*172*, 417.
Taborsky, G. (1972), *Biochemistry 11*, 729.
Takano, T., Swanson, R., Kallai, O. B., and Dickerson, R. E. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 397.
Takemori, S., Wada, K., Ando, K., Hosokawa, M., Sekuzu, I., and Okunuki, K. (1962), *J. Biochem. (Tokyo) 52*, 28.
Tzagoloff, A., and MacLennan, D. H. (1965), *Biochim. Biophys. Acta 99*, 476.
Ulmer, D. D. (1965), *Biochemistry 4*, 902.
Vanderkooi, J., Erecińska, M., and Chance, B. (1973a), *Arch. Biochem. Biophys. 154*, 219.
Vanderkooi, J., Erecinska, M., and Chance, B. (1973b), *Arch. Biochem. Biophys. 157*, 531.
Wada, K., and Okunuki, K. (1968), *J. Biochem. (Tokyo) 64*, 667.
Wada, K., and Okunuki, K. (1969), *J. Biochem. (Tokyo) 66*, 249.
Wharton, D. C., and Griffiths, D. E. (1962), *Arch. Biochem. Biophys. 96*, 103.
Yonetani, T. (1967), *Methods Enzymol. 10*, 332.

# Peptide Antibiotic–Nucleotide Interactions. Nuclear Magnetic Resonance Investigations of Complex Formation between Actinomycin D and Deoxyguanosine 5′-Monophosphate in Aqueous Solution†

Dinshaw J. Patel

ABSTRACT: The structure of the 1 : 2 complex of actinomycin D with deoxyguanosine 5′-monophosphate (d-pG) has been investigated by nuclear magnetic resonance (nmr) spectroscopy in aqueous solution. The $^{1}H$, $^{13}C$, and $^{31}P$ chemical shifts of d-pG were monitored on addition of actinomycin D and the complexation shifts analyzed in terms of ring current effects of the phenoxazone ring of actinomycin D. The $^{13}C$ nmr data suggest a range of stacking geometries for the purine ring of d-pG relative to the phenoxazone ring for actinomycin D in solution. A comparison of the proton chemical shifts, temperature coefficients, and line widths of the exchangeable protons of d-pG, actinomycin D, and the 1 : 2 actinomycin D–d-pG complex at superconducting fields suggests that the $NH_2$ proton(s) of d-pG participate in intermolecular hydrogen bonds with acceptor group(s) on the actinomycin D in the complex in solution.

The peptide antibiotic actinomycin D consists of a phenoxazone ring system to which are attached two cyclic pentapeptide lactones. Actinomycin D binds reversibly to double-helical DNA (for a review, see Reich and Goldberg, 1964) and inhibits RNA synthesis. Actinomycin D exhibits a specificity for guanosine residues (Reich and Goldberg, 1964) and its attachment to guanosine-rich regions in the DNA sequence has been attributed to base stacking between the phenoxazone and the nucleic acid bases (Sobell *et al.*, 1971; Muller and Crothers, 1968; Waring, 1970), hydrogen bonding by the amino guanosine proton with an acceptor on the actinomycin D molecule (Sobell *et al.*, 1971; Hamilton *et al.*, 1963) and hydrophobic interactions between groups on the peptide and the nucleotide (Sobell *et al.*, 1971).

The recent X-ray investigation of the 1 : 2 complex between actinomycin D and deoxyguanosine (d-G) defines the conformation in the crystal and the interactions responsible for the stability of the complex (Sobell *et al.*, 1971; Jain and Sobell, 1972; Sobell and Jain, 1972). The cyclic pentapeptide lactones are on either side of the phenoxazone ring and exhibit twofold symmetry in the crystal. The lactone rings are related to each other by two intramolecular hydrogen bonds between the D-Val N proton of one ring and the D-Val carbonyl of the other ring. The d-G ring stacks on either side of the phenoxazone ring and the complex is further stabilized by two strong intermolecular hydrogen bonds between the guanine amino group and the carbonyl of L-Thr, two weak intermolecular hydrogen bonds between the N-3 ring proton of guanine and the N proton of L-Thr, and hydrophobic interactions between the sugar ring of d-G and the side chain of L-MeVal residue.

The structure of the antibiotic has also been approached by the application of conformational calculations (DeSantis *et al.*, 1972) which take into acount the available nuclear magnetic resonance (nmr) and infrared (ir) experimental data in nonaqueous solution (Victor *et al.*, 1969; Arison and Hoogsteen, 1970).

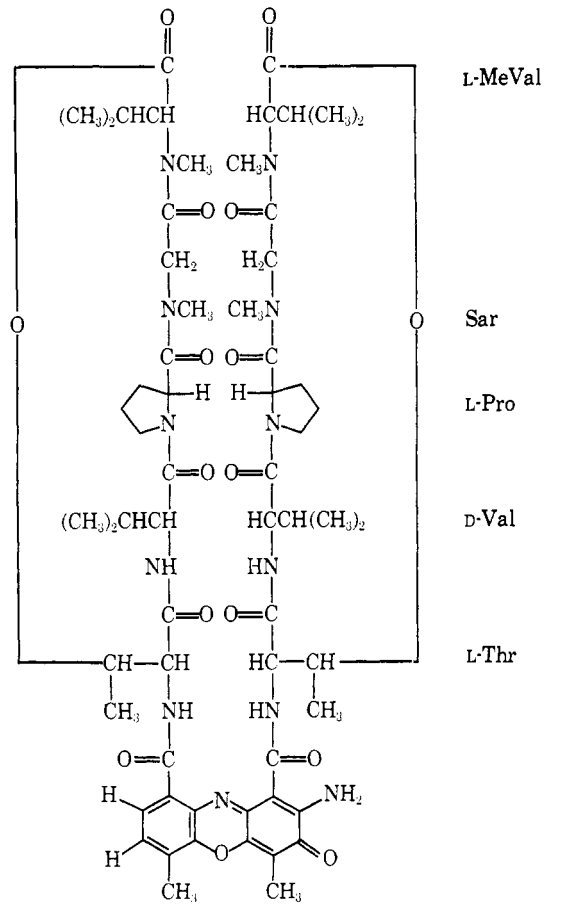
Actinomycin D dimerizes in aqueous solution through vertical stacking of phenoxazone rings (inverted relative to each other) with a dimerization equilibrium constant of $1.4 \times 10^{3}$ $M^{-1}$ at 18° (Angerman *et al.*, 1972; Krugh and Neely, 1973).

