For a conformationally heterogeneous system of dimers, the same equation will apply with k_2^+ replaced by a weighted sum of the forward rate constants for the individual dimerizations.

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A ^{13}C Nuclear Magnetic Resonance Study of Gramicidin A in Monomer and Dimer Forms†

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ABSTRACT: Carbon-13 chemical shifts and spin–lattice relaxation times (T_1) have been determined for the random coil and helical states of the linear pentadecapeptide, gramicidin A. Assignments of the resonances were accomplished with the aid of model compounds and gramicidin A analogs from which various amino acids were deleted. The T_1 measurements show that, in DMSO-d_6, the gramicidin A peptide backbone undergoes slowest motion near the center of the molecule (τ_R = 5.0 nsec), while the N-terminal residue moves more rapidly (τ_R = 0.7 nsec). In methanol-d_4, a solvent in which it has been shown that gramicidin A exists predominantly as helical dimer (Veatch *et al.*, 1974, Veatch and Blout, 1974), the T_1 measurements show that motion in the backbone of the molecule has been greatly reduced (τ_R = 30 nsec). This extreme decrease in motion in the peptide backbone going from random coil to dimer form is consistent with the proposed (Veatch *et al.*, 1974) double helical dimer model.

Recently, the first examples of ^{13}C nuclear magnetic resonance (nmr) studies of complex peptides and proteins have been reported. Potentially, chemical shift data, carbon–hydrogen coupling data, and relaxation data should all contribute useful information relating to the structure of the molecules studied. Chemical shift data should be sensitive to local chemical and electronic environmental factors. Although there is information on the chemical shifts of peptides and proteins in the literature (Voelter *et al.*, 1971; Deslauriers *et al.*, 1972; Glushko *et al.*, 1972; Gurd *et al.*, 1972; Jung *et al.*, 1972; Zimmer *et al.*, 1972; Dorman and Bovey, 1973), with minor exceptions it remains largely uninterpretable in terms of specific conformational features. Coupling data potentially contain information regarding angular relationships between atoms. The necessary calibration of coupling constants with bond angles is now beginning to appear (Lemieux *et al.*, 1972). Successful application of relaxation phenomenon to illuminate structural features, such as flexibility and segmental motion of molecules, is becoming more commonplace (Allerhand *et al.*, 1971a,b; Glushko *et al.*, 1972; Komorski and Allerhand, 1972; Levine *et al.*, 1972; Torchia and Piez, 1973; Allerhand and Oldfield, 1973; Brewer *et al.*, 1973; Chiên and Wise, 1973; Torchia and Lyerla, 1974).

The linear peptide antibiotic, gramicidin A, posed structural questions that ^{13}C nmr could help to answer. The amino acid sequence of gramicidin A is given in Table II. All of the amino acid residues are hydrophobic. Although the peptide contains 15 amino acids, it contains only five chemically different amino acids. In addition, if Gly can be designated as a potential D residue, the amino acids in the sequence alternate between L and D configuration. The two preceding papers (Veatch *et al.*, 1974, Veatch and Blout, 1974) have demonstrated that, at high concentration in nonpolar solvents, gramicidin A exists as a family of inter-

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converting aggregates, all of which may be dimers. The circular dichroism spectra of the major class of these isolated conformational species strongly suggests that they are helical. Various models for helical dimers were presented and evaluated.

The ^{13}C nmr work presented here provides information relating to the structure of these aggregated species and about the structure of the monomer which is favored in more polar solvents. For this purpose we have studied the ^{13}C nmr spectra of gramicidin A in dimethyl-d_6 sulfoxide, a solvent in which the molecule is probably a monomer having little or no secondary structure, and in methanol-d_4, a solvent which supports the dimer structures (Veatch *et al.*, 1974, Veatch and Blout, 1974). Resonances in the ^{13}C spectra have been assigned (where possible) to particular carbon atoms, and spin–lattice relaxation measurements were made in both solvents.

Materials and Methods

Gramicidin A mixture (obtained from S. B. Penick and Co. and Nutritional Biochemicals Co.) was crystallized from ethanol. *N*-Acetylamino acid amides were obtained from Fox Chemical Co.; *N*-formylethanolamine and *N*-acetylethanolamine were obtained from Eastman Organic Chemicals. Preparation and characterization of chain-shortened gramicidin A analogs have been described elsewhere (Miroshnikow *et al.*, 1973).

^{13}C spectra were obtained on a Varian XL-100-15 spectrometer equipped with Fourier transform and interfaced with a Varian 620i 16K computer and a Computer Operations tapedeck. Other ^{13}C spectra were obtained on a Varian CFT-20 spectrometer equipped with a 16K computer and a Brucker HX-270 spectrometer equipped with a 16K computer. XL-100 spectra were obtained at 5000-Hz sweep width, while CFT-20 spectra were obtained at 4000-Hz sweep width and HX-270 spectra at 15,000-Hz sweep width. With the XL-100 12-mm sample tubes were used with 3-ml samples in cases where the sample availability was not limiting. In cases where sample was limited, a Teflon spacer was placed in the bottom of the tube to fill space not seen by the receiver coil, and a Teflon vortex plug was used above the sample. This configuration reduced the sample volume required to approximately 1.6 ml. With the CFT-20 and HX-270 10-mm sample tubes were used with the Teflon spacer and vortex plug reducing the necessary sample size to 1.0 ml.

All spectra were obtained using a 90° pulse which had a pulse width of 36–37 μsec on the XL-100, 19 μsec on the CFT-20, and 15 μsec on the HX-270. Acquisition times were typically 0.8 sec on the XL-100 and 1.02 sec on the CFT-20. In some cases a pulse delay of up to 1.2 sec was used. The spectra of gramicidin A and analogs required accumulation of 3,000–30,000 FID's. In all cases a mild "sensitivity enhancement" was applied to the accumulated FID before Fourier transformation. The time constants for this digital filtering were 0.4 sec on the XL-100 and 0.5 sec on the CFT-20.

Chemical shifts are expressed as ppm upfield from CS_2, references by measuring the position of the CS_2 resonance in a concentric capillary tube with the instrument locked on the deuterium of each of the deuterated solvents used.

The measurement of T_1's was accomplished by the usual (180°-τ-90°) pulse sequence (Vold *et al.*, 1968), interspaced with a pulse delay (PD), so that the sequence was acutally (PD-180°-τ-90°)$_n$, with PD $\geq$ 5 times the longest T_1. Approximately 18 hr were required to acquire data at five τ values for the determination of T_1. During such a time period, it is expected that there may be some drift in instrumental conditions, such as field homogeneity and probe temperature, as well as other less obvious parameters. If, for example, all the FID's for the first τ value were accumulated at the beginning of the 18-hr time period and all of the FID's for the last τ value were accumulated at the end of the last time period, there would be a good chance that some drift in parameters would occur, making the two points not strictly comparable and introducing unnecessary error into the determination of T_1. To avoid this problem, as well as to allow for automated operation during T_1 determination, a computer program, described below, was utilized.

