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Phase separation and hexagonal H_{II} phase formation by gramicidins A, B and C in dioleoylphosphatidylcholine model membranes. A study on the role of the tryptophan residues

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The role of the tryptophan-residues in gramicidin-induced H_{II} phase formation was investigated in dioleoylphosphatidylcholine (DOPC) model membranes. ^{31}P-NMR and small angle X-ray diffraction measurements showed, that gramicidin A and C (in which tryptophan-11 is replaced by tyrosine) induce a similar extent of H_{II} phase formation, whereas for gramicidin B and synthetic analogs in which one tryptophan, either at position 9 or 11 is replaced by phenylalanine, a dramatic decrease of the H_{II} phase inducing activity can be observed. Modification of all four tryptophans by means of formylation of the indole NH group leads to a complete block of H_{II} phase formation. Sucrose density centrifugation experiments on the various peptide/lipid samples showed a quantitative incorporation of the peptide into the lipid. For all samples in a 1/10 molar ratio of peptide to lipid distinct bands were found, indicative of a phase separation. For the gramicidin A′/DOPC mixture these bands were analyzed and the macroscopic organization was determined by ^{31}P-NMR and small-angle X-ray diffraction. The results demonstrate that a quantitative phase separation had occurred between a lamellar phase with a gramicidin/lipid ratio of 1/15 and a hexagonal H_{II} phase, which is highly enriched in gramicidin. A study on the hydration properties of tryptophan-*N*-formylated gramicidin in mixtures with DOPC showed that this analog has a similar dehydrating effect on the lipid headgroup as the unmodified gramicidin. In addition both the hydration study and sucrose density centrifugation experiments showed that, like gramicidin also its analogs have a tendency to aggregate, but with differences in aggregation behaviour which seemed related to their H_{II} phase inducing activity. It is proposed (i) that the main driving force for H_{II} phase formation is the tendency of gramicidin molecules to self-associate and organize into tubular structures such as found in the H_{II} phase and (ii) that whether gramicidin (analogs) form these or other types of aggregates depends on their tertiary structure, which is determined by intra- as well as intermolecular aromatic-aromatic stacking interactions.

Introduction

Gramicidins are linear hydrophobic peptides, produced by *Bacillus brevis* strain ATCC8185 [1], prior to sporulation [1–4]. They are believed to be involved in gene regulation [2,5–9] by binding to the σ-subunit of RNA polymerase [5–8], or, to-

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Abbreviations: DOPC, dioleoyl phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; DMSO, dimethylsulphoxide; $\Delta\nu_q$, quadrupolar splitting.

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gether with tyrocidine, by affecting the superhelical structure of DNA [9].

Biosynthesis of gramicidin occurs via multienzyme complexes, resulting in the production of the peptide as a mixture of gramicidins A, B and C in a molar ratio of about 80 : 5 : 15. The structure of the main component, gramicidin A, is:

1 5
HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-
10 15
D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$

Gramicidins B and C differ from gramicidin A in that the tryptophan at position 11 is replaced by a phenylalanine and tyrosine residue, respectively [10]. Furthermore, in 5–20% of the gramicidin molecules Val-1 is substituted by an isoleucine residue [10].

The main interest in gramicidin has arisen from its ability to form cation-specific transmembrane channels in biological as well as in model membranes [11,12]. These channels are generally believed to consist of two gramicidin monomers, in a left-handed $\beta^{6.3}$ conformation, linked together at their N-terminal parts by hydrogen bonding [13,14].

Another unique feature of the peptide was only recently discovered; gramicidin can act as an extremely potent modulator of lipid structure. When mixed with a detergent-like molecule such as lysophosphatidylcholine, which in excess water organizes in micelles, a lamellar complex is formed [15,16]. Moreover, incorporation of gramicidin in model membranes of typical bilayer forming lipids, like the zwitterionic phosphatidylcholine [17] and even the negatively charged phosphatidylserine and phosphatidylglycerol, leads to the formation of a hexagonal H_{II} phase [18,19]. This observation is in particular intriguing in view of the possible involvement of non-bilayer lipid structures in specific aspects of membrane functioning and the role of lipid–protein interactions therein (for recent reviews, see Refs. 20 and 21).

It may be clear that the various functional abilities of gramicidin must be due to specific features in its chemical structure. In particular the tryptophan residues seem to play a key role, since these were found to be essential for the binding of gramicidin to the σ-subunit of RNA polymerase [8], for channel formation [22] and also for its H_{II} phase inducing activity in dioleoylphosphatidylcholine (DOPC) model membranes [23]. For the latter it was shown, that modification of the tryptophan residues by means of formylation of the amide protons of the indole moieties completely eliminates H_{II} phase formation. It was proposed that the gramicidin molecules by lateral self-association involving intra- as well as intermolecular tryptophan–tryptophan interactions can form ordered aggregates, which themselves prefer to organize into the type of tubular structures that are found in the H_{II} phase [18,23]. Strong indications for this tendency to aggregate were obtained by differential scanning calorimetry (DSC) [24] and by a study on the hydration properties of gramicidin/DOPC systems [18], which in addition showed that H_{II} phase formation is preceeded by a macroscopic phase separation between a peptide-poor and a peptide-rich phase, of which the latter converts into the H_{II} phase.

To get more insight into the molecular mechanism of gramicidin-induced H_{II} phase formation and the role of the tryptophan residues therein, we isolated gramicidins A, B and C from the natural mixture and we investigated the interaction of these and other, chemically prepared, tryptophan-substituted gramicidins with DOPC, using ^{31}P-NMR, small angle X-ray diffraction and DSC. Sucrose density centrifugation experiments were carried out in order to determine the extent of incorporation of these analogs and to further study the importance of aggregation and phase-separation for gramicidin-induced H_{II} phase formation in DOPC model membranes. By chemical analysis of the various fractions obtained after centrifugation and by structural determinations using ^{31}P-NMR and small angle X-ray diffraction, we will show in this paper that a quantitative separation can be obtained between a gramicidin-containing bilayer and an H_{II} phase, that is highly enriched in gramicidin. Finally, in order to get a clearer picture of the specific features of the gramicidin molecule, which are responsible for H_{II} phase formation, we used ^{2}H- and ^{31}P-NMR to investigate the properties of tryptophan-*N*-formylated gramicidin/DOPC mixtures, in which no H_{II} phase formation is induced, as a function of the water content and we compared the results with those earlier obtained for mixtures of DOPC with unmodified gramicidin in a similar study [18].

The results are discussed in the light of the possible implications of tryptophan–tryptophan interactions for the structure of gramicidin and its consequences for H_{II} phase formation as well as for the other functional activities of the peptide.

Materials and Methods

Chemicals

1,2-Dioleoyl-*sn*-glycero-3-posphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were synthesized according to the procedure of Van Deenen and De Haas [25]. The lipids were purified by preparative high performance liquid chromatography on silicagel, using chloroform/methanol/water/ammonia (68 : 28 : 2 : 2, by vol.) as eluent [26]. Dimethylsulphoxide (DMSO-d_6), 99.8% deuterated, was obtained from Merck (Darmstadt, F.R.G.). All other reagents were of analytical grade. Methanol and chloroform were freshly distilled before use.

Gramicidin (analogs)

The natural mixture of gramicidins from *Bacillus brevis* (gramicidin A′) was obtained from Sigma (St. Louis, MO, U.S.A.). ^{14}C-labeled gramicidin A′, with the label on the N-terminal formyl-group (spec. act. 30.3 μCi/mg) was a kind gift of Dr. H. Tournois. The method of preparation will be described elsewhere. [[1-^{13}C]Phe^{9}]-Gramicidin and [[1-^{13}C]Phe^{11}]-gramicidin (a kind gift of Drs. Urry and Prasad), in which, respectively, the tryptophans at positions 9 and 11 are replaced by ^{13}C-enriched phenylalanine, were synthesized via the solid phase method on a Vega Biochemicals Model 50 Automated Peptide Synthesizer (Vega Biochemicals, Tucson, AZ, U.S.A.). Synthesis and purification were carried out as described before in detail [27,28].

