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THE INTERACTION OF BEE MELITTIN WITH LIPID BILAYER MEMBRANES

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Summary

The influence of melittin and the related 8-26 peptide on the stability and electrical properties of bilayer lipid membranes is reported. Melittin, unlike the 8-26 peptide, has a dramatic influence on lipid membranes, causing rupture at dilute concentrations. The circular dichroism of melittin demonstrated that under physiological conditions, in water, melittin is in extended conformation, which is enhanced in aqueous ethanol. However in 'membrane-like' conditions it is essentially α-helical. Secondary structure predictions were used to locate possible α-helical nucleation centres and a model of melittin was built according to these predictions. It is postulated that melittin causes a wedge effect in membranes.

Introduction

The principal peptides of bee venom are the melittins [1—3]. This group of strongly basic peptides comprises about 50% of the dried venom and possesses a strong haemolytic property [4]. Indeed, melittin appears to be able to lyse a variety of cell membranes, including those of leukocytes [5], lysosomes [6] and mitochondria [7], as well as artifical lipid membranes [8].

There are many toxic proteins and peptides that have been shown to cause lysis of membranes [9]. With some bacterial toxins [10] a complicated structural disruption, rather like that of complement haemolysis [11] is

† Most of the artificial membrane experiments were performed by C.R. Dawson, who was tragically killed on December 26, 1975. The death of this young man is a great loss to his scientific colleagues.

Abbreviation: LP_{II}, hydrophobic copolymer of laurylmethacrylate and vinylpyrrolidone. For structure see Ash, P.S., Bunce, A.S., Dawson, C.R. and Hider, R.C. (1978) Biochim. Biophys. Acta 510, 216—229.

thought to be involved, whereas others, like surfactin [12], are strongly surface active and are thought to possess a detergent-like activity [13]. This later mechanism has been suggested for the mode of action of melittin [8,14] which is also highly surface active and experiences strong interaction with phospholipids [14—16]. However, there is no simple relationship between surface activity and the lytic properties of melittin and its derivatives [17], some synthetic fragments of melittin retain more than 50% of the surface activity of the parent peptide, but possess very low haemolytic potency [18]. In particular, a melittin II peptide (7-27) retains most of the surface activity but possesses virtually no haemolytic activity. There are also differences in the kinetics and osmotic influences with melittin and detergent-induced haemolysis [19]. Furthermore, melittin-induced haemolysis results in relatively intact ghosts, whereas haemolysis induced by many detergents at high concentration does not yield recognizable ghosts [4].

Recently, Shipolini (personal communication) has isolated a minor fraction (0.01% of dry weight) of bee venom which corresponds to the 8-26 sequence of melittin (*Apis mellifera*) and, consequently, contains the highly basic C-terminus as well as the majority of the hydrophobic amino acids. It was anticipated that this peptide would behave like the synthetic melittin II fragment (7-27) [18] and, if so, would offer an opportunity to establish some of the features of the melittin molecule that are essential for its haemolytic properties.

Materials and Methods

Chromatographically pure egg yolk phosphatidylcholine was isolated by the method of Wren [20]. Cholesterol (British Drug Houses) was recrystallized from ethanol. Melittin and the 8-26 peptide, isolated from *Apis mellifera* were kindly supplied by Dr. R.A. Shipolini. Melittin was also purchased from Sigma Chemical Co. together with polylysine (M_r = 1—4000 and >70 000). The polymer LP_{II} was synthesized by the method of Ash, Bunce, Dawson and Hider [21].