This program depends on the interfaced tapedeck for storage of FID's during operation. It allows one to take n FID's at τ value, τ_A, where n is a small number compared to the total number of FID's to be taken at τ_A; then records them on tape, proceeds to take n FID's at τ value, τ_B, records them on tape, etc. After taking n FID's at τ value, τ_X, and recording them on tape, the program starts over. It takes n more FID's at τ value, τ_A, but this time adds them on tape to the FID's already taken at τ value, τ_A. It then continues through all τ values, adding all FID τ_A's to FID τ_A's FID τ_B's to FID τ_B's, etc., until interrupted at which point it continues through τ_X and halts. The resulting accumulated FID's are then weighted with the sensitivity enhancement function and Fourier transformed.

The T_1 values were calculated by entering peak intensities of each τ value into a nonlinear, two parameter, least-squares fit program. This program gave the best value of T_1 as well as the standard deviation. The differences in Overhauser enhancement in the spectra in methanol-d_4 and in DMSO-d_6 are apparent from inspection of Figure 1a and c. However, accurate measurement of the NOE in these solvents was accomplished by employing a gated decoupler on the CFT-20, where the decoupler was on during accumulation and off during a delay between pulses. The spectrum obtained from this procedure was compared to a spectrum obtained with all conditions identical except that the decoupler remained on during both accumulation and delay. "Automatic intensity" was employed to make the vertical scaling comparable between the two cases. Peak intensities were then compared directly to evaluate the NOE.

Results and Discussion

We began our ^{13}C nmr investigation with the knowledge that in DMSO-d_6 gramicidin A is 70–80% in monomeric form at 100 mg/ml (Veatch and Blout, 1974), but bearing in mind that earlier workers proposed that gramicidin A existed as a structured dimer in this solvent (Glickson *et al.*, 1972; Urry *et al.*, 1972). In methanol-d_4 it had been shown that gramicidin A exists as a mixture of four slowly interconverting dimers present in unequal amounts (Veatch and Blout, 1974). At 100 mg/ml in methanol-d_4 gramicidin A is 80% in dimeric form (Veatch and Blout, 1974), and thus any spectrum is a spectrum of a mixture of at least four conformationally different dimers. In order to distinguish as many separate resonances as possible, ^{13}C spectra were obtained both in DMSO-d_6 and in methanol-d_4 at 20.0, 25.16, and 67.88 MHz. While the 20.0- and 25.16-MHz spectra were essentially identical, several resonances which were overlapping at these frequencies were resolved at

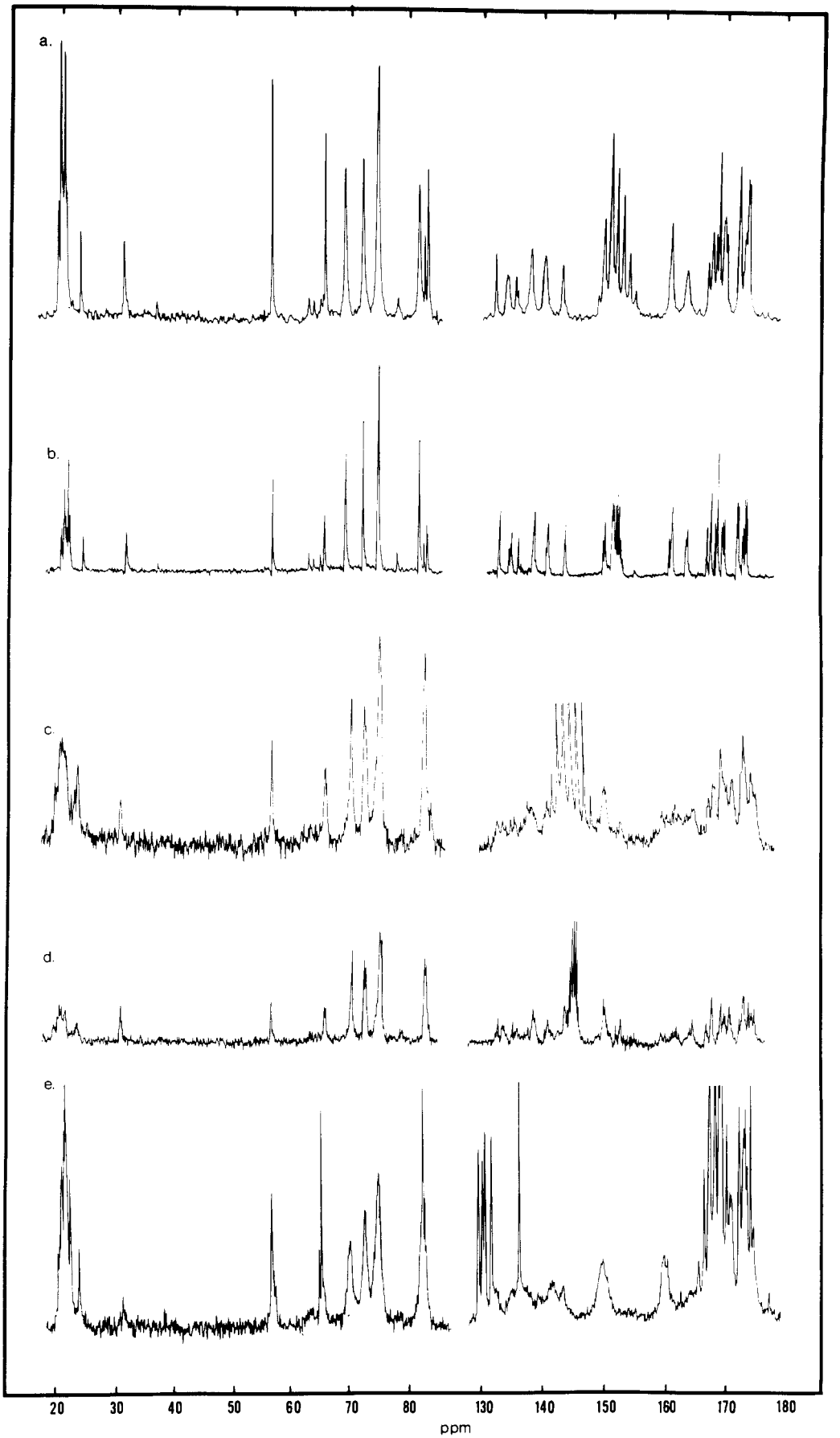
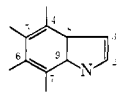


FIGURE 1: (a) ^{13}C nmr spectrum of gramicidin in DMSO-d_6 at 20 MHz. Approximately 30,000 transients were accumulated. The once-crystallized gramicidin mixture was dissolved to a concentration of 100 mg/ml. The downfield portion of the spectrum is enlarged 1.6 times more than the upfield portion. (b) ^{13}C nmr spectrum of gramicidin in DMSO-d_6 at 67.88 MHz. Approximately 32,000 transients were accumulated. The sample was the same used in Figure 1a. (c) ^{13}C nmr spectrum of gramicidin in methanol-d_4 at 20 MHz. Approximately 30,000 transients were accumulated. The sample was made at a concentration of 100 mg/ml of the once-crystallized gramicidin mixture. The downfield portion of the spectrum is enlarged 1.6 times more than the upfield portion. (d) ^{13}C nmr spectrum of gramicidin in methanol-d_4 at 67.88 MHz. Approximately 32,000 transients were accumulated. The sample was the same used in Figure 1c. (e) ^{13}C nmr of dimeric species 3 (Veatch *et al.*, 1974) in 2-propanol-d_8 at 20 MHz. Approximately 30,000 transients were accumulated. Crystalline dimer species 3 was dissolved at room temperature in 2-propanol-d_8 to a concentration of 150 mg/ml. The downfield portion of the spectrum is enlarged 1.3 times more than the upfield portion. Chemical shifts are relative to external CS_2 at 0 ppm for all spectra.

