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The effect of changes in gramicidin conformation on bilayer lipid properties

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The effects of two different gramicidin conformations on lipid phase behaviour and dynamics are compared. Samples of chain-perdeuterated dimyristoylphosphatidylcholine containing gramicidin were first prepared with gramicidin in a state having a circular dichroism spectrum generally identified as corresponding to the non-channel conformation. The effects, on bilayer lipid properties, of gramicidin in this conformation were then determined using deuterium nuclear magnetic resonance measurements of acyl chain orientational order and transverse relaxation times as a function of temperature. These samples were then incubated at 65 °C to convert the gramicidin to a state with a circular dichroism spectrum of the type generally identified with the channel conformation. The nuclear magnetic resonance measurements were then repeated. In the gel phase, it was found that transverse relaxation time and chain orientational order of the lipid were insensitive to gramicidin conformation. In the liquid crystalline phase, gramicidin in the channel conformation was found to have a slightly larger effect on transverse relaxation and orientational order than gramicidin in the non-channel conformation. The perturbation of the phase behavior by gramicidin was found to be relatively insensitive to gramicidin conformation.

Introduction

The structure of the conducting channel form of gramicidin in the membrane has been the subject of many investigations. A variety of conformations have been proposed [1–3]. It is now generally accepted that the channel conformation is a dimer of single-stranded helices hydrogen bonded at the amino ends [4–10].

Gramicidin has been widely used in studies of lipid–protein interaction as a model for the bilayer-spanning portion of intrinsic membrane proteins. In particular, gramicidin has been found to have a strong effect on lipid bilayer phase behaviour [11–13]. A number of other lipid-protein studies employing gramicidin have been summarized elsewhere [12].

Recent studies using circular dichroism (CD) and nuclear magnetic resonance (NMR) have examined the effects of solvent history and thermal history on the tendency of gramicidin to adopt either the channel or the non-channel conformation in bilayers [14–16]. The ability to prepare bilayers containing a particular gramicidin conformation has been exploited in a number of comparative studies [17,18]. Sawyer et al. [20] have recently demonstrated that the single channel properties of gramicidin show no solvent-history dependence.

Recently, deuterium (^{2}H) NMR has been used to examine the effect of gramicidin on the phase behavior [12] and slow motions [21] of perdeuterated dimyristoylphosphatidylcholine (DMPC-d_{54}) in bilayers containing gramicidin. The present study was undertaken to determine the extent to which gramicidin-induced changes in lipid properties depend on the polypeptide conformation. Bilayer samples were prepared containing gramicidin with a CD spectrum which has previously been associated with the non-channel state [14,15]. ^{2}H-NMR spectral moments and transverse relaxation times, T_{2e}, were obtained as a function of temperature for samples in this state. The gramicidin in the sample was then converted, by incubation, to a

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different state having CD spectrum which has previously been identified with the channel state [14,15]. The NMR experiments were then repeated. Using the same samples to obtain data with the polypeptide in two different conformations was essential in order to prevent conformation-dependent effects from being masked by the large concentration-dependent effects.

Materials and Methods

Myristic acid was perdeuterated using the method of Hsiao et al. [22] and used in the synthesis of DMPC-d_{54} following the method of Gupta et al. [23]. Gramicidin D (Dubos) was purchased from Sigma (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.). Two samples of DMPC-d_{54} containing gramicidin were prepared. In one sample, the polypeptide and lipid were codissolved in trifluoroethanol (Eastman Organic). This sample had a gramicidin concentration of 3.8 mol%. A second sample, with a gramicidin concentration of 8.2 mol%, was prepared using chloroform/methanol (2:1, v/v) as the cosolubilizing solvent. The solvents were removed quickly once the lipid and polypeptide were in solution and both samples were found to give rise to CD spectra consistent with the gramicidin being in the non-channel conformation. The dependence of gramicidin conformation on solvent and thermal history has been systematically investigated elsewhere [14–16].

The samples were dried to thin films using rotary evaporation followed by overnight pumping at room temperature. Samples with dry weights of about 35 mg were placed into 8 mm NMR tubes, hydrated with about 250 μl of 50 mM phosphate buffer at a pH of 7.0, and stirred with a fine glass rod. A portion of each dried sample was dissolved in ethanol and the gramicidin concentration determined using phosphorus analysis and absorbance at 280 nm to obtain lipid and gramicidin amounts, respectively. A known quantity of gramicidin in ethanol was used as a standard for the absorbance measurements.