The vertical bilayer lipid membrane system as described by Ash et al. [21] was utilized throughout this study, in order to determine capacitance and electrical resistance of phosphatidylcholine/cholesterol membranes. This membrane system is extremely susceptible to hydrostatic rupture and therefore the addition of fractions (1 μl) to the aqueous medium was standardized as indicated in Fig. 1. For melittin concentrations of 10^{-4} g · ml^{-1}, solid material was added to the bathing medium. Using these methods, control additions never caused membrane rupture. All the results were reproduced at least three times and wherever possible, the same fraction of phosphatidylcholine was used, thus ensuring consistency of phospholipid composition of bilayers for a given test. The Teflon sections of the apparatus were cleaned using chloroform in a soxhlet extractor, the perspex sections were cleaned in distilled water and air dried. All experiments were carried out at 37.0 ± 0.5°C in the presence of sodium chloride (150 mM) buffered to pH 7.4 by Tris (10 mM). With experiments involving the polymer LP_{II}, the following brush mixture was used: Phosphatidylcholine (5 mg)/cholesterol (2.5 mg)/LP_{II} (5 mg)/*n*-decane (200 μl).

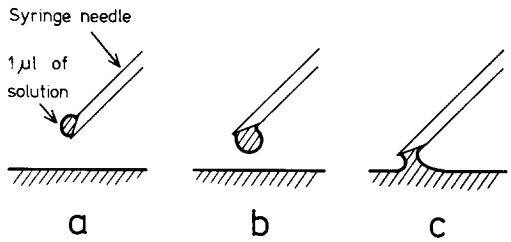

Fig. 1. Technique employed for the addition of small volumes of fluid to one side of a bilayer lipid membrane preparation. This method rarely caused hydrostatic rupture of membranes.

Circular dichroism measurements were performed using a Jouan Dichrographe CD 185 (Mark II) or a Jasco J40 CS. The results are expressed in terms of molar ellipticity based on an average monomer molecular weight of 110, the units are degree · cm^2 · mol^{-1}. Absorption spectra of the same solutions were recorded with a Cary 17 spectrophotometer.

Results

Bilayer lipid membranes. Not surprisingly, initial studies showed that melittin, at concentration of 10^{-5} g · ml^{-1} caused the rapid rupture of bilayer lipid membranes composed of phosphatidylcholine and cholesterol. A typical response is demonstrated in Fig. 2a, where melittin was added to the negative side of the membrane (—50 mV). After approximately 1 min the membrane

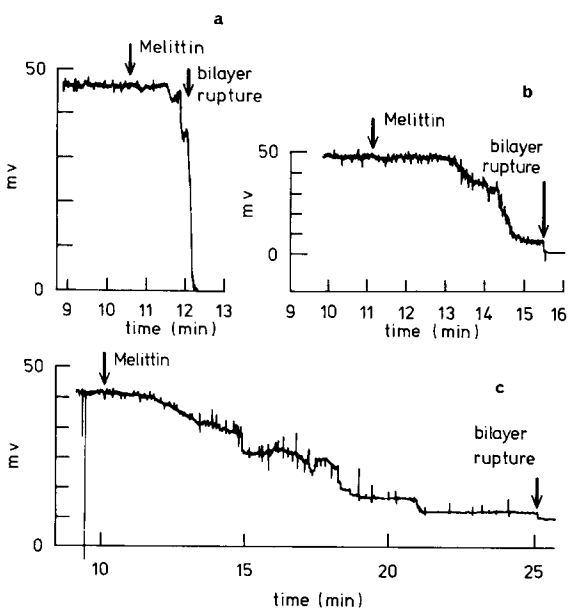

Fig. 2. The rupture of bilayer lipid membranes induced by melittin. Flat bed recorder traces of the variation of membrane potential with time: a, melittin (10^{-5} g · ml^{-1}) added to the negative side of the membrane; b, melittin (10^{-6} g · ml^{-1}) added to the negative side of the membrane; c, melittin (10^{-5} g · ml^{-1}) added to the positive side of the membrane.

