide adopts a wedge conformation with a severe bend so that although the peptide is not in a transbilayer conformation the helical segments remain approximately parallel to the bilayer normal. There is no evidence that melittin can adopt such a conformation. Broadly similar conclusions were reached in a study of the orientation of the melittin helices in DOPC and DPPC using Attenuated Total Reflectance (ATR) IR spectroscopy [78]. In this technique the melittin/lipid mixture is deposited from organic solvent ($\text{CHCl}_3/\text{MeOH}$) onto a germanium crystal and orientational order and dichroism in the peptide IR absorption bands determined. The conclusion that the helix axis lies roughly perpendicular to the membrane plane in these studies may reflect the method of sample preparation, the fact that the lipid is in the gel phase and the extremely low water content of the oriented bilayers that probably limits the possibility for hydration of polar groups required for a surface orientation. A recent study [33] of the orientation of the melittin helix in oriented egg PC and DTPC bilayers at 97% relative humidity (30%, w/w, water/lipid) used the difference in the CD spectrum of the peptide in oriented membranes and unoriented vesicles to determine helix order parameters. Like the previous studies using oriented bilayers, the axis of the melittin helix was calculated to be oriented approximately perpendicular to the plane of the membrane. In contrast to these conclusions, neutron diffraction of oriented, fluid phase DOPC bilayers containing *N*-deuteromethylated melittin shows that the neutron scattering intensity (melittin-bound deuterons) are located at the bilayer surface (Bradshaw, J., Dempsey, C.E. and Watts, A., unpublished data). This indicates that under these conditions the deuterated peptide finds a location where all the amino groups are at the membrane surface.

A class of less direct studies have been carried out on amino-group modified melittins to probe their accessibility to the aqueous phase (and thus, by inference, the location of the helix); these studies have uniformly been interpreted in terms of a surface location for the melittin helix. Reductive methylation of melittin has been used to introduce ^{13}C - [22] or ^2H -labels [56] in *N*-dimethyl derivatives of melittin for NMR studies. *N*-Methylation of melittin by reductive methylation only slightly reduces the hemolytic activity of the peptide [56]. These studies were done with samples of *N*-methylated melittin in unoriented, fully solvated vesicles suspended in buffer, the ^{13}C -NMR studies using sonicated egg phosphatidylcholine vesicles and the ^2H -NMR studies using unsonicated multilamellar suspensions of DMPC. pH titrations show that the pK_a values of the dimethylamino groups of the membrane-bound peptides are similar to the pK_a values for the peptide in water, except that one lysine dimethylamino group has a pK_a

reduced by about 0.4 pH units [22] and the N-terminal deuteromethylamino group has a pK_a reduced by about 0.5–0.8 pH units consistent with a tendency for this group to move into the acyl group region of the bilayer (Lemmon, M.A. and Dempsey, C.E., unpublished data). From ^{13}C relaxation measurements and by determining the accessibilities of labeled amino groups to aqueous shift reagents, the lysine *N* $^\epsilon$ -dimethylamino groups were shown to be located at the membrane surface freely solvated in the aqueous environment [22]. The ^{13}C relaxation times, ^2H order parameters and measured pK_a values indicated that the dimethylamino groups of the peptide do not interact strongly with the lipid phosphates. No effect of the ^{13}C shift reagent was observed when added to the opposite side of the membrane to which the ^{13}C -labeled melittin was added, precluding a transbilayer conformation for the peptide in which the *N* $^\epsilon$ -dimethylamino group of Lys-7 is exposed on the trans side of the membrane [22]. Studies with nitroxide spin-labeled melittins, specifically labeled on single amino groups [79], lead to similar conclusions. The addition of membrane impermeant chromium oxalate to the aqueous solution above the membrane resulted in relaxation broadening of the electron spin resonance spectra from melittin spin labeled at each of the amino groups, indicating accessibility to the aqueous solution. The relative broadening was in the order $7 > 21 > \text{N}^\alpha > 23$ which parallels the relative exposure of the groups to the aqueous environment if the peptide adopts a helical conformation with the helix axis parallel to the membrane surface and the hydrophobic side of the helix facing the membrane interior [80] as in the model of Fig. 12 (See also Fig. 1A).