^{2}H-NMR spectra were obtained using the quadrupole echo sequence [24] with a pulse separation of 35 μs and $\pi/2$ pulse lengths between 2 and 3 μs. Transverse relaxation was studied using quadrupole echo spectra collected for a series of pulse separations ranging from 25 μs to 185 μs. The initial echo decay was used to determine the transverse relaxation rate averaged over the chain, $\langle T_{2e}^{-1} \rangle$. Details of the NMR spectrometer have been reported elsewhere [21].

Following preparation of the samples containing gramicidin in the 'non-channel' conformation, NMR measurements were performed over a series of temperatures beginning at 32 °C and descending to 3 °C. Each sample was then incubated for at least 12 h at 65 °C in order to convert the gramicidin to the 'channel' conformation. The NMR experiments were then repeated on the sample. Gramicidin conformations were confirmed by inspection of circular dichroism spectra made prior to a given series of NMR experiments.

Samples used for circular dichroism measurements were obtained by withdrawing 15-μl portions from the NMR samples. The preparation of these samples for CD was modeled on the method described by Lo-Grasso et al. [15]. They were first diluted in 1.2 ml of distilled water. The aqueous mixtures were then dispersed by sonication using a Branson Sonicator (model 185) set to a power level of 5 W with a duty cycle of 50% over a period of 5 min. The sample was kept on ice during this procedure to minimize gramicidin conversion. Following sonication, the samples were centrifuged in an Eppendorf centrifuge to remove titanium particles at $12000 \times g_{av}$. The supernatant was collected and the protein concentration determined by the method of Bradford [25] using weighed gramicidin to prepare the standard curve. CD spectra were recorded on a Jasco J-500A spectropolarimeter (Jasco Inc., Easton, MD) using a 0.1 cm cell at 25 °C.

Results and Discussion

The primary goal of this study was to examine the extent to which the effect of gramicidin on the properties of lipids in the bilayer changes with gramicidin conformation. To separate the influence of gramicidin conformation from that of gramicidin concentration, NMR measurements were made on a sample containing gramicidin in one conformation and then, after conversion of the gramicidin to a different conformation, repeated on the same sample. The NMR data are thus correlated to the type of CD spectrum observed. Accordingly, the comparisons to be drawn are between NMR data sets corresponding to different CD spectra obtained from a single sample having a particular gramicidin concentration. The entire protocol was repeated with a second sample having a different gramicidin concentration. While we have no direct evidence regarding the functional state of gramicidin in these experiments, it is convenient to categorize the states, indicated by CD in this experiment, as 'non-channel' and 'channel', based on comparison with spectra presented elsewhere [14,15].

Non-channel to channel conversion is effectively irreversible within the bilayer [14]. The observance of a non-channel-like CD spectrum from gramicidin in a sample prepared with TFE, presumably the result of allowing only a short equilibration time in the solvent [14], provided an opportunity for a comparative study of the type reported here. A second sample, made using a more conventional solvent for preparation of gramicidin in the non-channel conformation, was used to confirm results obtained with the first and to test for