TABLE I: ^{13}C Nmr Chemical Shift Values[a] of Model Compounds in DMSO-d_6 and Methanol-d_4.

Compound	DMSO-d_6				Methanol-d_4			
	C_α	C_β	C_γ	C_δ	C_α	C_β	C_γ	C_δ
N-Acetyl-Gly-amide	150.4				151.2 *obscured by solvent*			
N-Acetyl-Ala-amide	144.4	174.1			176.2			
N-Acetyl-Val-amide	134.9	162.2	173.0 174.4		134.6	162.8	174.2 176.1	
N-Acetyl-Leu-amide	141.5	151.4	168.0	169.4 170.8	142.6	152.3	168.4	171.0 172.5
N-Acetyl-Trp-amide	139.0	164.4			139.1	165.4		
N-Formylethanolamine	152.3	132.6			152.7	132.9		



	DMSO-d_6	Methanol-d_4
C_9	56.2	56.4
C_8	65.2	65.6
$C_{2,4,5,6,7}$	68.9	70.0
	71.5	72.0
	74.0	74.6
	81.0	82.2
C_3	81.8	83.3

[a] All chemical shift values reported are reproducible to at least ±0.05 ppm.

67.88 MHz. In addition, a ^{13}C spectrum of a single dimeric species was obtained; this conformational species, designated "species 3" in the preceding paper (Veatch *et al.*, 1974), is conformationally stable in 2-propanol-d_8 over a time period necessary for obtaining a ^{13}C nmr spectra.

Chemical Shifts in DMSO-d_6. In DMSO-d_6 assignment of ^{13}C resonances was accomplished in a first approximation through use of model molecules consisting of one structural unit; *e.g.*, *N*-acetylamino acid amides, *N*-formylethanolamine, and *N*-acetylethanolamine. These chemical shifts are tabulated in Table I. In order to confirm the assignments and to evaluate the effect of subtle sequence changes on chemical shifts, the ^{13}C spectra of eight synthetic chain-shortened analogs of gramicidin A were measured. In these analogs several amino acid residues, varying from two to eight, were deleted from the sequence. These peptides (chain-shortened gramicidin A analogs), therefore, varied in length from 7 to 13 residues. The sequences of these peptides and their relationship to gramicidin A are indicated in Table II. The numbers in the names of the analogs refer to the residues which are omitted from the gramicidin A sequence.

Resonances were found in the gramicidin A spectrum which corresponded to the resonances of model molecules, usually within ±0.3 ppm, with the largest difference between peptide and model amino acid being 0.7 ppm. The ^{13}C spectrum of gramicidin A in DMSO-d_6 is shown in Figure 1a and b at both 20.0 and 67.88 MHz. In the 67.88-MHz spectrum several resonances were resolved in the upfield region which were not resolved at 20.0 and 25.16 MHz. Table III shows a comparison of the chemical shifts of the corresponding resonances in the model molecules and in gramicidin A.

To verify and refine the chemical shifts assignments which were based on model molecules, we undertook analysis and comparison of the spectra of the eight chain-shortened analogs (Figure 2) with that of gramicidin A in DMSO-d_6. This solvent minimizes chemical shift differences due to conformational features of the peptide by favoring a random conformation. Differences in chemical

TABLE II: Sequences and Amino Acid Composition of Chain-Shortened Analogs (from Miroshnikov *et al.*, 1973).

		L 1	D 2	L 3	D 4	L 5	D 6	L 7	D 8	L 9	D 10	L 11	D 12	L 13	D 14	L 15					
																	Gly	Ala	Val	Leu	Trp
Valine-gramicidin A	Form-V	G	A	L	A	V	V	V	T	L	T	L	T	L	T-EA		1	2	4	4	4
GD(9-10)	Form-V	G	A	L	A	V	V	V	-	-	-	-	-	-	T	L	T	L	T-EA	1	2	4	3	3
GD(9-12)	Form-V	G	A	L	A	V	V	V	-	-	-	-	-	-	-	-	-	T	L	T-EA	1	2	4	2	2
GD(6-9)	Form-V	G	A	L	A	-	-	-	-	-	-	-	-	-	L	T	L	T	L	T-EA	1	2	1	4	3
GD(3-6)	Form-V	G	-	-	-	-	-	-	-	-	-	-	V	V	T	L	T	L	T	L	T-EA	1	0	3	3	4
GD(6-11)	Form-V	G	A	L	A	-	-	-	-	-	-	-	-	-	-	-	-	L	T	L	T-EA	1	2	1	3	2
GD(3-8)	Form-V	G	-	-	-	-	-	-	-	-	-	-	-	-	T	L	T	L	T	L	T-EA	1	0	1	3	4
GD(6-13)	Form-V	G	A	L	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	T-EA	1	2	1	2	1
GD(3-10)	Form-V	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	L	T	L	T-EA	1	0	1	2	3

TABLE III: Comparison of Gramicidin and Model Compound ^{13}C Nmr Chemical Shift Values[a] in DMSO-d_6.

Carbon	Residue	ppm: Model Compound	ppm: Gramicidin
Carbonyl	*N*-Formyl group	30.5	31.0
Indole C_9	Trp	56.2	56.1
C_8		65.2	65.1
$C_{2,4,5,6,7}$		68.9 71.5 74.0 81.0	68.5 71.5 74.1 81.0
C_3		81.8	82.0 82.5 82.7
CH_2OH	Ethanolamine	132.6	132.5
$C_\alpha H$	Val	134.9	134.2, 134.6 135.8
	Trp	139.0	138.5
	Leu	141.5	140.8, 140.7, 140.6
	Ala	144.4	143.7
$C_\alpha H_2$	Gly	150.4	150.3
	Ethanolamine	152.3	150.5
$C_\beta H_2$	Leu	151.4	151.4
$C_\beta H$	Val	162.2	161.6 162.0
$C_\beta H_2$	Trp	164.4	164.6 164.5
$C_\gamma H$	Leu	168.0	168.0 168.6
$C_{\delta_1} H_3$	Leu	169.4	169.3 169.7
$C_{\delta_2} H_3$	Leu	170.8	170.4 170.7 170.9
$C_{\gamma_1} H_3$	Val	173.0	173.3 173.1
$C_{\gamma_2} H_3$	Val	174.4	174.3 174.6
$C_\beta H_3$	Ala	174.1	174.2 174.0

[a] Relative to external CS_2.

shift from the expected values are then the result of other factors, such as differences in the chemical nature of adjacent groups. It is of value to discuss illustrative reference amino acid analogs by type.