Complex formation between actinomycin D and deoxyguanosine 5′-monophosphate (d-pG) has been investigated by proton nmr spectroscopy in $D_2O$ solution (Arison and Hoogsteen, 1970; Danyluk and Victor, 1970; Krugh and Neely, 1973). Upfield shifts observed for proton resonances of the phenoxazone groups in the complex relative to those in the antibiotic were interpreted to support the intercalation model.

## Experimental Section

d-pG was purchased from Collaborative Research, Inc., Waltham, Mass., and was passed through a Chelex column prior to use. Actinomycin D was purchased from Merck.

† From the Bell Laboratories, Murray Hill, New Jersey 07974. *Received September 18, 1973.*




$^{13}C$ and $^{31}P$ were run on a Varian XL-100 spectrometer interfaced to a Nicolet 1080 computer in the Fourier transform mode. The spectrometer was locked to the deuterium resonance of solvent $D_2O$. $^{13}C$ chemical shifts at 25.165 MHz were referenced relative to internal dioxane which was assigned a chemical shift of 126.3 ppm relative to $CS_2$. $^{31}P$ chemical shifts at 40.51 MHz were referenced relative to external 16% phosphoric acid in $D_2O$. $^1H$ nmr spectra were run on a Varian 300 MHz superconducting spectrometer operating in either the field sweep or frequency sweep mode. $^1H$ chemical shifts were referenced to either internal sodium 3-(trimethylsilyl)-1-propanesulfonate in $D_2O$ or to the HOH signal in $H_2O$. The chemical shift of the HOH resonance relative to sodium 3-(trimethylsilyl)-1-propanesulfonate as a function of temperature was previously calibrated. The spectra were time averaged where necessary on a Nicolet 1074 computer employing 4K channels. Spin-decoupling and indor experiments were undertaken in the frequency-sweep mode utilizing a Varian decoupler with its own frequency synthesizer.

## Results

Two approaches are available for investigation of complex formation between actinomycin D and d-pG in aqueous solution. Krugh and Neely (1973) added incremental amounts of d-pG to actinomycin D in aqueous solution. Since actinomycin D dimerizes in water (Angerman *et al.*, 1972), the proton nmr shifts on complexation required correction.

*$^1H$ Nmr Studies.* The 300-MHz proton nmr spectrum of the 1:2 complex of actinomycin D–d-pG in $D_2O$, pH 7.7, 26°, has been studied, and the resonances of the peptide and nucleotide can be identified by inspection, spin-decoupling, and indor techniques. The chemical shifts are summarized in Table I along with those reported by Angerman *et al.* (1972) for actinomycin D in aqueous solution under conditions where dimerization is negligible. Neglecting shifts of <0.1 ppm, the $CH_3$ and H protons of the phenoxazone ring of the antibiotic are shifted upfield on complexation, consistent with earlier reports (Arison and Hoogsteen, 1970; Danyluk and Victor, 1970; Krugh and Neely, 1973). Consistent with the data of Arison and Hoogsteen (1970), the H-1′, H-4′, and H-5′ protons in the sugar ring of d-pG shift upfield by 0.1–0.15 ppm on complexation.

TABLE I: Proton Chemical Shifts in Parts per Million Relative to Standard Sodium 3-(Trimethylsilyl)-1-propanesulfonate for the Resonances of Actinomycin D, d-pG, and the 1:2 Actinomycin D–d-pG Complex in Aqueous Solution.

| Residue | Proton | Act-D[a] 4° | d-pG[b] pH 7.4, 26° | 1:2 Act-D–d-pG Complex[c] pH 7.7, 26° |
|---|---|---|---|---|
| L-N-MeVal | $CH_3$ | 0.82, 0.99 | | 0.81, 0.97 |
| | N-$CH_3$ | 3.01, 3.05 | | 3.00 |
| | $H^\beta$ | 2.55 | | ~2.47 |
| | $H^\alpha$ | 3.40 | | 3.16 |
| Sar | N-$CH_3$ | 2.90 | | 2.89 |
| | $H^\alpha$ | 3.70, 3.77 | | 3.63, 3.67 |
| D-Val | $CH_3$ | 0.85, 1.10 | | 0.89, 1.13 |
| | $H^\beta$ | 2.15 | | 2.23 |
| L-Thr | $CH_3$ | 1.38 | | 1.48 |
| | $H^\beta$ | 5.30[d] | | 5.29 |
| Phenoxazone | H-7,8 | 7.56[e] | | 7.30 |
| | $CH_3$-4,6 | 2.61,[e] 2.23[e] | | 2.31, 1.97 |
| Purine | H-8 | | 8.12 | 8.05 |
| Sugar | H-1′ | | 6.27 | 6.13 |
| | H-2′ | | 2.76, 2.50 | 2.79, 2.47 |
| | H-3′ | | 4.70 | 4.69 |
| | H-4′ | | 4.20 | 4.09 |
| | H-5′ | | 3.93 | 3.80 |

[a] The concentration and pH of this solution were not reported (Angerman *et al.*, 1972); Act-D = actinomycin D. [b] 0.04 M. [c] 0.02 M. [d] This investigation. [e] Chemical shifts reported for the actinomycin D monomer at pH 7.23, 18° (Angerman *et al.*, 1972).

