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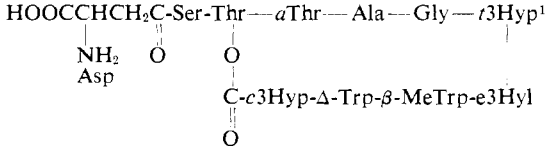
Conformational Analysis of the Polypeptide Antibiotic Telomycin by Nuclear Magnetic Resonance†

N. G. Kumar and D. W. Urry*

ABSTRACT: The solution conformation of the polypeptide antibiotic telomycin in Me_2SO-d_6 has been studied through the use of 220-MHz ^1H nuclear magnetic resonance (nmr) spectroscopy. In addition to the information provided by chemical shifts and the vicinal α-CH–NH coupling constants in elucidating conformation, three methods—proton–deuteron exchange, temperature dependence of peptide proton chemical shift, and methanol–trifluoroethanol solvent mixture dependence of peptide proton chemical shift—are used to delineate peptide protons in terms of exposure to solvent. All three methods clearly define the threonine, hydroxyleucine, and β-methyltryptophan peptide protons as solvent shielded. These data are utilized in discussing a conformation of telomycin in which there are three hydrogen-bonded rings of ten atoms, two of which are typical β turns.

Telomycin, an undecapeptide antibiotic, was isolated from the culture broth of an unidentified streptomyces by Misiek *et al.* (1957–1958). Its primary structure was determined by Sheehan *et al.* (1963, 1968).



It is the purpose of this work to present proton magnetic resonance (pmr) results on the conformational aspects of telomycin. The spectral features for distinguishing between solvent-exposed and solvent-shielded peptide protons (Kopple *et al.*, 1969; Ohnishi and Urry, 1969; Urry and Ohnishi, 1970; Pitner and Urry, 1972a) are discussed as they apply to telomycin. Detailed discussion of the solution conformation compatible with the experimental nmr data is given.

Experimental Section

Pmr spectra were recorded on a Varian Associate HR-220 spectrometer, as is described elsewhere (Kumar and Urry, 1973). The chemical shift difference between resonances of ethylene glycol or methanol was used to determine the probe temperature. Chemical shifts were measured relative to tetramethylsilane as an internal reference, unless otherwise indicated. Telomycin was shown to be homogeneous by thin-layer chromatography (Kumar and Urry, 1973).

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[1] Abbreviations used are: *trans*-3Hyp and *cis*-3Hyp, *trans*- and *cis*-3-hydroxyproline, respectively; Δ-Trp, α,β-didehydrotryptophan; e3Hyl, *erythro*-3-hydroxyleucine. All amino acids in telomycin are reported to be of the L configuration (Sheehan *et al.*, 1963, 1968).

TABLE I: Pmr Parameters of the Proton Resonances of Telomycin.[a]

Amino Acid Residue	Coupling Constant[b] ($J_{\alpha\text{-CH-NH}}$, Hz)	Chemical Shift[c] (ν_{NH}, Hz) (at 220 MHz)	$J_{\alpha\beta}$	$J_{\beta\beta'}$
Δ-Trp	0.0	2205		
Gly	6.5 ± 0.3 ($J_{\alpha-N}$)	1925		
	3.8 ± 0.3 ($J_{\alpha'-N}$)			
Ser	~0 (v small)	1890		
*a*Thr	9.0	1904	10.0	
Thr	8.1 ± 0.2	1706	10.8	
Ala	8.0 ± 0.2	1693	6.5 ($J_{CH_3-\alpha\text{-CH}}$)	
3Hyl	9.2 ± 0.2	1635	9.5 ± 0.5	
β-MeTrp	7.3 ± 0.2	1465	10.5 ± 0.5	
Asp			11.0 ± 0.5 ($\alpha\beta'$)	12.5 ± 0.5
			3.8 ± 0.2 ($\alpha\beta$)	

[a] Pmr parameters of the proton resonances of all the telomycin residues are listed in detail in an earlier paper (Kumar and Urry, 1973). Listed in Table I are only those pmr parameters which are helpful in the discussion of the peptide backbone conformation and side chains α–β conformers. [b] Coupling constant within experimental error remained constant between 30–68°. [c] Spectra were recorded in Me_2SO-d_6 at 40°.

Results

Spectral Assignments. The 220-MHz proton magnetic resonance spectrum of telomycin (Table I) in Me_2SO-d_6 at 46° is presented in Figure 1, together with resonance assignments. The analysis of the nmr spectra of telomycin in Me_2SO-d_6 and the assignment of proton resonances to specific hydrogens of the constituent amino acids have been discussed in detail in an earlier paper (Kumar and Urry, 1973). The peptide proton resonances are well resolved at 40°, and are shown on an expanded scale in Figure 2. Amide resonances 1, 2, 3, 4, 5, 6, 7, and 8 have been assigned to Gly, *a*Thr, Ser, Thr, Ala, 3Hyl, β-MeTrp and Δ-Trp residues, respectively (Kumar and Urry, 1973).

H–D Exchange Rates. The amide resonances, well separated at 40°, allow satisfactory delineation between the exchange rates of the peptide protons in Me_2SO-d_6 containing 10% D_2O. The C_7 or C_4 indole CH proton of β-MeTrp was used as an internal intensity standard (1.00 proton) for estimating the number of unexchanged protons from the peptide NH integrals. When 10% D_2O was added to a solution of telomycin in Me_2SO-d_6 at 40°, after 10 min, the indole NH protons of β-MeTrp and Δ-Trp and the peptide NH protons corresponding to resonances 2, 3, 5, and 8 had almost completely exchanged. The peptide NH proton corresponding to resonance 1 had slightly slower exchange rate than the resonances 2, 3, 5, and 8, and had a half-life of 8 min (after 10 min, 40% remained unexchanged relative to C_7 or C_4 indole CH proton of β-MeTrp). The remaining peptide protons corresponding to resonances 4, 6, and 7 exchanged slowly and had half-lives of greater than 180 min, 156 min ($k_1 = 4.44 \times 10^{-3}$ min^{-1}), and 77 min ($k_1 = 9.00 \times 10^{-3}$ min^{-1}), respectively.

