that the predominant complex should be the one in which the guanine is bound in the 4-binding site, while the adenine is bound in the 6-binding site (i.e., the complex on the right in Figure 6B). A more detailed discussion will be presented in the following paper (Chiao and Krugh, 1977).

Supplementary Material Available

Figure 2, 5, and 9 (4 pages). Ordering information is given on any current masthead page.

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.

Chiao, Y-C. C., and Krugh, T. R. (1977), *Biochemistry 16,* following paper in this issue.

Crothers, D. M., Sabol, S. L., Ratner, D. I., and Müller, W. (1968), *Biochemistry 7,* 1817.

Danyluk, S. S., and Victor, T. A. (1970), Symposium on Quantum Chemistry and Biochemistry, II, Jerusalem, The Israel Academy of Sciences and Humanities, p 394.

Giessner-Prettre, C., and Pullman, B. (1976), *Biochem. Biophys. Res. Commun. 70,* 578-581.

Hollstein, U. (1974), *Chem. Rev. 74,* 625.

Hyman, R. W., and Davidson, N. (1971), *Biochim. Biophys. Acta 228,* 38-48.

Krugh, T. R. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1911.

Krugh, T. R., and Chen, Y.-C. (1975), *Biochemistry 14,* 4912.

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

Krugh, T. R., and Neely, J. W. (1973b), *Biochemistry 12,* 4418.

Meienhofer, J., and Atherton, E. (1973), *Adv. Appl. Microbiol. 16,* 203-300.

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

Patel, D. J. (1974), *Biochemistry 13,* 1476.

Schara, R., and Müller, W. (1972), *Eur. J. Biochem. 29,* 210.

Sobell, H. M. (1973), *Prog. Nucleic Acid Res. 13,* 153.

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

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

Van Geet, A. L. (1970), *Anal. Chem. 42,* 679-680.

Wang, J. C. (1971), *Biochim. Biophys. Acta 232,* 246.

Waring, M. (1970), *J. Mol. Biol. 54,* 247-279.

Wells, R. D., and Larson, J. E. (1970), *J. Mol. Biol. 49,* 319.

Actinomycin D Complexes with Oligonucleotides as Models for the Binding of the Drug to DNA. Paramagnetic Induced Relaxation Experiments on Drug–Nucleic Acid Complexes†

Yu-Chih Chen Chiao and Thomas R. Krugh*,‡

ABSTRACT: Mn(II) ions have been used as a paramagnetic probe to investigate the geometry of drug-oligonucleotide complexes. Nuclear magnetic resonance and electron spin resonance experiments show that Mn(II) ions bind approximately two orders of magnitude stronger to the 5′-terminal phosphate group than to the 3′-5′ phosphodiester linkage of deoxydinucleotides. By using mixtures of nucleotides in which only one nucleotide contains a terminal phosphate group, the location of the Mn(II) ion in the drug-nucleotide-Mn(II) complexes may be preselected. The paramagnetic induced relaxation of the nuclear spin systems in these complexes has been used to investigate the geometry of these complexes. These data confirm that actinomycin D is able to recognize and preferentially bind guanine (as opposed to adenine) nucleotides in the quinoid portion of the phenoxazone ring, while both adenine and guanine will bind to the benzenoid portion of the phenoxazone ring. These results suggest that stacking forces are primarily responsible for the general requirement of a guanine base when actinomycin D binds to DNA.

In the preceding manuscript (Krugh et al., 1977) we have investigated the geometry of the complexes formed between actinomycin D and mixtures of deoxymono- and dinucleotides by analyzing the changes in the proton chemical shifts of the actinomycin D and nucleotide resonances. In the present manuscript we show that the paramagnetic Mn(II) ion binds much stronger to terminal phosphate groups than to phosphodiester groups, and we utilize this phenomenon to specifically locate the paramagnetic probe in actinomycin D-nucleotide-manganese(II) complexes. Paramagnetic metal ions have been previously used as a probe to obtain structural information in a variety of biological systems. Electron spin resonance (ESR)[1] may be used to directly monitor changes in the environment (e.g., ligation) of the paramagnetic metal ion,

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ESR, electron spin resonance.

TABLE I: The ESR Spectral Intensities of Mn(II) Solutions Relative to the Intensity of Uncomplexed Mn(II) Ion at 25 °C.[a]

Substrate Added[b]	$I_{mixture}/I_{free} \times 100\%$
Act D	100
dGMP(pdG)	44
Act D-(dGMP) (1:1)	43
dA-dC	95
Act D-(dA-dC) (1:1)	95
Act D-(dA-dC)-(dGMP) (1:1:1)	42
dG	96
Act D-(dG) (1:1)	96
pdA-dC	58
Act D-(pdA-dC) (1:1)	58
Act D-(pdA-dC)-dG (1:1:1)	55
dAMP	58
Act D-d(AMP) (1:1)	58
pdG-dC[c]	30
Act D-(pdG-dC) (1:2)[d]	30
dG-dC[c]	90
Act D-(dG-dC) (1:2)[d]	90
pdC[a]	55
pdC-dG[a]	61
pdC-dA[a]	62

[a] All the solutions contain 3 mM Mn(II), except pdC, pdC-dG, and pdC-dA where the Mn(II) and nucleotide concentrations were both 1 mM. [b] All the substrates are 3 mM, except where noted. [c] The concentration of the substrate is 6 mM. [d] The concentration of the Mn(II) is 3 mM, and the concentration of the nucleotide is 6 mM.

while nuclear magnetic resonance (NMR) techniques may be used to determine structural information on the metal ion complex (e.g., see the reviews by Krugh, 1976; Mildvan and Cohn, 1970; Swift, 1973; Dwek, 1973). A variety of workers have studied the binding of paramagnetic metal ions to nucleosides, nucleoside mono-, di-, and triphosphates, and to DNA (e.g., see Cohn and Hughes, 1962; Shulman et al., 1965; Reed et al., 1971; Anderson et al., 1971; Wee et al., 1974, Kotowycz and Hayamizu, 1973; Reuben and Gabbay, 1975; and the many references therein). These nuclear magnetic resonance experiments have shown that the Mn(II) ion binds to the phosphate group of nucleotides and forms a second ligand site at the N(7) position of the base, either directly or through a water bridge. The unpaired electrons of Mn(II) induce relaxation in the nuclear spin system. The relaxation rate for the direct interaction of a nuclear spin with a paramagnetic ion is proportional to r^{-6}, where r is the separation between the nuclear spin and the electron spin. Thus the use of paramagnetic metal ions provides the opportunity of gaining geometric information on the complexes. In the present experiments we add Mn(II) ions to solutions of actinomycin D with various mixtures of nucleotides in order to gain independent information on the ability of actinomycin D to preferentially bind guanine and adenine nucleotides.