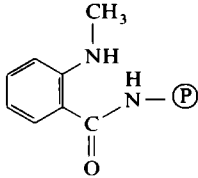
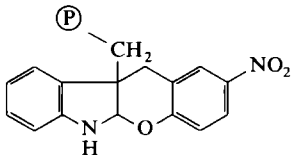
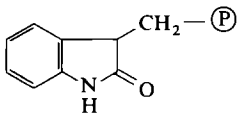
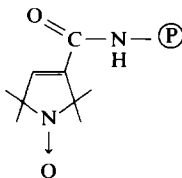
The variation in results obtained in different studies may result from true variation in the orientation of the melittin helix in membranes depending on the lipid composition of the bilayer and the lipid phase and hydration level. Results obtained from fluid phase bilayers at high levels of hydration are of greatest interest because the effects of melittin (other than gel-phase-induced micellization) occur in fully hydrated, fluid phase bilayers. Although most of the studies done under these conditions support a model such as that shown in Fig. 12 in which the axis of the helix lies parallel to the membrane surface, there may be circumstances where transbilayer, helical orientations are preferred.

IV-C The association state of melittin in membranes

The association state of melittin in membranes is an important issue regarding the voltage-gated channel forming properties of the peptide which presumably requires self-association of monomers to produce a 'pore'. It is not known whether 'pore'-forming melittin aggregates pre-exist in the membrane in the absence of a membrane potential, and several studies to determine

TABLE III

Summary of Studies on the Self-Association of Melittin in Model Membranes

Lipid (phase)	Melittin donor/acceptor	lipid/melittin ratio	association state (<i>n</i>)	Ref
DMPC (L_α)		> 50 1 (2 M NaCl)	unassociated	[81]
DMPC (L_α)		1000 1	associated (<i>n</i> = 4)	[30]
DMPC eggPC PG (all L_α)		3700 1 100 1	low I-unassociated high I-associated (0.5 M NaCl) (<i>n</i> = 3–10)	[82]
DOPC DOPC/DOPA (9:1) (all L_α)		200 1	unassociated	[79]

the association state of melittin in model membranes have produced contradictory results. The experiments employ melittins chemically modified to introduce spin-labels or fluorophores, and the measurement of spin-spin interaction or fluorescence energy transfer as a measure of probe proximity and thus peptide self-association. The studies are summarized in Table III. In DMPC bilayers above the phase transition temperature and at lipid/peptide ratios above 50:1, no energy transfer could be detected between melittin and melittin labeled with methyl anthraniloyl (Table III) at a single undetermined amino group [81]. Conversely, by measuring energy transfer from the melittin tryptophan to a modified melittin (Table III) in fluid phase DMPC bilayers (at lipid/melittin ratios of 1000:1), Vogel and Jähnig [30] observed significant transfer between melittins with native and modified tryptophan. The transfer data was best fitted to tetrameric aggregates, based on the assumption that the peptide exists in a bent helical conformation with the average helix axis aligned roughly along the bilayer normal. A related study in which the melittin tryptophan fluorescence acceptor was an *N*-bromosuccinimide-oxidised tryptophan derivative of melittin (Table III) indicated that the association state of melittin in fluid phase DMPC, PG or egg phosphatidylcholine was dependent on the ionic strength of the solution above the membrane [82]. In the absence of

salt the weak transfer could be accounted for by random collisions of melittin monomers. In solutions of 0.5 M NaCl self-association was apparent based on the observation of energy transfer between unlabeled and modified melittin. The association state could be fit to degrees of association varying between 3 and 10 monomers per aggregate. Apart from the observation that the membrane-bound melittin aggregates exhibit markedly different transfer efficiencies from the water-soluble tetramer, indicating a different spatial organization, no conclusive information on the molecular organization of the aggregates was obtained. Using spin-labeled amino derivatives of melittin, Altenbach and Hubbell [79] were unable to observe spin-spin interactions in membrane-bound spin-labeled peptide at lipid/protein ratios of about 200:1 in either DOPC or DOPC/DOPA (9:1) at low or high ionic strengths, indicating insignificant self-association in the membrane under these conditions.