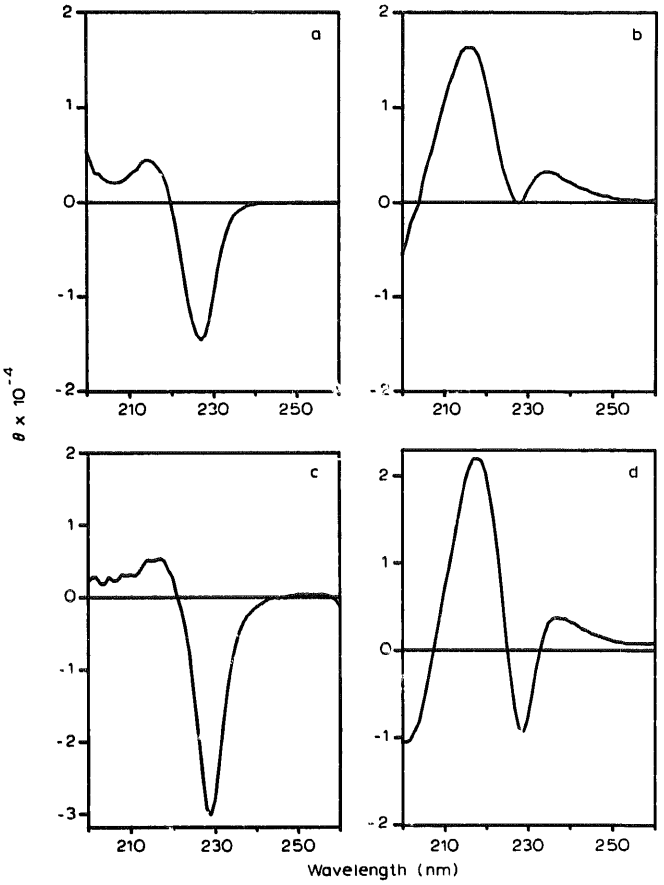

Fig. 1. (a) Circular dichroism spectrum for 3.8 mol% gramicidin in DMPC-d_{54} prior to the sample being incubated. (b) Circular dichroism spectrum for the same sample following incubation at 65 °C for 12 h. (c) Circular dichroism spectrum for 8.2 mol% gramicidin in DMPC-d_{54} prior to the sample being incubated. (d) Circular dichroism spectrum for the same sample following incubation at 65 °C for 24 h.

conversion from 'non-channel' to 'channel' conformations during the NMR experiments. In the following discussion, the sample with 3.8 mol% gramicidin, prepared from trifluoroethanol, is referred to as sample 1 and the sample with 8.2 mol% gramicidin, prepared from chloroform/methanol (2:1, v/v) is referred to as sample 2. Each sample was subjected to the same series of experiments and gave rise, independently, to qualitatively similar conclusions regarding the relative effects of the channel and non-channel conformations of gramicidin on bilayer properties.

Figs. 1a and 1c show the circular dichroism spectra, obtained prior to the first NMR experiments, for samples 1 and 2, respectively. The negative ellipticity just below 230 nm is consistent with the presence of the non-channel conformation based on comparison with the spectra presented by LoGrasso et al. [15]. We note that there is a difference in the magnitude of the negative ellipticity in Figs. 1a and 1b. This may reflect differences in the amount of optical flattening and scattering in the two samples or may indicate that the proportion of 'non-channel' conformation in sample 1 was somewhat less. A portion of sample 2 was extracted following the first NMR measurements, but prior to incubation, in order to check for changes in conformation during the first series of NMR experiments. The CD spectrum was still indicative of gramicidin in the 'non-channel' conformation indicating that little conversion from 'non-channel' to 'channel' conformation occurred over the course of the NMR measurements.

Fig. 1b shows the circular dichroism spectrum of sample 1 following incubation at 65 °C for about 12 h. Fig. 1d shows the circular dichroism spectrum of sample 2 following incubation at 65 °C for about 24 h. Following incubation, sample 1 appears to have converted almost completely to the 'channel' conformation. For sample 2, the spectrum, following incubation at 65 °C, is dominated by the channel conformation but the persistence of negative ellipticity just below 230 nm suggests that conversion may be less complete. In both cases, however, it is clear that a significant change in gramicidin conformation has occurred.

Fig. 2 shows representative normalized ^{2}H-NMR spectra at 28 °C. Spectrum (A), obtained with pure DMPC-d_{54}, displays the superposition of axially symmetric powder patterns normally associated with chain-perdeuterated lipid in the liquid crystalline phase. The splitting between the sharp features in a given doublet, $\delta\nu_q$, is proportional to the orientational order parameters, $S_{CD} = \frac{1}{2}\langle 3\cos^2\theta - 1\rangle$, where θ is, in effect, the angle between the carbon-deuterium bond axis and the bilayer normal and the average is taken over the motions of the carbon-deuterium bond. Spectra (B) and (C) were obtained from sample 1 prior to, and following, incubation at 65 °C. They thus correspond to samples containing gramicidin in the 'non-channel' and 'channel' conformations respectively. Comparison of spectra (B) and (C) with the pure lipid spectrum (A) illustrates the gramicidin-induced increase in orientational order along the chain. The difference spectrum (D) compares the influences of the two gramicidin conformations at the same concentration. Due to the narrowness of the central doublets, due to chain terminal methyl groups, small differences in splitting can give rise to large spikes in the difference spectrum and such features in the center of the difference spectrum are not surprising. The most notable features in the difference spectrum are the 'dips' near the ±17 kHz 'plateau' region of the spectrum corresponding to deuterons near the headgroup end of the chain. It would appear that, in the liquid crystalline state of this sample, the 'channel' conformation of gramicidin induces slightly more ordering in the chain that the 'non-channel' conformation. This difference is particularly apparent at the end of the chain contributing to the 'plateau' region of the spectrum.