Experimental Section

Actinomycin D was obtained from Merck, Sharp, and Dohme Research Laboratories. The deoxymononucleotides were purchased from Sigma Chemical Co., the deoxydinucleotides were purchased from Collaborative Research Inc. Actinomycin D and the nucleotides were passed through separate 1 × 10 cm columns of Chelex 100 (100–200 mesh, Bio-Rad) to remove paramagnetic impurities. After lyophilization, the samples were dissolved in distilled D_2O (Bio-Rad). The pH was checked and adjusted with NaOD or DCl. $MnCl_2 \cdot 4H_2O$ (Fisher Scientific Co., analytical grade) was used as the source of Mn(II) ion. The pH of the manganous solution was adjusted to pH_{meter} 7.01 (pD 7.41) with NaOD and DCl. Manganous concentration was determined by weight. The concentrations of other solutions were determined spectrophotometrically. For actinomycin D, an extinction coefficient of 23 500 at 425 nm was used. The extinction coefficients listed in P-L Biochemicals catalog No. 103 for the deoxydinucleotide monophosphates were used for all the dinucleotides and dinucleoside monophosphates. The presence of the terminal phosphate in the deoxydinucleotides is not expected to change significantly the magnitude of the extinction coefficients at λ_{max} for the respective deoxydinucleoside monophosphates.

The ^1H nuclear magnetic resonance spectra were recorded on a JEOL PFT-100 NMR spectrometer interfaced with an EC-100 computer. A 180°-τ-90° (WEFT) pulse sequence was used to minimize the residual water magnetization (Patt and Sykes, 1972; Benz et al., 1972; Mooberry and Krugh, 1975; Krugh and Schaefer, 1975). Varying amounts of a 0.001 M solution of $MnCl_2 \cdot 4H_2O$ were added to the nucleotide and actinomycin D–nucleotide solutions. The spectrum was recorded after each addition. The temperature in all cases was probe temperature (25 °C). Sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$ (Merck) was used as a chemical shift reference and as a line width reference to correct for the broadening due to the magnetic field inhomogeneity.

The *X*-band electron spin resonance spectra were recorded at 9.45 GHz on a JEOL 3BX spectrometer equipped with a cyclindrical cavity, using a field modulation frequency of 100 kHz. Due to the high dielectric constant of aqueous solutions, 1-mm (inner diameter) capillary tubes were used as sample containers. The ESR spectra were recorded at room temperature (~22 °C). The ESR spectra of different concentrations of free manganous ion were measured to calibrate the intensity measurements.

Results

ESR Binding Studies. Electron spin resonance provides a convenient monitor of the formation of Mn(II) complexes because a change in the symmetry of the aquated Mn(II) ion results in a broadening of the ESR lines. The Mn(II) ion has a $3d^5$ electron configuration ($S = 5/2$), while the ^{55}Mn nucleus has a nuclear spin of 5/2 ($I = 5/2$), which results in a six-line ESR spectrum. The line width is strongly influenced by the zero-field splitting, which is determined by the deviation from spherical symmetry of the electric field on the ion (e.g., Reed et al., 1971; Basosi et al., 1975). The change in the symmetry that results from replacing a solvated water molecule with a ligand (such as a nucleotide) results in both a broadening and a reduction in the peak to peak amplitude of the ESR resonances. The ESR spectrum of a 1 mM Mn(II) solution in the presence of a large excess of pdG (300 mM) gave a spectrum of the bound Mn(II), which was identical with previously published spectra (e.g., Reed et al., 1971). The peak-to-peak amplitude of the inner lines (e.g., the third line) of a dGMP bound Mn(II) ion is only a few percent (< 3%) of the peak-to-peak amplitude of the same line for an aqueous Mn(II) solution. As a result, the observed amplitude of the ESR resonances of Mn(II) solutions is essentially proportional to the concentration of *uncomplexed* Mn(II) ions.

Thus the measurement of the peak-to-peak amplitudes provides a convenient means of monitoring complex formation, as illustrated by the data in Table I. The ESR spectra of a Mn(II) solution and a Mn(II) solution with actinomycin D are identical, which shows that Mn(II) does not bind to actino-

TABLE II: Effect of Mn(II) Ion on the Line Widths of Selected Protons of pdG-dC and dG-dC.

Proton	$\Delta\nu$ (Hz)[a]					
	3 μM Mn(II)		10 μM Mn(II)		26 μM Mn(II)	
	pdG-dC[b]	dG-dC[b]	pdG-dC	dG-dC	pdG-dC	dG-dC
G-H(8)	2.5	0	13	0	34	0
C-H(6)	0	0	0.5	0	≥2[c]	0
H(1′)	0	0	1	0	3[c]	0
H(1′)	0	0	1	0	3[c]	0
C-H(5)	0	0	1.5	0	≥2[c]	0

[a] The full change in width at half-height of the peak. The uncertainty is 0.5 Hz. [b] Nucleotide solutions (10 mM) in D_2O, pH 7, 25 °C. [c] These values have a larger uncertainty due to overlapping peaks.

mycin D. The data in Table I also show that the nucleotides lacking a terminal phosphate (dG, dA-dC, and dG-dC) bind the Mn(II) ion only weakly under the present experimental conditions. On the other hand, the nucleotides that have a terminal phosphate will form a complex with Mn(II) ions. The observation that Mn(II) ion binds to a terminal phosphate group much stronger than to an internucleotide phosphate group is consistent with the double negative charge of the terminal phosphate group as opposed to the single negative charge of the internucleotide phosphate group (at pH 7.0).

The data in Table I also illustrate that the binding of Mn(II) to the nucleotides is not significantly affected by the formation of a complex with actinomycin D (to form an actinomycin D–nucleotide–Mn(II) complex). This is consistent with the geometry of the actinomycin D–nucleotide complex as well as the Mn(II)–nucleotide complex, as will be discussed later. The data in Table I can also be used to calculate approximate binding constants for the formation of Mn–nucleotide complexes and, for example, we find that $K \sim 10^3$ M^{-1} for the binding of Mn(II) ion to pdG, as compared with a $K \sim 10$ M^{-1} for the binding of Mn(II) to dG (deoxyguanosine).