Tryptophan. Of the four L-tryptophans in gramicidin A, two have D-leucine both preceding and following them in the sequence, one has D-valine preceding and D-leucine following, and one has D-leucine preceding and ethanolamine following. These situations exemplify where differences in chemical shift could result from differences in the nature of adjacent groups if the conformations are random. While all of the rest of the indole carbon resonances appear as single lines and are within 0.3 ppm of their model compound value, the indole C_3 carbon near 81.8 ppm appears as three nonequivalent resonances in gramicidin A. As noted above, there are three types of L-tryptophan as distinguished by the chemical nature of adjacent groups. From Figure 2 it may be seen that GD(6-13) (h) with one L-tryptophan shows one C_3 resonance, while GD(9-12) (c) and GD(6-11) (f) with two L-tryptophans have two resonances. In GD(9-10) (b) and GD(3-10) (i), which have three tryptophans, there are three resonances observed with the upfield pair barely resolved. In GD(6-9) (d) the single upfield resonance is of double height, probably due to an unresolved pair of peaks. For gramicidin A and the analogs with four L-tryptophans (GD(3-6) (e) and GD(3-8) (g)), at most three resonances are resolved.

Valine. The four valines in gramicidin A are divided in the sequence into two groups. The first is a single L-valine-1 positioned between the *N*-formyl group and the glycine. The second region is the trivaline sequence, L-Val-D-Val-L-Val, in position numbers 6, 7, and 8. There are three peaks in the gramicidin A spectrum which correspond to the C_α resonance in the valine model compound (134.9 ppm). One of these peaks is broadened (134.6 ppm), the others sharp (134.2 and 135.8 ppm). In the spectra of the analogs (GD(6-9) (d), GD(6-11) (f), GD(3-8) (g), GD(6-13) (h), and GD(3-10) (i) (Figure 2)), it is seen that those

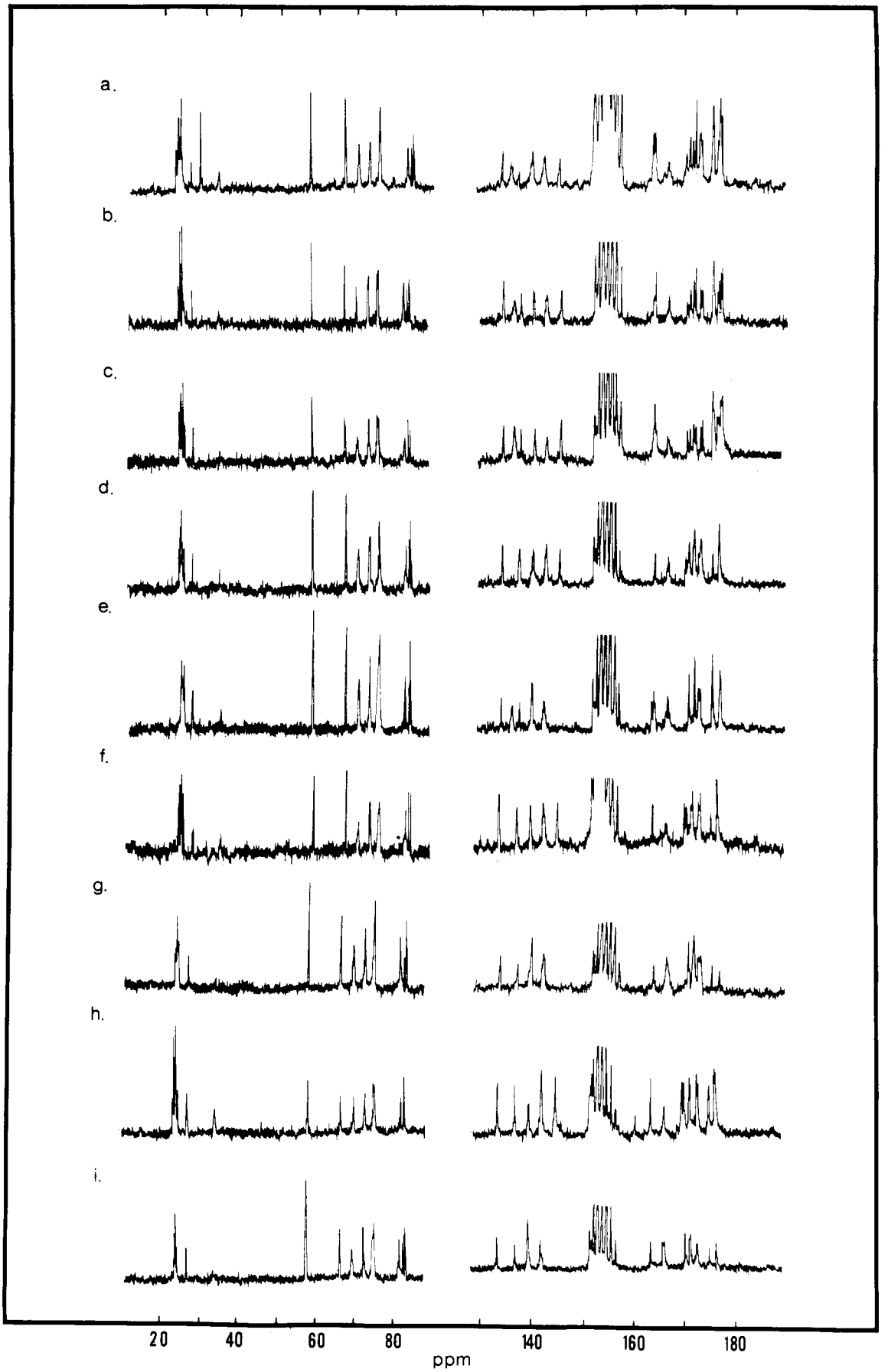


FIGURE 2: ^{13}C nmr spectra of chain-shortened analogs in DMSO-d_6 at 25.16 MHz. About 50 mg of each of the chain-shortened analogs were dissolved in about 1.5 ml for each sample. Typically, about 18,000 transients were accumulated. The gramicidin spectrum is included in (a) for comparison. (a) Gramicidin mixture; (b) GD(9-10); (c) GD(9-12); (d) GD(6-9); (e) GD(3-6); (f) GD(6-11); (g) GD(3-8); (h) GD(6-13); (i) GD(3-10). Chemical shifts are relative to external CS_2 at 0 ppm.