electrical resistance began to decrease and after a further 1 min the membrane ruptured. During the period between the melittin addition and subsequent membrane rupture there was no obvious change in the appearance of the membrane or of its electrical capacitance. Similar effects were obtained throughout the concentration range 10^{-4}—10^{-6} g · ml^{-1} but there was a slight delay in the onset of the lytic process with lower melittin concentrations. With doses of 10^{-6} g · ml^{-1} a decrease in electrical resistance from $5 \cdot 10^9$ Ω/mm^2 to $2 \cdot 10^7$ Ω/mm^2 occurred over a 3 min period, generating a relatively permeable membrane which appeared to be very unstable (Fig. 2b). The electrical capacitance of these unstable membranes was always found to be similar to that of the bilayer before melittin addition. Concentrations below 10^{-6} g · ml^{-1} failed to generate reproducible results, presumably due to difficulties with the melittin binding to Teflon and perspex surfaces of the cell. No lytic effects were obtained with concentrations below 10^{-8} g · ml^{-1}. When melittin was added to the earth potential side of the membrane or when no electrical potential was applied to the membrane, lysis took longer to achieve; for instance, at a melittin concentration of 10^{-5} g · ml^{-1} the membrane resistance gradually decreased over a period of 12 min, the membrane rupturing when the resistance reached a value close to 10^7 Ω/mm^2 (Fig. 2c). In the absence of additions, or with control additions, the membrane lifetimes were normally well in excess of 1 h.

Once membranes had been lysed by melittin, attempts were made to reform bilayers without cleaning the cells. Under these circumstances, melittin would be present in both bathing solutions due to diffusion, which would be further facilitated by the cell magnets. In general, such reformed membranes were very short lived, for instance, reforming membranes in low concentrations of melittin (10^{-6} g · ml^{-1}) resulted in slowly thinning films lasting for approximately 10 min (Fig. 3). With higher concentrations of melittin the membranes were even less stable. As in the formation of normal bilayers, there was an initial period when swirling diffraction patterns were observable to reflected light, followed by the formation of black spots which gradually spread across the diffraction pattern. Normally with phosphatidylcholine/cholesterol membranes this phenomenon lasts for 5—15 s, but in the presence of melittin, the black regions remained localized in the diffraction pattern until the membrane broke (Fig. 3). Membranes often persisted in this state for periods in excess of 10 min. The phenomenon was observed in all melittin experiments, thus indicating that melittin slows the drainage of decane during the thinning process.

The 8-26 melittin peptide (10^{-4}—10^{-8} g · ml^{-1}) failed to lyse membranes or to have any of the other effects reported for melittin. After a number of such additions, melittin (10^{-5} g · ml^{-1}) would be routinely added to ensure that the membrane was not behaving atypically. Clearly, under the conditions used, the 8-26 melittin peptide is not a lytic effector. Addition of both the high and low molecular weight polylysines also failed to produce any observable change in membrane resistance or stability in the concentration range 10^{-4}—10^{-6} g · ml^{-1}.

When membranes were prepared containing the hydrophobic polymer LP_{II} a dramatic difference resulted. In the absence of melittin, stable membranes

