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Nuclear Magnetic Resonance Spectral Studies on Actinomycin D. Preliminary Observations on the Effect of Complex Formation with 5′-Deoxyguanylic Acid*

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ABSTRACT: The complete assignment of the nuclear magnetic resonance spectrum of actinomycin D is described. This compound was studied in various organic solvents, in water at low temperature, at room temperature, and in aqueous solutions containing various small amounts of dimethylformamide.

Solvent effects, unusual chemical shifts, and the behavior of the active hydrogens were used to establish specific geometric relationships in the pentapeptide configuration. Spectral studies of this compound in aqueous solutions in the presence of 5′-deoxyguanylic acid have shown that complex formation occurs by base stacking; the pyrimidine ring of 5′-deoxyguanylic acid is located above and below the phenoxazone chromophore of actinomycin D.

Actinomycin D (Figure 1) is of considerable biochemical interest. It binds strongly to double-stranded DNA and inhibits the DNA-dependent RNA synthesis (Waksman, 1968). Two models have been proposed for the mode of binding to DNA, one by Hamilton *et al.* (1963), where the molecule binds externally to the DNA molecule with the NH_2 group and the quinone oxygen of the phenoxazone ring system forming three hydrogen bonds with deoxyguanine. A different model, which involves intercalation of the phenoxazone ring between the base pairs in DNA has been proposed by Müller and Crothers (1968). As the first step to arrive by nuclear magnetic resonance techniques at a more detailed understanding of the mode of binding of actinomycin D we have completed the assignment of its nuclear magnetic resonance spectrum.

Because the solubility of actinomycin D in water at room temperature is too low for direct spectral observation, various organic solvents were used first. Additional spectra were taken in water at low temperatures, at room temperature in supersaturated solution and in aqueous solutions containing various small amounts of dimethylformamide. Finally, the extrapolated chemical shifts in water were used to study the effect of 5′-deoxyguanylic acid on the spectrum of actinomycin.

Experimental Section

All spectra were determined on a Varian HA 100D spectrometer operating at a probe temperature of 32°. Concentrations were in the 5–10% range. Chemical shifts are reported in parts per million relative to internal tetramethylsilane (TMSi) (organic solvents) and internal 2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt (aqueous media). Peak positions were determined by measuring the differences between the manual and sweep frequency oscillator settings and are reliable to ±1 Hz. Actinomycin D was obtained from Merck and Co. A purity of >98% was indicated by thin-layer chromatography and phase solubility analyses.

Results

The 220-MHz spectrum of actinomycin D in $CDCl_3$ is illustrated in Figure 2.[1] Although the 100-MHz spectrum was sufficiently resolved for a complete analysis, the 220 was chosen for illustration because of the improvement in resolution of the sarcosine and Thr-α-CH protons (τ 5.2–5.5) and for a somewhat better definition of the proline α- and γ-proton resonances. All chemical shifts in this report refer to the 100-MHz spectrum.

The nuclear magnetic resonance parameters of actinomycin D in $CDCl_3$, acetone, pyridine, dimethylformamide,

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[1] We thank Mr. L. F. Johnson of Varian Associates for kindly supplying us with this spectrum.

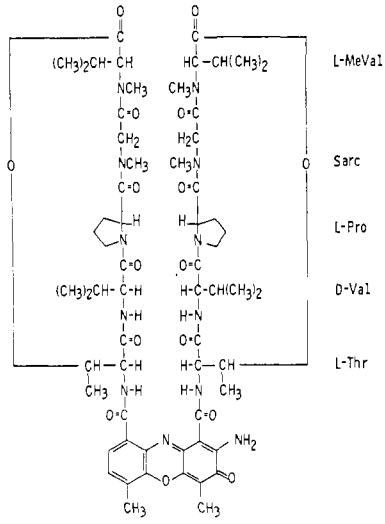

FIGURE 1: Actinomycin D.

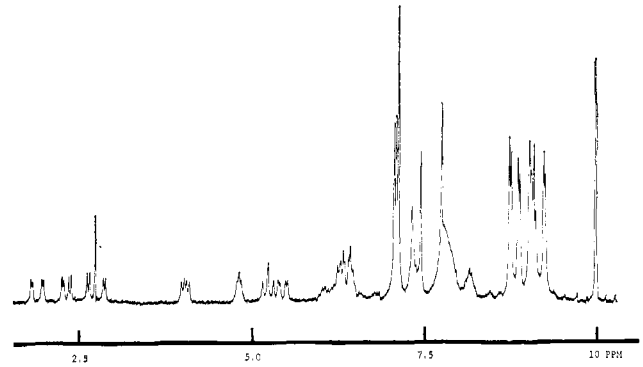

FIGURE 2: 220-MHz nuclear magnetic resonance spectrum of actinomycin D ($CDCl_3$).

and D_2O (extrapolated from dimethylformamide–D_2O systems) are presented in Table I. Assignments were made by spin decoupling in conjunction with a consideration of chemical shifts (Jardetzky and Jardetzky, 1958, Li *et al.*, 1962), multiplicities, and area relationships. Since this approach was common to all solvents, only the analysis of the spectrum in chloroform is discussed in detail.