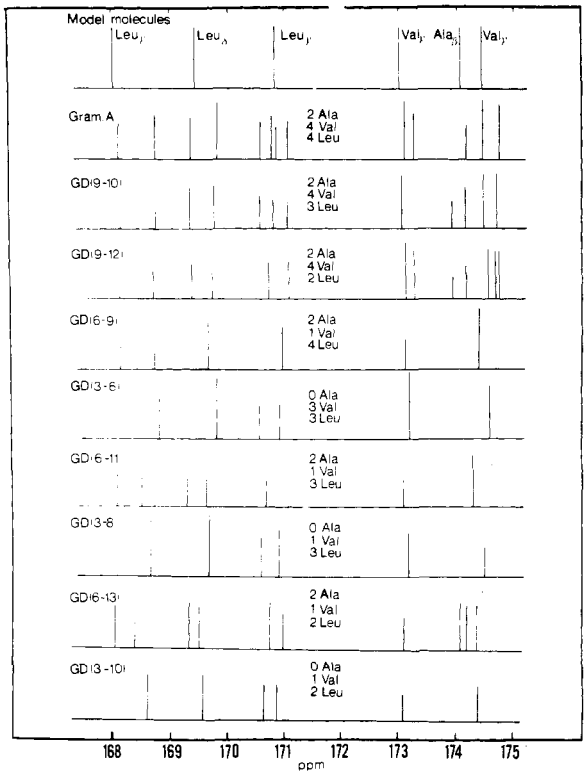


FIGURE 3: Line diagram of expanded upfield ^{13}C nmr spectra of gramicidin A, chain-shortened analogs, and model molecules. This diagram allows a comparison of the chemical shift values for the major peaks of the leucine C_γ's and C_δ's, the valine C_γ's, and the alanine C_β's. The chemical shifts are in ppm relative to external CS_2. The relative height of the peaks in a given spectrum is proportional to the length of the lines.

which lack the trivaline sequence also lack the broader downfield peak but retain the sharp peak at 135.8 ppm. This sharp upfield peak at 135.8 ppm, therefore, belongs to the *N*-formylvaline.

The methyl resonances of leucine, valine, and alanine in gramicidin A, in model molecules, and in the chain-shortened analogs (all in DMSO-d_6) are presented diagrammatically in Figure 3. It is seen that marked differences exist in chemical shifts between analogs. Multiple resonances often occur corresponding to an amino acid being represented with multiple frequency in the molecule. These chemical shift differences and multiple resonances are indicative of subtle differences in environments of the methyl carbon atoms.

Chemical Shifts in Methanol-d_4. Chemical shift assignments were made to a first approximation (Table IV) by the same method used for assignments in DMSO-d_6. That is, spectra of model molecules, such as *N*-acetylamino acid amides and *N*-formylethanolamide, were taken in methanol-d_4. These chemical shifts are tabulated in Table I. However, with few exceptions, it was not possible to make simple and unequivocal assignments of resonances in methanol-d_4, as the nearly exact correspondence between model molecules and peptide, which was noted in DMSO-d_6, does not exist in methanol-d_4. Instead, broad resonances and scattered multiple resonances were found. The spectra at 20.0 and 67.88 MHz are shown in Figure 1c and d. In the spectrum in methanol-d_4 at 67.88 MHz, many resonances are resolved which were unresolved at the lower frequencies. The situation in methanol-d_4 solution is quite different from that in DMSO-d_6 solution. The largely homogeneous chemical shifts in DMSO-d_6 have been replaced by marked chemical shift nonequivalence. It is interesting, however, to compare by type some of the resonances of the component amino acids of gramicidin A in the two solvents.

L-*Tryptophan.* The indole carbon resonances appear as single, but broadened, lines. The indole C_3 carbon resonance near 82.5 ppm no longer appears as a multiple resonance. This appearance may be due to broadened lines overlapping. Four of the five protonated carbons of the indole ring, which appear as single resonances in DMSO-d_6, break into double resonances in methanol-d_4 at 67.88 MHz. The fifth, at 69.9 ppm, remains as a single line. The β-carbon resonances are assigned from the model (165.4 ppm) to be a series of several peaks ranging from 164.4 to 166.0 ppm.

Valine. It is difficult to distinguish between the N-terminal valine and the central trivaline group in methanol-d_4. The C_α resonance in the *N*-acetyl-L-valinamide occurs at 134.6 ppm. In gramicidin A a group of seven resonances are distinguishable at 67.88 MHz: 133.7, 134.1, 134.3, 135.8, 136.1, 136.3, and 136.6 ppm. The valine C_β occurs at 162.8 in the *N*-acetyl-L-valineamide, and a group of eight peaks occur in the gramicidin A between 160.7 and 163.6 ppm.

Spectra of "Crystal Species 3" in 2-Propanol-d_8. The dimer obtained from crystals, referred to in the previous paper (Veatch *et al.*, 1974) as "crystal species 3," was found to be stable for at least 18 hr when dissolved in 2-propanol-d_8 at 25°. A solution of this material in 2-propanol was made and immediately placed in the CFT-20 spectrometer with probe temperature of 25°. A spectrum was obtained within 6.5 hr, a time in which little of the material would have interconverted. This spectrum (Figure 1e), then, is of a single conformational dimer, suggested to be an antiparallel-β double helix (Veatch *et al.*, 1974) as compared to the spectrum of gramicidin A in methanol-d_4 which is of a mixture of conformationally different dimers.

Comparison of the α-carbon region in the two alcohol solvents shows that, while the spectra do differ, a similar broadness and lack of sharply defined peaks is a common feature to both. The broad peaks centered at approximately 150 ppm are similar in the two cases. However, despite the region which is obscured by solvent (166.0–171.8 ppm), the comparison of the spectra in the aliphatic side chain region (159–175 ppm) reveals that the spectrum in 2-propanol is sharper and more simple. This indicates that at least in this region the marked chemical shift heterogeneity in the methanol spectra has as its origin—at least in part—the multiple forms in which the dimer is present in methanol.

Spin–Lattice Relaxation Studies. Spin-lattice relaxation time (T_1) measurements offer a useful method for assessing molecular motion. The relationship between T_1 for protonated ^{13}C nuclei and molecular rotation is derived by assuming that the relaxation mechanism is dominated by ^{13}C–^1H dipole–dipole interaction (Doddrell *et al.*, 1972). In the case where assignment of resonances to particular carbon atoms is possible, as in gramicidin A, it is possible to estimate the magnitude of molecular motion at particular positions in the molecule. In addition, measurement of T_1's of gramicidin A in DMSO-d_6 and in methanol-d_4 affords information concerning the motion of various carbon atoms in the molecule in both the monomer and dimer form.

The relationship between τ_R and T_1 (and T_2) has been presented graphically in the literature (Doddrell *et al.*, 1972). These theoretical relations are shown in Figure 4 for a 23.5-kG field and are plotted for singly protonated ^{13}C

TABLE IV: ^{13}C Nmr Assignments for Gramicidin A in Methanol-d_4.