Proton Magnetic Resonance Results. The proton magnetic resonance spectra of an actinomycin D solution show no appreciable changes in the line widths of the actinomycin D resonances when 1×10^{-4} M Mn(II) ion is added. The ESR data also showed that there is no interaction between actinomycin D and Mn(II) ion under the present conditions. However, Mn(II) does bind to the phosphate group of nucleotides and nucleic acids, with the N(7) of the purines serving as a secondary ligand site (e.g., Anderson et al., 1971; Lam et al., 1974; Reuben and Gabbay, 1975; Shulman et al., 1965; Kotowycz and Hayamizu, 1973; Reed et al., 1971; Wee et al., 1974; Cohn and Hughes, 1962; and references therein). The spectra in Figure 1 (Figure 1 is in the supplementary material; see paragraph at the the end of this paper concerning supplementary material) illustrate the effect of Mn(II) on the spectra of pdG-dC and dG-dC. The preferential broadening of the G-H(8) resonance as compared with the C-H(6) resonance in the pdG-dC spectra serves to locate the binding of the Mn(II) ion at the terminal phosphate group. The line width data as a function of added Mn(II) are listed in Table II. We also note that the G-H(8) resonance in pdG-dC is further downfield than the G-H(8) resonance in dG-dC; this results from the electrostatic deshielding due to the 5′-phosphate group in pdG-dC, which indicates that the guanine base is in an anti conformation (e.g., see Ts'o, 1974).

The ^{1}H NMR line widths of pdA-dC and dA-dC as a function of Mn(II) concentration are listed in Table III (in supplementary material). In the pdA-dC spectra the Mn(II) ion preferentially broadens the A-H(8) resonance as compared with the A-H(2) resonance, which is consistent with the Mn(II) binding to the terminal phosphate group with the adenine base in the anti conformation. The ^{1}H NMR and ESR results both show that only a small amount of Mn(II) binds to dA-dC under the present experimental conditions.

The ESR (Table I) and the ^{1}H NMR line width data (not included) show that Mn(II) ions bind to the terminal phosphate group of pdC and pdC-dG, which again illustrates that it is the presence of the terminal phosphate group and not the nature of the base that is the determining factor for the binding of Mn(II).

Actinomycin D–2(pdG-dC) and Actinomycin D–2(dG-dC) Complexes. Previous results have shown that actinomycin D binds either two pdG-dC or two dG-dC molecules to form a miniature double helical complex in which the phenoxazone ring of actinomycin D is intercalated between two G·C base pairs (Krugh, 1972; Krugh and Neely, 1973b; Schara and Müller, 1972; Patel, 1974; Krugh and Chen, 1975), as schematically illustrated in Figure 2. The addition of Mn(II) to the 2:1 pdG-dC–Act D solution results in a broadening of both the nucleotide resonances and the actinomycin D resonances (Table IV). The actinomycin D resonances which were monitored are the 4-CH_3 and the 6-CH_3 groups, the H(7) and H(8) protons, the MeVal N-CH_3, the Sar N-CH_3, and the Thr-CH_3 groups (see Figure 1 of Krugh et al., 1977). Since the Mn(II) ion is bound to the nucleotide (Figure 2), we can be certain that the increases in the line width of the actinomycin D resonances are a result of dipolar relaxation (see, for example, Swift, 1973). As a result, the increase in the line width is proportional to r^{-6}, where r is the distance between the Mn(II) ion and the proton(s) whose line width is being measured. Even at the highest Mn(II) concentration used, there is a large excess of nucleotide binding sites so that only a small fraction of the actinomycin D–2(pdG-dC) complexes have one Mn(II) ion bound and a negligible fraction have two Mn(II) ions bound. The magnitude of the manganese binding constants and the appearance of the ^{1}H NMR spectra as a function of added Mn(II) show that the Mn(II) is in the rapid exchange limit on the ^{1}H NMR time scale.

The G-H(8) proton of pdG-dC broadens rapidly as a function of added Mn(II) (Table IV) and then disappears into the baseline. The paramagnetic induced broadening of the 4-CH_3 and the 6-CH_3 resonances is approximately the same (within experimental error) in the actinomycin D–2(pdG-dC) complex. The H(7) and H(8) proton resonances, which exhibit a well-resolved AB pattern in the initial actinomycin D–2(pdG-dC) solution, are differentially broadened upon the addition of Mn(II) ion as illustrated in Figure 3. The MeVal N-CH_3, the Sar N-CH_3, and the Thr-CH_3 resonances do not exhibit any measurable line broadenings.

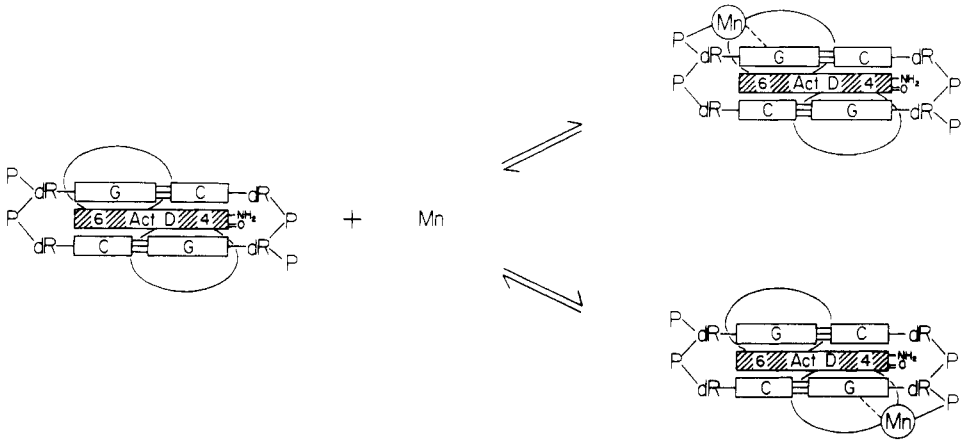

FIGURE 2: Schematic illustration of the binding of Mn(II) ion to the two terminal phosphate groups of an actinomycin D-2(pdG-dC) complex. The dashed line between the Mn(II) ion and the guanine base represents the secondary ligation to the guanine N(7). In this complex the guanine N(7) is out of the plane of the figure and away from the pentapeptide rings of actinomycin D.