Tryptophan *N*-formylated gramicidin, i.e. gramicidin A′ in which the indole protons of all tryptophan residues are replaced by a formylgroup, was prepared by incubation of gramicidin A′ in HCl-saturated formic acid, as described before [23]. The purity was checked with high-performance thin-layer chromatography (HPTLC) on silica HPTLC plates (Merck, Darmstadt, F.R.G.) with chloroform/methanol/water (65 : 25 : 4, by vol.) as eluent. After spraying the plates with 10% sulphuric acid in water and charring at 180°C, a single spot was detected with an R_F value of 0.83. The molar absorption coefficient in methanol was 13700 $cm^{-1} \cdot M^{-1}$, measured on a Shimadzu UV-110-02 spectrophotometer at 280 nm. This value was based on a molecular weight of 1986. Gramicidins A, B and C were isolated from the natural mixture of gramicidins by reversed-phase HPLC on a Si-100-Polyol-RP-18 (Serva, Heidelberg, F.R.G.) column (350 × 50 mm, 10 μm particle size, 100 Å pores), using methanol/water (80 : 20, by vol.) as eluent. Gramicidin A′ was applied to the column in aliquots of 250 mg, dissolved in 13 ml of eluent. A flow rate of 20 ml/min was used. The column was connected to a UV-detector (Kratos analytical, spectroflow 757 absorbance detector) set at 280 nm. Fractions of 100–300 ml were collected and their composition was judged with HPTLC, which allows detection of gramicidins A, B and C as separate spots with R_F values of 0.74, 0.76 and 0.71, respectively. Gramicidin C eluted first from the column, at an elution volume of about 1200 ml, closely followed by gramicidin A. No baseline-resolution could be achieved and only those fractions were collected which contained more than 80% pure gramicidin A or C, as judged from HPTLC. After both species were eluted from the column at a total elution volume of 6000–7000 ml, the polarity of the eluent was decreased by raising the methanol/water ratio to 85 : 15 (v/v). Gramicidin B then eluted in pure form from the column after an additional elution volume of about 1500 ml. For further purification of gramicidins A and C the crude fractions of several runs were pooled and, after removal of the solvent under reduced pressure, and dissolving the peptide in a small volume of the eluent, both species were separately rechromatographed. This time a maximal amount of 130 mg of peptide was applied to the column and only fractions containing pure gramicidin A or C, as judged from HPTLC, were collected. After HPTLC the various fractions were dried by removal of the solvent under reduced pressure followed by overnight storage under high vacuum. During all runs 95–100% of the peptide was recovered after elution. Per initial amount of 100 mg of gramicidin A′, during the first run an average quantity of 75 mg of impure gramicidin A and 7.5 mg of impure gramicidin C were collected, which, after rechromatography yielded amounts of 41 and 6 mg, respectively, of the pure compounds. The average yield of gramicidin B after one run was 5 mg per 100 mg of the applied mixture. The molar absorption coefficients of the isolated species in methanol at 280 nm were determined to be: 21 200 $\mathrm{cm}^{-1} \cdot \mathrm{M}^{-1}$ for gramicidin A′, based on a molecular weight of 1880, 22 600 $\mathrm{cm}^{-1} \cdot \mathrm{M}^{-1}$ for gramicidin A (M_r 1885), 14 100 $\mathrm{cm}^{-1} \cdot \mathrm{M}^{-1}$ for gramicidin B (M_r 1845) and 19 500 $\mathrm{cm}^{-1} \cdot \mathrm{M}^{-1}$ for gramicidin C (M_r 1861). These values are in fair agreement with those reported by Hinton et al. [29].

^{1}H-Nuclear magnetic resonance (^{1}H-NMR)

4 to 10 mg of peptide was dissolved in 600 μl of DMSO-d_6. ^{1}H-NMR spectra were recorded at 500.13 MHz on a Bruker WM 500 spectrometer at 30°C. 16 to 64 free induction decays were accumulated, using 16K data points, a spectral width of 6.25 kHz, a 14 μs 90° pulse and an interpulse time of 1.1 s. Fourier transformation was performed without application of line-broadening or resolution enhancement techniques.

Preparation of gramicidin (analogs)/DOPC dispersions

Dry films of DOPC and the appropriate amount of peptide were prepared in small pyrex tubes (length 5 cm, outer diameter 0.8 cm) as described before [18]. Since samples with a low water content generally give rise to better lineshapes with ^{31}P-NMR, the films were hydrated with an amount of buffer (150 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA (pH 7.0)) equal to the sample weight. Under these conditions the bilayers are maximally swollen and the samples are fully hydrated [18]. For the hydration experiments with tryptophan-*N*-formylated gramicidin/DOPC mixtures, controlled amounts of ^2H_2O were used to hydrate the lipid/peptide films [18]. Samples for sucrose-density centrifugation experiments were prepared by addition of water to the dry films to a final lipid concentration of 60 mM. For radioactivity measurements ^{14}C-labeled gramicidin was used, diluted with unlabeled gramicidin A′ to a specific activity of 0.45 μCi/μmol. Following hydration the samples were incubated at 37°C and spun down as described before [18]. For ^{2}H and ^{31}P-NMR measurements the tube with the pelleted samples as inserted into a conventional 10 mm tube.

^{31}P-NMR

Proton-noise decoupled ^{31}P-NMR spectra were recorded at 25°C either at 36.4 MHz on a Bruker WH 90 spectrometer or at 81.0 MHz on a Bruker WP 200 spectrometer, under conditions as described elsewhere [18]. To the accumulated free induction decays an exponential multiplication was applied, corresponding to a linebroadening of 50 Hz. Relative amounts of bilayer and H_{II} component were estimated as follows. In the composite spectrum the shape of the bilayer component was estimated and both the lineshape of the composite spectrum and the bilayer component were then digitized by taking points at every 50 Hz. Upon substraction of the 'bilayer' from the composite spectrum a set of points was obtained through which a curve could be drawn corresponding to an H_{II} type of spectrum. Small adjustments in the estimation of the bilayer component could be made in order to obtain optimal theoretical lineshapes for both the bilayer component and the H_{II} component, which remained after substraction. Finally the areas of both these components were determined by integration and related to the area of the total spectrum. The maximal error in calculated percentages was estimated to be about 5% in absolute terms. The residual chemical shift anisotropy (csa) was measured as three times the distance between the chemical shift position of isotropically moving phospholipids and the high and low field peak in spectra of phospholipids organized in a lamellar and H_{II} phase, respectively. The estimated maximal error in the chemical shift anisotropy is 1 ppm.

^{2}H NMR

^{2}H-NMR spectra were obtained at 30.7 MHz on a Bruker WP 200 spectrometer at 25°C as described before [18]. Prior to Fourier transformation an exponential multiplication was applied, resulting in a 20 Hz line-broadening.

Small angle X-ray diffraction

X-ray experiments were performed at 25°C on a Kratky camera as previously described [24]. The exposure time was 5–10 minutes.

Sucrose-density centrifugation

Hydrated lipid/peptide samples were vortexed and loaded on top of a 3–20% linear sucrose gradient. Centrifugation was performed at 150 000 × g in a SW 41 Beckman rotor at 4°C for 16–20 h. Isolated fractions were carefully collected with a Pasteur pipet. The sucrose-density of each fraction was determined with a refractometer (Bausch and Lomb, Abbe 3-L). The peptide/lipid ratio in the various fractions was obtained with similar results either by chemical analysis of the isolated fractions or it was derived indirectly from the density of the fraction with the use of a calibration curve. For chemical analysis of the samples the amount of lipid was determined by using the method of Rouser [30]. The peptide content was determined spectrophotometrically by measuring the absorbance at 280 nm after dilution of the sample with methanol or, with identical results, by using ^{14}C-labeled gramicidin and measuring the radioactivity in a Packard Liquid Scintillation Counter (Prias, PLD, Tricarb).