Assignment			ppm	
			Gramicidin	Model Compound
Carbonyls			20.6–21.3	
Formyl			30.5	
Indole		C_9	56.1	56.4
		C_8	65.1, 65.4	65.6
		$C_{2,4,5,6,7}$	69.9	70.0
			72.0, 72.2	72.0
			74.6, 74.9	74.6
			82.2	82.2
		C_3	83.0	
			83.1	83.3
CH_2OH			132.9	132.9
C_α	Val		133.7, 134.0	
			135.3, 135.7	
			136.1, 136.2	134.6
			136.6	
	Trp		137.3, 137.9, 138.7, 139.1	139.1
	Leu		140.6, 141.2–142.0	142.6
	Ala		144.1	
	Gly + EA		150.6, 151.0	151.2
C_β Leu			152.9, 153.6	152.3
C_β Val			160.6, 161.4, 161.8, 162.1, 162.5, 163.1, 163.1, 163.5	162.8
C_β Trp			165.2, 165.4, 165.8	165.4
C_γ Leu			168.1, 169.0	168.4
C_{δ_1} Leu			170.6, 171.2, 171.3, 172.0, 172.3, 173.7	171.0
C_{δ_2} Leu				172.5
C_β Ala			174.4, 174.6, 175.3, 175.7, 175.9	176.2
C_{γ_1} Val				174.2
C_{γ_2} Val			176.4	176.1

nuclei in a rigid molecule undergoing isotropic rotational diffusion with correlation time, τ_R. A carbon-bearing N proton would have a T_1 value of $1/N$ times that of a comparable carbon with one proton; therefore, the number of protons times T_1, NT_1, is used to express the relaxation data for protonated carbons. Inspection of Figure 4 reveals that for every T_1 value, except the minimum, two possible values of τ_R may be assigned from the theoretical curve. In order to assign a correlation time, it is necessary to choose between the two possible values. There are, fortunately, two independent ways of choosing between the two τ values (Doddrell *et al.*, 1972; Allerhand and Oldfield, 1973). The nuclear Overhauser enhancement (NOE) of the intensity of a signal is dependent on the correlation time, τ_R, varying at 23.5 kG from 3.0 for τ_R of 2×10^{-10} sec to 1.1 for τ_R of 2×10^{-7} sec. Also, T_2, as is seen in Figure 4, is single valued with respect to τ_R. Values of T_2 may be measured directly from the line width, as peak width at half-height is equal to $(\pi T_2)^{-1}$. Caution must be exercised in measuring T_2 from resonances which, while appearing to be single peaks, represent more than one chemically nearly equivalent carbon atom. Such resonances may be broadened by slight chemical shift nonequivalence. In such a case the line width measured would be greater than the true line width. The τ_R value determined by measurement of T_2 from the line width would then not agree with τ_R determined from T_1 and NOE. If resonances are not broadened by slight chemical shift nonequivalence, the τ_R value from the measured line width should be similar to the one obtained from measurement of the NOE. This τ_R value should agree with one of the two τ_R values derived from NT_1.

Nuclei which are relaxed only through molecular tumbling, and not at all through segmental motion, will have the slowest rotational motion and the smallest τ_R. Depending on which side of the minimum of the T_1 curve τ_R occurs, large T_1 values could represent either rapid or slow motion. If we are on the left-hand branch of the T_1 curve (Figure 4), the atoms with slowest motion will have the shortest T_1 values; however, if we are on the right-hand branch of the T_1 curve, the atoms with the slowest motion will have the longest T_1 values. Any additional motions due to flexibility or segmental motion will serve to increase T_1 on the left-hand branch and to decrease T_1 on the right-hand branch.

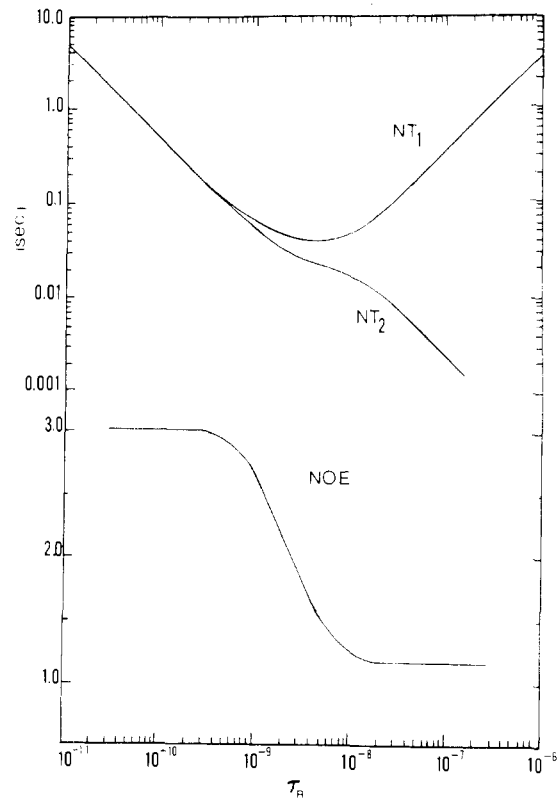


FIGURE 4: Plot of NT_1 and NT_2 and NOE *vs.* τ_R for 23.5 kG field (^{13}C frequency of 25.16 MHz).

In order to derive τ_R from a treatment such as that represented by Figure 4, it is necessary that the molecules being studied tumble isotropically. The dimensions of the proposed double helical models vary between approximately 20 and 25 Å in diameter, including side chains, and between 25 and 32 Å in length (Veatch *et al.*, 1974). A dimer with such a ratio of length to diameter is nearly symmetric in shape and, therefore, will be undergoing isotropic rotational diffusion.

Examination of NT_1 values for various carbon atoms of gramicidin A in DMSO-d_6 and methanol-d_4 shows that the T_1 values for given atoms in the two solvents are not greatly different (Table V). As discussed above, however, atoms with similar T_1 values do not necessarily have correspondingly similar correlation times. NOE, and possibly T_2, may be used to determine which of the τ_R values defined by T_1 is the actual τ_R value. The appearance of the spectra of the molecule in the two different solvents gives the distinct, and correct, impression that the lines in the spectrum in methanol-d_4 are much broader (shorter T_2) than corresponding lines in DMSO-d_6. The NOE was measured (Table VI) for gramicidin A in methanol-d_4 and in DMSO-d_6. The values at 20 MHz found in methanol-d_4 varied for different resonances between values of 1.05 and 1.2. In DMSO-d_6 the values varied between 2.1 and 2.5. A value of 1.1 corresponds to the minimum value of NOE and indicates that τ_R is no faster than 15 nsec. A NOE of 2.3 corresponds to τ_R of 1.5 nsec. In addition, line widths measured in the two spectra (Table VI) show that the T_1 values of gramicidin A in DMSO-d_6 fall on the left-hand branch of the curve (Figure 4), while the T_1 values of gramicidin A in methanol-d_4 fall on the right-hand branch of the curve. Note that the τ_R values derived from T_1 and those derived from T_2 agree relatively well.

The result of this analysis, as the NOE demonstrates and T_2 confirms, is that the values of T_1 of gramicidin A in DMSO-d_6 fall on the left-hand branch of the T_1 curve with τ_R values ranging from 0.7 to 5 nsec, while the values in methanol-d_4 fall on the right-hand branch of the T_1 curve with τ_R values ranging from 14 to 20 nsec.

The T_1 values of the α-carbon atoms are of particular interest because these atoms reflect the mobility of the peptide backbone of the molecule. In the structure in DMSO-d_6 the trivaline sequence at the middle of the molecule has a T_1 value of 41 msec which corresponds to the absolute minimum value of the theoretical curve. Other α-carbon atoms on either side of the trivaline sequence have T_1 values of approximately 50 msec, except for the valine in position number 1 which has a value twice that of the internal valines (80 msec). Thus, the α carbons of the trivaline sequence have a τ_R of about 5 nsec, while the alanine, tryptophan, and leucine residues, all of which are closer to the ends of the chain, have τ_R's of approximately 2 nsec. The α carbon of the N-terminal valine has a τ_R of about 1 nsec.