At 40° in Me_2SO-d_6, the peptide NH 5 resonates at a slightly upfield position relative to peptide proton 4. The chemical shift difference between the two protons is ~13 Hz. But, on addition of 10% D_2O to Me_2SO-d_6 at 40°, these assignments are reversed. The chemical shift difference between the two protons is about the same (~14 Hz). Therefore, in Me_2SO-d_6 containing 10% D_2O, the downfield resonance is peptide proton 5 and upfield resonance 4. That this solvent-induced change in chemical shifts did not indicate significant alteration in conformation was verified from spin-decoupling experiments and from temperature dependence of chemical shift in Me_2SO-d_6 containing 10% H_2O. The coupling constants and temperature dependence of peptide proton chemical shifts were essentially unchanged on addition of 10% H_2O to Me_2SO-d_6.

At 23° in Me_2SO-d_6 containing 10% D_2O, delineation between H–D exchange rates of the indole NH protons of Δ-Trp

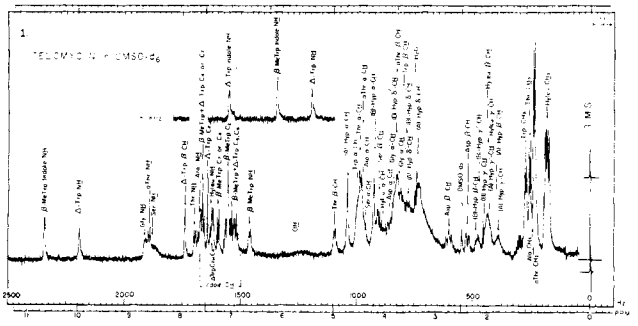

FIGURE 1: 220-MHz pmr spectrum of 7% (w/v) telomycin in dimethyl sulfoxide-d_6 at 46° (assignments from Kumar and Urry, 1973).

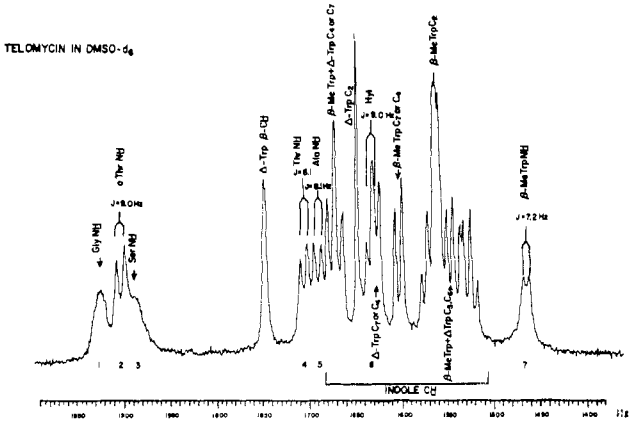

FIGURE 2: 220-MHz pmr spectrum of the peptide proton resonances in dimethyl sulfoxide-d_6 at 40°.

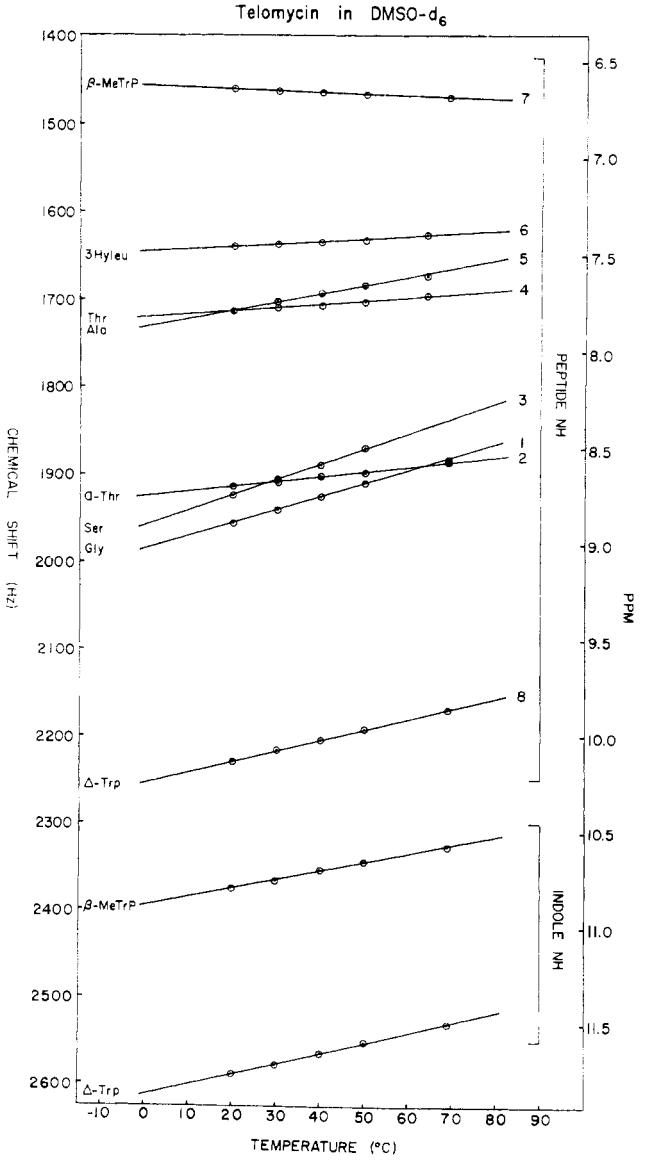

FIGURE 3: Chemical shift temperature dependence of the peptide proton resonances in dimethyl sulfoxide-d_6.

and β-MeTrp was achieved. The indole NH of Δ-Trp completely exchanged after ~12 min, whereas that of β-MeTrp after ~40 min (half-life of β-MeTrp indole NH ~20 min). At 23°, the peptide NH proton corresponding to resonance 1 has a half-life of 205 min. The peptide protons 2, 3, 5, and 8 exchanged fast. The remaining peptide protons corresponding to resonances 4, 6, and 7 exchanged very slowly.