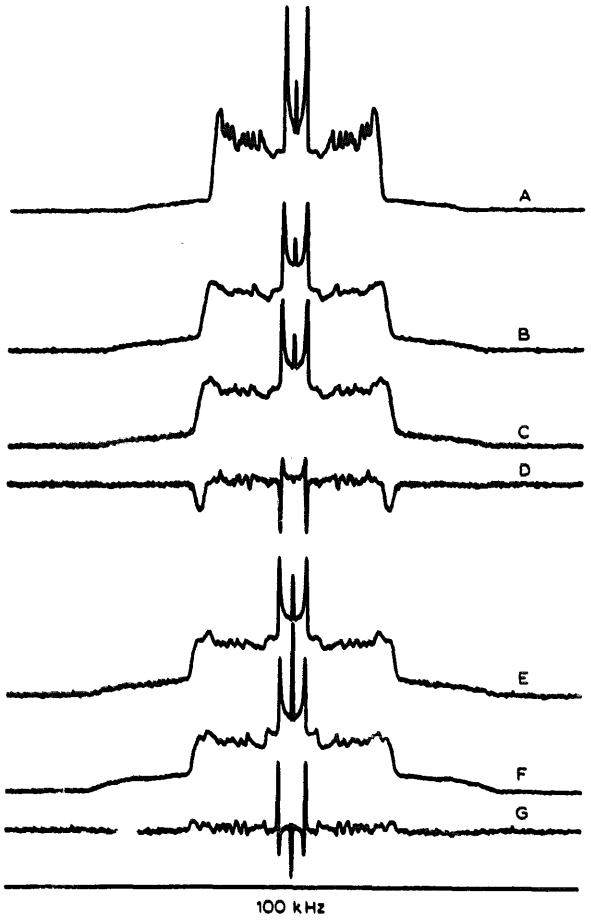

Fig. 2. ^{2}H-NMR spectra at 28 °C. (A) Pure DMPC-d_{54}. (B) 3.8 mol% gramicidin in DMPC-d_{54} before incubation. The corresponding CD spectrum is given in Fig. 1(a). (C) The same sample as in spectrum (B) but following incubation at 65 °C. The corresponding CD spectrum is given in Fig. 1(b). (D) The difference spectrum obtained by subtracting (C) from (B). (E) 8.2 mol% gramicidin in DMPC-d_{54} before incubation. The corresponding CD spectrum is given in Fig. 1(c). (F) the same sample as in spectrum (D) but following incubation at 65 °C. The corresponding CD spectrum is given in Fig. 1(d). (G) the difference spectrum obtained by subtracting (F) from (E).

Spectra (E) and (F) were obtained from sample 2 prior to, and following, incubation. Again, by comparison with spectrum (A), gramicidin is seen to increase ordering of hydrocarbon chains in the bilayer. While the difference spectrum (G) is not flat, the absence of significant dips indicates that any additional ordering caused by the change in gramicidin conformation is small for this sample. It is possible that for samples containing a higher gramicidin concentration, and thus already displaying a large increase in chain order, any additional increase in ordering associated with the observed change in gramicidin conformation is relatively small.

Fig. 3 shows normalized ^{2}H-NMR spectra at 12 °C. Spectrum (A) was obtained using pure DMPC-d_{54} and is characteristic of chain-perdeuterated lipid in the gel phase. Spectra (B) and (C) were obtained from sample 1 prior to, and following, incubation. The flatness of the difference spectrum (D) suggests that chain orientational order in the gel phase is insensitive to differences between the observed conformations of gramicidin. Spectra (E), (F) and (G) illustrate the same point for sample 2.