The T_1 values of gramicidin A in methanol-d_4, although similar to those in DMSO-d_6, fall on the other arm of the T_1 curve corresponding to much slower τ_R's. The T_1 values for all the α-carbon atoms in methanol-d_4 are identical (within experimental error) and have an average T_1 of 60 msec. A 60-msec T_1 value on the right-hand branch of the curve corresponds to a τ_R of 20 nsec.

To compare quantitatively the τ_R's in methanol-d_4 with those in DMSO-d_6 and with values from the literature (of molecules in water), one may take into account the viscosity of the solvents. The viscosity of methanol is 0.60 cP (20°) and of DMSO-d_6 is 1.98 cP (25°). In order to compare the τ_R values in these solvents, we normalize them to water (Table VII). The normalized value of the trivaline sequence in DMSO-d_6 is 2.5 nsec, while the normalized value in methanol-d_4 is 25 nsec. The normalized values of the other α-carbon atoms in DMSO-d_6 range from 0.35 to 1 nsec, while the normalized values of these α-carbon atoms in methanol-d_4 are about 25 nsec.

The observation that the normalized τ_R values of α-carbon atoms in gramicidin A change by about an order of magnitude going from DMSO-d_6 to methanol-d_4 has bearing on the structure of the molecule in both solvents. The values of τ_R in DMSO-d_6 when compared to τ_R values of peptide random coils establish that in DMSO-d_6 the flexibility of the peptide chain is similar to that of random coil peptides. Other investigations of gramicidin A (Veatch *et al.*, 1974, Veatch and Blout, 1974) have suggested that in methanol-d_4 gramicidin A exists as a family of interconverting dimers. Circular dichroism measurements (Veatch *et al.*, 1974) on isolated conformational species indicate that the major class of these dimeric species is helical. The large increase in τ_R of the α-carbon atoms in going from DMSO-d_6 to methanol-d_4 supports these data as the proposed dimer structures would be expected to be highly rigid.

It is useful to compare the normalized τ_R values (*vide supra*), normalized to water, to some τ_R values of other peptides. The correlation times, τ_R, of the α-carbon atoms in poly(L-proline) and poly(L-Pro-Gly) in random coil form in water were found to be 0.3–0.6 nsec. The samples were found to be undergoing approximately isotropic reorientation (Torchia and Lyerla, 1974). In a study on a homogeneous collagen fragment containing 36 amino acids (Torchia *et al.*, 1974), a τ_R is found for carbons at the center of the chain of approximately 0.6 nsec when the molecule is in

TABLE V: ^{13}C T_1 Values of Protonated Carbons for Gramicidin in DMSO-d_6 and Methanol-d_4 at 25.16 MHz.

	DMSO-d_6		Methanol-d_4	
^{13}C Assignment	ppm	T_1 ± SD (msec)	ppm	T_1 ± SD (msec)
Formyl carbonyl	31.0	50 ± 20	30.5	60 ± 10
Indole CH	68.5	50 ± 5	69.9	65 ± 7
	71.5	48 ± 6	72.1	62 ± 4
	74.1	48 ± 4	74.7	56 ± 3
	81.0		82.2	71 ± 8
CH_2OH	132.5	140 ± 10	132.9	100 ± 30
C_α Val 6, 7, 8	134.3	41 ± 4	133.8–135.8	54 ± 9
1	135.8	80 ± 14		
Trp	138.5	48 ± 8	137.8–139.6	48 ± 7
Leu	140.8	48 ± 6	141.0–141.8	56 ± 5
Ala	143.7	50 ± 4		
$C_\beta H_2$ Leu + $C_\alpha H_2$ Gly, EA			149.3–150.6	48 ± 2
$C_\beta H$ Val	162.0	87 ± 10		64 ± 4
				68 ± 9
			161.3–163.5	85 ± 15
				86 ± 8
				61 ± 6
$C_\beta H_2$ Trp	164.6	30 ± 4		36 ± 10
			165.2–166.0	30 ± 4
				38 ± 7
$C_\gamma H$ Leu 4	168.0	99 ± 2		106 ± 11
10, 12, 14	168.6	87 ± 6	168.2–169.6	110 ± 7
				82 ± 6
$C_{\delta_1}H_3$ Leu 4	169.3	230 ± 30	170.6–171.0	280 ± 30
10, 12, 14	169.7			
$C_{\delta_2}H_3$ Leu	170.9	220 ± 30	171.6–172.6	250 ± 10
$C_{\gamma_1}H_3$ Val	173.1	220 ± 10		
$C_{\gamma_2}H_3$ Val	174.5	230 ± 20	176.2	220 ± 50
			176.4	230 ± 20
$C_\beta H_3$ Ala			176.5	210 ± 10

the random coil form and from 50 to 200 nsec for the native helical structure. Anisotropic reorientation of this highly elongated helical molecule makes comparison with the gramicidin A analysis complicated. In a study on poly(γ-benzyl-L-glutamate), Allerhand and Oldfield (1973) find a τ_R for the random coil α-carbon atoms of 1 nsec and a τ_R for these same carbon atoms in the helical form to be 24 nsec for a polypeptide with the same number of residues as a gramicidin A dimer. However, the length of the α-helix of this molecule (approximately 45 Å) makes it longer by a factor of 2 than any of the gramicidin A double helix models and, therefore, more susceptible to anisotropic reorientation. The normalized values of τ_R for gramicidin A in DMSO-d_6 of 0.35–2.5 nsec (monomer random coil) and in methanol-d_4 of 25 nsec (helical dimers) are consistent with the previous observations for τ_R values of polypeptide molecules in the random coil and helical states.

Correlation times of 24 nsec for the α-helical form of

TABLE VI: Line Widths and NOE Measurements in DMSO-d_6 and Methanol-d_4.

	DMSO-d_6	Methanol-d_4
τ_R calculated from T_1	0.9×10^{-9} sec[b]	
	5.0×10^{-9} sec[c]	15.0×10^{-9} sec[d]
NOE (range over all resonances)	2.1–2.5	1.05–1.2
τ_R from NOE (calculated from average value)	1.5×10^{-9} sec	15×10^{-9} or greater
Line width	5.5 Hz	17 Hz
τ_R calculated from T_2 indole protonated carbon[a]	1.0×10^{-9} sec	13×10^{-9} sec

[a] Resonance located at 69.9 ppm in methanol-d_4 and at 68.5 ppm in DMSO-d_6. [b] From N-terminal valine α-carbon resonance. [c] From the central trivaline α-carbon resonance. [d] From average of all the α-carbon resonances.

TABLE VII: Viscosity Normalized τ_R Values of α-Carbon Atoms τ_R(nsec).