In CD_3OD–Me_2SO-d_6 (7:3 v/v), H–D exchange between the solvent deuterons and peptide protons is relatively faster than in F_3EtOH-d_3–Me_2SO-d_6 (1:1 v/v). In F_3EtOH-d_3–Me_2SO-d_6 (1:1 v/v) at 22°, and in CD_3OD–Me_2SO-d_6 (7:3 v/v at 7°, the slowly exchanging peptide NH protons corresponded to resonances 4, 6, and 7. Their identity was confirmed from spin-decoupling experiments and temperature dependence of the peptide proton chemical shifts in F_3EtOH-d_3–Me_2SO-d_6 (1:1 v/v) and CH_3OH–Me_2SO-d_6 (7:3 v/v).

On the basis of exchange rates with deuterium oxide, the NH hydrogens fall into three classes. Class I consists of the indole NH protons of β-MeTrp and Δ-Trp, and peptide protons corresponding to resonances 2, 3, 5, and 8 which exchanged rapidly. Class II consists of peptide proton 1 which exchanged at an intermediate rate. Class III consists of slowly exchanging peptide protons corresponding to resonances 4, 6, and 7.

Temperature Dependence. The temperature dependence of the peptide proton resonances and indole NH resonances of telomycin in Me_2SO-d_6 is presented in Figure 3. The resonances are numbered in the order of increasing field as they appear in Me_2SO-d_6 except resonance 8. Amide resonances 4 and 5 overlap at 30°, but are well resolved at 40°; whereas resonance 6 partially overlaps with the C_7 or C_4 indole CH of Δ-Trp at 40° but is resolved at 50°. The peptide NH resonances 2 and 3 overlap at 20°. As the temperature is increased resonance 3 moves upfield and exhibits line broadening. At 50°, peptide NH proton 3 resonates at 1870 Hz as a broad resonance and is located upfield to resonance 2. Amide resonance 8, which is in conjugation with the α,β-unsaturated double bond of Δ-Trp, also exhibits line broadening as the temperature is increased. At 68°, the line width at half-height is ~17.5 Hz as compared to ~10 Hz at 20°. On increasing the temperature, the indole NH proton of Δ-Trp also broadens out relative to the indole NH of β-MeTrp. At 68°, the line widths at half-heights are 14.5 and 5.8 Hz, respectively; whereas at 20° the line widths are about the same (~8.7 Hz). Differential broadening of the indole NH proton signals of Δ-Trp and β-MeTrp can result from different relaxation rates of the corresponding protons (an increase in temperature causes the relaxation time of the indole NH proton of Δ-Trp to decrease). This may be due to quadrupole relaxation of the ^{14}N or peptide proton exchange.

Increasing the temperature of a complex and compact molecule such as telomycin causes a change in the vibrational states of the molecule, so that on repopulation the average magnetic environment experienced by the peptide protons could change. Therefore, it is important to conduct temperature-dependence experiments in different solvents in which relative chemical shift temperature dependence has been shown to be different for solvent-exposed and solvent-shielded peptide protons (Pitner and Urry, 1972a,b). The temperature coefficients of the peptide NH protons in Me_2SO-d_6, F_3EtOH–Me_2SO-d_6 (1:1 v/v),[2] CH_3OH–Me_2SO-d_6 (7:3 v/v) and H_2O–Me_2SO-d_6 (7:3 v/v) are listed in Table II. For gramicidin S the ratios of the temperature coefficients of solvent exposed to the coefficients of intramolecularly hydrogen-bonded peptide protons are 1.6 and 3.3 in F_3EtOH and methanol, respectively. For some of the peptide NH protons of telomycin, delineation between the ratios of the temperature coefficients of solvent exposed to the coefficients of solvent-shielded peptide protons in F_3EtOH–Me_2SO-d_6 (1:1 v/v)[2] and Me_2SO-d_6 is as clear-cut as for gramicidin S, even though the temperature dependence of the peptide protons of telomycin was determined only in 50% F_3EtOH. For example, ratios of the temperature coefficients of resonances 1 to 4; 1 to 6; 3 to 4; and 3 to 6 in F_3EtOH–Me_2SO-d_6 (1:1 v/v) and Me_2SO-d_6 are 2.6, 4.1; 2.5, 5.6; 1.8, 4.9; 1.7 and 6.6, respectively. As expected, in F_3EtOH–Me_2SO-d_6 (1:1 v/v) solvent mixture, exposure of peptide protons to solvent has little effect on the temperature dependence. Therefore, differentiation between the temperature coefficients of solvent-exposed and solvent-shielded peptide protons is not as clear-cut as in Me_2SO-d_6.

In Me_2SO-d_6 the downfield peptide protons 1, 3, 8 and indole NH protons exhibited large temperature coefficients,

[2] Chemical shift temperature dependence of the amide resonances in 100% F_3EtOH could not be determined, as telomycin is only slightly soluble in F_3EtOH.

TABLE II: Temperature Dependence of Peptide Proton Chemical Shifts in Different Solvents.

Peptide and Indole NH Protons	Me_2SO-d_6		Me_2SO-d_6–F_3EtOH (1:1 v/v)		MeOH–Me_2SO-d_6 (7:3 v/v)		H_2O–Me_2SO (7:3 v/v)	
	Temp Coef (ppm/°C)	0° Intercept, Hz (ppm)	Temp Coef (ppm/°C)	0° Intercept, Hz (ppm)	Temp Coef (ppm/°C)	0° Intercept, Hz (ppm)	Temp Coef (ppm/°C)	0° Intercept, Hz (ppm)
Gly (1)	−0.0067	1984 (9.02)	−0.0054	2014 (9.15)	−0.0051	2020 (9.18)	−0.0053	2006 (9.12)
*a*Thr (2)	−0.0025	1924 (8.75)	−0.0040	1862 (8.46)	−0.0039	1896 (8.62)	−0.0079	1997 (9.08)
Ser (3)	−0.0079	1958 (8.90)	−0.0036	1887 (8.58)	−0.0044	1920 (8.73)	−0.0060	1922 (8.74)
Thr (4)	−0.0016	1720 (7.82)	−0.0020	1761 (8.00)	−0.0020	1765 (8.02)	−0.0033	1826 (8.30)
Ala (5)	−0.0038	1728 (7.85)	−0.0059	1812 (8.24)	−0.0067	1822 (8.28)	−0.0077	1913 (8.70)
Hyl (6)	−0.0012	1644 (7.47)	−0.0021	1714 (7.79)	−0.0027	1709 (7.77)	−0.0033	1738 (7.90)
β-MeTrp (7)	+0.0009	1457 (6.62)	*a*	*a*	*c*	*c*	+0.0029	1651 (7.50)
Δ-Trp (8)	−0.0055	2253 (10.24)	*b*	*b*	−0.0074	2251 (10.23)	−0.0038	2130 (9.68)
Indole NH								
β-MeTrp	−0.0045	2395 (10.88)	*b*	*b*	−0.0052	2319 (10.54)	−0.0033	2256 (10.26)
Δ-Trp	−0.0054	2612 (11.87)	*b*	*b*	−0.0062	2536 (11.53)	−0.0023	2460 (11.18)