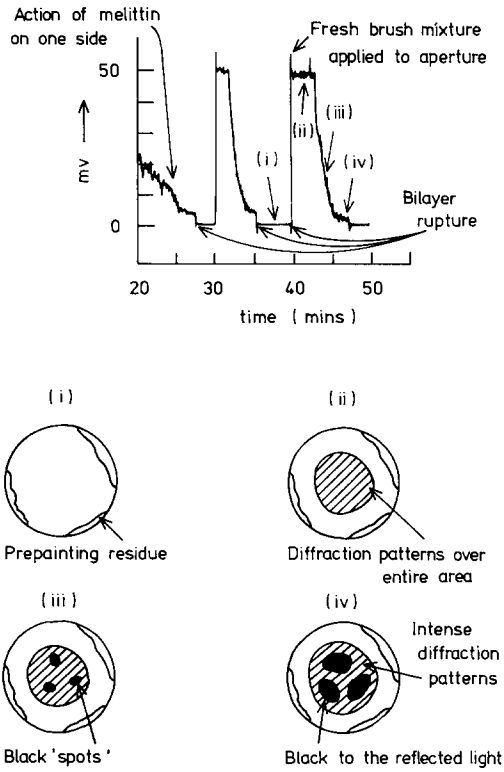

Fig. 3. The effect of melittin on the thinning of bilayer lipid membranes. Flat bed recorder trace of the variation of membrane potential with time. Initially, melittin was applied to one side of a bilayer lipid membrane, after lysis a new membrane was formed, the membrane was unstable and broke within 5 min. The procedure was repeated. The lower portion of the figure represents the schematic appearance of the membrane at various stages (i)—(iv) viewed by reflected light using a microscope. The membranes never became completely black to reflected light.

were formed which possessed lifetimes well in excess of 120 min. When melittin was added to the bathing solution on the negative side of these bilayers to a final conentration of 10^{-4} g · ml^{-1} rapid rupture of the membrane was not observed. Instead, a slow decrease in electrical resistance occurred resulting in rupture 50—70 min after melittin addition (Fig. 4). Thus, the stabilizing effect of the polymer [21] dramatically increases the resistance of the bilayer to melittin-induced rupture.

Secondary structure analysis of melittin. The secondary structure of melittin (*A. melifera*) was analyzed by Dufton and Hider's modification [22] of Chou and Fasman's method [23]. The molecule is predicted to possess two extensive structured regions separated by a proline 'hinge' (Table I). There were no strongly predicted β-turns. The mean conformational parameters $<P_\alpha>$ and $<P_\beta>$ for the predicted nucleation centres of the melittins isolated from *Apis dorsata* and *Apis florea* [3] are very similar to those of *A. melifera* (Table II), although *A. dorsata* melittin would be predicted to possess a β-turn at peptide 10-13, associated with serine-10.

Circular dichroism. The circular dichroism (CD) spectra of melittin in various

TABLE I

SEQUENCE AND CONFORMATIONAL PARAMETERS OF MELITTIN (*APIS MELIFERA*)

N_α and N_β are defined by Dufton and Hider [22]. $< P_\alpha >$ and $< P_\beta >$ are defined by Chou and Fasman [23].

					5					10					15					20				25			
N_α	G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	R	K	R	Q	Q	NH_2
	0.62	1.26	1.43	3.62	2.05	1.47	0.58	0.73	0.38	0.41	0.67	0.81	1.21	1.03	1.75	1.29	0.76	0.81	0.81	0.84	0.98						
					$< P_\alpha > = 1.07$										$< P_\alpha > = 1.11$												
N_β	1.68	2.53	1.17	2.38	3.00	2.18	2.15	2.35	1.74	0.88	0.71	0.73	1.43	0.85	1.62	2.68	1.62	0.91	0.94	0.71	0.55	0.91					
					$< P_\beta > = 1.16$										$< P_\beta > = 1.15$												

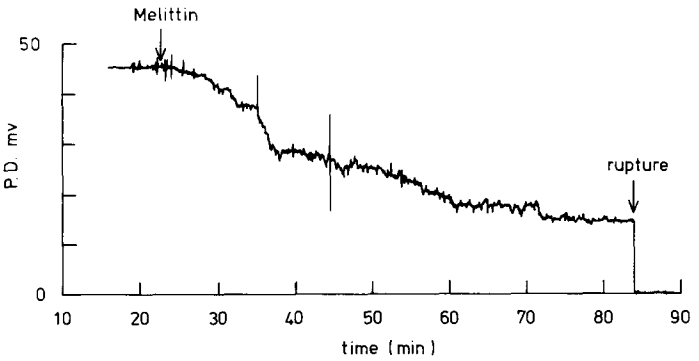

Fig. 4. The effect of hydrophobic polymers on melittin-induced membrane rupture. Flat bed recorder trace of the variation of membrane potential with time. Melittin (10^{-4} g · ml^{-1}) was added to the negative side of the membrane.