The marked variation in these experimental results is most readily explained by differences in association properties of the different labeled melittins and by the different conditions of ionic strength and membrane compositions employed. Overall, the results indicate that in fluid phase membranes at low ionic strength melittin is unassociated at peptide:lipid molar ratios below about 1:100. The effect of increasing ionic

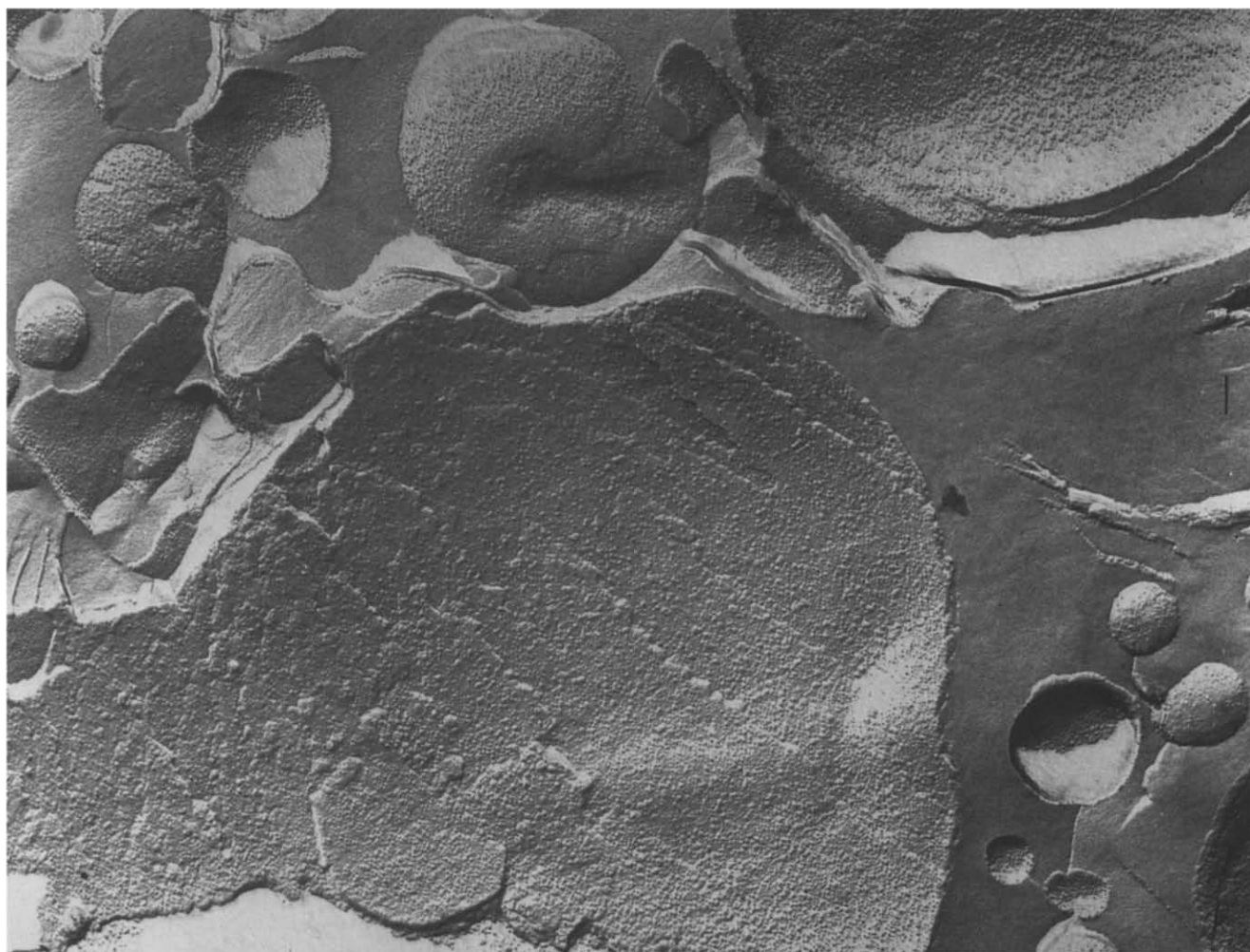


Fig. 13 Freeze fracture electron micrograph of DMPC bilayers containing 5 mol% melittin obtained after rapid cooling from a temperature (27°C) above the lipid phase transition temperature. The scale bar represents 100 nm. Shadowing was from right to left across the micrograph. (From Dr B. Sternberg, Friedrich-Schiller-Universitat, Jena, G D R.)

strength in increasing self-association (in Ref. 82, but not Ref. 81) is analogous to the salt-induced self-association of aqueous melittin and indicates that electrostatic repulsion between melittins promotes dispersion of the peptide in bilayers. It is interesting that high ionic strength [82], but not the presence of negatively charged lipids [79,82] promotes self-association, suggesting that melittin does not bind to the negatively charged head group of DOPA reducing melittin-melittin charge repulsion.