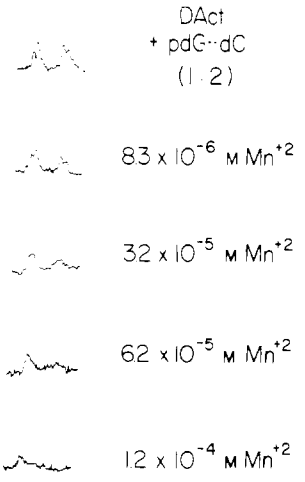

FIGURE 3: The effects of Mn(II) ion on the H(7) and H(8) resonances of actinomycin D in a 3 mM actinomycin D-2(pdG-dC) solution in D_2O at 25 °C. The manganese was incrementally added to the initial actinomycin D-2(pdG-dC) solution.

The paramagnetic induced line broadening data for the incremental addition of Mn(II) to an actinomycin D-2(dG-dC) solution are also given in Table IV. The deoxydinucleoside monophosphate dG-dC lacks a terminal phosphate group and consequently we observe only a small paramagnetic induced line broadening at the higher concentrations of Mn(II).

Actinomycin D. Complexes with pdG and dG. In previous experiments (Krugh and Neely, 1973a; Krugh and Chen, 1975), we have shown that the actinomycin D-2(dGMP) and the actinomycin D-2(dG) complexes have a conformation similar to the actinomycin D-2(dG) crystal structure reported by Sobell and co-workers (Sobell et al., 1971; Sobell and Jain, 1972). The paramagnetic induced line broadenings observed for the incremental addition of Mn(II) to an actinomycin D-pdG (1:1) solution are listed in Table V. Actinomycin D will form a complex with two pdG(5′-dGMP) molecules in solution (e.g., Arison and Hoogsteen, 1970; Krugh, 1972; Krugh and Neely, 1973a) with approximately equal binding constants. However, in preparation for the experiments to be described in the next sections, we have monitored the paramagnetic induced broadening in a 1:1 actinomycin D complex with pdG. The data in Table V show that the 4-CH_3 and the 6-CH_3 resonances are broadened to the same extent by the addition of Mn(II). This observation is consistent with the crystalline geometry of the actinomycin D-deoxyguanosine complex (Sobell et al., 1971) and the observation that dGMP has approximately equal binding constants for the two nucleotide binding sites on the phenoxazone ring of actinomycin D. The ^1H NMR spectrum of an actinomycin D-deoxyguanosine (1:1) solution remains unchanged when Mn(II) is added, except at high concentrations of Mn(II) (>50 μM) where the G-H(8) resonance begins to broaden. This result is consistent with the ESR data (Table I) and confirms that Mn(II) binds much stronger to nucleotides containing a terminal phosphate group.

The H(7) and H(8) protons are accidentally degenerate in both the actinomycin D and the actinomycin D·pdG spectra, and thus only a single resonance is observed (e.g., Arison and Hoogsteen, 1970; Krugh and Neely, 1973a). Upon the addition of Mn(II), the H(7) and H(8) resonance gradually broadens until the Mn(II) concentrations is approximately 1.3×10^{-4} M. At higher concentrations of Mn(II), the line width of the H(7) and H(8) resonance appears to decrease slightly (Figure 4 in supplementary material). This phenomenon can be explained if the H(7) and H(8) protons have different degrees of paramagnetic induced broadening, as was observed for the H(7) and H(8) doublets in the actinomycin D-2(pdG-dC) spectra (Figure 3).

Actinomycin D Complex with pdA(5′-dAMP), pdA-dC, and dA-dC. The ^1H NMR spectrum of an actinomycin D-(dA-dC) (1:1) solution was unchanged by the addition of 54 μM Mn(II), as expected. However, a solution of actinomycin D and pdA-dC (1:1) does exhibit paramagnetic induced line broadening when Mn(II) is added, as illustrated by the data in Table VI. The paramagnetic induced line width data for the addition of Mn(II) to a (1:1) actinomycin D-(pdA) solution are listed in Table VII. In both the pdA (i.e., 5′-dAMP) and pdA-dC experiments, we note that the 6-CH_3 resonance is broadened substantially more than the 4-CH_3 resonance. This is consistent with our previous observation that dAMP binds stronger to the 6-binding site than to the 4-binding site of actinomycin D. However, these experiments are not definitive because we are uncertain of the relative orientation of the Mn(II) ions with respect to the 4-CH_3 and 6-CH_3 groups when the adenine nucleotides are bound in the 4- and 6-binding sites. This problem is circumvented by using mixtures of nucleotides, as presented below, especially those mixtures with pdG since the experiments presented in the last sections showed equal

TABLE IV: Effect of Mn(II) Ion on the Line Widths of Selected Protons of the Act D-2(pdG-dC) Complex and the Act D-2(dG-dC) Complex.[a]

Proton	$\Delta\nu$ (Hz) for the Act D-2(pdG-dC) Complex[b]			
	3 μM Mn(II)	6 μM Mn(II)	9 μM Mn(II)	14 μM Mn(II)
G-H(8)	3	12	14	24
G-H(6)	0	0	1	1
H(8)[c]			1	2
H(7)[d]			2	5
C-H(5)	0	0.5	1	1
6-CH_3	1	2[e]	3[e]	7[e]
4-CH_3	0.5	2	3	6

Proton	$\Delta\nu$ (Hz)[e] for the Act D-2(dG-dC) Complex[f]			
	3 μM Mn(II)	6 μM Mn(II)	32 μM Mn(II)	62 μM Mn(II)
G-H(8)	0.5	1.5	2	>2
C-H(6)	0	0	0	0
H(8)[g]	0	0	0	0
H(7)[h]	0	0	0	0
H-(1′)	0	0	0	0
C-H(5)	0	0	1	1
6-CH_3	0	0	0.5[e]	1[e]
4-CH_3	0	0	0	1[e]

[a] No line width changes were observed for the Sar NCH_3, MeVal NCH_3, and Thr CH_3 resonances of actinomycin D. The uncertainty in the line width measurements is estimated to be ~0.5 Hz. [b] The spectra were recorded on a 5 mM Act D-2(pdG-dC) solution in D_2O. pH 7, 25 °C. [c] The downfield proton resonance of the AB pattern in the Act D-2(pdG-dC) complex. [d] The upfield proton resonance of the AB pattern in the Act D-2(pdG-dC) complex. [e] These values have a larger uncertainty due to overlapping peaks. [f] The spectra were recorded on a 3 mM Act D-2(dG-dC) solution in D_2O, pH 7, 25 °C. [g] The downfield proton resonance of the AB pattern in the Act D-2(dG-dC) complex. [h] The upfield proton resonance of the AB pattern in the Act D-2(dG-dC) complex.