For structural assignments of separate fractions, samples containing 100 μmoles of lipid were loaded on the gradient, resulting in a similar density distribution as observed for smaller quantities of lipid. Isolated fractions were washed twice with 10 ml of water by centrifugation at 4°C at 150 000 × g for 20–60 min. After ^{31}P-NMR and small angle X-ray diffraction measurements on the pelleted samples, both the lipid and the peptide content were determined. Control experiments showed no significant effect of sucrose on the ^{31}P-NMR characteristics and the X-ray diffraction profiles of gramicidin/lipid dispersions, even at 20% sucrose.

Results

^{1}H-NMR characterization of the gramicidin analogs

The various gramicidin analogs were first characterized by ^{1}H-NMR in DMSO. Fig. 1A shows the various resonances originating from the different protons of the peptide backbone and the side chains of the aromatic amino acids in the natural mixture of gramicidins. Assignments are based on data of Glickson et al. [31] and Bundi et al. [32]. Since the phenylalanine ring protons at 7.22 ppm as well as the *o*-CH protons of tyrosine, visible as a doublet at 6.62 ppm, give rise to well resolved peaks, measurement of the intensity of these peaks in relation to the total intensity of the tryptophan indole N-H peaks at 10.80 and 10.74 ppm allows an accurate determination of the composition of gramicidin A′. The molar ratio of gramicidins A, B and C in the natural mixture was thus determined to be 80 : 5 : 15, with an estimated error of 2%. The isolated gramicidin A (Fig. 1B) appeared to be free of gramicidin B, but at the resonance position of the tyrosine *o*-CH protons still an intensity was visible, corresponding to a relative amount of approx. 3% of gramicidin C. In the spectrum of gramicidin B (Fig. 1c) the presence of gramicidin C could not be detected. Due to the broad base of the peak, originating from the phenylalanine ring protons, which have slightly different chemical shift positions [32] it was difficult to estimate from this spectrum the purity of gramicidin B in terms of a possible contamination with gramicidin A. The most prominent features in the spectrum of gramicidin C (Fig. 1D) are the high intensities of the tyrosine-associated resonances, i.e. the hydroxyl proton at 9.13 ppm, the *m*-CH protons at 6.99 ppm and the *o*-CH protons at 6.62 ppm. An amount of approx. 2% of gramicidin A was determined to be present, whereas no gramicidin B could be detected.

Formylation of the tryptophan indole amide protons drastically affects the ^{1}H-NMR spectrum of gramicidin (Fig. 1E). The tryptophan NH protons disappear and resonances of the formyl protons occur at 9.66 and 9.23 ppm. Furthermore, all resonances, originating from the tryptophan indole ring seem to have shifted in the low field direction. Not only the *o*-CH but also the *m*-CH resonances of tyrosine and the resonances of the ring protons of phenylalanine are now clearly visible. For a number of resonances, especially those associated with the tryptophan residues, the linewidth is markedly increased. The reason for this is at yet unknown. It is interesting to note, that the tryptophan fluorescence, which is completely lost after formylation [23,33] is already

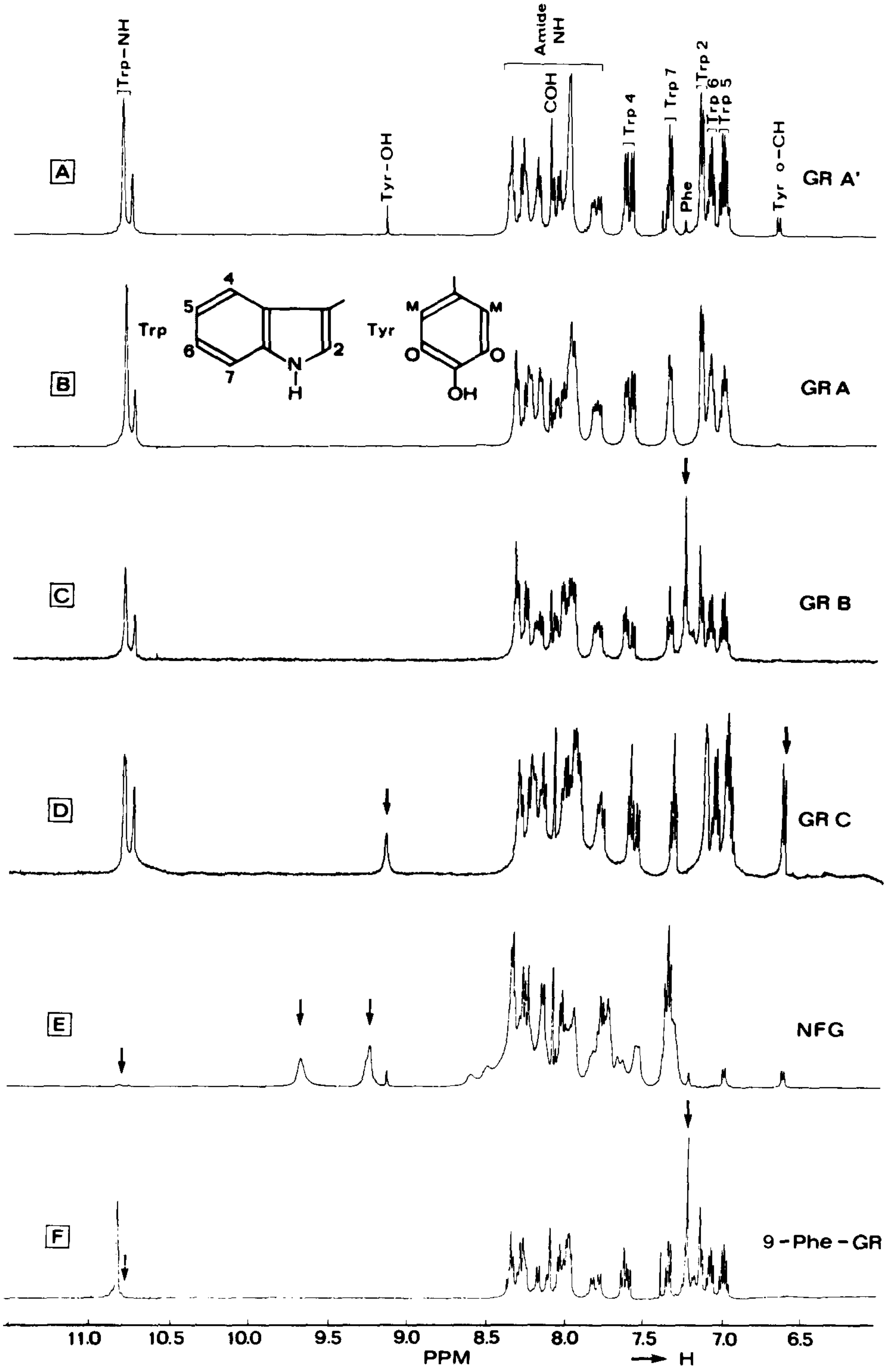

Fig. 1. Low field part of 500.13 MHz ^{1}H-NMR spectra of gramicidin A′ (A), the isolated species gramicidin A, B and C (B–D), tryptophan-*N*-formylated gramicidin (E) and the synthetic [Phe^9]-gramicidin (F) in DMSO-d_6. Peak positions are measured relative to tetramethylsilane (TMS).

reduced to zero when ^{1}H-NMR spectra showed 25% of the tryptophan residues to be still intact. The full recovery of the tryptophan fluorescence after deformylation [23] shows that this effect is not due to an irreversible damaging of the tryptophan residues. Most likely this behaviour is related to the occurrence of stacking interactions between for instance tryptophans 9 and 15 [34,35,27], resulting in fluorescence quenching. This is in line with a recent observation, that not all four tryptophan residues contribute to the fluorescence of the gramicidin molecule [35]. That such stacking interactions occur is also strongly suggested by the ^{1}H-NMR data in Fig. 1. The indole amide protons of tryptophan give rise to two peaks with intensity ratios of 3 : 1 for gramicidin A and 2 : 1 for gramicidins B and C, suggesting that the resonance position of the NH proton of one of the tryptophans, but not of tryptophan-11, has been shifted upfield. Such a shift occurs, when the local field around the proton is affected by the ring current of the indole moiety of another tryptophan residue. The proposition that tryptophan-9 is involved in these stacking interactions is supported by the observation, that in the spectrum of the synthetic [Phe^9]-gramicidin (Fig. 1F), which in other respects is almost identical to that of gramicidin B, none of the tryptophan NH resonances is shifted in the upfield direction.