Irradiation of the two-proton multiplet at 4.82 resulted in collapse of signals at 5.38 (1 H), 5.49 (1 H), and 8.73 (6 H). This established at CH_3CHCH fragment and identified the threonines, since these are the only residues characterized by two types of low-field CH protons. Subsequent irradiation of the individual Thr-α-CH then linked the amide doublets at 2.18 and 2.80 with the lower and higher α-CH signals, respectively.

The location of the D-Val-α-CH protons could now be determined by searching the complex region between 6.0 and 6.5 with the irradiating frequency, until collapse of the two D-Val-NH doublets at 1.91 and 2.06 was achieved. Since both amidic protons behaved identically over the decoupling range of 6.35–6.40, the chemical shifts of the 2-D-Val-α-hydrogens must be almost the same.

In a similar fashion both the D-Val-β-H and methyl protons were located when irradiation at 7.75–7.85 (*i.e.*, the β-CH) resulted in simultaneous collapse of the D-Val-α-CH and the methyl doublets at 8.87 and 9.09. Consequently, the two remaining methyl doublets at 9.04 and 9.24 must be associated with the MeVal methyl protons. Simultaneous collapse of these doublets and the doublets at 3.97 and 4.02 occurred upon irradiation at 7.25–7.40 which thus defined the position of the MeVal α and β protons, respectively.

The sarcosine methylene protons appear as two pairs of doublets (J = 17.5 cps) at (5.21, 6.37) and (5.28, 6.37). Assignment of the individual NCH_3 peaks could not be arrived at directly in view of the small differences in chemical shift. However, a pairwise association was obtained from a chloroform–benzene dilution study, since the two lowest field signals sustained large diamagnetic shifts in the aromatic solvent. Assignment of the solvent-sensitive pair to the sarcosine NCH_3 protons is favored for reasons discussed later.

The signals corresponding to the protons of the phenoxazone ring system were identified on the basis of known chemical shifts; the resonances at 2.36, 2.63, 7.44, and 7.76 were assigned to the protons at C_8, C_7, 6-CH_3, and 4-CH_3, respectively. Supportive evidence for these assignments is indicated by the selective broadening of the 2.63 and 7.44 peaks which results from long-range spin coupling between the C_7H and the 6-CH_3 protons. Similar weak interactions, in the order of 0.1–0.3 Hz, are commonly observed in para-substituted aromatic systems (Varian Associates, 1962, 1963).

The 2-amino protons appear as a low-lying broad resonance in the region of the chloroform line. The line width has been found to be quite variable which accounts for its apparent absence in the 220-MHz tracing.

With the contributions from the other residues known at this stage, all proline signals could be now identified from area and chemical shift considerations. The region from 7.6 to 8.0 is composed in part of the 4-CH_3 and the two β protons of the D-Val pair. Since the total area corresponds to eleven protons, the balance must contain six proline protons, which are most reasonably assigned to protons on C_3 and C_4. The one remaining methylene group of this set appears as a diamagnetically shielded multiplet near 8.2, and is believed to represent C_4 rather than C_3 protons. (Some spin-decoupling data appear to bear this out, although not conclusively.)

By similar reasoning, four proline protons are located in the multiplets between 5.95 and 6.50. This region also contains the D-Val-α-H and two sarcosine hydrogens, all four of which are located between 6.2 and 6.5. The absorption between 5.95 and 6.2, which must then arise solely from proline, appears to be composed of two partially overlapping single proton multiplets at 6.0 and 6.1. The better-resolved lower field signal, characterized by a fairly symmetrical contour with *ca.* 8-Hz spacings, is consistent with an α-hydrogen designation. A geminally split δ proton is a less likely alternative since the relatively large J/σ ratio would require a markedly different area distribution. Because of line-shape similarity with the 6.0 signal, the 6.1 multiplet is associated with the second Pro-α-CH.

With six of the eight signals in this region now assigned, the remaining two-proton resonance centered at about 6.30 defines the location of a proline CH_2N. The second proline CH_2N is a moderately broad "singlet" at 7.35 ($W_{1/2}$:6–7 Hz). Since this signal is virtually independent of field strength,

TABLE I: Actinomycin D Chemical Shifts.