[a] Resonance 7 is under the C_5,C_6 indole CH protons of β-MeTrp and Δ-Trp. [b] Temperature dependence of resonance 8 and indole NH protons was not determined in F_3EtOH–Me_2SO-d_6 (1:1 v/v), only multiscans of the peptide proton region from 2100 to 1400 Hz was averaged. [c] Resonance 7 is under the solvent.

ranging from −0.005 to −0.008 ppm per °C. The peptide protons 2 and 5 are somewhat less temperature sensitive (−0.0025 and −0.0038 ppm per °C), but the upfield peptide protons 4, 6, and 7 (−0.0016, −0.0012, and +0.0086 ppm per °C) clearly have low temperature coefficients. The small negative temperature coefficient values of amide resonances 4, 6, and small positive temperature coefficient of resonance 7 are typical for internal (buried or intramolecularly H bonded) protons, and large negative coefficient values of the peptide protons 1, 3, 8, and indole NH protons are observed for external (exposed to solvent) NH protons (Ohnishi and Urry, 1969; Urry and Ohnishi, 1970; Kopple *et al.*, 1969, 1972; Torchia *et al.*, 1972a,b; Kopple and Ohnishi, 1969).

F_3EtOH–Me_2SO-d_6 Solvent Mixture. Chemical shift dependence as a function of mole per cent of trifluoroethanol in dimethyl sulfoxide-d_6 was followed to 50 mol % of F_3EtOH because telomycin is only slightly soluble in F_3EtOH. On increasing the mole per cent of trifluoroethanol from 0 to 50, indole NH protons of β-MeTrp and Δ-Trp, and peptide protons 2 and 3 show large upfield shifts of 75, 73, 70, and 44 Hz, respectively, as did the solvent-exposed peptide protons of gramicidin S. It is worthy of note that these large upfield shifts correspond to 50 mol % of F_3EtOH and not 100 mol %. There are six peptide NH resonances 1, 5, 8, 4, 6, and 7 which shift definitely downfield by 41, 83, 31, 43, 67, and 72 Hz, respectively, as did the intramolecularly hydrogen-bonded protons of gramicidin S (Pitner and Urry, 1972a). On increasing the mole per cent of F_3EtOH, very little change in the $J_{\alpha\text{-CH-NH}}$ coupling constants is observed.

Discussion

Delineation of Peptide Protons. Three methods for identification of the solvent-exposed and solvent-shielded peptide protons—temperature dependence of chemical shift, proton–deuterium exchange rates, and chemical shift dependence on F_3EtOH–solvent mixtures—have been shown to be effective, when dealing with small, relatively rigid cyclic polypeptides (Kopple *et al.*, 1969; Ohnishi and Urry, 1969; Urry and Ohnishi, 1970; Ramachandran *et al.*, 1971; Bystrov *et al.*, 1969; Pitner and Urry, 1972a; Stern *et al.*, 1968; Bovey *et al.*, 1972; Hassall and Thomas, 1971). For gramicidin S, agreement between the three methods is very good for the identification of the solvent-exposed and intramolecularly hydrogen-bonded peptide protons. For the larger telomycin molecule, agreement between the methods for the peptide protons 4, 6, and 7 (solvent shielded or intramolecularly hydrogen bonded) is very good, as with gramicidin S. However, the delineation of other peptide proton resonances is not as clear-cut as with gramicidin S. This demonstrates the utility of all three methods and provides an opportunity to compare the behavior of each amide resonance. It also allows discussion of possible problems that may be encountered when dealing with large complex molecules.

Once assignments are made proton magnetic resonance studies provide three classes of information as constraints for CPK space-filling model building. Dihedral angles, *e.g.*, H–C_α–N–H and H–C_α–C_β–H are obtained from the corresponding $^3J_{\alpha\text{-CH-NH}}$ and $^3J_{\alpha\beta}$ coupling constants; the presence of magnetically anisotropic vicinal moieties is implied by the variance of a chemical shift from a characteristic range of values for a specific proton, and the presence of stable intramolecular hydrogen bonds are detected by the three methods noted above for delineating peptide protons. As will be discussed below, nmr experimental data points to a folded conformation for the nine amino acid residue lactone ring of telomycin. With such a folded conformation, one must be careful in interpreting chemical shift temperature-dependence results; slight rotation about the bonds can alter relative spatial orientations of peptide protons and nearby peptide moieties and can cause significant chemical shift changes due to magnetic anisotropy of the nearby peptide moiety or due to ring currents from the nearby indole rings. Also, slight alterations proximal to the α,β-unsaturated double bond of Δ-Trp can cause perturbation in the field positions of vicinal peptide protons. However, for telomycin, the $^3J_{\alpha\text{-CH-NH}}$ coupling constants, within experimental error, remained constant throughout the temperature range of 30–

68°, indicating that the peptide backbone conformation, as reflected in the α-CH–NH dihedral angles, remained essentially unchanged on the nmr time scale.