The proton nmr spectra of the 1:2 complex of actinomycin D–d-pG in $H_2O$ at pH 3.5 and 7.05 are presented in Figure 1. The exchangeable protons at 6.38 and 6.59 ppm can be

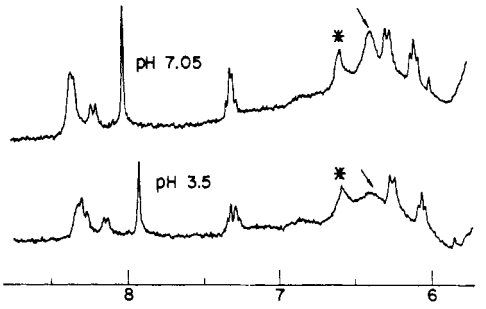



FIGURE 1: The proton nmr spectra of the 1:2 actinomycin D–d-pG complex in $H_2O$ at 28°. The top spectrum is at pH 7.05 and the bottom spectrum at pH 3.5. Sample concentrations were 0.025 M.

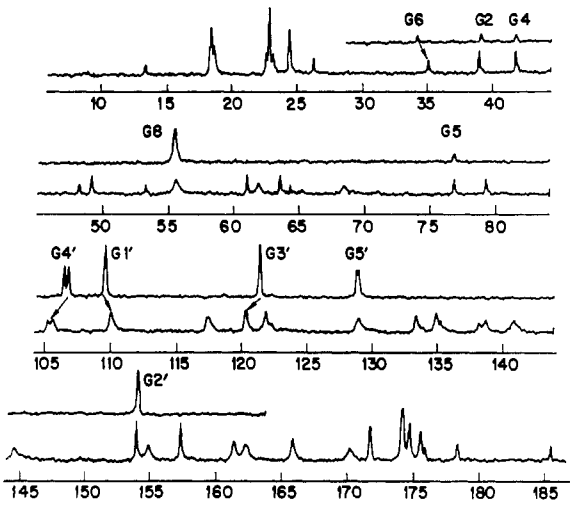



FIGURE 2: The carbon nmr spectra of 0.22 M d-pG in $D_2O$ (pH 7.3) at 30° (top), and 0.062 M 1:2 actinomycin D–d-pG in $D_2O$ (pH 7.3) at 30° in the region 0–200 ppm relative to standard $CS_2$.

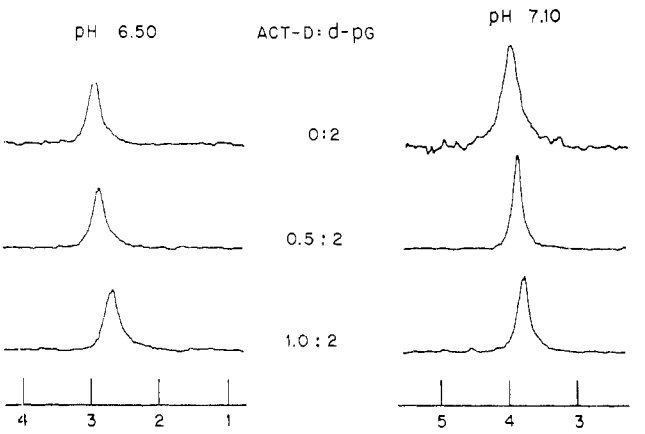



FIGURE 3: Changes in the $^{31}P$ chemical shift of 0.3 M d-pG on gradual addition of actinomycin D in $D_2O$ at 30°. Spectra are shown at pH 6.50 and 7.10 and referenced relative to 16% phosphoric acid as standard.

readily identified from a comparison with the spectrum in $D_2O$. These resonances exhibit pH dependent line widths.

*$^{13}C$ Nmr Studies.* The $^{13}C$ nmr spectra at 25 MHz of d-pG and the 1:2 complex of actinomycin D and d-pG are presented in Figure 2 and referenced relative to $CS_2$. Since there is no information available on the assignment of carbon chemical shifts for actinomycin D in aqueous solution, Table II compares the carbon chemical shifts of d-pG in the free and complexed forms. Since d-pG aggregates in aqueous solution, spectra were run at two different concentrations and the $^{13}C$ chemical shifts extrapolated to infinite dilution (Table II). At a given concentration and temperature, the $^{13}C$ chemical shifts of d-pG were independent of added EDTA. The purine C-6 carbon of d-pG shifts upfield by 1.15 ppm on complexation while the remaining four purine carbons are shifted upfield by 0.15–0.3 ppm (Table II). The sugar ring C-1′, C-3′, and C-4′ carbon resonances of d-pG shift 0.5–1.2 ppm on complexation while the C-2′ and C-5′ carbons are shifted by smaller amounts.

*$^{31}P$ Nmr Studies.* A pH study of the $^{31}P$ nmr resonance of d-pG in $D_2O$ at 30° has been undertaken. The phosphate group exhibits a $pK_a \sim 6.5$ and titrates over a 3.5-ppm range between pH 5 and 8.

The chemical shift of the $^{31}P$ resonance was investigated as a function of actinomycin D concentration at pH 6.5 and 7.1 and the data are presented in Figure 3 and Table III. The $^{31}P$ resonance shifts as an average signal with increasing actinomycin D concentration. An upfield shift of 0.2 ppm was detected for the $^{31}P$ signal in the 1:2 antibiotic–nucleotide complex.

*Hydrogen Bonding*

For actinomycin D, the exchangeable resonances are the peptide protons ($H^N$) of D-Val and L-Thr and the phenoxazone ring $NH_2$ protons (A-$NH_2$). For d-pG, the exchangeable resonances are the C-2′ hydroxyl proton and the purine ring NH (G-NH) and side-chain $NH_2$ (G-$NH_2$) protons. Since the actinomycin D–deoxyguanosine interaction involves intermolecular hydrogen-bond formation between G-$NH_2$ and a peptide carbonyl of L-Thr, investigations of the chemical shift and temperature coefficients of the exchangeable resonances on complex formation were undertaken.