Electron micrographs of melittin-containing membranes obtained by freeze-fracture after rapid quenching from the fluid phase in egg PC, DPPC [53] or DMPC bilayers (Fig. 13) clearly show the presence of intramembranous particles that are not observed in melittin-free bilayers. The nature of these particles has not been uniquely defined but they seem likely to be melittin aggregates. The observation of these particles on the membrane fracture face (the plane of the bilayer centre) indicates that the melittin aggregates penetrate

at least half way into the bilayer. While there is evidence that melittin self-associates and penetrates more deeply into PC bilayers when the temperature is lowered into the gel phase ([55], subsection III-D), rapid freezing techniques are expected to preserve the association state of proteins in the fluid phase. Whether the intramembranous particles in melittin/PC freeze-fracture electron micrographs are melittin aggregates that either pre-exist in the fluid phase or form by rapid redistribution of the peptide during the freezing process remains to be determined.

IV-D Selective interactions of melittin with different lipids

Biological membranes are composed of mixtures of lipids and the possibility that melittin may interact selectively with different lipid types has been explored in several studies. The affinity of melittin for membranes composed of negatively charged lipids is about 100-fold greater than for zwitterionic lipids [83], indicat-

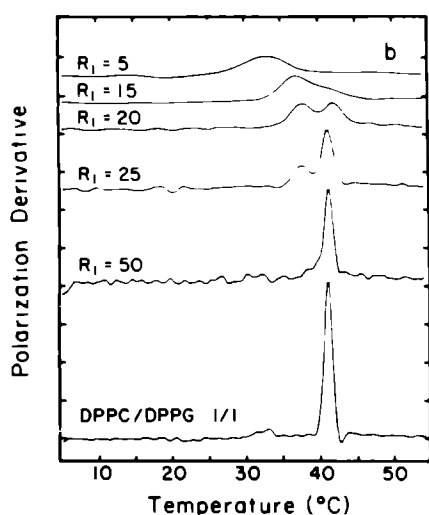


Fig. 14 Derivatives of the diphenylhexatriene fluorescence polarization as a function of temperature for mixed DPPC/DPPG bilayers containing increasing melittin concentrations (R_1 is the lipid/melittin mole ratio). Melittin-induced phase separation below T_m is characterized by the observation of two thermal transitions (From Ref 89, with permission)

ing the potential for a strong electrostatic component in the binding of melittin to membranes. The stoichiometry for interaction of melittin with negatively charged lipids is about 4–5 lipids per melittin for singly charged lipids (PS and PG; [57,84,85] and two lipids per melittin for the doubly anionic lipids phosphatidic acid and cardiolipin [86,87]. This is consistent with the finding that melittin has a net charge of +5–6 in membranes around neutral pH [22].

The affinity of melittin for negatively charged lipids has stimulated efforts to determine melittin-induced phase separation of negative lipids that might be important for the membrane activities of the peptide. Separation of melittin-enriched phases in single lipid bilayers composed of DPPG, DPPS or DPPA has been detected by DSC and fluorescence anisotropy when the bilayers are cooled into the gel phase [87,88]. Similar melittin-induced gel phase immiscibility has been detected in mixtures of DPPG and DSPC or DPPC with the formation of peptide-free domain enriched in PC on

cooling below the lipid phase transition temperature [89] (Fig. 14). Phase separation in fluid phase lipid under isothermal conditions is more difficult to detect and only one study has been reported to date. In DMPC/DMPS mixtures containing melittin at up to 6 mol% relative to total lipid, all the components were shown (using deuterium NMR) to be in rapid diffusional exchange with no formation of complexes of melittin with the anionic lipid having lifetimes longer than microseconds [57]. This result does not preclude the possibility of observing phase separation in fluid phase lipid mixtures of different compositions. The possibility of melittin-induced lipid phase separation in biological membranes has not yet been addressed experimentally.