TABLE V: Effect of Mn(II) Ion on the Line Widths of Selected Protons of an Equimolar Mixture of Actinomycin D with dGMP.[a]

Proton	$\Delta\nu$ (Hz)[b]		
	3 μM Mn(II)	9 μM Mn(II)	14 μM Mn(II)
G-H(8)	14	c	c
H(7) and H(8)	0.5	2	3
6-CH_3	2	5	>10
4-CH_3	2	5	>10

[a] No line width changes were observed for the Sar NCH_3, MeVal NCH_3, and Thr CH_3 resonances. [b] The full change in width at half-height of the peak. The uncertainty is estimated to be 0.5 Hz. [c] The peak was very broad and disappeared into the baseline.

TABLE VI: Effect of Mn(II) Ion on the Line Widths of Selected Protons of Equimolar Mixtures of Actinomycin D and pdA-dC.[a]

Proton	$\Delta\nu$ (Hz)[b]				
	3 μM Mn(II)	14 μM Mn(II)	28 μM Mn(II)	41 μM Mn(II)	54 μM Mn(II)
A-H(8)	2	10	c	c	c
A-H(2)	0	1	2	3	3.5
H(7) and H(8)	0	0.5	1	2	3
6-CH_3	0	2	5	8[d]	>10[d]
4-CH_3	0	1	2	4	7[d]

[a] The data are from a 5 mM 1:1 mixture of actinomycin D and pdA-dC in D_2O, pH 7.0 at 25 °C. No line width changes were observed for Sar NCH_3, MeVal NCH_3, and Thr CH_3 peaks. The C-H(5) and C-H(6) doublets were not well resolved (due to broadening) so the line widths were not measured. [b] The change in width at half-height of the peak. The uncertainty is estimated to be 0.5 Hz. [c] The peak was very broad and disappeared into the baseline. [d] These values have large uncertainties due to overlapping resonances.s040

broadening of the 4-CH_3 and 6-CH_3 groups when Mn(II) is added to either actinomycin D-(pdG) or actinomycin D-(pdG-dC) solutions.

Actinomycin D Complexes with dG and pdA-dC. The data have illustrated that Mn(II) binds much stronger to nucleotides containing a terminal phosphate than to nucleotides which do not have a terminal phosphate group. Thus, in an actinomycin D complex with a mixture of nucleotides in which only one nucleotide has a terminal phosphate group, the Mn(II) will bind exclusively to the terminal phosphate containing nucleotide. A measurement of the paramagnetic induced line broadening will provide an estimation of the amount of the

TABLE VII: Effect of Mn(II) Ion on the Line Widths of Selected Protons of an Equimolar Mixture of Actinomycin D with dAMP.[a]

Proton	$\Delta\nu$ (Hz)[b]				
	3 μM Mn(II)	13 μM Mn(II)	20 μM Mn(II)	42 μM Mn(II)	62 μM Mn(II)
A-H(8)	1.5	21	>20	[c]	[c]
A-H(2)	0	1.5	2	4	7
H(7) and (8)	0	1	1.5	2.5	4
6-CH_3	0	2	3.3	5	≥10
4-CH_3	0	1	1.6	3	6

[a] A mixture of actinomycin D and dAMP (5 mM, 1:1) in D_2O pH 7.0 at 25 °C. No line width changes were observed for Sar NCH_3, MeVal NCH_3, and Thr CH_3 peaks. [b] The change in width at half-height of the peak. The uncertainty is estimated to be 0.5 Hz. [c] The peak was very broad and disappeared into the baseline.

TABLE VIII: Effect of Mn(II) Ion on the Line Widths of Selected Protons of 1:1:1 and 1:2:2 Actinomycin D-(pdA-dC)-(dG) Mixtures.[a]

Proton	$\Delta\nu$ (Hz) for the 1:1:1 Act D-(pdA-dC)-(dG) Mixture[b]			
	3 μM Mn(II)	16 μM Mn(II)	32 μM Mn(II)	62 μM Mn(II)
A-H(8)	1	>15	>20	[c]
A-H(2)	0.5	2.5	3	5
G-H(8)	0	1	1.5[e]	[e]
C-H(6)	0	1	1.5[f]	[f]
H(7) and (8)	0	[d]	[d]	[d]
C-H(5)	0	1	1.5[f]	[f]
6-CH_3	0	4	7.5	>10
4-CH_3	0	0.5	0.5	1

	$\Delta\nu$ for the 1:2:2 Act D-(pdA-dC)-(dG) Mixture[g]			
	11 μM Mn(II)	34 μM Mn(II)	50 μM Mn(II)	81 μM Mn(II)
A-H(8)	1	18	28	[c]
A-H(2)	0	1	2	5
G-H(8)	0	0	1[e]	[e]
C-H(6)	0	0.5	1	[f]
H(7) or H(8)	0	0	0.5	1
H(7) or H(8)	0	0	1	2.5
C-H(5)	0	0.5	1	[f]
6-CH_3	0	1	3	6
4-CH_3	0	0	0	1

[a] No line width changes were observed for the Sar NCH_3, MeVal NCH_3, and Thr CH_3 resonances. [b] A mixture of actinomycin D, pdA-dC, and dG (1:1:1, 5 mM) in D_2O, pH 7.0, at 25 °C. $\Delta\nu$ is the change in width at half-height of the peak. The uncertainty is estimated to be 0.5 Hz. [c] The peak was very broad and disappeared into the baseline. [d] It was difficult to measure the line width change because of the unresolved AB pattern. [e] The G-H(8) peak overlaps with the A-H(2) peak so this value has larger uncertainty. [f] The doublet was not well resolved due to the broadening, so the line width could not be measured. [g] A mixture of actinomycin D, pdA-dC, and dG (1:2:2, 5 mM Act D) in D_2O, pH 7.0, at 25 °C. $\Delta\nu$ is the change in width at half-height of the peak. The uncertainty is estimated to be 0.5 Hz.