*Chemical Shifts.* The effect of gradual addition of actinomycin D to the exchangeable G-$NH_2$ resonance of d-pG in $H_2O$ at pH 7.4 at 10 and 26° is presented in Figure 4. The G-$NH_2$ signal shifts downfield on complexation from 6.29 ppm in d-pG to 6.39 ppm in the complex at 26° (Table IV). The phenoxazone ring $NH_2$ proton (A-$NH_2$) exhibits a chemical shift independent of d-pG concentration in the complex.

*Temperature Coefficients.* The temperature dependence of the observable exchangeable protons of the 1:2 actinomycin D–d-pG complex in $H_2O$ (pH 7.4) is shown in Figure 5. The peptide N protons of D-Val and L-Thr resonate between

TABLE II: $^{13}C$ Chemical Shifts in Parts per Million Relative to Standard $CS_2$ for the Resonances of d-pG and the 1:2 Actinomycin D–d-pG Complex in Aqueous Solution.

| Guanosine Carbons | d-pG[a] $D_2O$, pH ~7, 30° | 1:2 Act-D–d-pG Complex[b] $D_2O$, pH ~7, 30° |
|---|---|---|
| C-6 | *33.93* | *35.09* |
| C-2 | 39.08 | 38.98 |
| C-4 | 41.56 | 41.79 |
| C-8 | 55.17 | 55.58 |
| C-5 | 76.73 | 76.94 |
| C-4′ | *106.68* | *105.47* |
| C-1′ | *109.40* | *110.10* |
| C-3′ | *121.32* | *120.40* |
| C-5′ | 128.74 | 128.99 |
| C-2′ | 154.08 | 153.94 |

[a] These carbon chemical shifts are assigned values at infinite dilution from an investigation of $^{13}C$ spectra of d-pG at 0.25 and 0.085 M concentrations. [b] 0.063 M.

TABLE III: $^{31}P$ Chemical Shifts in Parts per Million Relative to Standard 16% Phosphoric Acid for the Resonance of d-pG on Gradual Addition of Actinomycin D in Aqueous Solution.

| | pH 6.50 | pH 7.10 |
|---|---|---|
| d-pG[a] | 2.95 | 3.98 |
| 1:4 Act-D–d-pG | 2.83 | 3.86 |
| 1:2 Act-D–d-pG[b] | 2.65 | 3.78 |

[a] 0.3 M. [b] 0.3 M.

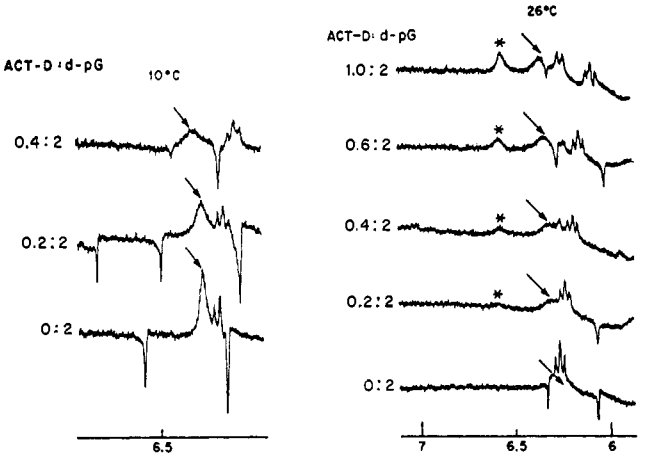



FIGURE 4: Chemical shift changes in the proton nmr spectrum of 0.05 M d-pG in $H_2O$ (pH 7.4) between 6 and 7 ppm, relative to standard sodium 3-(trimethylsilyl)-1-propanesulfonate, on gradual addition of actinomycin D at 10° (left) and 26° (right). The arrow indicates the exchangeable G-$NH_2$ protons and the asterisk the exchangeable A-$NH_2$ protons.

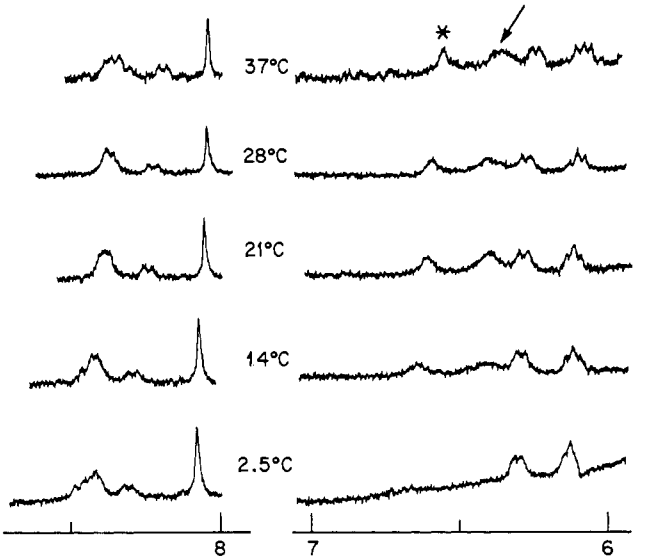



FIGURE 5: The temperature dependence (0–40°) of the proton chemical shifts between 6 and 9 ppm relative to standard sodium 3-(trimethylsilyl)-1-propanesulfonate of 0.05 M 1:2 actinomycin D–d-pG complex in aqueous solution. The asterisk indicates the A-$NH_2$ protons and the arrow the G-$NH_2$ protons.

8.2 and 8.5 ppm, the A-$NH_2$ protons are at ~6.6 ppm, and the G-$NH_2$ protons are at ~6.3 ppm.

The temperature coefficient of $3.3 \times 10^{-3}$ ppm/°C for the G-$NH_2$ resonance in d-pG in $H_2O$ (pH 7.4) decreases to a value of $2.4 \times 10^{-3}$ ppm/°C in the complex in $H_2O$ (pH 7.6) (Figure 6). The latter value may be compared with a temperature coefficient of $3.5 \times 10^{-3}$ ppm/°C for the A-$NH_2$ resonance in the complex (Figure 6).