Also, on increasing the mole per cent of F_3EtOH from 0 to 50 in Me_2SO-d_6, very little experimentally observed change is detected in the $J_{\alpha CH-NH}$ coupling constants, again suggesting that the gross conformation remained essentially the same. At 50 mol % of F_3EtOH, solvent-exposed indole NH protons of β-MeTrp and Δ-Trp, and solvent-exposed peptide protons 2 and 3 did exhibit large upfield shifts. The solvent-shielded peptide protons 4, 6, and 7 (buried or internally hydrogen bonded) exhibited downfield shifts. A proposed conformation of telomycin should be consistent with this detailed information provided by the nmr studies.

The rate at which peptide NH protons are replaced by deuterium, when 10% D_2O is added to dimethyl sulfoxide-d_6, may reflect the degree of their exposure to the medium, the strength of intramolecular hydrogen bondings, and differences in rigidity of the peptide backbone. Restricted access to solvent retards the rate of proton exchange. Exchange is a rate process which may take place as the molecule unfolds for a short period of time through energetically minor changes, *e.g.*, slight rotation of the planes of peptide bonds may suffice to allow exposure of peptide protons to solvent and permit exchange. In telomycin, H–D exchange results indicate that the most solvent-exposed peptide protons are 2, 3, 5, 8, and indole NH protons. Peptide proton 1 is slightly less exposed to the solvent. Peptide protons 4, 6, and 7 are shielded extremely well from the solvent, possibly through the formation of strong intramolecular hydrogen bonding. In Me_2SO-d_6 containing 10% D_2O at 40°, the half-lives of resonances 4, 6, and 7 are >180, 156, and 77 min, respectively. Hence, stability of hydrogen-bonded configurations would be $4 > 6 > 7$.

When comparing the methods of delineating peptide protons, it is important to emphasize that H–D exchange results are observations of kinetically controlled processes, dependent on transient activated state, whereas the temperature dependence of peptide proton chemical shifts and F_3EtOH–solvent titration results are observations of thermodynamically controlled processes dependent on average property of states in equilibria. The peptide protons 4, 6, and 7 with the slowest exchange rates shift downfield as the mole per cent of F_3EtOH is increased; amide resonances 4 and 6 exhibit very small negative temperature coefficients and peptide proton 7 exhibits a small positive temperature coefficient (Table II). These three spectral patterns are consistent with the results of the internally hydrogen-bonded protons of gramicidin S.

The results on peptide protons 5 and 8 are complicated in that they exchange at a very fast rate indicative of solvent exposure, but shift downfield as the mole per cent of F_3EtOH is increased as occurs when solvent occluded. In nonaqueous solvent systems peptide proton 8 has a large negative temperature coefficient and that of proton 5 is slightly smaller. Peptide proton 1 exhibits a large negative temperature coefficient but exchanges at a slightly slower rate than protons 2, 3, 5, and 8 and shifts downfield with increase in F_3EtOH concentration. In agreement with the H–D exchange results, peptide protons 2 and 3 exhibit upfield shifts as the mole per cent of F_3EtOH is increased. The results of these two methods are consistent with the results of the solvent-exposed protons of gramicidin S. Also consistent with solvent exposure, the temperature study shows that proton 3 has a large negative coefficient, however, the temperature coefficient of proton 2 is not large (−0.0025 ppm/°C). Accordingly, there are ambiguous cases where one method may indicate solvent exposure whereas another may indicate that the same peptide proton is buried. When all three methods indicate solvent exposure, this is a reasonable conclusion. When all three methods indicate occlusion of a peptide proton from the solvent, the proton in question becomes a candidate for a reasonably stable intramolecular hydrogen bond. The decision as to whether a buried peptide proton is actually intramolecularly hydrogen bonded will depend on whether the coupling constant and chemical shift data are consistent with the bond angles and proximity of groups required for one of its several possible intramolecular hydrogen bonds. The ambiguous cases likely reflect rather subtle conformational features.

The solvent-exposed indole NH protons of β-MeTrp and Δ-Trp exchange very rapidly and exhibit large upfield shift as the mole per cent of F_3EtOH is increased from 0 to 50. They also have large negative temperature coefficients.

As in the case of gramicidin S (Ohnishi and Urry, 1969; Urry and Ohnishi, 1970) the spatial relationship of solvent-shielded peptide protons to magnetically anisotropic groups, such as peptide moieties, will affect the spectral position of peptide proton resonances. The peptide protons 4, 6, and 7 of telomycin, solvent shielded by the temperature-dependence criterion, H–D exchange rates, and F_3EtOH–Me_2SO-d_6 mixture titration, are also the highest field peptide protons, the order being $7 > 6 > 4$.

All these spectral features, when taken together, suggest a structure in which these particular peptide protons are shielded as when intramolecularly hydrogen bonded in β turns. This possibility must also conform to the dihedral angles implied by the experimental vicinal coupling constants.

Solution Conformation in Dimethyl Sulfoxide-d_6. In proposing conformational models, one must be aware of the fact that there may be more than one conformation which agrees with experimental data. Usually the term "conformation" stands for an "ensemble average on the nmr time scale;" *i.e.*, one is dealing with an ensemble of conformations which may be in rapid equilibrium, so that the resulting pmr spectrum is determined by a weighted time average of spectral parameters of the conformations. With these considerations in mind, two possible conformations will be discussed which satisfy the steric requirements and experimental nmr data. CPK space-filling models were used in constructing the proposed conformational model of telomycin.

The primary structure of telomycin (Sheehan *et al.*, 1963, 1968) contains a lactone ring of nine amino acid residues. The ester linkage is formed between the carboxyl group of L-*c*3Hyp and the hydroxyl group of L-Thr. Being cyclic this ring system has the distinct possibility of being folded using ten-atom hydrogen-bonded rings, *e.g.*, β turns (Venkatachalam, 1968; Geddes *et al.*, 1968; Urry and Ohnishi, 1970).