Effect of the gramicidin analogs on lipid structure

First, ^{31}P-NMR spectra of dispersions of DOPC and mixtures of DOPC and various gramicidin analogs were recorded. Fig. 2A shows the axially symmetric ^{31}P-NMR spectrum, with a low-field shoulder and a high-field peak, obtained for pure DOPC dispersions. Such a lineshape is characteristic for lipids in an extended lamellar phase, in which there is incomplete motionial averaging of the orientation of the phospholipid headgroups [36,37]. When organized in the hexagonal H_{II} phase the lipids undergo additional rapid motion around the tubes of which this phase is composed, resulting in a ^{31}P-NMR spectrum with a reversed asymmetry, i.e. a low-field peak and a high-field shoulder, and with a chemical shift anisotropy, which, in the absence of changes in local order, is reduced by a factor of 2 [36,37]. The presence of both a bilayer and a hexagonal H_{II} phase in a sample results in the type of composite spectra as observed for mixtures of DOPC with gramicidin A′ or any of the isolated species (Fig. 2B–E). Fig. 3 shows that the ability to induce H_{II} phase formation is in the order of gramicidin A > gramicidin A′ > gramicidin C ≫ gramicidin B, while no H_{II} phase formation is observed in mixtures of the lipid with tryptophan-*N*-formylated gramicidin (Fig. 2F). As shown before for gramicidin A′ [18] the extent of H_{II} phase formation is highly dependent on the peptide concentration (Fig. 3). For the gramicidin mixture and for gramicidins A and C the formation of a hexagonal H_{II} phase is induced upon increasing the peptide/lipid ratio from 1 : 50 to 1 : 25. A considerable increase in the extent of H_{II} phase formation is observed when the peptide content is raised to 10 mole%. With this amount of peptide present also gramicidin B is able to induce H_{II} phase formation, to a similar extent as the synthetic [Phe^{11}]-analog. While this analog behaves similarly as the isolated gramicidin B fraction (also compare Figs. 2C and 2H), substitution of the tryptophan at position 9 by phenylalanine seems to result in a different behaviour: superposed on the bilayer type of spectrum, a rather broad isotropic signal now is observed, possibly with a small underlying hexagonal component. The remaining amount of 'bilayer' signal in this sample was estimated to be 89%. In all cases incorporation of the peptide leads to a reduction of the csa of the bilayer component of about 10%, in agreement with previous observations [19].

Next the various samples were subjected to small-angle X-ray diffraction measurements. Table I shows the distances of the reflections as found in the pure DOPC dispersion and in the peptide/lipid samples with a molar ratio of 1/10. In agreement with the ^{31}P-NMR data in the pure DOPC as well as in the mixture of lipid with tryptophan *N*-formylated gramicidin only two reflections are present at distances which relate as 1 : 1/2, indicative for a bilayer organization of the lipids. In all other peptide/lipid samples an additional '$1/\sqrt{3}$' reflection is present and in some cases even a '$1/\sqrt{7}$' reflection can be detected, both characteristic for the organization of lipids in a hexagonal phase [38]. In all samples similar values of the first order spacing were measured for the bilayer and the hexagonal H_{II} phase, in agreement with earlier data [18,19]. The intensities of the H_{II} phase specific '$1/\sqrt{3}$' reflections in all samples showed a good correlation with the percentage of H_{II} phase as determined with ^{31}P-NMR. An example is given in Fig. 4, which shows the diffraction profiles of mixtures of DOPC with the isolated species of gramicidin A, B and C and with

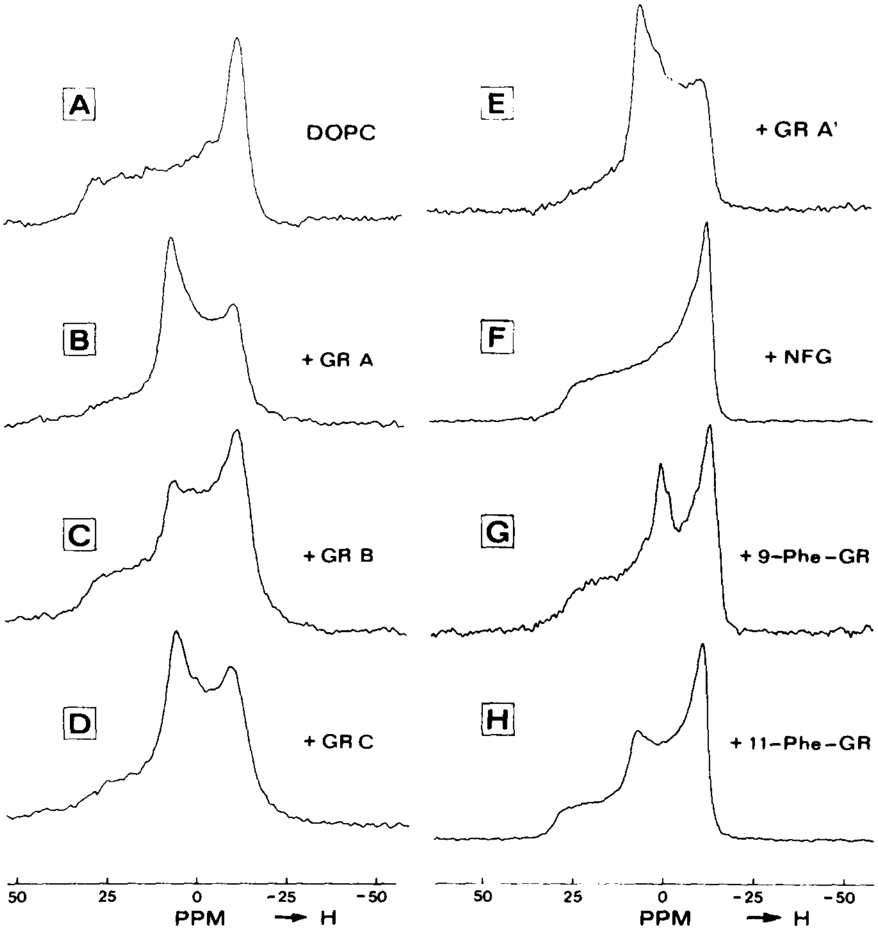

Fig. 2. Proton-noise decoupled 36.4 MHz (A–D) and 81.0 MHz (E–H) ^{31}P-NMR spectra of aqueous dispersions of DOPC (A) and of mixtures of DOPC with gramicidin A (B), gramicidin B (C), gramicidin C (D), the natural mixture of gramicidins (E), tryptophan-*N*-formylated gramicidin (F) and with the synthetic [Phe^{9}]- (G) and [Phe^{11}]-gramicidin (H) in a molar ratio of peptide to lipid of 1 to 10.

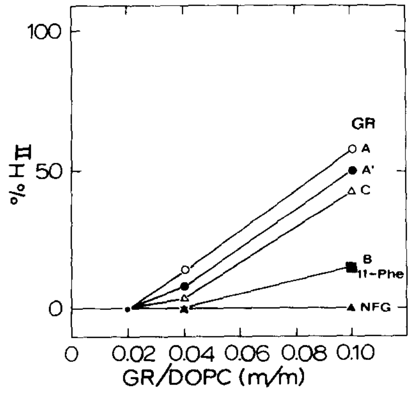

Fig. 3. Percentage of H_{II} phase formation in DOPC model membranes as a function of the peptide content upon incorporation of gramicidin A′ (●), gramicidin A (○), gramicidin B (×), gramicidin C (△) and tryptophan-*N*-formylated gramicidin (▲). For the synthetic [Phe^{11}]-analog (■) only a molar ratio of peptide of lipid of 1 : 10 was measured.