The interaction of melittin with lipids having different head group structures has been shown to affect lipid morphologies in a manner that can be understood in terms of simple ideas about the geometry of packing of lipids and proteins [90]. Melittin perturbs phosphatidylcholine bilayers in a manner consistent with a wedge effect, inducing convex structures as the bilayer surface region are expanded due to the presence of melittin in the head group region (subsection III-C). In bilayers composed of negatively charged lipids, melittin induces the formation of inverted lipid systems (H_{II} , or hexagonal phase) which is proposed to arise partly from the deeper penetration of the melittin helix into the membrane surface [40,90]. The opposing effects of melittin on bilayer organization of phosphatidylcholine membranes and negatively charged lipids (Fig. 15) may account for the stabilization of DMPC bilayers to the micellizing effects of melittin when DMPS is added to the bilayers [57]. In addition to its bilayer disrupting effects, melittin can stabilize bilayer structure in fluid phase mixtures of DMPC, lysophosphatidylcholine and myristic acid that form micelles in the absence of melittin [38] and in phosphatidylethanolamine membranes under conditions where the PE normally adopts the hexagonal phase [83]. Some of the selective effects of melittin on the morphologies of different lipid types are summarized in reference [83].

V. Summary

The molecular mechanisms underlying the various effects of melittin on membranes have not been completely defined and much of the evidence described indicates that different molecular mechanisms may underlie different actions of the peptide. Ideas about the formation of transbilayer aggregates of melittin under the influence of a transbilayer potential, and for bilayer structural perturbation arising from the location of the peptide helix within the head group region of the membrane have been made based on the crystal structure of the peptide, the kinetics and concentration dependence

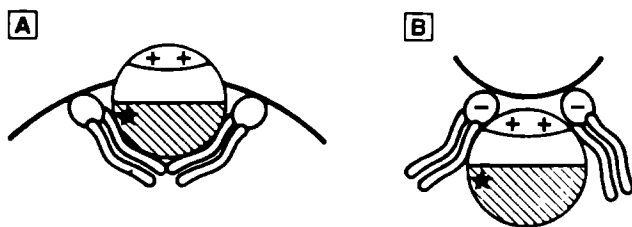


Fig. 15 Schematic illustration of the effect of melittin in inducing convex (A) or concave (B) structures in zwitterionic and negatively charged lipids, respectively. Deeper penetration of the melittin into negatively charged lipids was determined from the resistance of the tryptophan residue (starred) to aqueous fluorescence quenchers (From Ref 90, with permission)

of melittins membrane actions, together with simple ideas about the conformational properties of amphipathic helical peptides and their interactions with membranes. Physical studies of the interaction of melittin with model membranes have been useful in determining the potential of the peptide to adopt different locations, orientations and association states within membranes under different conditions, but the relationship of the results obtained to the actions of melittin in cell membranes or under the influence of a membrane potential are unclear. Experimental definition of the interaction of melittin with more complex membranes, including the erythrocyte membrane or in bilayers under the influence of a transmembrane potential, will require direct study in these membranes. Experiments employing labeled melittins for ESR, NMR or fluorescence experiments are promising both for their sensitivity (ESR and fluorescence) and the ability to focus on the peptide within the background of endogenous proteins within cell membranes. The study of melittin in model membranes has been useful for the development of methodology for determination of membrane protein structures. Despite the structural complexity of integral membrane proteins, it is interesting that in some respects their study be more straightforward, lacking as they do the elusive properties of melittin (and other structurally labile membrane peptides) which limit the possibility of defining their interaction with membranes in terms of a single conformation, location, orientation and association state within the membrane.