solvents are given in Fig. 5. At first sight the CD spectrum in an aqueous solution of sodium chloride (150 mM), buffered to pH 7.4 with Tris (10 mM), appeared to represent the type of situation discussed by Greenfield and Fasman [24] with melittin describable as a mixture of approximately 80% random coil with some minor fraction of α-helix and β-sheet structures. Careful analysis reveals this is not the case. Indeed, Fasman, Hoving and Timasheff [25] have cast doubt on the earlier treatment. The increase in CD in ethanolic solution is constant overall wavelengths and, in particular, the increase in the 223 nm negative shoulder does not show a concomitant decrease in the 198 nm peak, leading to positive CD below 200 nm, a condition required, if α-helix or β-sheet were present. On this basis, the Greenfield and Fasman analysis [24] is precluded. In fact, the CD spectra of melittin resemble those discussed by Fasman et al. [25] who observed CD spectra of films of previously called random coil proteins and peptides which were quite different to those in solution. More recently, it has been demonstrated that this type of film CD spectrum could, under certain circumstances, be seen in solution [26]. It is now fully appreciated that random conformations are better described as extended structures [27]. The CD of melittin in water and aqueous ethanol is not inconsistant with these ideas.

However, it is clear that on going from water (ϵ = 80) to 77% aqueous ethanol (ϵ = 36) there is a large change in CD, which in turn indicates a change

TABLE II

EXTENT AND CONFORMATIONAL PARAMETERS OF THE TWO NUCLEATION CENTRES IN MELITTIN MOLECULES ISOLATED FROM DIFFERENT SPECIES OF BEES

	N.Terminal region				C-Terminal region			
	α-Helix		β-Sheet		α-Helix		β-Sheet	
	Extent	$< P_\alpha >$	Extent	$< P_\beta >$	Extent	$< P_\alpha >$	Extent	$< P_\beta >$
Apis melifera	2—11	1.07	1—13	1.16	15—21	1.11	15—21	1.15
Apis dorsata	2—11	1.05	1—13	1.13	15—21	1.11	15—21	1.15
Apis florea	2—11	1.09	1—13	1.14	15—21	1.03	15—21	1.18

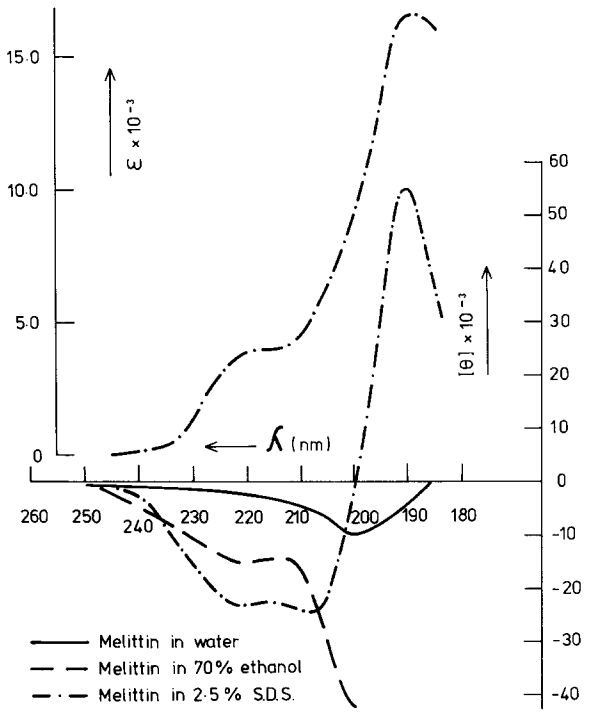

Fig. 5. The circular dichroism and absorption spectra of melittin. The concentration of melittin was 1 mg/ml throughout. The ultraviolet spectra were unchanged for the three experimental conditions. In aqueous solution, in the absence of other salts, the average sodium dodecyl sulphate (SDS) micelle contains 62 molecules [29]. Thus, under the conditions of the experiment, the micelle : melittin monomer ratio is 4 : 1.

in protein conformation without any evidence for the presence of either α-helix or β-sheet (Fig. 5). In order to obtain a better approximation to the hydrophobic membrane environment ($\epsilon < 10$) and also to generate large areas of interface, the CD spectrum of melittin was measured in an aquous solution of sodium dodecyl sulphate (25 mg/ml). A remarkable change was observed, with all the features associated with the α-helix being observed (Fig. 5) [28].