	DMSO-d_6	Water Normalized	Methanol-d_4	Water Normalized
Trivaline	5 ± 0.8	2.5 ± 0.4	16 ± 3	27 ± 5
Valine-1	0.7 ± 0.3	0.35 ± 0.15		
Leucine	2 ± 0.2	1 ± 0.1	16 ± 3	27 ± 5
Tryptophan	2 ± 0.2	1 ± 0.1	14 ± 4	23 ± 7
Alanine	2 ± 0.2	1 ± 0.1		

poly(γ-benzyl-L-glumate) and 25 nsec for a structured gramicidin A dimer, at least intuitively, seem much too large for molecules of their size and shape and rigidity. Indeed, calculation of the correlation time expected for helical dimers results in smaller τ_R's.[1] The difference between the expected smaller values and the values determined experimentally can partially be explained by extensive solvation of the helical peptides. At the high concentrations necessary for these studies (100 mg/ml for gramicidin A in methanol-d_4), the viscosity of the solution is somewhat higher than that of pure solvent. This factor would serve to lower the normalized τ_R values.

Comparison of the correlation times of gramicidin A in DMSO-d_6 and methanol-d_4 with the literature cited above establishes that the random coil in DMSO-d_6 is transformed into a much more rigid structure in methanol-d_4. This more rigid structure, which was shown by other investigations to be helical and dimeric (Veatch *et al.*, 1974, Veatch and Blout, 1974), has a correlation time consistent with that of other established helical polypeptides. The magnitude of the change in correlation times, corresponding to the transition from random coil to helical dimer forms, is suggestive of a highly rigid structure, such as the family of double helical gramicidin A dimers proposed elsewhere (Veatch *et al.*, 1974) or other helical dimer structures.

Conclusion

The ^{13}C chemical shift and relaxation studies reveal much about the structure of gramicidin A in solution. In DMSO-d_6 the chemical shifts of the various carbon atoms fall very close to the chemical shifts of the model amino acid amides. Any deviations from this generalization occur in situations where there are clear differences in the chemical environment of given atoms; *e.g.*, the C_α of the *N*-formylvaline is clearly different from the C_α of the central trivalyl sequence. The close correspondence of the chemical shifts in gramicidin A to those of the model compounds indicate an averaging of environments through relatively rapid rotations around most bonds. This conclusion is borne out by relaxation studies which show that the correlation time behavior of the gramicidin A peptide backbone in DMSO-d_6 is similar to that of a peptide random chain.

In methanol-d_4, in contrast to the behavior in DMSO-d_6, the close correspondence of gramicidin A chemical shifts to those of model molecules is no longer observed. The spectra of the mixture of gramicidin A dimers shows extensive chemical shift heterogeneity. Part of this chemical shift heterogeneity can be ascribed to the conformational differences between dimer types. This is demonstrated by the fact that the spectrum of "species 3" in 2-propanol-d_8 is a more simple spectrum. However, some of the heterogeneity may be ascribed to carbon atoms residing in chemically equivalent, but magnetically nonequivalent, environments. This magnetic nonequivalence would result from nonaveraging among conformationally possible states in the relatively rigid dimer states. The conclusion from the relaxation studies in methanol-d_4 is that the C_α atoms of the peptide backbone are all held with equal rigidity and that their correlation time is similar to the correlation times of established helical molecules. These findings are inconsistent with certain other models that have been proposed for gramicidin A but are consistent with the proposed (Veatch *et al.*, 1974) double helical models for gramicidin A in methanol.

[1] If helical dimers of the type considered in a preceding paper (Veatch *et al.*, 1974) are approximated as prolate ellipsoids of revolution, then only the correlation time for end-over-end tumbling will contribute to the C_α relaxation times because the $C_\alpha H$ vector is nearly parallel to the major axis. Calculation of this correlation time (Woessner, 1962) for such ellipsoids, using a reasonable range of volume (including side chains) and axial ratio, yields no values greater than about 10 nsec in water.

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The Role of Histones in the Conformation of DNA in Chromatin as Studied by Circular Dichroism†

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ABSTRACT: This paper is an investigation of the role of histone in the conformation of DNA in chromatin. Quantitative acrylamide gel electrophoresis of proteins from chromatins isolated from various tissues and dissociated with solutions of sodium chloride–5 mM phosphate of ionic strengths 0.6, 1.0, 1.2, and 3.0 indicates that the response of each chromatin to dissociation is different as to the amount and type of histone removed at each of the sodium chloride concentrations, the exception to this being the unique removal of histone I from all the chromatins with 0.6 ionic strength solutions. Correlation of these observations with the circular dichroism (CD) above 260 nm of each of the depleted nucleoproteins shows that no change occurs in the CD of the chromatins with removal of the lysine-rich histone I; furthermore, the changes which occur with removal of the other (slightly lysine- and arginine-rich) histones are found to be approximately linear with the amount, not the type, of total histone in the depleted nucleoprotein sample. This suggests that the secondary structure of chromatin DNA is determined equally and independently by each of the slightly lysine-rich and arginine-rich histone fractions. This point is supported by an inverse, linear correlation observed between the magnitude of the CD above 260 nm of depleted nucleoproteins and the amount of slightly lysine- and arginine-rich histones bound to a DNA as estimated by analysis of thermal denaturation data. In contrast, the data show that the nonhistone proteins do not effect the CD of DNA in chromatin although they do bind the DNA.

Isolated chromosomes of higher plants and animals—usually studied in the form isolated from interphase nuclei, chromatin—are found to consist of DNA, protein, and some RNA. The major constituent of the protein component is the histone proteins, a set of five small, basic proteins which are characterized by their high content of lysine and arginine, and are ultimately bound to the DNA (Stellwagen and Cole, 1969; Georgiev, 1969; Hearst and Botchan, 1970; DeLange and Smith, 1971; Elgin *et al.,* 1971; Huang and Hjelm, 1974). The interactions of histones and their relationship to the structure of the chromosome have been the focus of many types of physical studies on the chromosome.

The integration of DNA into chromatin involves a change in the secondary structure of the DNA, and it is usually assumed that this is a direct result of the constraints placed on the DNA topology by the folding or coiling of the DNA duplex into a chromatin fibril 100–250 Å in diameter (Ris and Kubai, 1970; DuPraw, 1970). One powerful method of probing the secondary structure of chromosomal DNA is circular dichroism (CD). The region of the chromatin spectrum above 250 nm reflects largely the conformation of the DNA component, though we have shown that RNA and nonhistone protein chromophores can contribute a small amount to the CD in this region (Hjelm and Huang, 1974).

It appears well established that the histone proteins are the major determinants of the structure of DNA in chromatin. This has been demonstrated, in part, by removing the histones either partially or completely with solutions of different concentrations of NaCl (Ohlenbusch *et al.,* 1967). As the histones are removed the properties of the complexed DNA become more and more like those of DNA free in solution. Since somewhat selective removal of the different histone fractions occurs at various NaCl concentrations, workers have attempted to determine by CD which histone, if any, has the greatest role in determining the secondary structure of DNA in chromatin (Henson and Walker, 1970b; Simpson and Sober, 1970; Wagner and Spelsberg, 1971). Similar studies have been carried out on the hydrodynamic (Henson and Walker, 1970a; Ohba, 1966) and dye binding (Kleiman and Huang, 1971; Angerer and Moudrianakis, 1972) properties of chromatin. The results of these and the CD studies have been interpreted as being due to the association of different histone fractions with distinct physical characteristics of chromatin. The CD studies show that the removal of the lysine-rich histone I does not affect

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