A ten-atom hydrogen-bonded ring can be constructed which contains the β-carbon of L-Thr (involved in the ester linkage) at one corner and L-*c*3Hyp at the other corner. In this β-like turn there is an end ester moiety (formed from the hydroxyl group of L-Thr and carboxyl group of L-*c*3Hyp). By analogy with the most sterically favored conformation of the end peptide moiety in such a β turn, the carbonyl oxygen of the end ester moiety may be given the correct orientation (Urry and Ohnishi, 1970). The β-like turn is formed by an intramolecular hydrogen bond between the L-Thr peptide NH proton (4) and carbonyl oxygen of the Δ-Trp. A similar ten-atom hydrogen-bonded ring, or the β-like turn, was proposed for stendomycin (Pitner and Urry, 1972b), and the

presence of this conformational feature in telomycin is supported by the following spectral features. The peptide proton of L-Thr (4) has the slowest exchange rate and low temperature coefficient, and resonates at a relatively high field as expected from its participation in a β turn (Table II). The shielding of the proton (4) is due to the magnetic anisotropy of the end ester moiety. The relatively high field position of peptide proton in the peptide region of the spectrum has been noted as a characteristic conformational feature for the intramolecularly hydrogen-bonded protons of β turns (Urry and Ohnishi, 1970). The $J_{\alpha\text{-CH-NH}}$ coupling constant is large (8.1 Hz, Table I), corresponding to a dihedral angle (θ) near 155°, as would be expected from the trans orientation of these two protons (Ramachandran *et al.*, 1971). By the above criteria, L-Thr peptide proton (4) is very well shielded from the solvent, and may reasonably be taken as internally hydrogen bonded. The relatively high field position of L-Thr peptide proton (4) and large $^3J_{\alpha\text{-CH-NH}}$ coupling constant are consistent with the presence of a β-like turn in which L-*c*3Hyp-L-Thr form the corners as indicated in Figure 4.

One possible β turn has L-*t*3Hyp and Gly at the corners of the turn with the carbonyl and nitrogen of the end peptide moiety in the most sterically favored orientation (Urry and Ohnishi, 1970; Venkatachalam, 1968). The peptide proton (number 6) of L-e3Hyl is internally hydrogen bonded to the carbonyl oxygen of L-Ala resulting in a ten-atom hydrogen-bonded ring for the standard β turn. This β turn utilizes proline as residue $i + 2$, in the same position as found for the gramicidin S type β turn. This conformational feature is supported by the following observations. The L-e3Hyl peptide proton (6) exhibits very slow exchange rate, and low temperature coefficient of chemical shift and downfield shift on addition of trifluoroethanol and occurs at a relatively high field position in the peptide region of the spectrum (Tables I and II). The high field position of peptide proton (6) implies shielding as by the magnetic anisotropy of an end peptide moiety. The magnitude of the $J_{\alpha\text{-CH-NH}}$ coupling constant is large (9.3 Hz, Table I) with a corresponding α-CH–NH dihedral angle (θ) for the peptide proton (6) near 180°, as expected from the trans orientation of the two protons (Ramachandran *et al.*, 1971). By the above criteria, the L-e3Hyl peptide proton (6) is well shielded from the solvent. In gramicidin S and other model systems, the above spectral features have been correlated with intramolecular hydrogen bonding (Urry and Ohnishi, 1970; Ohnishi and Urry, 1969; Pitner and Urry, 1972a). Hence, it is reasonable to propose that the peptide proton (6) is intramolecularly hydrogen bonded in a β turn.

Another feature of interest is the nonequivalence of the α-CH protons of Gly, which is positioned at one corner of the β turn (L-*t*3Hyp-Gly form the corners, Figure 4). The chemical shift difference between the α-CH and α'-CH protons is ~22 Hz (Kumar and Urry, 1973). The $J_{\alpha\text{-CH-NH}}$ coupling constants for the glycine residue are 3.8 and 6.5 Hz (Table I), with corresponding dihedral angles (θ) near 43 and 140°, respectively (Ramachandran *et al.*, 1971).

Another β turn can be formed which utilizes L-*t*3Hyp and L-e3Hyl at the corners. The end peptide moiety would be formed by L-*t*3Hyp-L-e3Hyl residues. The β turn results from an intramolecular hydrogen bond between the peptide NH proton (number 7) of L-β-MeTrp and carbonyl oxygen of Gly. This conformational feature is suggested by the very slow exchange rate, by the low temperature coefficient, by the downfield shift on addition of F_3EtOH and by the high field position of peptide proton 7. It is possible that the shielding of

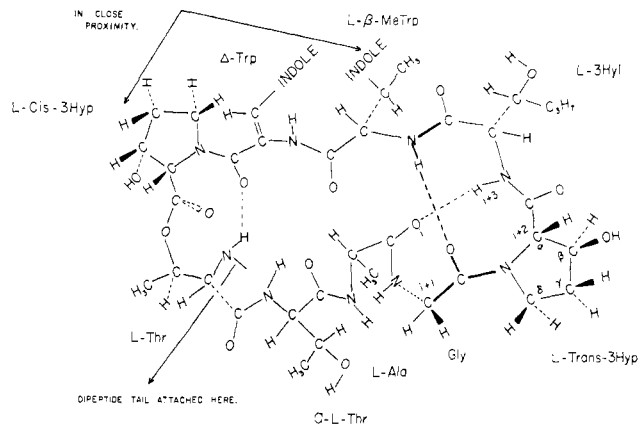

FIGURE 4: One possible conformation for the lactone ring of telomycin. The ring is partially unfolded to reveal β-conformational aspects (all amino acids are of L configuration).

the peptide proton (7) resulting in the highest field position of all the peptide protons is due to ring-current effects of both the indole ring of Δ-Trp and the peptide carbonyl moiety of L-Ala. Two additional features may be mentioned. The $J_{\alpha\text{-CH-NH}}$ coupling constant of L-β-MeTrp is large (~7.5 Hz, Table I) corresponding to a dihedral angle (θ) near 150°, which is consistent with the trans orientation of these two protons (Ramachandran *et al.*, 1971). The large magnitude of the observed vicinal coupling constant between the α-CH and β-CH protons ($J_{\alpha\beta}$ = 9.5 ± 0.5 Hz, Table I) suggests a conformer for the C^α–C^β bond of the L-e3Hyl residue in which the relative orientation of the α and β protons is trans. The above two β turns combine to form one turn of right-handed 3_{10} helix. The assignment of the *cis*- and *trans*-3-hydroxy-L-proline residues is assisted by the above discussed conformation (Figure 4). The skewing of the lactone ring, which results from the above proposed ten-atom hydrogen-bonded rings, places the indole moiety of L-β-MeTrp and L-*c*3Hyp in close proximity, thereby explaining the relatively large $J_{\alpha\beta}$ coupling constant and the large ring-current upfield shifts exhibited by the β, γ, and δ protons of (A)-3Hyp (Kumar and Urry, 1973) relative to the (B)-3Hyp. Hence, it appears from this conformation (Figure 4), that the hydroxyproline residue labeled as (A)-3Hyp can be assigned to L-*c*3Hyp. While we view the above discussed conformation (Figure 4) as the most consistent with the present nmr data other conformations may be possible.