Sephadex LH-20 chromatography. The elution volume for melittin was determined on a Sephadex LH-20 column equilibrated with water or ethanol/water (77 : 23, by vol.). Cytochrome *c* (M_r = 12 400) and ferrichrome A (M_r = 1100) were used to calibrate the column (Fig. 6). It has been established by Habermann [4] and Shipolini (personal communication) that melittin forms a tetramer in aqueous solution with a molecular weight of approximately 12 000. However, on LH-20 equilibrated with water, melittin is eluted after cytochrome *c* (Fig. 6A). This probably results from the absorption of exposed hydrophobic regions of the melittin tetramer on the LH-20 gel. In aqueous ethanol, melittin is eluted after the much smaller ferrichrome A molecule. Again, adsorption of melittin occurs, but is unlikely that adsorption alone is responsible for this retention as only one tryptophan is present on each molecule [30]. Furthermore, hydrophobic interaction is likely to be weaker in

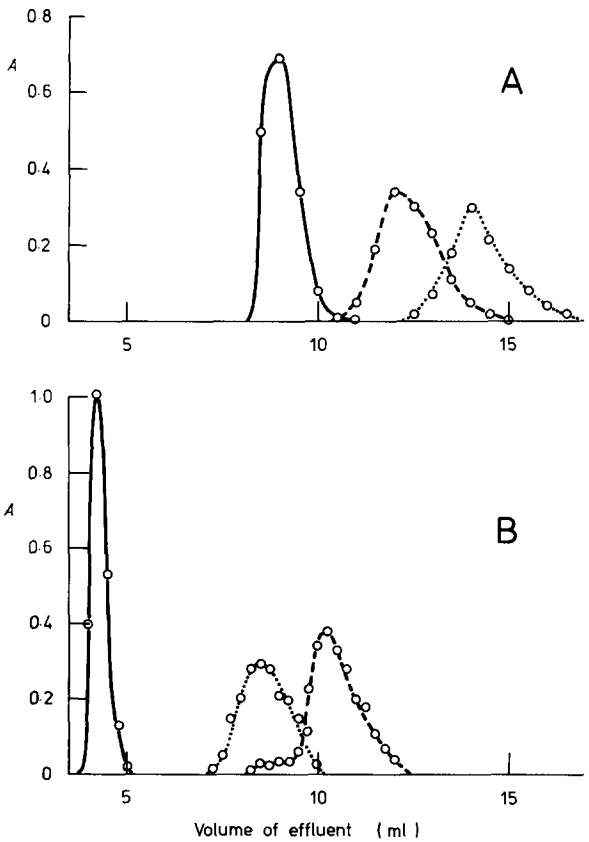

Fig. 6. Chromatography on Sephadex LH-20. Chromatography was performed on a 28 cm column (1 cm internal diameter). Cytochrome *c* (M_r = 12 400) was monitored at 450 nm, melittin was monitored at 270 nm and ferrochrome A (M_r = 1100) was monitored at 425 nm. Cytochrome *c* was dissolved in an acidified ethanolic solution.

aqueous ethanol than in water. The most probable explanation for the increased retention observed in aqueous ethanol is that tetrameric melittin dissociates to the monomeric form in media of relatively low dielectric constant.

Discussion

The initial interaction between melittin and lipid membranes is probably electrostatic, the highly basic C-terminal end forming salt links with the lipid phosphate anions. Indeed, at 10^{-4} M both melittin and its C-terminal peptide (mellitin 20-26) have profound effects on the mobility of the phospholipid molecules [16,31]. However, melittin 20-26 does not possess lytic properties, nor, as is shown in this study, do melittin 8-26 or polylysine. Thus the electrostatic interaction probably serves just to anchor melittin to the membrane surface.