Both peptide N protons of D-Val exhibit a temperature coefficient of $\sim 3 \times 10^{-3}$ ppm/°C while those of L-Thr have values of $3.8 \times 10^{-3}$ and $4.7 \times 10^{-3}$ ppm/°C for the 1 : 2 complex in $H_2O$ (pH 7.6) (Table IV).

Finally, inspection of Figure 5 indicates that on lowering the temperature from 40 to 0°, the exchangeable protons of A-$NH_2$ and G-$NH_2$ of the complex broaden significantly.

## Discussion

### *Stacking in Solution*

*Ring Current Models.* Giessner-Prettre and Pullman (1970) have evaluated the ring-current shifts in a plane parallel and at a distance of 3.4 Å from the guanosine purine ring (Figure 7). Giessner-Prettre and Pullman (1970) have estimated the ring currents in oxidized and reduced isoalloxazine ring systems, and found them to be similar. The ring current model derived for the oxidized isoalloxazine ring system is utilized for the phenoxazone ring system in this article.

TABLE IV: Proton Chemical Shifts in Parts per Million and Temperature Coefficients in ppm/°C for the Exchangeable Resonances A-$NH_2$ and G-$NH_2$ in the Spectra of d-pG and 1 : 2 Actinomycin D–d-pG in $H_2O$ Solution.

| | A-$NH_2$ | G-$NH_2$ |
|---|---|---|
| Chemical shifts (ppm) | | |
| d-pG[a] | | 6.29 |
| 1 : 2 Act-D–d-pG[b] | 6.60 | 6.39 |
| Temperature coefficients (ppm/°C) | | |
| d-pG | | $3.5 \times 10^{-3}$ |
| 1 : 2 Act-D–d-pG | $3.3 \times 10^{-3}$ | $2.4 \times 10^{-3}$ |

[a] 0.06 M, pH 7.4, 26°. [b] 0.025 M, pH 7.7, 26°.

*Ring Current Predictions.* The stacking geometries of the two guanosine purine rings relative to the phenoxazone ring of the antibiotic in the 1 : 2 actinomycin D–d-G complex determined by X-ray crystallography in the crystal are shown in Figure 8A (Jain and Sobell, 1972).

Let us first consider the ring currents from the guanosine purine ring on the chemical shifts of nuclei in the phenoxazine ring on complex formation. The $CH_3$-4 and $CH_3$-6 protons are predicted to be shifted upfield by ~0.2 ppm with a larger shift for $CH_3$-6. The H-7 proton is predicted to be shielded by

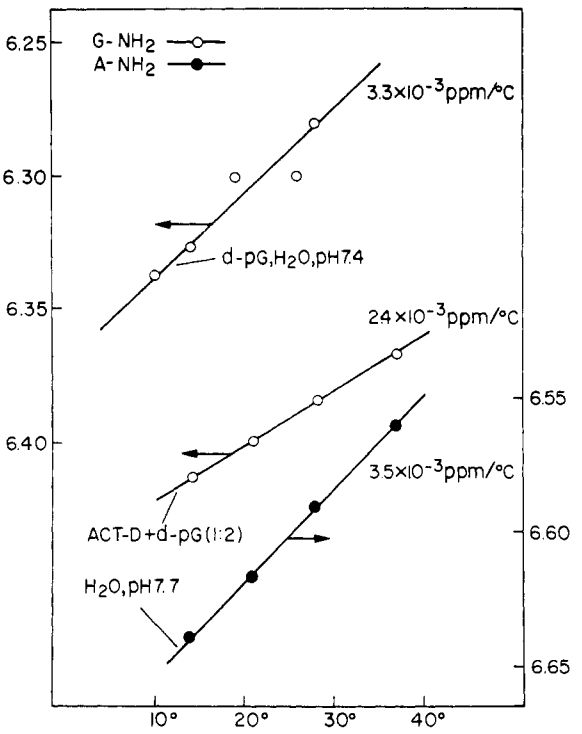



FIGURE 6: Plots of the temperature dependence of the proton chemical shifts between 6.0 and 6.7 ppm of the exchangeable resonances of 0.05 M d-pG and 0.05 M 1:2 actinomycin D–d-pG in aqueous solution.

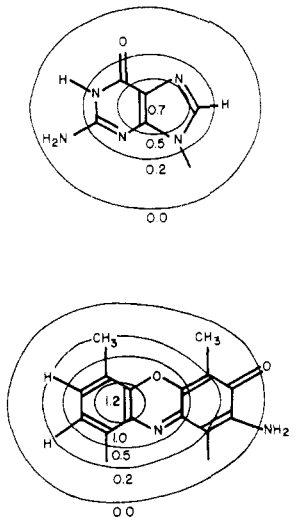


FIGURE 7: Ring-current contours (Giessner-Prettre and Pullman, 1970) in a plane parallel and at a distance of 3.4 Å from the guanosine purine ring (top). Approximate ring-current contours in a plane parallel to and at a distance of 3.4 Å from the phenoxazone ring (bottom).

0.2 ppm while the H-8 proton is predicted to be shielded by <0.2 ppm (Table V).

Let us next consider the ring current shifts from the phenoxazine ring on the guanosine $^1H$ and $^{13}C$ chemical shifts. Since the phenoxazone ring lacks an element of symmetry and the stacking geometry in the crystal is such that one purine ring intercalates over the benzenoid ring while the other purine intercalates over the quinonoid ring of the phenoxazone, an average ring current due to the two geometries will have to be considered.[1] The carbon chemical shifts of the purine ring carbons at positions 2, 4, 5 and 6 in guanosine are predicted to be upfield shifted by 0.6–0.7 ppm on complexation.