Another possible conformation (Figure 5) is obtained by reversing the assignment of the *cis*- and *trans*-3-hydroxy-L-proline residues, and replacing the L-e3Hyl by D-e3Hyl. The nmr spectrum of neotelomycin (*c*-3Hyp is replaced by proline residue) suggests that hydroxyproline residue labeled as (A)-3Hyp may be assigned to *t*-3Hyp. This possible conformation (Figure 5) has the same three hydrogen-bonded rings of ten atoms, one of which is a β-like turn and two are typical β turns, utilizing the same residues in the end peptide moieties, and intramolecular hydrogen bonds between the peptide NH protons and peptide carbonyl moieties. The above two β turns combine to form one turn of left-handed 3_{10} helix instead of a right-handed helix. Clearly, the presence of the β turn utilizing L-*t*3Hyp and D-e3Hyl at the corners (Figure 5) required D-e3Hyl residue and it is necessary that this point be clarified. The following observations are the basis for this suggestion. The e3Hyl peptide proton (6) exhibits large $J_{\alpha\text{-CH-NH}}$ coupling constant (9.2 Hz, Table I). This corresponds to a value of α-CH–NH dihedral angle (θ) near 180°, and requires a trans

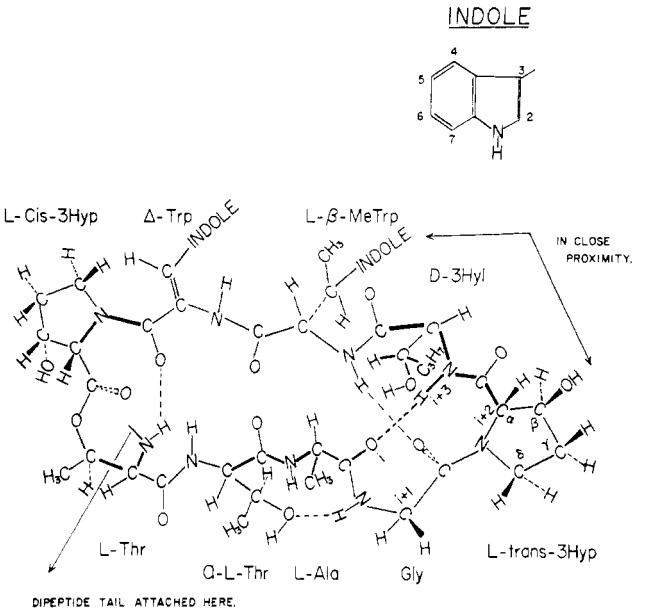

FIGURE 5: Another possible conformation for the lactone ring of telomycin. The ring is partially unfolded to reveal β-conformational aspects (in this conformation e3Hyl is of D configuration).

orientation of the two protons (Ramachandran *et al.*, 1971). This requirement is easily met if, the absolute configuration of e3Hyl residue is D, and also if there is no steric crowding between the bulky isopropyl and indole groups of e3Hyl and L-β-MeTrp, respectively. On the other hand, substitution of D-e3Hyl by L-e3Hyl cannot satisfy the trans orientation of the α-CH and NH protons as indicated by the large $J_{\alpha\text{-CH-NH}}$ vicinal coupling constant, and in addition to this there are severe nonbonded steric interactions between the bulky isopropyl and indole moieties of L-e3Hyl and L-β-MeTrp, respectively. Another observation, however, is consistent with the D configuration, the skewing of the lactone ring, which results from the above-proposed ten-atom hydrogen-bonded rings and places the indole moiety of L-β-MeTrp and L-*t*3Hyp in close proximity, thereby explaining the large ring-current upfield shifts exhibited by the β, γ, and δ protons of (A)-3Hyp (Kumar and Urry, 1973) relative to the (B)-3Hyp. Hence, the D configuration of the e3Hyl residue and reversed assignment of *c*- (B) and *t*3Hyp (A) can satisfy the steric requirements and additional experimental details of unusual chemical shifts. The point of the above discussion is to demonstrate that an error in absolute configuration of a key residue can negate a proposed conformation and lead to an alternate model which is quite consistent with the nmr data.

Additional Conformational Features. The Gly peptide proton (1) exhibits a large temperature coefficient (Table II) as expected for a solvent-exposed proton, but a slightly slower exchange rate (see Results) than peptide protons 2, 3, 5, and 8 (solvent-exposed protons). As seen with space-filling models, skewing of the lactone ring which results from the above-discussed ten-atom hydrogen-bonded rings (Figure 4) places the Gly N–H and L-Asp carbonyl oxygen in close proximity. This allows the peptide proton (1) of Gly to be hydrogen bonded to the carbonyl oxygen of L-Asp, and could explain the slowed exchange rate of the peptide proton 1 (a $t_{1/2}$ of 205 min at 23° in Me_2SO-d_6 containing 10% D_2O) as compared to the peptide protons 2, 3, 5, and 8. The second possible conformation (Figure 5) could also explain slightly slower exchange rate of Gly peptide proton (1). The skewing of the lactone ring which results from the above β-like and β turns places the Gly N–H and L-*a*Thr O–H in close proximity, thereby allowing the Gly N–H to be hydrogen bonded to the hydroxyl oxygen of L-*a*Thr.