Melittin exists as a tetramer in aqueous solution [4], presumably shielding

many of the hydrophobic residues from the aqueous phase. However, the hydrophobic portions of the melittin peptide have been shown to enter the membrane hydrocarbon matrix as demonstrated by a variety of spectroscopic techniques [14,15,32,33]. Thus, the tetramer must undergo extensive rearrangement on the membrane surface. This rearrangement could be triggered by the gradation of the dielectric properties of the immediate aqueous environment of the membrane. A drop in the dielectric constant from 80 to 10 occurs over a distance of 2 nm, corresponding to the Gouy-Chapman layer. The gel permeation and circular dichroism experiments in 77% ethanol (ϵ = 36, Figs. 5 and 6) indicate that this major rearrangement could be the dissociation of tetrameric melittin.

In view of the amphiphilic properties of melittin, it is not surprising to find that it is strongly surface active, forming air-water monolayers both in the presence and absence of lipids [8]. It is tempting, therefore, to equate melittin with simple detergents, however, this concept does not explain the total absence of lytic properties by the melittin 8-26 peptide. Clearly, the entire length of melittin is required for its toxicity. Environments of low dielectric constant favour the α-helical conformation over those of random coil [34,35] and β-sheet [36]. Trauble, Middelhoff and Brown [37] have demonstrated a large increase in the helical content of a lipoprotein, when added to lipid bilayers. These concepts are amply demonstrated by the CD results reported in this paper, which suggest that once melittin is anchored on the membrane and in an environment similar to the interior of sodium dodecyl sulphate micelles, it can adopt an α-helical conformation. The Chou and Fasman analysis [22,23] identified the regions of the melittin molecule with high α-helical tendencies. Two clear regions, the segments 2-11 and 15-21, which together amount to 65% of the peptide, are predicted to be α-helical. A molecular model of melittin (Fig. 7), incorporating the predicted α-helical regions, was constructed using the technique of Ashworth and Fieldhouse [38]. The resulting 'hinge-like' molecule possessed clear regions of hydrophobic and hydrophilic character. The C-terminal helix is almost completely hydrophobic, whereas the N-terminal helix is amphipathic, glycine-3, lysine-7, threonine-10 and threonine-11, all being orientated to one side of the helix. Similar examples of amphipathic helices have been demonstrated in a plasma lipoprotein [39] and an outer membrane lipoprotein isolated from *Escherichia coli* [40]. The hydrophobic index [41] for the C-terminal helix, together with the hinge region and the hydrophobic portion of the N-terminal helix is 3.1 (15 residues). This value is higher than those corresponding to the intramembranous domain of the MN-glycoprotein (2.6; 23 residues) [41] and to the hydrophobic segment of a bacteriophage coat protein (2.9; 19 residues) [41], although somewhat lower than that corresponding to gramicidin A (2.6; 15 residues) [41]. This high value for the hydrophobic regions of the melittin helices suggests that the most favourable orientation of this molecule in a biological membrane is of the type outlined in Fig. 8. Thus, the melittin molecule would be predicted to produce a 'wedge' effect known to weaken the bilaminar structure of lipid membranes [42,43]. Conversion of melittin to the 8-26 peptide is associated with the removal of a region of relatively low hydrophobic index (1.8) together with two positive charges, thus destroying the