The first spectral moment, M_1, of the ^{2}H-NMR spectrum for perdeuterated lipid chains is proportional to $\langle S_{CD} \rangle$ where the average extends over all deuterons on the chain. An indication of the nature of the phase change can be obtained by observing the behavior of M_1 through the region of the liquid crystal to gel transition. Earlier work [12], using samples prepared in ethanol and thus presumably containing gramicidin in the non-channel conformation [14,16], indicated that the presence of gramicidin causes the first order gel to liquid crystal transition to be replaced by a continuous phase change showing no discontinuity in M_1. Figs.

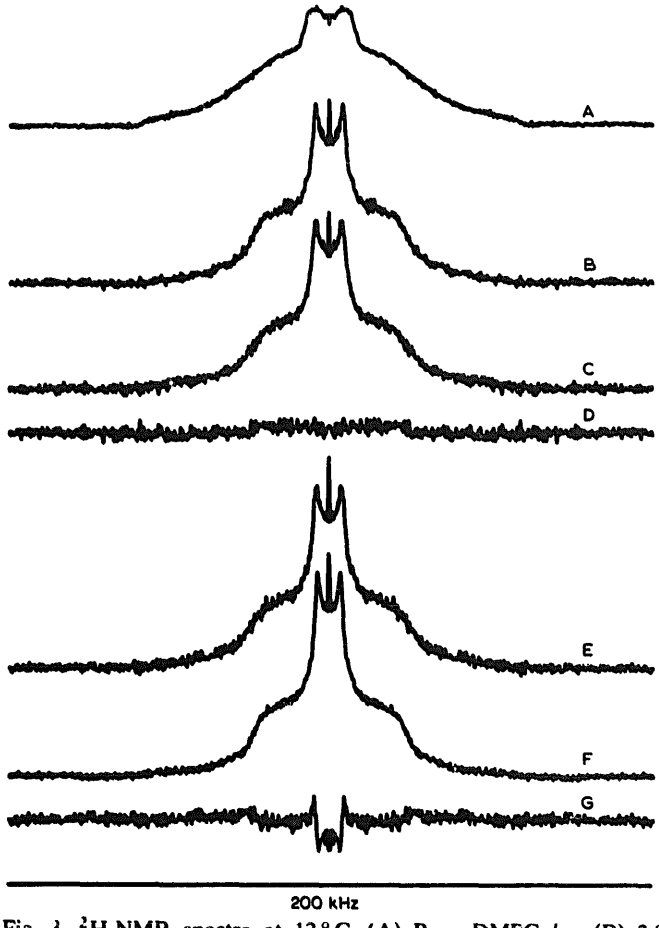

Fig. 3. ^{2}H-NMR spectra at 12 °C. (A) Pure DMPC-d_{54}. (B) 3.8 mol% gramicidin in DMPC-d_{54} before incubation. The corresponding CD spectrum is given in Fig. 1(a). (C) The same sample as in spectrum (B) but following incubation at 65 °C. The corresponding CD spectrum is given in Fig. 1(b). (D) The difference spectrum obtained by subtracting (C) from (B). (E) 8.2 mol% gramicidin in DMPC-d_{54} before incubation. The corresponding CD spectrum is given in Fig. 1(c). (F) the same sample as in spectrum (D) but following incubation at 65 °C. The corresponding CD spectrum is given in Fig. 1(d). (G) the difference spectrum obtained by subtracting (F) from (C).