TABLE I

X-RAY DIFFRACTION CHARACTERISTICS OF AQUEOUS DISPERSIONS OF DOPC AND MIXTURES OF DOPC AND GRAMICIDIN (ANALOGS) IN A MOLAR RATIO OF PEPTIDE TO LIPID OF 1 TO 10

Sample	Reflections (Å)			
DOPC	63.9	–	31.8	–
GR A′/DOPC	63.3	36.5	+	23.5
GR A/DOPC	64.2	36.7	+	24.0
GR B/DOPC	68.7	37.4	33.9	–
GR C/DOPC	62.6	35.9	+	23.4
[Phe^9]-GR/DOPC	63.1	34.8	31.6	–
NFG/DOPC	68.9	–	34.2	–

the synthetic [Phe^9]-analog in a 1 to 10 molar ratio of peptide to lipid. The presence of a '$1/\sqrt{3}$' reflection in the latter case demonstrates, that in this sample indeed part of the lipids is organized in the H_{II} phase. The exact nature of the isotropic component observed in the corresponding ^{31}P-NMR spectrum (Fig. 2G) is unknown.

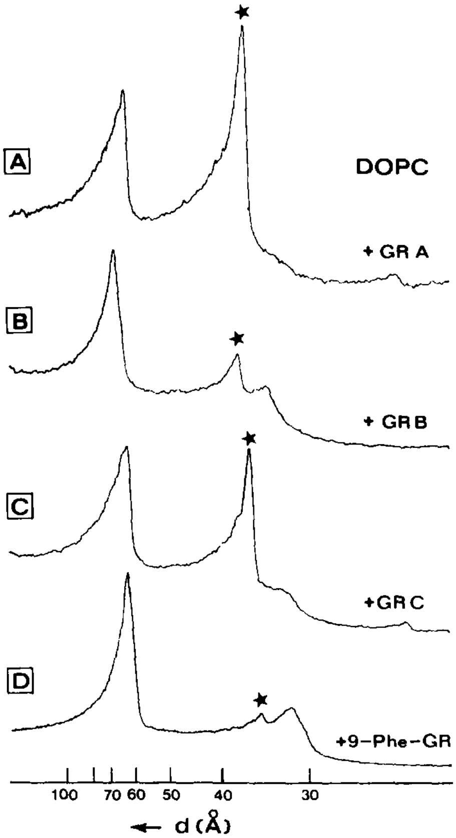

Fig. 4. Small-angle X-ray diffraction patterns of aqueous dispersions of mixtures of DOPC with gramicidin A (A), gramicidin B (B), gramicidin C (C) and the [Phe^9]-analog of gramicidin (D) in a peptide to lipid molar ratio of 1:10. The '$1/\sqrt{3}$' reflection is labelled with an asterisk.

A relatively high intensity of the '$1/\sqrt{3}$' reflection as compared to the intensity of the first-order spacing such a shown in Fig. 4 for the gramicidin A/DOPC mixture has been observed before in mixtures of DOPC with gramicidin A′ [18]. It should be realized, that this is an unusual behaviour for lipids organized in the H_{II} phase, which typically show a much lower intensity of the '$1/\sqrt{3}$' reflection. The reason for this behaviour, however, is up till now still unknown.

Sucrose density centrifugation

First a series of experiments were performed on mixtures of DOPC with increasing amounts of gramicidin A′. For pure DOPC a single band was found on the sucrose gradient at a density of 1.014 g/ml (Fig. 5A). Upon incorporation of increasing amounts of the peptide up till a molar ratio of about 1/40 of peptide to lipid still only one band was observed, of which the equilibrium density increased with the gramicidin content. In the peptide concentration range of 1:40 to 1:10 (molar ratio of gramicidin to DOPC) this band

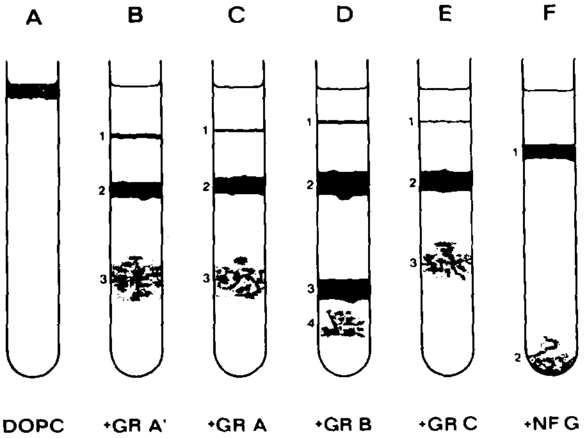

Fig. 5. Density distribution in tubes with a 3–20% linear sucrosegradient after centrifugation of aqueous dispersions of DOPC (A) and mixtures of DOPC with gramicidin A′ (B), gramicidin A (C), gramicidin B (D), gramicidin C (E), and tryptophan-*N*-formylated gramicidin (F) in a molar ratio of peptide to lipid of 1/10. Samples contained approx. 4 μmol of lipid.

increased in density, till a maximum value of about 1.028 g/ml was reached (data not shown). Simultaneously, however, at a peptide/lipid molar ratio of 1 : 40 two additional fractions appeared, one at a lower density (d = 1.017 g/ml) and one at a higher density (d = 1.041 g/ml), the latter with a granular-like appearance. Upon increasing the initial gramicidin content to 10 mole%, the intensity of these two bands increased, but their density remained unchanged (data not shown). A schematic representation of the density distribution of gramicidin A′/DOPC mixtures in a 1 : 10 molar ratio is shown in Fig. 5B. At higher initial peptide concentrations both lower density fractions gradually disappeared, until at a gramicidin/lipid ratio of about 1 : 7 only the high-density band remained at d = 1.041 g/ml (data not shown). The density of this band increased upon further raising the gramicidin concentration.

The behaviour of gramicidin A′/DOPC mixtures on sucrose density gradients indicates, that at intermediate peptide concentrations structural heterogeneity occurs in the samples. Similar behaviour was observed for mixtures of DOPC with gramicidins A and C. From Fig. 5 it can be seen, that centrifugation of these samples in a 1 : 10 molar ratio of peptide to lipid results in a similar density distribution on the sucrose gradient as obtained from gramicidin A′/DOPC mixtures. Gramicidin B incorporation leads to a different distribution pattern in the high-density region, where now two fractions are observed, one with a smooth appearance (d = 1.050 g/ml) and one of a granular material (d = 1.061 g/ml). The tryptophan-*N*-formylated gramicidin/DOPC mixture shows an altogether different behaviour: only two bands are formed, one at intermediate density (d = 1.021 g/ml) and one, which sediments on the bottom of the tube. After centrifugation of this sample on a gradient with a higher density (10–30% sucrose), the density of this latter fraction could be determined to be 1.082 g/ml.

The analysis of the various fractions, as shown in Fig. 5, is given in Table II.