Proton	$CDCl_3$ δ[a]	$CDCl_3$ J	Benzene δ	Acetone δ	Pyridine δ	Dimethylformamide δ	D_2O[b] δ
NHC=O D-Val	1.91	5.7	1.14	1.74	1.25	1.79	
NHC=O D-Val	2.06	6.0	1.37	1.82	1.35	1.79	
NHC=O Thr (1)	2.18	6.2	1.84	2.18	1.51	1.90	
NHC=O Thr (2)	2.80	6.8	2.25	2.50	1.74	2.06	
ArNH_2	2.6–2.7			~2.5	1.78	2.60	
ArC_8H	2.36	7.8	2.33	2.41	2.22	2.53	2.49
ArC_7H	2.63	7.8	3.17	2.57	2.70	2.53	2.49
α-CH-MeVal	3.97	~7.5	~3.64	3.81	3.31	3.74	3.83
α-CH-MeVal	4.02	~7.5	~3.66	3.85	3.39	3.74	3.83
β-CH-Thr	4.79	2.0–2.5 ($\beta\alpha$)	4.28	4.77	4.26	4.80	
β-CH-Thr	4.85	6.0 ($\beta\gamma$)	4.28	4.77	4.26	4.80	
α-CH-Thr (1)	5.38	~6.5 (α-NH)	4.94	5.29	4.83	5.29	
α-CH-Thr (2)	5.49	2.0–2.5 ($\alpha\beta$)	4.94	5.35	4.83	5.29	
Sar (1)	5.21 6.37	17.8	5.51 7.08	5.33 6.07	5.13 6.12	5.40 5.88	
Sar (2)	5.28 6.37	17.8	5.55 7.08	5.33 6.07	5.13 6.12	5.40 5.88	
α-CH-Pro	~6.0	Ind[c]	~5.7–6.1	~6.0	5.6–5.7	~5.9–6.0	
α-CH-Pro	~6.1	Ind[c]	~5.7–6.1	~6.4?	5.6–5.7	~5.9–6.0	
α-CH-D-Val	6.38	Ind[c]	~6.0	~6.32	5.8–5.9	6.14	
α-CH-D-Val	6.38	Ind[c]	~6.0	~6.32	5.8–5.9	6.14?	
CH_2N-Pro (2 H)	6.25–6.30		~6.0	~6.32	5.25–5.40	6.3–6.4	
NCH_3	7.06 (3 H)		7.23	7.11 (3)	7.24 (3 H)	7.06 (6 H)	6.98 (6 H)
			7.25	7.14 (3)	7.20 (3 H)	7.08 (6 H)	
NCH_3	7.11 (9 H)		7.80	7.18 (6)	7.00 (6 H)	7.17 (6 H)	7.09 (6 H)
			7.85				
CH_2N-Pro (2 H)	7.33 (m)		7.2–7.5	7.1–7.3?	6.88	6.92	
			7.56 (J gem 9.0)		7.2	~7.2–7.3	
β-CH-MeVal	7.25–7.40		7.3–7.4	7.34–7.44	7.15–7.25	~7.48	
6-CH_3	7.44		7.91	7.48	7.65	7.46	7.44
4-CH_3	7.76		8.13	7.96	7.85	7.83	7.84
β-CH-D-Val	~7.8		7.5–7.6	7.79–7.89	~7.35–7.45	~7.90	
3 + 4-CH_2-Pro	7.8–7.9		7.8–8.2	~7.9	7.7–8.0	~7.85	
4-CH_2 (2 H) Pro	8.15[d]		~8.2[d]	8.2[d]	7.7–8.0	8.17[d]	
Thr-CH_3	8.73	6.0	8.35	8.70	8.38	8.71	8.64
				8.72		8.73	
D-Val-CH_3	8.87	6.5	8.49	8.86	8.48	8.88	8.91[d]
D-Val-CH_3	9.09	6.5	8.52	9.11	8.74	9.14	9.13[d]
MeVal-CH_3	9.04		9.04 9.08	9.04	8.94	9.03	9.02[d]
MeVal-CH_3	9.24		9.49 9.50	9.24	9.26	9.24	9.19[d]

[a] τ scale. [b] Relative to internal dimethylsilapentanesulfonic acid. [c] Indeterminate. [d] Tentative.

its peculiar line shape cannot be attributed to geminal nonequivalence. Neither does a conformational averaging seem a likely explanation, since no change is evident over a 5–55° temperature interval. It can be rationalized, however, by assuming weak coupling with the vicinal C_4 protons. The possibility of such an arrangement is supported by the data in benzene, pyridine, and dimethylformamide, in which one of the two protons always appears as a relatively sharp, geminally split doublet (J = 9–10 Hz). The absence of additional splitting establishes that coupling with both C_4 protons is negligible. The remaining proton, in contrast, must engage extensively in vicinal coupling, since it is not readily observed.