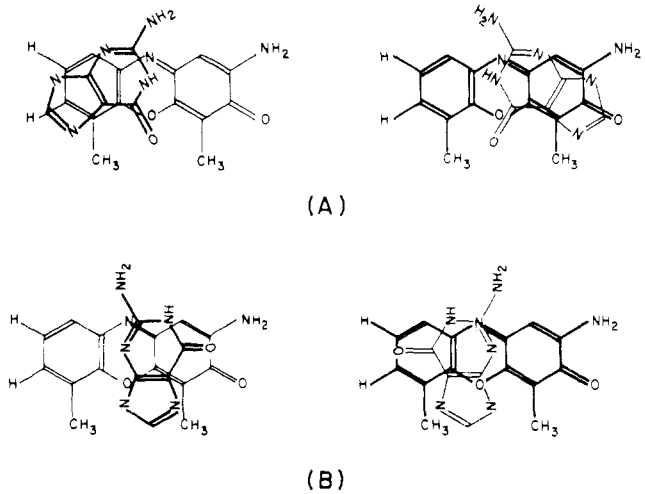


FIGURE 8: (A) Stacking orientation of the phenoxazone ring relative to the two purine rings of guanosine in the 1:2 actinomycin D–d-G structure determined in the crystal (Jain and Sobell, 1972); (B) alternate stacking geometry. In both parts A and B the darker rings are toward the viewer.

[1] This assumes that the two modes of d-pG have equal binding affinity. To this effect it has been observed that during the gradual addition of actinomycin D to d-pG, the $^1H$ and $^{13}C$ chemical shifts of actinomycin D are independent of d-pG concentration suggesting that all the actinomycin D in the presence of d-pG is in the form of the 1:2 actinomycin D–d-pG complex.

TABLE V: Predicted Ring-Current Shifts for the Sobell Model of the 1:2 Actinomycin D–d-pG Complex Based on the Ring-Current Contributions from the Purine Ring of Guanosine and the Phenoxazone Ring of Actinomycin D.

| Phenoxazone Residues | Upfield Shifts (ppm) |
|---|---|
| $CH_3$-4 | 0.2 |
| $CH_3$-6 | 0.2 |
| H-7 | 0.3 |
| H-8 | 0.1 |

| | Ring Currents | | |
|---|---|---|---|
| Guanosine Residues | Benzenoid | Quinonoid | Av |
| H-8 | 0.1 | 0 | 0.05 |
| H-2′ | 0.2 | 0 | 0.1 |
| H-3′, 4′, 5′ | 0 | 0 | 0 |
| C-2 | 0.8 | 0.4 | 0.6 |
| C-4 | 1.2 | 0.2 | 0.7 |
| C-5 | 1.0 | 0.2 | 0.6 |
| C-6 | 0.8 | 0.5 | 0.65 |
| C-8 | 0.3 | 0 | 0.15 |
| C-1′ | 0.2 | 0 | 0.1 |
| C-3′, 4′, 5′ | 0 | 0 | 0 |

*Experimental Ring-Current Shifts and Stacking Geometry.* Arison and Hoogsteen (1970) observed upfield shifts for the $CH_3$-4, $CH_3$-6, H-7, and H-8 phenoxazone protons on complexation with d-pG in aqueous solution. The data were interpreted to be consistent with an intercalation model. Krugh and Neely (1973) reported similar data and suggested that the magnitude and sign of the shifts in solution supported the published X-ray model of Jain and Sobell (1972) for the complex in the crystal.

Inspection of CPK models of the X-ray structure of the complex suggests that the guanosine amino protons at position 2 (G-$NH_2$) can hydrogen bond with the carbonyls at position 1 or 9 of the phenoxazone in addition to the carbonyl group of L-Thr.

Starting with the geometry of the complex in the crystal (Figure 8A; Jain and Sobell, 1972), and the ring current contours for the phenoxazone ring (Figure 7), the carbon resonances at C-2, C-4, C-5, and C-6 of the purine ring of d-pG are predicted to shift upfield by ~0.65 ppm on complexation with actinomycin D (Table V). These shifts are larger than the phenoxazone proton shifts discussed above and are more sensitive indicators of orientation(s) of the stacking residues. The experimental data in Table II show that the C-8, C-6, C-4, and C-5 carbons shift upfield by 0.3, 1.15, 0.2, and 0.2 ppm, respectively, while the C-2 carbon chemical shift remains essentially unchanged.

To explain the selective upfield shift of the d-pG C-6 carbon resonance on complexation in solution,[2] alternate stacking geometries from that determined in the crystal must be considered. One such geometry for intercalation in solution is presented in Figure 8B, and is consistent with a large upfield shift for the C-6 purine carbon resonance. For this arrangement one of the G-$NH_2$ protons can hydrogen bond with a

[2] A referee has suggested that the large shift of the C-6 of d-pG on complex formation may be due to subtle electronic and geometric changes in the ring system. This alternative interpretation cannot be ruled out since there is little experience in interpreting ring current shifts (carbon) induced by stacking aromatic rings together.

phenoxazone carbonyl group, though peptide–nucleotide hydrophilic interactions are absent for this conformation since the sugar residue is exposed to solvent.

It is therefore proposed that the purine ring of d-pG stacks in several orientations relative to the actinomycin D phenoxazone ring (of which the arrangements in Figure 8A,B are two possibilities), and that the interconversion between these orientations is rapid on the nmr time scale.

*Experimental Ring-Current Shifts and Sugar Geometry.* The chemical shifts of the sugar carbons and protons are predicted to be unaffected (0–0.1-ppm shifts) by the ring currents of the phenoxazone ring (Table V) in the crystalline structure of the complex. This conclusion remains unchanged if alternate stacking geometries (Figure 8B) are considered. Experimentally, the sugar protons shift ~0.1 ppm on complexation (Table I; Arison and Hoogsteen, 1970). More dramatic are the 0.7-ppm upfield shift for the C-1′ carbon and ~1-ppm downfield shifts for C-3′ and C-4′ carbons of the sugar on complexation. These shifts do not arise from ring currents of the phenoxazone ring. Though no quantitative interpretation of these experimental data is currently feasible, the observed carbon chemical shift changes for the sugar ring carbons C-1′, C-3′, and C-4′ (there is a small chemical shift change for side chain carbon C-5′) on complexation suggest that the torsion angle about the glycosidic bond and/or the sugar pucker for d-pG in the complex is different from that for the free nucleotide in solution.