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References

- Habermann, E (1972) *Science* 177, 314–322
- Habermann, E (1980) in *Natural Toxins* (Eaker, D and Wadstrom, T. eds.), pp 173–181, Pergamon Press, New York
- Bernheimer, A.W. and Rudy, B (1986) *Biochim Biophys Acta* 864, 123–141
- Habermann, E. and Jentsch, J (1967) *Hoppe-Seyler's Z. Physiol Chem* 248, 37–50
- Podo, F., Strom, R., Crifo, C. and Zulauf, M (1982) *Int J Pept Protein Res* 19, 514–527
- Segrest, J.P., Jackson, R.L., Morrisett, J.D. and Gotto, A.M., Jr (1973) *FEBS Lett* 38, 247–253
- Kaiser, E.T. and Kezdy, F.Z. (1984) *Science* 223, 249–255
- Eisenberg, D (1984) *Annu Rev Biochem* 53, 595–623
- Gauldie, J., Hanson, J.M., Rumjanek, F.D., Shipolini, R.A. and Vernon, C.A. (1976) *Eur J Biochem* 61, 369–376
- Faucon, J.F., Dufourcq, J. and Lussan, C. (1979) *FEBS Lett* 102, 187–190
- Talbot, J.C., Dufourcq, J., De Bony, J., Faucon, J.-F. and Lussan, C. (1979) *FEBS Lett* 102, 191–193
- Lauterwein, J., Brown, L.R. and Wuthrich, K. (1980) *Biochim Biophys Acta* 622, 219–230
- Brown, L.R., Lauterwein, J. and Wuthrich, K. (1980) *Biochim Biophys Acta* 622, 231–244
- Quay, S.C. and Condie, C.C. (1983) *Biochemistry* 22, 695–700
- Schubert, D., Pappert, G. and Boss, K. (1985) *Biophys J* 48, 327–329
- Dawson, C.R., Drake, A.F., Helliwell, J. and Hider, R.C. (1978) *Biochim. Biophys. Acta* 510, 75–86
- Knoppel, E., Eisenberg, D. and Wickner, W. (1979) *Biochemistry* 28, 4177–4181
- Bello, J., Bello, H.R. and Granados, E. (1982) *Biochemistry* 21, 461–465
- Drake, A.F. and Hider, R.C. (1979) *Biochim Biophys Acta* 555, 371–373
- Lubke, K., Matthes, S. and Kloss, G. (1971) *Experientia* 27, 765–767
- Quay, S.C. and Tronson, L.P. (1983) *Biochemistry* 22, 700–707
- Stanislowski, R. and Ruterjans, H. (1987) *Eur Biophys J* 15, 1–12
- Brown, L.R. and Bradbury, J.H. (1975) *Eur J Biochem.* 54, 219–227
- Terwilliger, T.C. and Eisenberg, D. (1982) *J Biol Chem* 257, 6010–6015
- Terwilliger, T.C. and Eisenberg, D. (1982) *J Biol Chem* 257, 6016–6022
- Terwilliger, T.C., Weissman, L. and Eisenberg, D. (1982) *Biophys J* 37, 353–361
- Bazzo, R., Tappin, M.J., Pastore, A., Harvey, T.S., Carver, J.A. and Campbell, I.D. (1988) *Eur J Biochem* 173, 139–146
- Dempsey, C.E. (1988) *Biochemistry* 27, 6893–6901
- Inagaki, F., Shimada, I., Kawaguchi, K., Hirano, M., Terasawa, I., Ikura, T. and Go, N. (1989) *Biochemistry* 28, 5985–5991
- Vogel, H. and Jahnig, F. (1986) *Biophys J* 50, 573–582
- Brown, L.R. and Wuthrich, K. (1981) *Biochim. Biophys Acta* 647, 95–111
- Brown, L.R., Braun, W., Kumar, A. and Wuthrich, K. (1982) *Biophys J* 37, 319–328
- Vogel, H. (1987) *Biochemistry* 26, 4562–4572
- Pastore, A., Harvey, T.S., Dempsey, C.E. and Campbell, I.D. (1989) *Eur Biophys J* 16, 363–367
- Shipolini, R.A., Callewaert, G.L., Cottrell, R.C., Doonan, S., Vernon, C.A. and Banks, B.E.C. (1971) *Eur J Biochem* 20, 459–468
- Banks, B.E.C., Dempsey, C.E., Pearce, F.L., Vernon, C.A. and Wholley, T.E. (1981) *Analyt Biochem* 116, 48–52
- Dasseux, J.-L., Faucon, J.-F., Lafleur, M., Pezolet, M. and Dufourcq, J. (1984) *Biochim. Biophys Acta* 775, 37–50
- Dempsey, C.E. and Watts, A. (1987) *Biochemistry* 26, 5803–5811
- Lafleur, M., Dasseux, J.-L., Pigeon, M., Dufourcq, J. and Pezolet, M. (1987) *Biochemistry* 26, 1173–1179
- Batenburg, A.M., Hibbeln, J.C.L. and De Kruijff (1987) *Biochim Biophys Acta* 903, 155–165
- Wille, B. (1989) *Anal Biochem* 178, 118–120
- Sessa, G., Freer, J.H., Colacicco, G. and Weissmann, G. (1969) *J Biol Chem* 244, 3575–3582
- Hider, R.C., Khader, F. and Tatham, A.S. (1983) *Biochim Biophys Acta* 728, 206–214
- DeGrado, W.F., Musso, G.F., Lieber, M., Kaiser, E.T. and Kezdy, F.J. (1982) *Biophys. J* 37, 329–338
- Tosteson, M.T., Holmes, S.J., Razin, M. and Tosteson, D.C. (1985) *J Membr Biol* 87, 35–44