Active Hydrogen. The behavior of the active hydrogens with regard to deuterium exchange, temperature variation, and hydrogen-bonding capabilities has been the subject of a recent publication (Victor *et al.*, 1969) and is therefore touched

upon only briefly here. The important finding reported by these workers that the D-Val-NH are difficult to exchange was noted in the present study. Their subsequent statement that these protons are less prone to form a hydrogen bond with acceptor solvents than those of the threonine is only partially supported by the solvent shift data in Table II. Downfield shifts relative to chloroform which are generally taken as indicative for hydrogen bonding are observed with one exception, for all amide hydrogens in the three acceptor solvents. The consistently larger deshielding of the higher field Thr-NH implies that it is more exposed to solvent influences than its twin. An explanation for this differential behavior lies in the possibility for the NH of the threonine attached to the quinoid side to form a hydrogen bond with the C_9 phenoxazone ketone. This configuration would also allow hydrogen-bond formation between the C_1 phenoxazone carbonyl and the 2-amino protons. Under these conditions the remaining Thr-NH, which faces outward, would not be involved in intramolecular hydrogen bonding. This arrangement thus favors the assignment of the low-field NH to the threonine attached to the quinoid side of the chromophore.

Solvent Effects. The benzene-induced solvent shifts (Table III) are distinguished less by some extreme displacements than by the indication of an apparent trend toward increased shielding with increasing distance from the phenoxazone ring. Relative to chloroform all protons on the threonine and D-valine residues experience marked deshielding in the aromatic medium. Protons in the adjacent Pro and MeVal pairs exhibit smaller shifts which are occasionally variable in sign. The two lower field sarcosine methylene protons sustain upfield shifts of 0.27–0.30 ppm. The higher field pair and (probably) the sarcosine NCH_3 protons show diamagnetic displacements between 0.69 and 0.79 ppm. Downfield shifts in excess of 0.3 ppm which characterized the Thr and D-Val protons are relatively rare. In general they are limited to instances where an occasional proton is favorably positioned with regard to one or preferably two negative groups (Williams and Wilson, 1966; Bowie *et al.*, 1967; Wilson and Williams, 1969). A zone of high electron density in the vicinity of the affected proton seems to be a requirement. The fact that major deshieldings are experienced by all Thr and D-Val protons is therefore given special significance. It implies that these residues are oriented so that most of their negative centers are located near the molecular periphery. It is thus reasonable to suggest that this region of the molecule presents a partial negative charge to the surroundings. This arrangement evidently does not apply to the remaining members of the two pentapeptides since they show a more conventional range of displacements.

By similar reasoning, the deshielding of the MeVal α-CH and the comparatively small diamagnetic shift on one of the sarcosine methylene protons follow from their distinctive low-field positions in chloroform which suggest that they must be located close to and approximately in the plane of a carbonyl group.

The decision to associate the two more strongly shifted NCH_3 groups with the sarcosine is based on several arguments. The sarcosines are the farthest removed from the chromophore and are therefore least likely to be affected by the deshielding influences just discussed. The fact that the sarcosine NCH_3 is adjacent to the higher shielded sarcosine methylene proton is supporting but not altogether compelling evidence since

TABLE II: Solvent Shifts of Amide Protons.

Proton	$\Delta\tau^a$ Pyridine	Acetone	Dimethylformamide
D-Val (low field)	−0.65	−0.17	−0.12
D-Val (high field)	−0.71	−0.24	−0.27
Thr (low field)	−0.67	0	−0.28
Thr (high field)	−1.06	−0.30	−0.74

[a] $\Delta\tau = \tau_{CDCl_3} - \tau_{solvent}$.

conformations are possible in which the MeVal NCH_3 group is similarly positioned. Finally, the differences in solvent shielding within any fully assigned amino acid do not exceed 0.6 ppm. To link the highly shielded NCH_3 to MeVal would confer an exceptionally large shift of 1.1 ppm for this residue and would also require that this difference be expressed over the comparatively short distance between the NCH_3 and the α hydrogen.