The $^{31}P$ nucleus of d-pG is predicted to be distant from the phenoxazone ring of actinomycin D from the structure of the 1 : 2 actinomycin D–d-G complex structure in the crystal (Jain and Sobell, 1972), and thus unaffected by ring-current shifts from the phenoxazone. Experimentally, upfield shifts of 0.2 ppm are observed in d-pG for the $^{31}P$ resonance on complexation with actinomycin D at pH 6.5 and 7.1 in aqueous solution. The magnitude of this shift is small compared to the range of $^{31}P$ chemical shifts.

*Hydrogen Bonding in Solution*

Hydrogen-bond formation of the guanosine amino group ($G\text{-}NH_2$) with carbonyl groups of acceptor molecules has been investigated by proton nmr in solution. Katz and Penman (1966) observed an 0.8-ppm downfield shift for the addition of an equimolar amount of cytosine in $Me_2SO$ at 16°C. The downfield shift was assigned to Watson-Crick base pairing involving hydrogen-bond formation between $G\text{-}NH_2$ proton and cytosine carbonyl group. In a parallel study, Shoup *et al.* (1966) observed a temperature dependence for this shift from 0.52 ppm at 40° to 0.72 ppm at −10° in $Me_2SO$–dimethylformamide (1 : 1) mixtures. This downfield shift decreased to 0.3 ppm in $H_2O$–dimethyl sulfoxide (2 : 98) mixtures (Chan *et al.*, 1972). Raszka and Kaplan (1972) have reported a 0.15-ppm downfield shift for the $G\text{-}NH_2$ protons of 0.2 M pG on complexation with an equimolar solution of pC in aqueous solution.

From the intercalation geometry of the complex in the crystal (Figure 8A) (Jain and Sobell, 1972), and the phenoxazone ring current contours (Figure 7), an average upfield shift of ~0.2 ppm is predicted for the $G\text{-}NH_2$ protons resulting from the ring currents of the phenoxazone ring.[3] Experimentally, the $G\text{-}NH_2$ protons shift downfield by 0.1 ppm from 6.29 to 6.39 ppm on complexation with actinomycin D in aqueous solution at 26° (Table IV). Correcting for the ring current contribution, the ~0.3-ppm downfield shift is assigned to hydrogen-bond formation between the $G\text{-}NH_2$ proton(s) and acceptor group(s) on the actinomycin D.

The $G\text{-}NH_2$ protons and the exchangeable phenoxazone amino protons ($A\text{-}NH_2$) are broadened out in the 1 : 2 actinomycin D–d-pG complex on lowering the temperature from 37 to 2.5° (Figure 5). Since broadening occurs on lowering the temperature, it is proposed that the rates of rotation of the amino groups of $A\text{-}NH_2$ and $G\text{-}NH_2$ about the C–N bonds are decreased to an intermediate nmr exchange condition in the complex compared to fast rotation on the nmr time scale for the uncomplexed species in solution. Such restricted rotations of amino groups could arise if their exchangeable hydrogens participated in hydrogen bonds or are buried.

The temperature dependence of the chemical shifts of exchangeable protons has been utilized extensively in polypeptide conformational studies to elucidate whether the proton is hydrogen bonded or buried, on the one hand, or exposed to solvent, on the other. Temperature coefficients of $\geq 6 \times 10^{-3}$ ppm/°C for the exchangeable proton suggest exposure to solvent while coefficients of $\leq 2 \times 10^{-3}$ ppm/°C suggest its participation in a hydrogen bond or its burial from solvent water (for a review, see Bovey *et al.*, 1972). The $G\text{-}NH_2$ resonance of d-pG in aqueous solution exhibited a temperature coefficient of $3.3 \times 10^{-3}$ ppm/°C (Figure 6). One explanation for this low value is that d-pG in aqueous solution has been shown to rapidly rotate about the glycosidic bond with equal populations of syn and anti conformers (Tran-Dinh Son *et al.*, 1972). Since the $G\text{-}NH_2$ protons can hydrogen bond with the phosphates in the syn conformation, the temperature coefficients could be reduced relative to the value characteristic of exposed exchangeable protons. Indeed, for d-G in dimethylformamide, where the phosphate group is absent, the $G\text{-}NH_2$ proton exhibits a temperature coefficient of $6.4 \times 10^{-3}$ ppm/°C.

The temperature coefficient of the $G\text{-}NH_2$ proton in the 1 : 2 actinomycin D–d-pG complex has a value of $2.4 \times 10^{-3}$ ppm/°C characteristic of hydrogen-bonded or buried exchangeable protons (Figure 6).[4]

The phenoxazone amino protons ($A\text{-}NH_2$) of the actinomycin D exhibit a temperature coefficient of $3.5 \times 10^{-3}$ ppm/°C for the complex in solution (Figure 8) suggesting shielding of these protons from solvent water. The $A\text{-}NH_2$ protons are exposed to solvent in the crystal structure of the complex (Jain and Sobell, 1972).

Table IV summarizes the temperature coefficients of the peptide NH protons of D-Val and L-Thr. The resonances of L-Thr exhibit a temperature coefficient of 4.2 ± 0.5 ppm/°C while those of D-Val show a value of $3.0 \times 10^{-3}$ ppm/°C. In the crystal structure of the complex, the D-Val NH protons are intramolecularly hydrogen bonded to the D-Val carbonyl groups on the other ring, and the L-Thr NH protons are weakly hydrogen bonded to the N-3 ring nitrogen of guanine residues (Jain and Sobell, 1972). The observed temperature coefficients are consistent with the predictions of the structure of the complex in the crystal.

*Comparison of the Results in Solution with Those Determined in the Crystal*

Upfield proton and carbon chemical shifts of the purine ring of d-pG on complexation with actinomycin D in solution

[3] Since the $G\text{-}NH_2$ resonances participate in intermolecular hydrogen bonds their position relative to the phenoxazone ring is approximately fixed even though the purine ring may rapidly interconvert among various stacking orientations relative to the phenoxazone ring.