terminal phosphate containing nucleotide bound in each of the binding sites. An example of this approach is the actinomycin D-(dG)-(pdA-dC) (1:1:1 and 1:2:2) solutions, for which the paramagnetic induced line-broadening data are given in Table VIII. The addition of Mn(II) to both the 1:1:1 and the 1:2:2 solutions results in substantially more broadening of the 6-CH_3 resonance than the 4-CH_3 resonance (Table VIII). The pronounced broadening of the A-H(8) resonance and the preferential broadening of the 6-CH_3 resonance when compared with the 4-CH_3 resonance shows that pdA-dC binds stronger to the 6-binding site than to the 4-binding site of actinomycin D. The magnitude of the paramagnetic induced broadening is much less in the 1:2:2 mixture than in the 1:1:1 mixture for two reasons: (1) the excess (unbound) pdA-dC will compete for the limited Mn(II) added to the solution, and (2) there is a twofold excess of nucleotides in the 1:2:2 mixture and thus the excess dG will compete with pdA-dC for the 6-binding site.

Actinomycin D Complexes with pdG and dA-dC. The paramagnetic induced broadening data for the 1:1:1 and 1:2:2 actinomycin D-(pdG)-(dA-dC) mixtures are listed in Table IX. The pronounced line broadening of the G-H(8) resonance as Mn(II) is added clearly shows that the terminal phosphate is on the guanine nucleotide in this experiment, which is a complementary experiment to the one described immediately above. In both the 1:1:1 and 1:2:2 actinomycin D-(pdG)-(dA-dC) mixtures the addition of Mn(II) results in a preferential broadening of the 4-CH_3 resonance as compared with the 6-CH_3 resonance. This provides independent evidence that dA-dC binds stronger to the 6-binding site than to the 4-binding site of actinomycin D. The Mn-dGMP complex will compete with dA-dC for the 6-binding site and thus the preferential broadening is not as dramatic in this mixture as it is in the actinomycin D-(dG)-(pdA-dC) mixture (Table VIII).

TABLE IX: Effect of Mn(II) Ion on Line Width Changes of Selected Protons of 1:1:1 and 1:2:2 Actinomycin D-(dA-dC)-(pdG) Mixtures.[a]

Proton	$\Delta\nu$ (Hz) for the 1:1:1 Act D-(dA-dC)-(pdG) Mixture[b]			
	16 μM Mn(II)	26 μM Mn(II)	45 μM Mn(II)	62 μM Mn(II)
A-H(8)	1	2	2[d]	4[d]
A-H(2)[c]	0.5	1	2[d]	2[d]
G-H(8)[c]	>5	>10	>20	[e]
C-H(6)	0	0	0.5	1
H(7) and H(8)	0	0	0.5	1.5
C-H(5)	0	0	0	0.5
6-CH_3	0	1	3	5
4-CH_3	4	5	7	>10
	$\Delta\nu$ (Hz) for the 1:2:2 Act D-(dA-dC)-(pdG) Mixture[f]			
	3 μM Mn(II)	17 μM Mn(II)	24 μM Mn(II)	34 μM Mn(II)
A-H(8)	0	1	2	3
A-H(2)[c]	0	1[c]	2[c]	3[c]
G-H(8)[c]	4	[g]	[g]	[g]
C-H(6)	0	0	0	0.5
H(7) and H(8)	0	0	0	0.5
C-H(5)	0	0	0	0.5
6-CH_3	0.5	3	4	6
4-CH_3	2	6	10	19

[a] No line width changes were observed for the Sar NCH_3, MeVal NCH_3, and Thr CH_3 peaks. [b] A mixture of actinomycin D, dA-dC, and dGMP (5 mM, 1:1:1) in D_2O (pH 7.0) at 25 °C. $\Delta\nu$ is the change in width at half-height of the peak. The uncertainty is estimated to be 0.5 Hz. [c] The A-H(2) peak overlaps with the G-H(8) peak, so these values have large uncertainties. [d] These values have larger uncertainties due to overlapping resonances. [e] The peak was very broad and disappeared into the baseline. [f] A mixture of actinomycin, dA-dC, and dGMP (5 mM, 1:2:2) in D_2O (pH 7.0) at 25 °C. $\Delta\nu$ is the change in width at half-height of the peak. The uncertainty is estimated to be 0.5 Hz. [g] These values could not be measured due to overlapping resonances.

Discussion

The use of Mn(II) to induce nuclear relaxation in adjacent nuclei has been extensively exploited over the past 5–10 years because of the important role of metal ions in biological systems. In the present experiments we have shown that the Mn(II) ion has a much higher binding constant for terminal phosphate groups as compared with the internucleotide group of dinucleotides. This observation makes it possible to know the location of the Mn(II) ion in the Mn(II)–nucleotide–drug complexes. It is also important to note that in the actinomycin D complexes with the nucleotides the addition of Mn(II) ion did not result in any observable changes in the chemical shifts, which suggests that the geometry of the actinomycin D–nucleotide complex is not affected by the binding of Mn(II) ions. This observation is consistent with the geometry of these complexes, as schematically illustrated for the actinomycin D–2(pdG-dC) complex in Figure 2, where we note that the Mn(II) ion can bind to the terminal phosphate group without changing the stacking of the guanine on the phenoxazone ring. This can be seen more clearly from a consideration of the geometry of an actinomycin D–2(pdG) complex which is made by adding terminal phosphate groups to the deoxyguanosine–actinomycin D complex of Sobell and Jain (1972). The N(7) of the guanine serves as a second ligand site, either directly or through a water bridge. Since the N(7) of guanine is located away from the cyclic pentapeptide groups, the Mn(II) ion has unrestricted access in binding to the nucleotide. In this configuration the Mn(II) ions are located approximately 6 Å from the plane of the phenoxazone ring in the area above either the 4-CH_3 and the 3-carbonyl groups or the 6-CH_3 and the H(7) proton, depending on whether the Mn(II) ion is bound to the nucleotide in the 4-binding site or the 6-binding site (Figure 2). We have observed a preferential broadening of the H(7) resonance when the H(7) and H(8) resonances can be resolved (e.g., in the actinomycin D–2(pdG-dC) complex (Figure 3)), which is consistent with the proposed location for the Mn(II) ion. The Mn(II) ion will preferentially broaden the methyl resonance in the same nucleotide site as opposed to the methyl resonance in the other nucleotide binding site. However, there will be some "cross relaxation" (e.g., the Mn(II) ion bound in the 6-binding site will induce nuclear relaxation of the 4-CH_3 group). We estimate that the paramagnetic induced relaxation of the methyl resonance located in the same nucleotide binding site as the Mn(II) ion will be >5 times larger than the induced relaxation of the methyl resonance in the other nucleotide binding site (i.e., the "cross relaxation").