Benzene-induced shifts of the chromophore protons are consistent with many earlier findings for aromatic systems (Bowie *et al.*, 1967; Klinck and Strothers, 1962; Ronayne and Williams, 1968) in that larger upfield shifts are experienced by protons at the more electron-deficient sites. The displacements in pyridine generally parallel those in benzene except for much smaller diamagnetic shifts. This is in keeping with previous observations (Williams and Wilson, 1966) and may be attributed primarily to the poorer donor qualitites of the heteroaromatic system. Shifts in acetone and dimethylformamide resemble each other and moreover show a fair correspondence with those of chloroform.

D_2O Spectra. Of the three approaches explored in efforts to determine the nuclear magnetic resonance parameters of actinomycin D in water, the D_2O–dimethylformamide system was found to be the most reliable. Detailed monitoring of the trends with changing solvent composition and the finding that the associative phenomena characteristic of actinomycin D in water are repressed in the presence of organic solvents (Müller and Emme, 1965; Crothers *et al.*, 1968) led to this appraisal. (Since the studies at low temperatures and in supersaturated solutions at room temperature were done in pure D_2O, these data are considered less reliable and are therefore not discussed further. It should be noted, however, that of the eleven proton types which had been assigned at the lower temperatures, eight agreed to within ±0.05 ppm with the extrapolated values; two differed by ±0.12 (6-CH_3) and −0.13 (MeVal α-CH), and only one (4-CH_3) experienced a major displacement of +0.3–0.4 ppm. This last shift is believed to be a consequence of self-association.)

Dimethylformamide was found to be effective in solubilizing actinomycin D. A concentration of 2×10^{-3} M, sufficient for nuclear magnetic resonance studies, could be maintained in water containing as little at 1 mole % of dimethylformamide. Additives in this general concentration range are believed to be without appreciable influence on the spectral parameters. To verify this point, partial nuclear magnetic resonance spectra of actinomycin D in D_2O containing 1.1, and 2.5,

TABLE III: Benzene Solvent Shifts.

Proton	$\Delta\tau^a$	Proton	$\Delta\tau^a$
NHC=O (D-Val)	−0.77	$N\text{-}CH_3$	+0.12
NHC=O (D-Val)	−0.69	$N\text{-}CH_3$	+0.14
NHC=O (Thr-1)	−0.34	$N\text{-}CH_3$	+0.69
NHC=O (Thr-2)	−0.55	$N\text{-}CH_3$	+0.79
ArC_8H	−0.03	CH_2N Pro (low field)	−(0.2–0.3)
ArC_7H	+0.54	CH_2N Pro (high field)	−0.1
α-CH (MeVal)	−0.33		+0.2
α-CH (MeVal)	−0.36	$6\text{-}CH_3$	+0.47
β-CH (Thr)	−0.54	$4\text{-}CH_3$	+0.37
α-CH (Thr-1)	−0.44	$3\text{-}CH_2$ (Pro)	0–0.2
α-CH (Thr-2)	−0.55	$4\text{-}CH_2$ (Pro)	~+0.1
Sar 1 (1 H)	+0.30	CH_3 (Thr)	−0.38
Sar 2 (1 H)	+0.27	CH_3 (Thr)	−0.34
α-CH (D-Val)	~−0.4	CH_3 (D-Val-1)	−0.38
α-CH (D-Val)	~−0.4	CH_3 (D-Val-1)	−0.35
Sar 1 and 2 (2 H)	+0.71	CH_3 (D-Val-2)	−0.36
β-CH (D-Val)	~−(0.2–0.3)	CH_3 (D-Val-2-)	−0.36
β-CH (MeVal)	~0	CH_3 (MeVal)	−0.01
α-CH (Pro)	~(−0.4–0)		+0.04
		CH_3 (MeVal)	+0.26

[a] $\Delta\tau = \tau_{benzene} - \tau_{CCl_3}$.

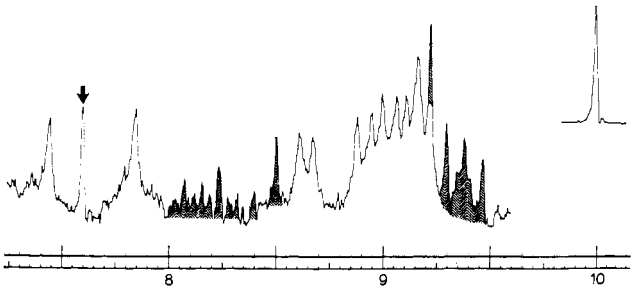

FIGURE 3: 100-MHz nuclear magnetic resonance spectrum of actinomycin D (2.4×10^{-3} M) in D_2O and dimethylformamide-d_7 (4 mole %).

and 3.8 mole % of dimethylformamide-d_7 were obtained and the results are presented in Table IV.[2] With the possible exception of the $4\text{-}CH_3$ signal, the data at all three levels of dimethylformamide agree to within experimental error. By analogy with the low-temperature data, it is possible that the small upfield displacement of the $4\text{-}CH_3$ resonance at the lowest dimethylformamide level represents self-association. Even if ultimately confirmed, it is apparent that the perturbation from this source is trivial. Further evidence that associative effects are small is indicated by the absence of severe line broadening in a typical spectrum (Figure 3). Compared to the internal dimethylsilopentanesulfonic acid and sodium succinate (arrow) standards, all methyl signals show additional broadening of only 1–2 Hz. The linearly extrapolated chemical shifts are therefore regarded as sufficiently reliable reference points for binding studies.