[4] An anti conformation exists about the glycosidic bond of the guanosine residue in the crystal of the 1 : 2 actinomycin D–d-pG complex (Jain and Sobell, 1972).

suggest that the purine and phenoxazone rings stack in the 1 : 2 actinomycin D–d-pG complex. The stacking geometry in the complex has been evaluated based on the ring currents of the phenoxazone ring on the purine carbon chemical shifts of d-pG. Compared to a unique orientation of stacked phenoxazone and purine rings in the structure of the complex in the crystal (Sobell and Jain, 1972), a range of geometries has been suggested in aqueous solution.

A strong nucleotide–peptide intermolecular hydrogen bond involving the 2-amino proton of guanine (G-$NH_2$) was observed in the structure of the complex in the crystal (Jain and Sobell, 1972). The presence of this intermolecular hydrogen bond in solution was verified by (i) the downfield shift in the G-$NH_2$ resonance of d-pG on complexation with actinomycin D, (ii) a temperature coefficient of $2.4 \times 10^{-3}$ ppm/°C for the G-$NH_2$ proton in the complex, and (iii) restricted rotation of the $NH_2$ group about the carbon–nitrogen bond in the complex as manifested in the temperature dependent line widths for this resonance.

Acknowledgment

I acknowledge the expert technical assistance of Miss L. L. Canuel.

References

Angerman, N. S., Victor, T. A., Bell, C. L., and Danyluk, S. S. (1972), *Biochemistry 11*, 2402.

Arison, B. H., and Hoogsteen, K. (1970), *Biochemistry 9*, 3976.

Bovey, F. A., Brewster, A. I., Patel, D. J., Tonelli, A. E., and Torchia, D. A. (1972), *Accounts Chem. Res. 5*, 193.

Chan, S. I., Lee, G. C. Y., Schmidt, C. F., and Kreishman, G. P. (1972), *Biochem. Biophys. Res. Commun. 46*, 1536.

Danyluk, S. S., and Victor, T. A. (1970), *Jerusalem Symp. Quant. Chem. Biochem. 2*, 395.

DeSantis, P., Rizzo, R., and Ughetto, G. (1972), *Biopolymers 11*, 279.

Giessner-Prettre, C., and Pullman, B. (1970), *J. Theor. Biol. 27*, 87.

Hamilton, L., Fuller, W., and Reich, E. (1963), *Nature (London) 198*, 538.

Jain, S. C., and Sobell, H. M. (1972), *J. Mol. Biol. 68*, 1.

Katz, L., and Penman, S. (1966), *J. Mol. Biol. 15*, 220.

Krugh, T. R., and Neely, J. W. (1973), *Biochemistry 12*, 1775.

Muller, W., and Crothers, D. M. (1968), *J. Mol. Biol. 35*, 251.

Raszka, M., and Kaplan, N. O. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2025.

Reich, E., and Goldberg, I. H. (1964), *Progr. Nucl. Acid Res. 3*, 183.

Shoup, R. R., Miles, H. Todd, and Becker, E. D. (1966), *Biochem. Biophys. Res. Commun. 23*, 194.

Sobell, H. M., and Jain, S. C. (1972), *J. Mol. Biol. 68*, 21.

Sobell, H. M., Jain, S. C., Sakore, T. D., and Norman, C. E. (1971), *Nature (London), New Biol. 231*, 200.

Tran-Dinh Son, Guschlbauer, W., and Gueron, M. (1972), *J. Amer. Chem. Soc. 94*, 7903.

Victor, T. A., Hruska, F. E., Hikichi, K., Danyluk, S. S., and Bell, C. L. (1969), *Nature (London) 223*, 302.

Waring, M. (1970), *J. Mol. Biol. 54*, 247.

# The Dissociation of Myosin in Acid†

Sara Szuchet* and C. Richard Zobel

ABSTRACT: Myosin has been dissociated into its constituent polypeptide chains by aqueous solutions of acetic acid (1–10 M). High-speed sedimentation equilibrium has been used to characterize the system. By using a very high speed (52,000 rpm), the molecular weights of the so-called "light subunits" could be measured in the presence of the "heavy" polypeptide chains. The system has proven to be complex; nevertheless, a rationale for the complexity has been given with the aid of information obtained from sodium dodecyl sulfate acrylamide gel electrophoresis. "Heavy" polypeptide chains free of the "light" counterpart have been obtained by fractionation of dissociated myosin on Sephadex G-200 using 1 M acetic acid. In the latter solvent "heavy" polypeptide chains exhibited molecular weight heterogeneity. This heterogeneity was greatly reduced and occasionally even disappeared (*e.g.*, at low concentrations), when the solutions were transferred to 10 M acetic acid. The apparent specific volume ($\phi'$) of "heavy" chains in 10 M acetic acid has been determined to be 0.704 ± 0.007 ml/g. Using this parameter we have obtained a weight average molecular weight of $[M_w]_{c=0}$ = 197,000 ± 2000. Our experiments suggest that (within experimental error) the two "heavy" polypeptide chains which make up the myosin molecule do not differ in molecular weight.

The quaternary structure of myosin has been the object of intense studies during the last 2 decades. As the techniques are more refined and the theory underlying them is better understood, so is the model for the molecule being refined too. Thus, according to the current model, the molecule is made up of two large polypeptide chains ("heavy" chains) and a number, yet to be exactly determined, of smaller polypeptide chains ("light" chains).

The dissociation of the "light" chains from the rest of the molecule is easily achieved, and a variety of physical and chemical means have been used for that purpose (Tsao, 1953; Kominz *et al.*, 1959; Wetlaufer and Edsall, 1960; Dreizen

† From the Department of Biophysical Sciences, State University of New York at Buffalo, Amherst, New York 14226. *Received July 11, 1973.* This Research was supported by Research Grants 50-8108-D from the G. R. S. of the State University of New York at Buffalo, GR-12-UB-70 from the United Health Foundation, and AM-07635 from National Institute of Health.