To get more insight into the structural organization of the lipids in the various fractions we isolated the three bands obtained for gramicidin A′/DOPC mixtures in a 1 : 10 molar ratio. ^{31}P-NMR spectra of two of the isolated fractions at

TABLE II

ANALYSIS OF THE DIFFERENT FRACTIONS OBTAINED AFTER SUCROSE-DENSITY CENTRIFUGATION OF AQUEOUS DISPERSIONS OF MIXTURES OF DOPC AND GRAMICIDIN (GR) (ANALOGS) IN A MOLAR RATIO OF PEPTIDE TO LIPID OF 1/10 (see Fig. 5)

Sample	Number of experiments	Fraction number	Density (g/ml) [a]	GR/DOPC (m/m) ± S.d.	Relative % P recovery [c]
GR A′/DOPC	5	1	1.0174	0.016 ± 0.008	1
		2	1.0284	0.062 ± 0.016	43
		3	1.0408	0.120 ± 0.027	56
GR A/DOPC	2	1	1.0162	0.011 ± 0.001	9
		2	1.0272	0.057 ± 0.001	18
		3	1.0468	0.150 ± 0.002	72
GR B/DOPC	2	1	1.0162	0.011 ± 0.004	2
		2	1.0380	0.073 ± 0.008	45
		3	1.0500	0.167 ± 0.025	27
		4	1.0608	0.222 ± 0.034	26
GR C/DOPC	2	1	1.0174	0.016 ± 0.003	4
		2	1.0208	0.061 ± 0.001	10
		3	1.0418	0.125 ± 0.005	86
NFG/DOPC	2	1	1.0209 [b]	0.031 ± 0.025	79
		2	1.0820	0.345 ± 0.025	21

[a] Mean value, with deviation from mean < 0.002.

[b] Deviation from mean < 0.005.

[c] The total recovery was in general about 70%, therefore the given values should be regarded only as an indication.

$d = 1.028$ g/ml and $d = 1.041$ g/ml are shown in Fig. 6. Whereas the former exhibits a characteristic bilayer type of spectrum, the high density fraction seems to consist almost purely of a hexagonal H_{II} phase. The presence of an H_{II} phase in the latter fraction was confirmed by X-ray analysis, which showed the H_{II} phase characteristic '$1/\sqrt{3}$' reflection with a high intensity, as observed also in the initial mixture (see Fig. 4). The distances at which the first- and second-order reflections occurred were 65.6 and 37.2 Å. From the lower-density fraction at $d = 1.028$ g/ml no X-ray diffraction pattern could be obtained, even after prolonged exposure times. The upper fraction at $d = 1.017$ g/ml did not contain enough material to allow a structural assignment with either ^{31}P-NMR or small angle X-ray diffraction. For the three fractions from low density to high density molar ratios of peptide to lipid were found of 1 : 61, 1 : 15 and 1 : 7, respectively. In agreement with previous observations [18] these data thus clearly show, that the H_{II} phase, induced by gramicidin in DOPC model membranes is highly enriched in gramicidin.

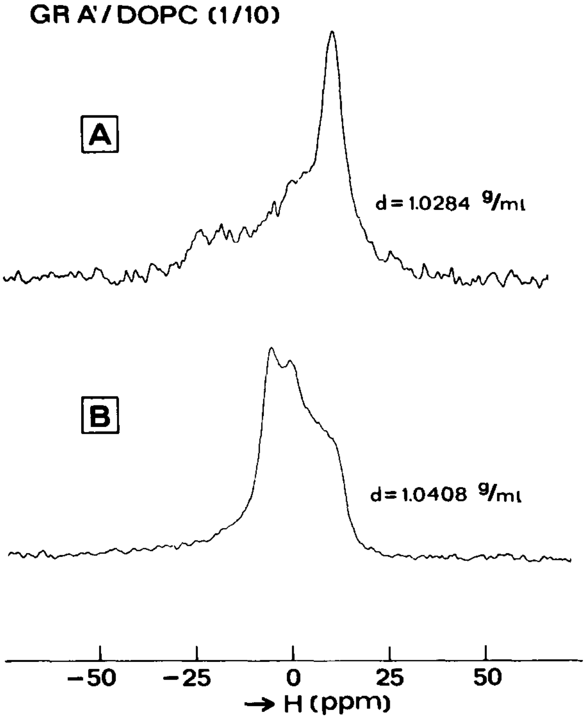

Fig. 6. Proton-noise decoupled 36.4 MHz ^{31}P-NMR spectra of fractions obtained by sucrose-density centrifugation of mixtures of DOPC with gramicidin A′ in a 1:10 molar ratio, showing the ^{31}P-NMR spectrum of the isolated band at intermediate density ($d = 1.028$ g/ml) (A) and of the high-density band at $d = 1.041$ g/ml (B).

Hydration properties of mixtures of tryptophan-N-formylated gramicidin and DOPC

Recently we reported experiments on the hydration properties of gramicidin A′/DOPC mixtures with a varying ^2H_2O content, which proved to be very useful to get insight into the mechanism of gramicidin induced H_{II} phase formation [18]. It was found that at low water content gramicidin dehydrates the lipid headgroup, but cannot induce the formation of a hexagonal H_{II} phase. At higher water content ($6 \leqslant N \leqslant 12$, N being the ^2H_2O/lipid molar ratio) phase separation occurs between a gramicidin-poor lamellar phase with similar properties as a lamellar phase of pure DOPC at the same stage of hydration, and a gramicidin-rich lamellar phase, characterized by a reduced ^{31}P chemical shift anisotropy. Upon further increasing the water content, the gramicidin-rich lamellar phase converts into a hexagonal H_{II} phase.

We now investigated in a similar way the hydration properties of tryptophan-*N*-formylated gramicidin/DOPC mixtures. ^{31}P-NMR spectra of these mixtures at various stages of hydration are shown in Fig. 7. The tryptophan-*N*-formylated gramicidin differs in two aspects from the parent molecule: (1) it does not induce the formation of a hexagonal H_{II} phase at any water content, and (2) at intermediate hydration states, the ^{31}P-NMR lineshape does not give any indication for phase separation. For DOPC dispersions in excess water a chemical shift anisotropy was measured of 43 ppm, which decreased with maximal 15% upon incorporation of the peptide in the highest molar ratio of 1 : 10 (peptide/lipid) or upon reducing the water content to its lowest value of N of about 2. Under conditions of both a high peptide concentration and a low water content the smallest value of the chemical shift anisotropy was measured of 35 ppm.

The behaviour of the ^2H_2O molecules in DOPC and gramicidin/DOPC systems at various stages of hydration can be directly monitored by ^{2}H-NMR. For pure DOPC it was shown that hydration with increasing amounts of ^2H_2O leads to a

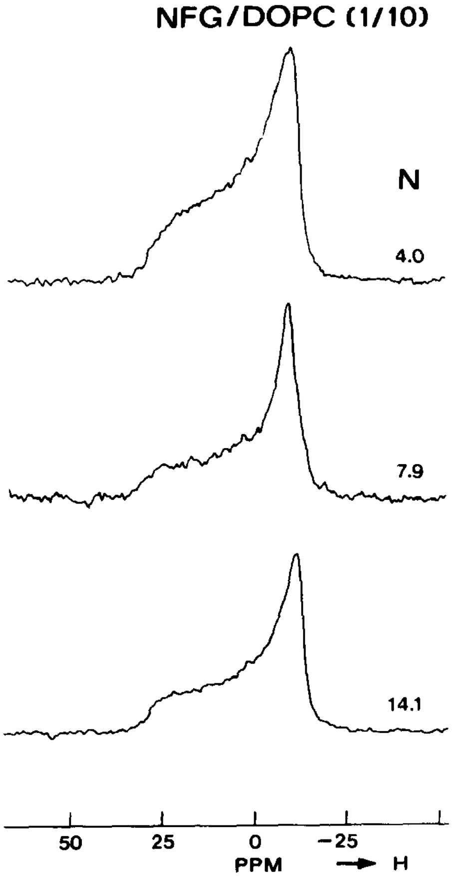

Fig. 7. Proton-noise decoupled 81.0 MHz ^{31}P-NMR spectra of aqueous dispersions of DOPC with tryptophan-*N*-formylated gramicidin in a 1 : 10 molar ratio at various stages of hydration. *N* is the number of ^2H_2O molecules per DOPC headgroup.

reduction in the quadrupolar splitting in a biphasic way (Fig. 9) [18,40]. Upon incorporation of tryptophan-*N*-formylated gramicidin at low water content a single quadrupolar splitting can be observed (Fig. 8) with a value of $\Delta\nu_q$ which decreases with increasing peptide concentrations. Similar behaviour was reported for gramicidin A′/DOPC mixtures [18] and was interpreted as follows. Upon hydration of the mixed film the peptide takes up water in preference to the lipid molecules. The peptide-associated ^2H_2O molecules move isotropically and are in fast exchange with water molecules bound to the lipid headgroup. The rate of exchange, though still fast on the ^{2}H-NMR timescale, most likely is slightly reduced, thus giving rise to the observed broadening of the spectra. The results indicate, that tryptophan-*N*-formylated gramicidin has a similar dehydrating effect on the lipid headgroup as gramicidin A′ at low water content.