The tentative assignments proposed for the MeVal and D-Val β and γ protons are based on a comparison to the data in chloroform, acetone and dimethylformamide (Table V). Since the individual protons undergo only small chemical shift changes in these solvents, it is unlikely that large displacements will be observed in water. Hence, the preferred assignments are those which correspond most closely

[2] The chemical shifts for the low-field protons in Table I were obtained from the solutions containing 1.1 mole % dimethylformamide.

TABLE IV: Chemical Shifts of 2×10^{-3} M Actinomycin D in Dimethylformamide–D_2O Systems.

Proton	Mole % Dimethylformamide			0% Dimethylformamide[c]
	3.8	2.5	1.1	
NCH_3	6.97	6.98	6.99	6.995
NCH_3	7.09	7.09	7.09	7.09
$6CH_3$	7.43	7.44	7.45	7.455
$4CH_3$	7.83	7.82	7.86	7.84
Thr-CH_3	8.63	8.64	8.64	8.645
D-Val-CH_3[b]	8.90	8.92	8.91	8.915
MeVal-CH_3[b]	9.01	9.03	9.02	9.025
D-Val-CH_3[b]	9.12	9.14	~9.13	9.135
MeVal-CH_3[b]	9.19	9.20	~9.19	9.19

[a] Relative to internal dimethylsilapentanesulfonic acid. [b] Tentative assignment. [c] Linear extrapolated values.

TABLE V: Chemical Shift Comparison of MeVal and D-Val β and γ Protons in Various Media.

Proton	$CDCl_3$	Acetone	Dimethyl-formamide	D_2O[a] (Actinomycin–dGMP)	D_2O[a]
MeVal-β-CH	7.25–7.40	7.34–7.44	~7.48	7.45–7.55	
D-Val-β-CH	7.8	7.79–7.89	~7.90	7.75–7.85	
D-Val-CH_3	8.87	8.86	8.88	8.86[b]	8.91
D-Val-CH_3	9.09	9.11	9.14	9.10[b]	9.13
MeVal-CH_3	9.04	9.04	9.03	9.01[c]	9.02
MeVal-CH_3	9.24	9.24	9.24	9.17[c]	9.19

[a] Tentative assignments. [b] Associated with higher field β-proton signal. [c] Associated with lower field β-proton signal.

to the findings in the organic systems. In the actinomycin–deoxyguanylic acid complex there are two alternatives which depend solely on the assignments of the β protons.[3] Only if these are assigned as indicated in Table V do the chemical shifts of all six proton types agree well with those in the organic solvents. The assignments for the four methyl signals in water were then based on the chemical shift similarity with the data for the complex.

Conformation of Actinomycin D. The nuclear magnetic resonance characteristics of actinomycin D provide new insights into the more detailed aspects of its conformation. Although a number of specific geometric relationships can be inferred from solvent effects, unusual chemical shifts, and behavior of active hydrogens, these alone do not lead to a uniquely defined conformation. This is not surprising in view of the variety of reasonable conformations suggested by molecular models. The problem is further complicated by the need to simultaneously account for the overall close similarity of the individual residue pairs and for the occasional large local differences which appear in the prolines and threonines. While a reasonable explanation can be offered for the latter in terms of hydrogen bonding (see section on Active Hydrogens), the basis for the former remains obscure.

In assessing different configurational possibilities, it became evident that the crowded and constrained physical makeup was conducive to the expression of long-range shielding phenomena. These were assumed to be a leading factor in contributing to the unusual chemical shifts listed in Table VI. In simple structures, displacements exceeding 0.2–0.3 ppm from a "normal" value are sufficient to suggest specific orientations between a proton and a proximate anisotropic group. A larger threshold is necessary for actinomycin D because of the uncertain contributions to the chemical shifts of steric effects and interring anisotropic influences. Hence, comment is confined to displacements exceeding ± 0.5 ppm.