- 46 Schroder, E., Lubke, K., Lahmann, M. and Beetz, I. (1971) *Experientia* 27, 764–765.
- 47 Tosteson, M.T. and Tosteson, D.C. (1981) *Biophys. J.* 36, 109–116.
- 48 Kempf, C., Klausner, R.D., Weinstein, J.N., Van Renswoude, J., Pincus, M. and Blumenthal, R. (1982) *J. Biol. Chem.* 257, 2469–2476.
- 49 Hanke, W., Metfessel, C., Wilmsen, H.-U., Katz, E., Jung, J. and Boheim, G. (1983) *Biochim. Biophys. Acta* 727, 108–114.
- 50 Tosteson, M.T., Caporale, L. and Tosteson, D.C. (1988) *Biophys. J.* 53, 9a.
- 51 Hall, J.E., Vodyanoy, I., Balasubramanian, T.M. and Marshall, G.R. (1984) *Biophys. J.* 45, 233–247.
- 52 Tosteson, M.T., Levy, J.L., Caporale, L., Rosenblatt, M. and Tosteson, D.C. (1987) *Biochemistry* 26, 6627–6631.
- 53 Dufourcq, J., Faucon, J.-F., Fourche, G., Dasseux, J.-L., Le Maire, M. and Gulik-Krzywicki, T. (1986) *Biochim. Biophys. Acta* 859, 33–48.
- 54 Dufourcq, E.J., Faucon, J.-L., Fourche, G., Dufourcq, J., Gulik-Krzywicki, T. and LeMaure, M. (1986) *FEBS Lett.* 201, 205–209.
- 55 Dufourcq, E.J., Smith, I.C.P. and Dufourcq, J. (1986) *Biochemistry* 25, 6448–6455.
- 56 Dempsey, C.E., Cryer, G. and Watts, A. (1987) *FEBS Lett.* 218, 173–177.
- 57 Dempsey, C., Bitbol, M. and Watts, A. (1989) *Biochemistry* 28, 6590–6597.
- 58 Dufourcq, E.J., Bonmatin, J.-M. and Dufourcq, J. (1989) *Biochimie* 71, 117–123.
- 59 Forte, T., Gong, E. and Nichols, A.V. (1974) *Biochim. Biophys. Acta* 337, 169–183.
- 60 Anantharamaiah, G.M., Jones, J.L., Brouillette, C.G., Schmidt, C.F., Chung, B.H., Hughes, T.A., Bhowan, A.S. and Segrest, J.P. (1985) *J. Biol. Chem.* 260, 10248–10255.
- 61 Jones, A.J.S., Epand, R.M., Lin, K.F., Walton, D. and Vail, W.J. (1978) *Biochemistry* 17, 2301–2307.
- 62 Inoue, K., Suzuki, S. and Najima, S. (1977) *J. Biochem. (Tokyo)* 81, 1097–1106.
- 63 Mandersloot, J.G., Reman, F.C., Van Deenen, L.L.M. and De Gier, J. (1975) *Biochim. Biophys. Acta* 382, 22–26.
- 64 Brouillette, C.G., Jones, J.L., Ng, C.T., Kercret, H., Chung, B.H. and Segrest, J.P. (1984) *Biochemistry* 23, 359–367.
- 65 Svenaga, M., Lee, S., Park, N.G., Aoyagi, H., Kato, T., Umeda, A. and Amako, K. (1989) *Biochim. Biophys. Acta* 981, 143–150.
- 66 Morgan, C.G., Williamson, H., Fuller, S. and Hudson (1983) *Biochim. Biophys. Acta* 732, 668–674.