Increasing the amount of water to $N \geqslant 5$ for gramicidin A′/DOPC mixtures results in a phase separation between a gramicidin-poor and a gramicidin-rich phase [18]. This leads to the appearance of two components in the ^{2}H-NMR spectra of ^2H_2O, one with a $\Delta\nu_q$ similar to that of the gramicidin-free bilayer and one with a largely reduced value of $\Delta\nu_q$. The intensity of the latter component was found to increase with both the peptide concentration and the water content [18]. For mixtures of DOPC with tryptophan-*N*-formylated gramicidin a completely different behaviour is observed (Fig. 8). At peptide/lipid ratios of 1 : 50 and 1 : 25 now still a single quadrupolar splitting is observed at intermediate water content, indicating that no phase separation occurs in these systems. For the sample with a peptide/lipid ratio of 1 : 25 the value of $\Delta\nu_q$ significantly increases upon raising *N* from 2 to about 6 (Figs. 8 and 9). Such an increase can be expected when the relative contribution of the peptide-associated ^2H_2O molecules (with $\Delta\nu_q = 0$) to the observed average $\Delta\nu_q$ largely decreases, and when the ratio of lipid bound (still with a large value of $\Delta\nu_q$) to isotropically moving ^2H_2O molecules thus drastically increases. At higher water content the effect of gramicidin on $\Delta\nu_q$ of ^2H_2O in DOPC bilayers becomes less pronounced (Fig. 9).

In the sample with a peptide/lipid molar ratio of 1 : 10 at $N = 6.0$ a large broadening can be observed, indicating a reduction of the rate of exchange between water molecules with a binding site at the lipid headgroup and peptide-associated water molecules. At higher water content ($N = 9.5$) even clearly a two component spectrum is present, suggesting that a phase separation has occurred. However, the broadening of both components indicates that still exchange occurs between both phases, on the timescale of the ^{2}H-NMR measurement. We consider it very well possible, that the spectral component giving rise to the broad isotropic signal, arises from aggregates of pure tryptophan-*N*-formylated gramicidin, present in the bilayer.

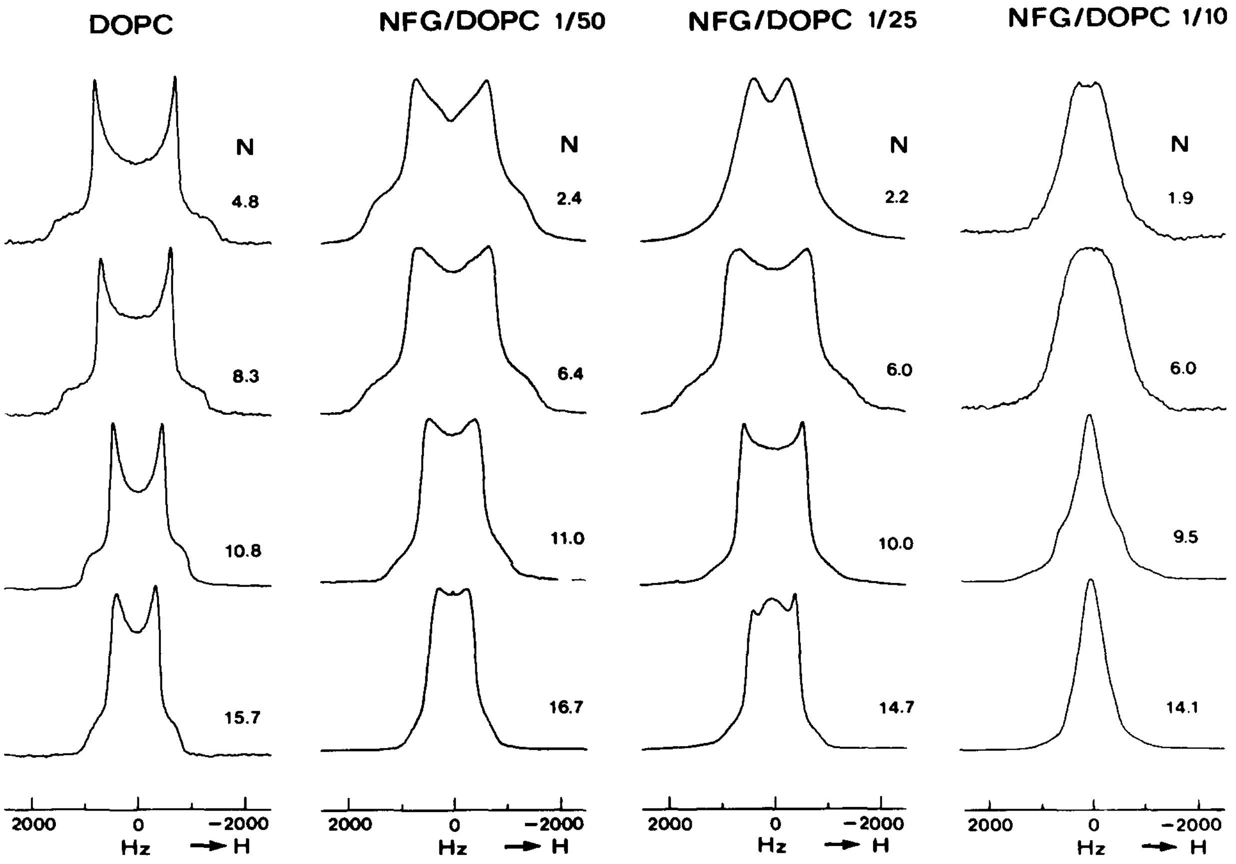

Fig. 8. 30.7 MHz ^{2}H-NMR spectra of DOPC/tryptophan-N-formylated gramicidin/^2H_2O samples. The molar ratios are as indicated in the figure.

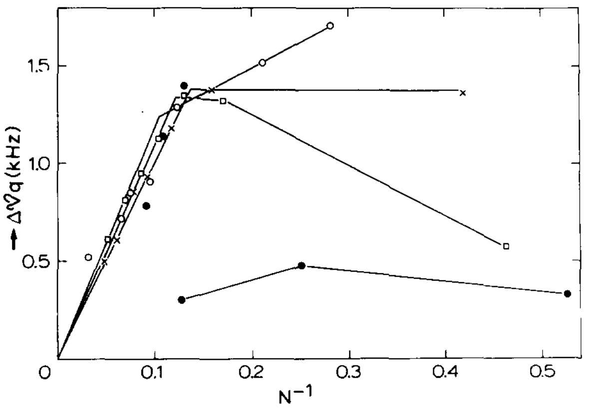

Fig. 9. Quadrupolar splitting ($\Delta\nu_q$) as a function of the ^2H_2O content for samples of tryptophan-N-formylated gramicidin and DOPC in molar ratios of 0 (○), 1 : 50 (×), 1 : 25 (□) and 1 : 10 (●).

At $N = 14.7$ also for the sample with a peptide/lipid ratio of 1 : 25 a two-component spectrum is observed. Tentatively, we interpret this as a result of the local formation of peptide-rich bilayers, due to sample heterogeneity.

The observation that two-component spectra in tryptophan-N-formylated gramicidin/ DOPC samples are found only at higher peptide content as compared to gramicidin A′ and that such spectra are not observed with ^{31}P-NMR, suggests that the different effects of the two peptides might be a result of differences in their aggregation behaviour in phosphatidylcholine bilayers as also indicated by the density centrifugation experiments.