The fact that the MeVal α-protons are deshielded by approximately 1.7 ppm (Morlino and Martin, 1968; Beecham and Ham, 1968) requires that they be located close to and in the plane of one or preferably two carbonyl groups. Such a relationship is stereochemically possible with the proline and sarcosine ketones. Since the proline CH_2N protons can lie over the planes of both the proline and D-valine carbonyl groups, this could well account for their large 1.0-ppm diamagnetic shift. The adjacent C_4 methylene protons would also be influenced by the same anisotropic centers but their upfield displacement should be smaller since they are farther from the shielding source. This particular model thus provides a physical basis for a displaced 4-CH_2 signal and also suggests that both perturbed proline signals arise from the same residue.

The anomalously weak vicinal coupling constants of the abnormal proline CH_2N protons are attributed to a steric effect of undetermined origin. In the absence of specific leads it is possible to specify only that factors which alter the dihedral angles in the direction of 80–90°, enlarge the HC–C′ and C–C′H′ bond angles, or increase the C–C′ bond length, will cause a reduction in the coupling constant (Karplus, 1959, 1963).

The internal (*i.e.*, geminal) chemical shift of about 1.0 ppm between the sarcosine methylene protons indicates a significant difference in their local environment. This is accounted for by a conformation in which one of the two protons is adjacent to the sarcosine carbonyl group. As

TABLE VI: Unusual Chemical Shifts in Actinomycin D Spectrum ($CDCl_3$).

Proton	τ_{obsd}	τ_{calcd}[a]	$\Delta\tau$
MeVal-α-CH	4.0	~5.7	−1.7
Pro-α-CH	~6.0	~5.5	+0.5
Pro abnormal CH_2N	7.33	~6.3	+1.0
Pro abnormal 4-CH_2	8.15	~7.9	+0.2–0.3
Sar-CH_2			
1 H	5.25 (av)	~6.0	−0.7
1 H	6.37	~6.0	+0.3
D-Val-α-CH	6.38	~6.1	+0.2–0.3
MeVal-β-CH	7.25–7.40	~7.7	−0.4–0.5

[a] Based either on chemical shifts in dipeptide or on values of the amino acid in cationic form. The error in the calculated τ values is believed to be within ± 0.1 ppm.

[3] The four methyl signals cannot be assigned independently since they have been linked in pairs by spin decoupling to the indicated β protons.

TABLE VII

Proton	Actinomycin D	Actinomycin D–dGMP[a]	$\Delta\tau$[b]
A. Effect of 5′-dGMP on Nuclear Magnetic Resonance Spectrum of Actinomycin D			
Ar($C_{7,8}$)	2.49	2.68	+0.19
MeVal-α-CH	3.83	~3.78	−0.05 ± 0.02
NCH_3 (6 H)	6.99	6.98	−0.01
NCH_3 (6 H)	7.09	7.10	+0.01
6-CH_3	7.45	7.69	+0.24
4-CH_3	7.84	8.05	+0.21
Thr-CH_3	8.64	8.53	−0.11
D-Val-CH_3[c]	8.91	8.86	−0.05
MeVal-CH_3[c]	9.02	9.01	−0.01
D-Val-CH_3[c]	9.13	9.10	−0.03
MeVal-CH_3[c]	9.19	9.17	−0.02
	5′-DGA	Actinomycin D–dGMP[a]	$\Delta\tau$[b]
B. Effect of Actinomycin D on Nuclear Magnetic Resonance Spectrum of 5′-dGMP			
C_8	1.89	1.99	+0.10
C_1'	3.73	3.89	+0.16
C_2'	~8.09	NO[d]	
	~8.30		
C_3'	5.31	NO[d]	
C_4'	5.77	5.91	+0.14
C_5'	6.04	~6.20	~+0.16

[a] Molar ratio actinomycin D/dGMP:1:2. [b] $\tau_{complex} - \tau_{actinomycin\ D}$. [c] Tentative assignment. [d] NO = not observed.

already noted, a similar arrangement was deduced from the benzene solvent shifts.

Active hydrogens provide an additional source of structural information. The significance of the differential behavior of the Thr-NH protons has already been covered (see section on Active Hydrogens). Two other features bear on the conformational problem: (1) the general deshielding in acceptor solvents and (2) the resistance of the D-Val-NH to deuterium exchange. The first point implies that all active hydrogens are reasonably accessible to the solvent molecules. Consequently, conformations in which the active hydrogens are "buried" between the pentapeptide chains would be unlikely. The second point serves as a reminder that there is no necessary relationship between the acceptor solvent shift and the rate of deuterium exchange. The lack of correlation is not surprising since one describes an interaction whereas the other involves a reaction rate. It is quite reasonable to propose that the slow rate of exchange is due to hydrogen bonding as suggested by Victor *et al.* (1966) and/or by steric effects.