- 67 Eytan, G.D. and Almary, T. (1983) *FEBS Lett.* 156, 29–32.
- 68 Murata, M., Nagayama, K. and Ohnishi, S. (1987) *Biochemistry* 26, 4056–4062.
- 69 DeGrado, W.J., Kezdy, F.J. and Kaiser, E.T. (1981) *J. Am. Chem. Soc.* 103, 679–681.
- 70 Reference deleted.
- 71 Mellor, I.R., Thomas, D.H. and Sansom, M.S.P. (1988) *Biochim. Biophys. Acta* 280–294.
- 72 Clague, M.J. and Cherry, R.J. (1988) *Biochem. J.* (1988) 252, 791–794.
- 73 Reference deleted.
- 74 Irmscher, G. and Jung, G. (1977) *Eur. J. Biochem.* 80, 165–174.
- 75 Bhakoo, M., Birkbeck, T.H. and Freer, J.H. (1985) *Can. J. Biochem.* 63, 1–6.
- 76 Dufton, M.J., Hider, R.C. and Cherry, R.J. (1984) *Eur. Biophys. J.* 11, 17–24.
- 77 Vogel, H. (1981) *FEBS Lett.* 134, 37–42.
- 78 Brauner, J.W., Mendelsohn, R. and Prendergast, F.G. (1987) *Biochemistry* 26, 8151–8158.
- 79 Altenbach, C. and Hubbell, W.L. (1988) *Proteins* 3, 230–242.
- 80 Altenbach, C., Froncisz, W., Hyde, J.S. and Hubbell, W.L. (1989) *Biophys. J.* 56, 1183–1191.
- 81 Hermetter, A. and Lakowicz, J.R. (1986) *J. Biol. Chem.* 261, 8243–8248.
- 82 Talbot, J.C., Faucon, J.-F. and Dufourcq, J. (1987) *Eur. Biophys. J.* 15, 147–157.
- 83 Batenburg, A.M., Van Esch, J.H. and De Kruijff, B. (1988) *Biochemistry* 27, 2324–2331.
- 84 Batenburg, A.M., Hibbeln, J.C.L., Verkleij, A.J. and De Kruijff, B. (1987) *Biochim. Biophys. Acta* 903, 142–152.
- 85 Dufourcq, J. and Faucon, J.-F. (1977) *Biochim. Biophys. Acta* 467, 1–11.
- 86 Batenburg, A.M., Van Esch, J.H. and Verkleij, A.J. (1987) *FEBS Lett.* 223, 148–154.
- 87 Bernard, E., Faucon, J.-F. and Dufourcq, J. (1982) *Biochim. Biophys. Acta* 688, 152–162.
- 88 Faucon, J.-F., Bernard, E., Dufourcq, J., Pezolet, M. and Bougis, P. (1981) *Biochimie* 63, 857–861.
- 89 Lafleur, M., Faucon, J.-F., Dufourcq, J. and Pezolet, M. (1989) *Biochim. Biophys. Acta* 980, 85–92.
- 90 Batenburg, A.M. and De Kruijff, B. (1988) *Biosci. Rep.* 8, 299–307.
- 91 Kanellis, P., Romans, A.Y., Johnson, B.J., Kercret, H., Chiovetti Jr., R., Allen, T.M. and Segrest, J.P. (1980) *J. Biol. Chem.* 255, 11464–11472.
- 92 Bradick, T.D. and Georgiou, S. (1987) *Biochim. Biophys. Acta* 905, 494–498.
- 93 Bradick, T.D., Freire, E. and Georgiou, S. (1989) *Biochim. Biophys. Acta* 982, 94–102.