The largest degree of preferential broadening was observed in the 1:1:1 actinomycin D–(pdA-dC)–(dG) solution (Table VIII), where the 6-CH_3 group was broadened approximately tenfold more than the 4-CH_3 group. These data clearly show that pdA-dC is binding exclusively at the 6-binding site. In the 1:1 actinomycin D–(pdA-dC) solution the Mn(II) ion preferentially broadened the 6-CH_3 group by a factor of two (Table V). Comparing these two experiments leads to the conclusion that in an equimolar mixture of pdA-dC and dG the deoxyguanosine binds much stronger than pdA-dC at the 4-binding site, whereas both dG and pdA-dC compete for the 6-binding site. In the 1:2:2 actinomycin D–(pdA-dC)–(dG) solution there is a twofold excess of nucleotides with respect to nucleotide binding sites on the phenoxazone ring of actinomycin D. Since the magnitude of the paramagnetic induced relaxation of the 4- and 6-methyl groups will depend upon the amount of pdA-dC bound to the phenoxazone ring, we would expect to observe a reduced broadening of the 6-CH_3 group because no more than half of the pdA-dC molecules can bind to the 6-

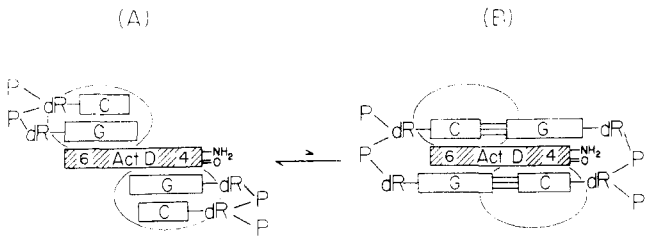

FIGURE 5: Schematic illustration of two complexes of actinomycin D with pdC-dG. (A) The stacked complex which predominates in solution and (B) the intercalated complex in which the phenoxazone ring is intercalated in a (dC-dG)·(dC-dG) sequence. All the spectroscopic evidence indicates that *less than* 10% of the actinomycin D molecules form an intercalated type complex with pdC-dG (under the conditions used for the NMR experiments).

binding site. pdA-dC and dG will compete for the 6-binding site so we would, in fact, expect less than one-half of the pdA-dC molecules to be bound to the 6-site in the 1:2:2 actinomycin D–(pdA-dC)–(dG) solution. The Mn(II) ion is approximately one-third as effective in broadening the 6-CH_3 resonance in the 1:2:2 mixture as in the 1:1:1 mixture (Table VIII), which is both consistent with this logic and also shows that pdA-dC does compete with dG for the 6-binding site. The same conclusions are obtained from an analysis of the line width data in the 1:1:1 and 1:2:2 actinomycin D–(dA-dC)–(pdG) solutions (Table IX) where the location of the terminal phosphate group has been switched from the A-C dinucleotide to the G nucleotide. It should be noted that we would not expect the same degree of preferential broadening in these spectra as observed in the actinomycin D–(pdA-dC)–(dG) experiments because the pdG (and therefore the Mn(II) ion) will bind to both the 4- and 6-binding sites and thus the preferential broadening will only reflect the competitive binding of dA-dC with pdG at the 6-binding site.

The paramagnetic induced relaxation data in this manuscript, as well as the chemical shift data in the preceding paper (Krugh et al., 1977), show that the two nucleotide binding sites of actinomycin D are quite distinct in their ability to recognize and preferentially bind the deoxymono- and dinucleotides. The 4-binding site (i.e., the quinoid portion of the phenoxazone ring) binds guanine nucleotides much stronger than adenine nucleotides, whereas the 6-binding site (i.e., the benzenoid portion of the phenoxazone ring) will bind both adenine and guanine nucleotides with approximately the same affinity. Actinomycin D exhibits an unusual requirement for the presence of a guanine base at the intercalation site, and it is interesting to consider the results of the present experiments in the context of a general discussion of the actinomycin D–DNA complex formation.

When an intercalating drug, such as actinomycin D, binds to DNA there will be both entropic and enthalpic contributions to the overall free energy change associated with complex formation. Actinomycin D is an unusual intercalating drug in that the formation of an actinomycin D–DNA complex is accompanied by a ΔH of ca. +2 kcal/mol (Quadrifoglio and Crescenzi, 1974; and references therein). Thus the net driving force for the formation of a stable actinomycin D–DNA complex is entropic in nature and presumably results from the release of the partially ordered water of hydration. In the present experiments with the deoxymononucleotides and/or deoxydinucleotides the formation of a complex with actinomycin D is accompanied by a *negative* enthalpy change (e.g., $\Delta H \approx -8.5$ kcal/mol for each dGMP bound (Quadrifoglio et al., 1976) as had been demonstrated by several groups (e.g., Gellert et al., 1965; Crothers et al., 1968; Krugh and Neely, 1973a). However, it should also be noted that, when actinomine (which is essentially an actinomycin molecule without the pentapeptide units) binds to DNA at low r values, the ΔH of complex formation is ca. −7 kcal/mol (Quadrifoglio et al., 1976). Thus the thermodynamics of binding to DNA are significantly influenced by the presence of the pentapeptides, which is not surprising in view of the excellent fit of the pentapeptide groups in the minor groove of DNA which was observed in the model building studies of Sobell and Jain (1972). The present experiments suggest that the stacking forces are important in the recognition and preferential binding of guanine (when compared with adenine binding) to the quinoidal portion of the phenoxazone ring. We suggest (as did Müller and Crothers (1968) when they first proposed that actinomycin intercalates) that it is these same stacking forces which are primarily responsible for the general requirement for a guanine base when actinomycin D intercalates into DNA.