The exchange rates of the L-*a*Thr NH (2) and L-Ala peptide protons (5) are fast. As discussed in the Results section, the temperature coefficient of the peptide proton (5) is large but not very large, and the coefficient of proton (2) is not large, but not low enough to be involved in intramolecular hydrogen bonding. It is quite conceivable that this anomalous temperature coefficient of proton 2 may be explained because it is sterically shielded by being buried in the interior of the molecule. The $J_{\alpha\text{-CH-NH}}$ coupling constants of L-*a*Thr and L-Ala are 9.0 and 8.0 Hz, respectively, which place the dihedral angles (θ) near 165 and 153°, respectively, consistent with the trans orientation of the α-CH and NH protons (Ramachandran *et al.*, 1971).

The peptide NH proton (3) of L-Ser residue which is in the dipeptide tail of telomycin exhibits near zero $J_{\alpha\text{-CH-NH}}$ coupling constant (very small). This corresponds to a dihedral angle (θ) near 90°.

Side Chains. Information concerning side-chain conformations can be made using the observed $J_{\alpha\beta}$ vicinal coupling constants to estimate the C^α–C^β dihedral angle. The observed $J_{\alpha\beta}$ coupling constants may correspond to fixed dihedral angles or reflect a population-weighted time average of the values corresponding to the rotational energy minima.

In L-Thr, L-e3Hyl, and L-β-MeTrp residues, the observed coupling constants between the α-CH and β-CH protons are 10.8, 9.5 ± 0.5, and ~11 Hz (Table I), respectively, define stereochemistry around the C^α–C^β bonds, and indicate major population of a α–β conformer in which the relative orientation of these two protons is trans ($\chi_1 \simeq 180°$). Also, the large magnitude of the vicinal coupling constant between the L-*a*Thr α-CH and β-CH protons ($J_{\alpha\beta}$ = 10.0 Hz, Table I) suggests major population of a α–β conformer for the C^α–C^β bond in which these two protons are in trans orientation.

The proposed conformation (Figure 4) allows L-Asp NH_2 to be hydrogen bonded to the carbonyl oxygen of *a*-L-Thr. For the L-aspartic acid residue, the β-CH proton resonates at a high field position ($\nu_{\beta\text{-CH}}$ = 526 Hz) and is shifted upfield by about 74 Hz relative to the β′-CH proton (Kumar and Urry, 1973). The geminal coupling between the β-CH and β′-CH protons is 13.0 Hz (absolute value), and vicinal coupling constants between the α-CH, β-CH and α-CH, β′-CH are 3.8 and 11.0 Hz (Table I), respectively. The observed $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ coupling constants support major population of a conformer in which the relative orientation of the α-CH and β′-CH protons is trans, and the α-CH and β-CH protons is gauche. This α–β conformer is also sterically favored over a conformer in which the relative orientation of the α-CH, β-CH and α-CH, β′-CH protons is gauche.

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Binding of Magnesium and Calcium Ions to the Phosphoglycoprotein Phosvitin†

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ABSTRACT: Dialysis equilibrium measurements were carried out at 25° at pH 6.5 and 4.5 to determine the binding of Mg^{2+} and Ca^{2+} by phosvitin. We find that at pH 6.5, in the concentration range of 0.2×10^{-3} to 2.0×10^{-3} M $MgCl_2$ or $CaCl_2$, phosvitin at 25° binds an average of 103 Mg^{2+} and 127 Ca^{2+}, respectively, whereas at pH 4.5 40 Mg^{2+} and 32 Ca^{2+} are bound. Optical rotatory dispersion and circular dichroism studies indicate that a conformational change occurs on interaction with the divalent cations. At pH 6.5, in the presence of Mg^{2+}, the mean residue rotation [m'] becomes more levorotatory, whereas interaction with Ca^{2+} has the opposite effect on [m'], implicating the importance of the ionic environment in controlling the polypeptide backbone conformation of phosvitin.

Studies of viscosity on the phosphoglycoprotein phosvitin have shown that this protein in some of its properties resembles a polyelectrolyte. Furthermore, it was also demonstrated that the presence of monovalent cations, Na^+ and K^+, has an effect on the optical rotatory dispersion (ORD) and circular dichroism (CD) (Grizzuti and Perlmann, 1970).

The purpose of the investigation described in this paper was to measure the binding of the divalent cations, Mg^{2+} and Ca^{2+}, by phosvitin and to study the effect of ion binding on the conformational characteristics of this protein. Dialysis equilibrium techniques and gel filtration have been used. We shall show that at the two pH values investigated the number of Ca^{2+} bound differs from that of Mg^{2+}, indicating a selectivity in the binding of divalent ions. In addition, a significant difference is found in the ORD patterns and CD spectra of this protein.

Materials and Methods

Materials. Phosvitin with a nitrogen content of 13.1% and phosphorus of 11.9% was isolated from fresh hens' eggs according to the procedure of Joubert and Cook (1958).

^{45}Ca was purchased as aqueous calcium chloride from Amersham/Searle; Aquasol, the scintillation counting fluid, was supplied by New England Nuclear Corp. Eriochrome Black T was purchased from Matheson, Coleman & Bell and Erio SE from Eastman Organic Chemicals. Sephadex G-25 was a product of Pharmacia. All other chemicals were reagent grade and were not further purified.

Methods. The binding of Ca^{2+} and Mg^{2+} was determined either by equilibrium dialysis at 25° or in some experiments by gel filtration on Sephadex G-25.

The *dialysis* experiments were carried out as follows: in the tests in which the protein concentrations were varied aliquots of a freshly prepared phosvitin solution (10 mg/ml) in either distilled water or sodium cacodylate buffer (pH 6.8), $\Gamma/2$ 0.02, were diluted to the desired protein concentration; 0.5 ml of each solution contained in bags of Visking sausage casing[1] were placed in large test tubes with 40 ml of the appropriate solvent. The tubes were placed on magnetic stirrers and mixing was obtained by gentle stirring. At specified time intervals the bags were withdrawn, carefully rinsed with distilled water, and wiped dry, and the contents were analyzed for protein and bound ion. Equilibrium at 25° was usually attained in 2–4 hr (see Results). Equilibrium controls consisting of Mg^{2+} or Ca^{2+}

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[1] Prior to use the dialyzing membranes were boiled in distilled water, followed by thorough rinsing and storage at 4°.