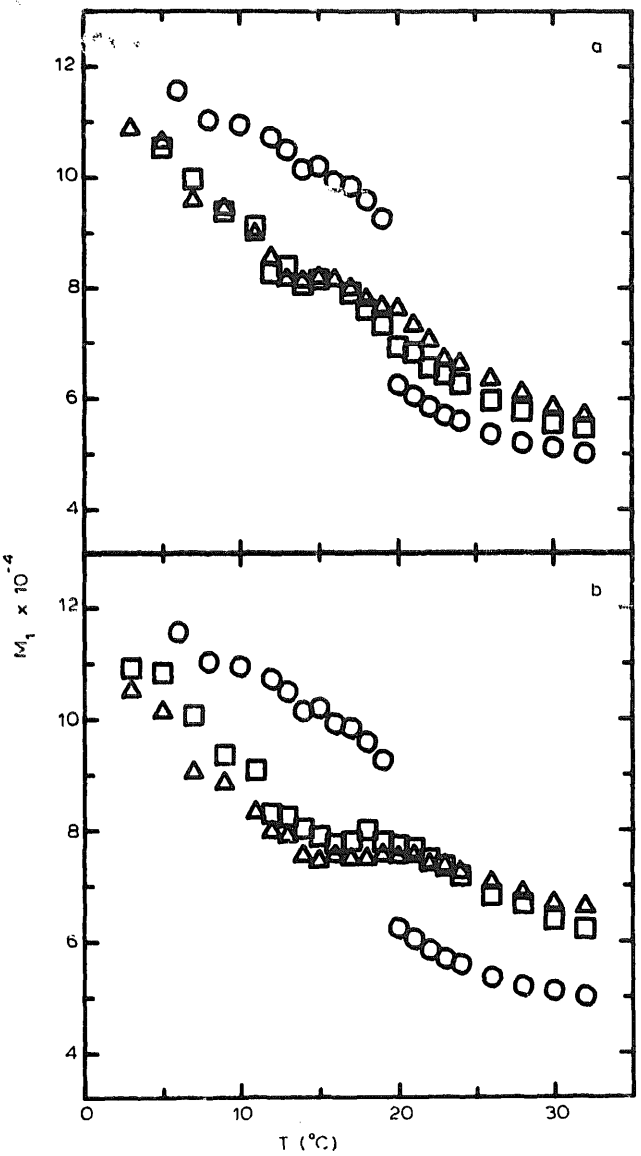

Fig. 4. (a) M_1 versus T for 3.8 mol% gramicidin in DMPC-d_{54}. □, Before incubation. The corresponding CD spectrum is given in Fig. 1(a). △, Following incubation at 65 °C for 12 h. The corresponding CD spectrum is given in Fig. 1(b). ○, Pure DMPC-d_{54}. (b) M_1 versus T for 8.2 mol% gramicidin in DMPC-d_{54}. □, Before incubation. The corresponding CD spectrum is given in Fig. 1(c). △, Following incubation at 65 °C for 24 h. The corresponding CD spectrum is given in Fig. 1(d). ○, Pure DMPC-d_{54}.

4(a) and (b) show the ^{2}H-NMR first spectral moment, M_1, as a function of temperature for sample 1 and sample 2, respectively. The squares in Fig. 4(a) and in Fig. 4(b) were obtained from the samples with gramicidin in the 'non-channel' conformation. The triangles were obtained after the samples had been incubated at 65 °C to change the gramicidin conformation. The circles in each figure are for pure DMPC-d_{54} and are presented for comparison. Both gramicidin conformations are seen to induce effectively the same large departure from pure lipid phase behavior as was reported earlier [12]. The lipid–polypeptide interaction which gives rise to the strong effect of gramicidin on bilayer phase behavior does not appear to display a specific sensitivity to details of the gramicidin conformation.

While the two conformations of gramicidin have qualitatively the same effect on bilayer phase behavior, they do appear to have slightly different effects on chain orientational order, and thus M_1, in the liquid crystalline phase. This small difference would likely be masked by uncertainties in sample composition if samples containing different conformations were prepared separately. For both samples in the liquid crystalline phase, the 'channel' conformation of gramicidin gives rise to a slightly larger M_1, implying slightly more chain ordering, than the 'non-channel' conformation. This suggests that the effect, on M_1, of gramicidin in the 'channel' conformation is equivalent to that of a slightly higher concentration of gramicidin in the 'non-channel' conformation.

Intrinsic proteins and polypeptides also affect slow lipid motions in the bilayer. This effect can be observed by measuring the transverse relaxation time, T_{2e}, for deuterated lipid chains using the quadrupole echo pulse sequence. The effect of gramicidin on transverse relaxation was previously examined using samples prepared with chloroform/methanol (2:1, v/v) [21] and thus expected to contain gramicidin in the non-channel conformation. Fig. 5 (a) and (b) show the transverse relaxation time as a function of temperature for the samples 1 and 2, respectively. The symbols have the same meaning as in Fig. 4. In the liquid crystalline phase, the two conformations of gramicidin have similar effects. Again, the 'channel' conformation appears to have more effect than the 'non-channel' conformation in the liquid crystalline phase. There is no significant difference between the influence of the two conformations on transverse relaxation in the gel phase.