Hydrogen bond formation will also stabilize complex formation. However, if the formation of the hydrogen bond between the guanine 2-amino group and the threonine carbonyl group of actinomycin D were the primary force involved in guanine recognition, then actinomycin D would be expected to bind to dG-dC sequences with a high degree of preference as a result of the symmetry of the two cyclic pentapeptide groups in actinomycin D (e.g., see Sobell and Jain, 1972). Although actinomycin D does appear to bind strongest to the alternating dG-dC copolymer, the apparent binding constant is less than twice as large as the binding constant to other sequences (Wells and Larson, 1970). The dinucleotide complexes with actinomycin D provide two clear examples of the interplay of stacking forces and hydrogen bond formation on the geometry of the complexes. The first example is the self-complementary deoxydinucleotide pdC-dG which forms a stacked complex with actinomycin D (Figure 5A) instead of the intercalated complex (Figure 5B) in spite of the fact that the formation of the intercalated complex would result in the formation of six hydrogen bonds (Watson–Crick type) between the two base pairs (see Krugh and Neely, 1973b, pp 4424 and 4425, for a more detailed discussion). The schematic illustrations in Figure 5 have been drawn to illustrate the important point that the stacking of the guanine rings with the phenoxazone ring are significantly different if actinomycin D is intercalated into a dC-dG sequence as opposed to a dG-dC sequence. These sequence dependent stacking patterns have been recognized as being important in the stabilization of the nucleic acid polymers, and we have proposed that they impart a sequence preference for the binding of the intercalating drugs to DNA (e.g., see Krugh and Reinhardt, 1975, and references therein).

The second example of the interplay of stacking forces and hydrogen bond formation is the complex of actinomycin D with the complementary dinucleotides pdG-dG and pdC-dC as presented in the preceding manuscript (Krugh et al., 1977). pdG-dG by itself forms a stacked complex with actinomycin D (similar to the pdC-dG complex). However, the addition of pdC-dC (which by itself does not form a complex with actinomycin D) results in the formation of an intercalated complex in which actinomycin D is intercalated in a (dG-dG)·(dC-dC) sequence. The six hydrogen bonds formed between the pdG-dG and pdC-dC nucleotides result in a switch from the stacked conformation to the intercalated complex (e.g., see Figure 12 of Krugh et al., 1977). A comparison of the pdG-dG + pdC-dC and the pdC-dG data illustrates the interplay of the stacking and hydrogen-bonding forces in determining the stability of

the complexes.

Recent experiments in our laboratory have shown that actinomycin D does bind strongly to poly(dA-dT)·poly(dA-dT) *in the presence of another intercalating drug* (Krugh et al., in preparation), which also supports the suggestion that it is the stacking forces and not hydrogen-bond formation which are primarily responsible for the unusual guanine requirement for the binding of actinomycin D to DNA. Further experiments on the binding of actinomycin D to oligonucleotides (in progress) should provide more detailed information.

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Supplementary Material Available

Figures 1 and 4 (spectra) and Table III (metal ion effect data) (4 pages). Ordering information is given on any current masthead page.

References

Anderson, J. A. Kuntz, G. P. P., Evans, H. H., and Swift, T. J. (1971), *Biochemistry 10,* 4368.

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

Basosi, R., Tiezzi, E., and Valensin, G. (1975), *J. Phys. Chem. 79,* 1725.

Benz, F. W., Feeney, J., and Roberts, G. C. K. (1972), *J. Magn. Reson. 8,* 114–121.

Cohn, M., and Hughes, T. R., Jr. (1962), *J. Biol. Chem. 237,* 176.

Crothers, D. M., Sabol, S. L., Ratner, D. I., and Müller, W. (1968), *Biochemistry 7,* 1817.

Dwek, R. A. (1973), Nuclear Magnetic Resonance in Biochemistry, London, Oxford University Press, pp 213–326.

Gellert, M., Smith, C. E., Neville, D., and Felsenfeld, G. (1965), *J. Mol. Biol. 11,* 445.

Kotowycz, G., and Hayamizu, K. (1973), *Biochemistry 12,* 517.

Krugh, T. R. (1972), *Proc. Natl. Acad. Sci. U.S.A., 69,* 1911.

Krugh, T. R. (1976), in Spin Labeling: Theory and Applications, Berliner, L. J., Ed., New York, N.Y., Academic Press, p 339.

Krugh, T. R., and Chen, Y.-C. (1975), *Biochemistry 14,* 4912.

Krugh, T. R., Mooberry, E. S., and Chiao, Y.-C. C. (1977), *Biochemistry 16,* preceding paper in this issue.

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

Krugh, T. R., and Neely, J. W. (1973b), *Biochemistry 12,* 4418.

Krugh, T. R., and Schaefer, W. C. (1975), *J. Magn. Reson. 19,* 99–107.

Lam, Y. F., Kuntz, G. P. P., and Kotowycz, G. (1974), *J. Am. Chem. Soc. 96,* 1834.

Mildvan, A. S., and Cohn, M. (1970), *Adv. Enzymol. 33,* 1.

Mooberry, E. S., and Krugh, T. R. (1975), *J. Magn. Reson. 17,* 128.

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

Patel, D. J. (1974), *Biochemistry 13,* 2388.

Patt, S. L., and Sykes, B. D. (1972), *J. Chem. Phys. 56,* 3182–3184.

Quadrifoglio, F., Ciana, A., and Crescenzi, V. (1976), *Biopolymers 15,* (595–597.

Quadrifoglio, F., and Crescenzi, V. (1974), *Biophys. Chem. 2,* 64–69.

Reed, G. H., Leigh, J. S., and Pearson, J. E. (1971), *J. Chem. Phys. 55,* 3311.

Reuben, J., and Gabbay, E. J. (1975), *Biochemistry 14,* 1230–1235.

Schara, R., and Müller, W. (1972), *Eur. J. Biochem. 29,* 210.

Shulman, R. G., Sternlicht, H., and Wyluda, B. J. (1965), *J. Chem. Phys. 43,* 3116.

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

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

Swift, T. J. (1973), in NMR of Paramagnetic Molecules, La Mar, G. N., Horrocks, W. DeW., Jr., and Holm, R. H., Ed., New York, N.Y., Academic Press, pp 53–83.

Ts'o, P. O. P. (1974), in Basic Principles in Nucleic Acid Chemistry I, Ts'o, P. O. P., Ed., New York, N.Y., Academic Press, p 453.

Wee, V., Feldman, I., Rose, P., and Gross, S. (1974), *J. Am. Chem. Soc. 96,* 103.

Wells, R. D., and Larson, J. E. (1970), *J. Mol. Biol. 49,* 319.