Discussion

In the present study we investigated the role of the tryptophan-residues of gramicidin in the peptide-induced H_{II} phase formation in DOPC model membranes. The data clearly show, that gramicidin A′, gramicidin A and gramicidin C all affect the structural parameters of the lipid in a comparable way and induce an almost similar extent of H_{II} phase formation. Replacement of only one tryptophan by phenylalanine however, as in gramicidin B or in the synthetic [Phe^9]- and [Phe^{11}]-gramicidin analogs, leads to a marked reduction in H_{II} phase formation. When all four tryptophan residues are modified by means of formylation of the indole rings, the H_{II} phase inducing activity of the peptide is completely blocked, in agreement with previous observations [23]. How to explain these strong changes in behaviour of the peptide upon applying such minor modifications?

Let us consider the possibilities with respect to the molecular mechanism of gramicidin-induced H_{II} phase formation (for recent review, see Ref. 41). One of the propositions was, that the geometrical shape of the molecule could be the driving force for H_{II} phase formation. Due to the presence of four bulky tryptophan residues the peptide, at least when present in a β-helical single-stranded conformation, has a pronounced cone shape, by which it fits very well into structures with a high curvature, such as the H_{II} phase. However, none of the modifications involves a significant change in the size of the aromatic amino acid side chains. On the contrary, N-formylation of the tryptophans would rather be expected to increase the bulkiness and thus result in an even more pronounced cone shape, thus enhancing H_{II} phase formation. Therefore, besides the geometrical shape of the peptide there must be additional factors that enable gramicidin to form an H_{II} phase with DOPC. One such factor might be lipid headgroup dehydration. It was proposed that, by taking up water in preference to the lipid headgroup, gramicidin could promote the formation of an H_{II} phase [18]. However, the present data clearly show that tryptophan-*N*-formylated gramicidin has a similar dehydrating activity as gramicidin A′ at low water content. In addition, the visual observation that gramicidin/DOPC films upon hydration disperse less readily then DOPC, and in this exhibit a similar behaviour as H_{II} type of lipids, holds as well for tryptophan-*N*-formylated gramicidin/DOPC systems.

Another factor of importance for the mechanism of gramicidin-induced H_{II} phase formation is the observed self-association of gramicidin molecules, which was proposed [18,23], to result in the formation of ordered aggregates with a tendency to organize into tubular structures such as found in the H_{II} phase. The sucrose-density centrifugation experiments give us further insight into the peptide aggregation and phase separation processes.

Let us first examine the results obtained with the gramicidin A′/DOPC (1 : 10, molar ratio) sample, in which three bands were found at different densities (Fig. 5B). An important observation was, that for the fraction occurring at intermediate density a peptide to lipid molar ratio of 1 : 15 was measured, while at the same time it was established that the lipids in this fraction were organized in a lamellar phase. Interestingly, it was exactly at this molar ratio that DSC measurements [24] suggested the onset of an aggregation process. That this ratio of 1 : 15 indeed corresponds to the maximum solubility of gramicidin in the PC bilayer, is furthermore suggested by the sucrose density centrifugation experiments, which showed that increasing the gramicidin content of the sample to molar ratios higher than 1 : 10 does not lead to an increase in the peptide/lipid molar ratio of 1 : 15 found for the band at intermediate density, but rather to a decrease in its intensity and to an increase in the intensity of the higher density fraction, characterized as an H_{II} phase. For this latter fraction a minimum peptide/lipid ratio of 1 : 7 was found, independent of the initial gramicidin content. Thus, gramicidin can dissolve in the bilayer up to a molar ratio of 1 : 15 of peptide to lipid. Upon exceeding this ratio aggregation of gramicidin occurs, resulting in the formation of a hexagonal H_{II} phase, with a maximal efficiency of seven DOPC molecules per gramicidin monomer. The solubility of the peptide in the H_{II} phase, however, is much higher than 1 : 7 (gramicidin/lipid, molar ratio).

In apparent contrast to this scheme is the observation, that H_{II} phase formation occurs to some extent already at gramicidin/lipid ratios of 1 : 25 (see Fig. 3) for which the bilayer solubility is not exceeded. We interpret this to be the result of film heterogeneity.

Another observation in apparent contrast to the depicted scheme is the occurrence of a low density band ($d = 1.017$ g/ml) upon centrifugation of samples with a gramicidin/lipid ratio in the range of 1 : 50 to 1 : 10. This band was found to appear simultaneously with the high-density band, suggesting that in this concentration range H_{II} phase formation is accompanied by the formation of a gramicidin-poor phase. This can be understood by the following scheme. Let us consider a dry film, in which the gramicidin/lipid ratio is such, that the bilayer solubility of the peptide is exceeded but yet more lipid is present than can be accomodated in the H_{II} phase with gramicidin. Upon hydration of such a film the peptide will aggregate, resulting in the formation of an H_{II} phase with a minimum gramicidin/lipid ratio of 1 : 7 and a bilayer with a maximum peptide/lipid ratio of 1 : 15. We now propose, that the interface between this H_{II} phase and the bilayer forms a nucleation site at which gramicidin molecules can go over from the lamellar phase into the H_{II} phase, thereby leaving a gramicidin-poor bilayer. Such a mechanism would account for the additional low density band found in the centrifugation experiments and indicates that the peptide prefers organization in the H_{II} phase, rather than being dissolved in the bilayer.

The density centrifugation experiments thus confirm the importance of aggregation and phase separation for gramicidin-induced H_{II} phase formation and support the idea [18,23] that it is the aggregated peptide itself which prefers organization in the H_{II} phase. Similar experiments with gramicidins A, B and C and tryptophan-*N*-formylated gramicidin showed, that also in samples of these analogs with DOPC aggregation and phase-separation occurred. However, the sucrose density centrifugation experiments and the hydration study revealed differences in the aggregation behaviour of the various analogs, which seemed to correlate with differences in their H_{II} phase inducing activity. It is therefore likely to assume, that the extent of H_{II} phase formation is determined by the type of macroscopic aggregates that are formed, which in turn depends on the nature of the aromatic amino acid side chains of the peptide. We propose, that the type of macroscopic aggregates that are formed by the various analogs of gramicidin, depends on their three-dimensional structure, which may be determined by intra- as well as intermolecular stacking interactions between the aromatic amino acid residues. In view of the suggestion, that stacking interactions can be explained in terms of dipole-induced dipole interactions [42] we furthermore propose, that the different behaviour of the various gramicidin analogs is related to differences in the nature of the dipolemoment of tryptophan and its substitutes.

There are a number of indications that modification of one tryptophan residue indeed can result in a change in the conformation of gramicidin. Circular dichroism (CD) measurements revealed differences in conformational behaviour between gramicidin A and B in a methanolic solution and also upon heat-incorporation into lysoPC micelles [27]. Under both conditions the spectrum of [Phe^{9}]-gramicidin differed slightly from that of gramicidin B. That tryptophan-*N*-formylated gramicidin has a completely different conformational behaviour than the other gramicidin analogs was shown by preliminary CD measurements in the presence and absence of Cs^{+} (Killian, J.A., unpublished observations).

The differences in conformation upon tryptophan-substitution or modification do not only correlate well with the H_{II} phase inducing activity of the various analogs in DOPC model membranes, but they are also reflected in the channel characteristics of the peptide. For gramicidins A and C similar channel properties were found [43,44], whereas for gramicidin B [27,43,44] and also for the synthetic [Phe^{9}]-analog [27] a markedly decreased conductivity was measured. In addition, a recent study on the Tl^{+} binding properties of gramicidins A, B and C [29] again revealed similar behaviour of gramicidins A and C and a much stronger binding of the cation to gramicidin B.

That the tryptophans of gramicidin are essential to form conductive channels was shown by Busath and Waldbillig [22] who demonstrated an irreversible loss of channel activity after exposure of the tryptophan to ultraviolet light.

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