Most of the conformational deductions obtained from solvent studies have been summarized in the section on solvent effects. Chemical shifts in chloroform, acetone, dimethylformamide, and water generally agree to better than ±0.1 ppm (Table I). A comparison of the available data in water to those in the three organic solvents shows the following. (1) On the average, protons in water resonate at slightly lower fields. (2) Chemical shift differences between water and the organic media tend to diminish with increasing dielectric constant of the organic solvent. These trends are consistent in magnitude and direction with those expected from reaction field effects alone (Buckingham, 1960; Buckingham *et al.*, 1960; Abraham, 1961; Watts *et al.*, 1963; Watts and Goldstein, 1966; Bhacca and Williams, 1964). Hence it is reasonable to conclude that the conformation of actinomycin D is essentially invariant in these solvents. (The aromatic solvents are excluded from this evaluation because of the added complication of long-range shielding effects.)

Although models suggest a fairly rigid structure, some degree of conformational flexibility is indicated by a variable vicinal coupling constant for the abnormal proline CH_2N signal in benzene–chloroform dilution studies. The geminally split doublet shows secondary splitting ranging from <1 to 6 Hz over the range of 50–80% benzene.

The overall similarity of the individual residue pairs implies a relatively high degree of molecular symmetry which is best achieved by orienting the pentapeptides so that their "best planes" are parallel to each other and perpendicular to that of the phenoxazone nucleus. Such an arrangement would also be consistent with the hydrogen-bonding scheme suggested for the Thr-NH protons since this would tend to anchor the pentapeptides in the proposed configuration. The location of the threonine methyls above the plane of the phenoxazone ring is strongly indicated from the study on the complex with deoxyguanylic acid.

At the present time, the nuclear magnetic resonance data do not seem powerful enough to favor any one clockwise or counterclockwise sequencing arrangement of the pentapeptides.

Actinomycin D–Deoxyguanylic Acid Complex. The effect of 5′-deoxyguanylic acid on the nuclear magnetic resonance spectrum of actinomycin D is summarized in Table VIIA. It is evident that on complex formation, all protons on the chromophore experienced upfield shifts while both of the threonyl methyl groups are deshielded. These displacements require that the base is positioned above the chromophore and is oriented so that the planes of the two systems are parallel. The nuclear magnetic resonance data thus clearly favor a base-stacking model for the complex. To the extent that an analogy is justified, this may be taken as support for the intercalated actinomycin D–DNA model recently proposed by Müller and Crothers on the basis of equilibrium, kinetic, and hydrodynamic studies (Müller and Crothers, 1968). Over an antibiotic concentration range of 3×10^{-3}–10^{-2} M the spectrum of the 2:1 mixture was essentially invariant. Addition of 2 mole % dimethylformamide to the most dilute solution resulted in a selective downfield displacement of 0.05 ppm of the 4- and 6-methyl signals. This is consistent with previous observations which indicate that the stacking arrangement is inhibited by organic agents (Crothers and Ratner, 1968). A variable-temperature study indicated that no significant chemical shift changes occurred in the actinomycin spectrum between 5 and 32°. The nuclear proton (C_8) in dGMP shifted downfield slightly with decreasing temperature ($\Delta\tau$ − 0.04 ppm at 5°). Temperatures above 32° could not be studied because of precipitation of the antibiotic.

Increasing the ratio of dGMP to actinomycin (3–5/1.) was without effect on the actinomycin spectrum but caused small downfield shifts (0.02–0.05 ppm) in the dGMP signals which increased with increasing amounts of dGMP. This is interpreted as reflecting a rapid exchange between free and complexed dGMP. Ratios less than 2:1 could not be investigated because of precipitation of actinomycin.

An explanation for the upfield shifts of the sugar protons (Table VIIB) would seem to require a shielding source other than the actinomycin chromophore. This could conceivably reflect self-association of dGMP. To explore the possible influence of the pentapeptides, plans are underway to study the interaction between dGMP and actinomycin-like substances lacking the pentapeptide chains, *e.g.*, actinocin and proflavin.

It is understood that the picture of the complex is a qualitative one at the present time. The involvement of the threonine residues in the binding process necessitates that the chemical shifts of the α and β protons be determined. This may be accomplished with the help of triple-resonance studies. No less important is the assignment of the remaining proton signals in water so that the influence of binding over all regions of the antibiotic can be evaluated. The findings obtained from this study will be applied to nuclear magnetic resonance investigations of the binding between actinomycin D and DNA.

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