By converting the gramicidin conformation within individual samples, the influence of gramicidin conformation has been separated from the effects of gramicidin concentration thus avoiding complications associated with uncertainty in sample composition. The results presented here suggest that the effect of gramicidin on bilayer phase behavior is largely independent of gramicidin conformation. Similarly, the effect of gramicidin on chain orientational order and slow motion in the gel phase in insensitive to gramicidin conformation. In the liquid crystalline phase, gramicidin in the channel conformation causes slightly more ordering of the lipid chains and slightly more enhancement of the slow motions responsible for transverse relaxation of the chain deuterons than gramicidin in the non-channel conformation. A comparison of the M_1 results for sample 1 and sample 2 suggest that the relative difference between the effects of gramicidin, in the two conformations, on orientational order may be slightly larger at smaller gramicidin concentration.

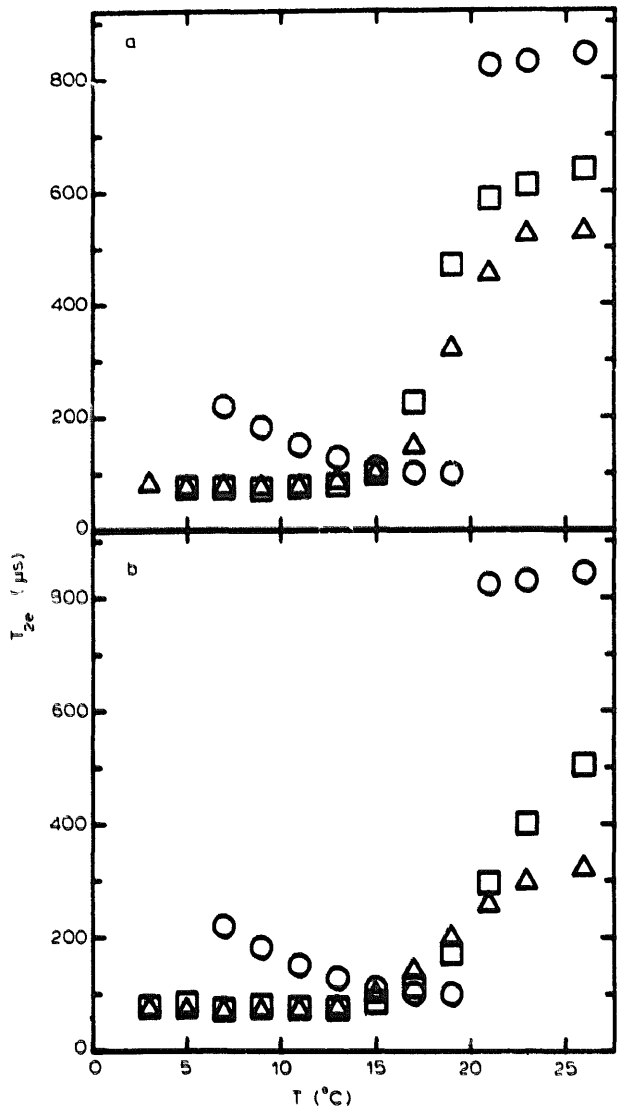

Fig. 5. (a) T_{2e} versus temperature for 3.8 mol% gramicidin in DMPC-d_{54}. □, Before incubation. The corresponding CD spectrum is given in Fig. 1(a). △, Following incubation at 65 °C for 12 h. The corresponding CD spectrum is given in Fig. 1(b). ○, Pure DMPC-d_{54}. (b) T_{2e} versus temperature for 8.2 mol% gramicidin in DMPC-d_{54}. □, Before incubation. The corresponding CD spectrum is given in Fig. 1(c). △, Following incubation at 65 °C for 24 h. The corresponding CD spectrum is given in Fig. 1(d). ○, Pure DMPC-d_{54}.

It is interesting to consider the results presented here in light of a recent report by Lafleur et al. [26] that the distribution of orientational order parameters along the acyl chain is strongly correlated to the magnitudes of the order parameters. This observation was interpreted as indicating that the correlation length for changes in order parameter along the acyl chain is greater than the separation between adjacent carbon atoms so that localized interactions with the chains give rise to perturbations spread along the entire chain. The observation, in the current work, that changing the conformation of gramicidin from 'non-channel' to 'channel' has relatively little effect on a range of bilayer properties supports the suggestion that the acyl chains are largely insensitive to details of the interaction with embedded molecules like gramicidin and respond primarily to the average perturbation.

Acknowledgements

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