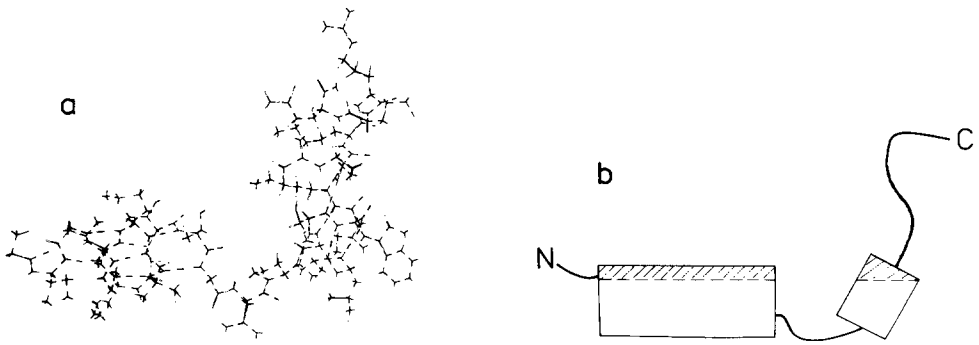

Fig. 7. Proposed structure for melittin monomer, when interacting with lipid membrane surface. (a) Model built with the Ashworth/Fieldhouse system [38] as predicted by the Chou and Fasman analysis presented in Table I. Leucine-13 and proline-14 can be seen in the hinge region, connecting the two α-helical regions. Tryptophan-19 is protruding from the second helix. The strongly basic C-terminal region, residues 21—26, is depicted as random coil. (b) Schematic representation of the model depicted in (a). The α-helical regions are represented by rectangles. The shaded areas represent the hydrophilic regions of the helices; for the N-terminal helix, the hydrophilic region contains glycine-3, alanine-4, lysine-7, threonine-10 and threonine-11; for the C-terminal helix, the hydrophilic region contains serine-18 and lysine-21. The unshaded regions represent the hydrophobic portions.

elongated amphipathic nature proposed for melittin. As a consequence, the 8-26 peptide would be expected to be orientated differently in the membrane surface (Fig. 8). The presence of the hydrophobic polymer LP_{II} is likely to buffer the wedge effect, as the polymer is thought to accumulate in the mid plane of the bilayer [21]. Thus, the effect of LP_{II} on lysis time finds a ready explanation.

It has been estimated that approximately 10 phosphatidylcholine molecules interact with each melittin molecule [31,44] and that their fatty acyl chains are more disordered than those of the bulk lipids [16]. As made clear by Lee [44] this disorder means that the fatty acyl chains adopt a wider distribution of angles with respect to each other than they would in a normal bilayer structure. This difference possibly accounts for the increase of ion permeability observed in liposomes and bilayer lipid membranes prior to rupture (Fig. 2 and 4). The proposed orientation of melittin in the bilayer (Fig. 8) offers an

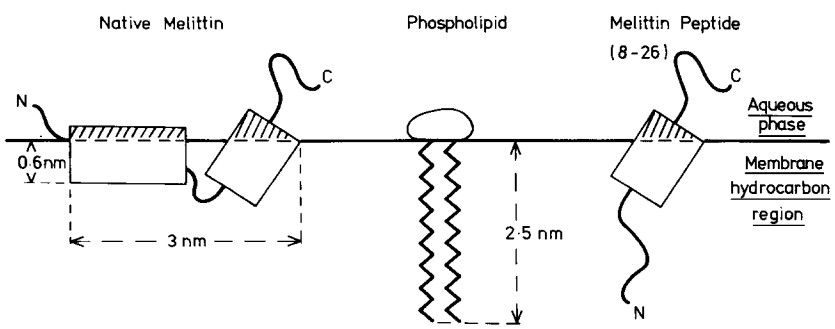

Fig. 8. Proposed orientations of melittin and the 8-26 peptide in a lipid membrane surface. The dimensions for melittin were calculated from the model depicted in Fig. 7a.

explanation for the gradation of fluidity changes observed for the annular lipids [14], as the peptide is predicted to enter the membrane hydrocarbon phase to a depth of less than 1 nm. Clearly, melittin would be expected to excert less influence over the acyl-chain methyl terminal ends than the corresponding polar ends.

This unique property of melittin explains the non-correlation between surface activity and haemolytic activity of the various melittin derivatives and fragments.

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