Interaction of Miracil D with Double-Stranded Poly(adenylic acid)·Poly(uridylic acid)†

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ABSTRACT: Miracil D [1-(2-diethylaminoethylamino)-4-methyl-10-thiaxanthenone] is a drug which is similar in structure to the acridines. It is an inhibitor of poly(nucleic acid) synthesis and is believed to exert its effect through noncovalent interactions with nucleic acids. High-resolution proton nuclear magnetic resonance (nmr) was used to study the interaction of Miracil D with double-stranded poly(adenylic acid)·poly(uridylic acid) (poly(A)·poly(U)) of about 20–25 nucleotides in length, prepared by mixing equimolar amounts of single-strand polymers. Formation of the double helix effected significant upfield shifts (0.7 ppm) for the base protons and produced a low-field nmr signal at 13.60 ppm in H_2O indicative of hydrogen bonding between the base pairs. Upon addition of Miracil D to double-stranded poly(A)·poly(U), the resonance of the 4-methyl group on the thiaxanthenone ring was significantly broadened and shifted about 0.3 ppm upfield. By comparison, the resonances for the two terminal methyl groups on the diethylaminoethylamino chain were not shifted or broadened. As the concentration of Miracil D was increased, the poly(A)·poly(U) base resonances were found to further broaden and shift to higher field. Double-stranded poly(A)·poly(U) appears to bind about 2–3 times more Miracil D than does an equivalent amount of single-stranded polymers. These results are consistent with a model in which the planar thiaxanthenone ring is intercalated with the stacked base pairs of poly(A)·poly(U), while the cationic chain portion of Miracil D extends to the outside of the helix, where it is probably bound to the anionic phosphate groups. Temperature studies indicate that Miracil D stabilizes the double-stranded poly(A)·poly(U) structure with respect to heat denaturation. The interaction of Acridine Orange and triethylamine with poly(A)·poly(U) was also studied. These two compounds are structurally analogous to the ring and chain portions of Miracil D. Results of the nmr studies indicate that triethylamine is most likely bound (as triethylammonium ion) to the outside of the poly(A)·poly(U) helix, while Acridine Orange appears to be intercalated within the stacked base pairs. The results in general demonstrate the usefulness of polynucleotides of limited chain lengths for nmr studies, in contrast to more highly polymerized polynucleotides or nucleic acids.

Many small compounds exert various biological effects through direct interaction with the nucleic acids of an organism. These compounds include drugs, antibiotics, mutagens, and possibly carcinogens. Compounds such as the acridines, ethidiums, and actinomycins interact with nucleic acids through electrostatic, hydrophobic, and hydrogen bonding forces. Upon binding, these compounds become effective inhibitors of nucleic acid synthesis. Extensive reviews of this subject have been given by Gale *et al.* (1972), Goldberg and Friedman (1971), Newton (1971), Goldstein *et al.* (1969), and Hahn (1971).

The interactions of these compounds with nucleic acids have been studied by various physical methods. In general, these methods point to an intercalation model for acridines, ethidiums, and actinomycins. A detailed understanding of these interactions, however, is far from complete (see above references).

Nuclear magnetic resonance (nmr) can be a particularly powerful method to study such interactions. Indeed, well-resolved proton nmr spectra are obtained for nucleotides, dinucleotides, and even high-molecular weight single-stranded nucleic acids (for reviews, see Bovey (1969) and Yamane (1971)). However, because of the rigid nature of double-stranded nucleic acids of high molecular weight, adverse relaxation effects, that is, strong dipolar interactions, cause broadening of the nmr resonances beyond detection (McDonald *et al.*, 1964, 1967). If small molecules that bind to the nucleic acid are added, in most cases their resonances are also broadened beyond detection. The problem associated with nmr studies of double-stranded nucleic acids has been overcome, to a certain degree, by using low molecular weight polynucleotides. Cross and Crothers (1971) have found that proton nmr spectra are observable for small double-stranded pentadeoxyribonucleotides. However, as the length of a double-stranded polymer is decreased, end effects become more significant; furthermore, lower temperatures are required to maintain the double helix.

Miracil D [1-(2-diethylaminoethylamino)-4-methyl-10-thiaxanthenone] is a compound which is similar to the acridines and ethidiums (Figure 1). This compound is bacteriostatic, mutagenic, and is a potent inhibitor of DNA-directed RNA synthesis. It is also an effective agent in the treatment of schistosomiasis in man, and has carcinostatic potency against many transplantable neoplasms in rodents (for review, see Weinstein and Hirschberg (1971)). Miracil D is believed to exert its biological effect through noncovalent binding to DNA by an intercalative type of interaction (Weinstein and Hirschberg, 1971).

In order to study the interaction of this type of compound with nucleic acids, we have prepared poly(adenylic acid) and poly(uridylic acid) of about 20–40 nucleotides in length. At

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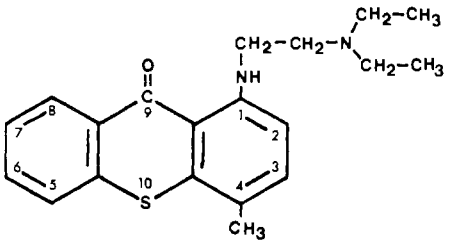

MIRACIL D

FIGURE 1: Miracil D [1-(2-diethylaminoethylamine)-4-methylthiaxanthenone]; also called lucanthone.

room temperature these polymers form a stable double-stranded helix that has an observable nmr spectrum. Because of the simple structure of Miracil D and fortuitous geometrical locations of the methyl groups, whose resonances appear at higher field than those of the polymer, much information can be obtained for the interaction of this compound with double-stranded poly(A)·poly(U).

Materials and Methods

Miracil D (lucanthone-HCl) was obtained from Calbiochem, Acridine Orange from K & K Laboratories, triethylamine from Pierce Chemical Co., and poly(adenylic acid) and poly(uridylic acid) from Sigma Chemical Co.

Poly(adenylic acid) and poly(uridylic acid) were degraded to smaller molecular weights by controlled alkaline hydrolysis (Bock, 1967). About 100 mg of polymer was added to 30 ml of 0.1 M NH_4HCO_3 (pH 10.0). Poly(A) was refluxed for 90 min at 100°, and poly(U) for 45 min at 90°. Samples were then lyophilized. The partially hydrolyzed polymers were fractionated on a 1.5 mm × 60 mm Sephadex G-50 column according to Stanley (1967). The buffer used was 8 M urea and 0.5 M ammonium bicarbonate (pH 8.6). A flow rate of 8.0 ml/hr was maintained, using a Sage Instrument tubing pump, Model 375. Elution was monitored by uv at 260 nm, and 2.6-ml fractions were collected.

The column was first calibrated using a mixture of poly(adenylic acid) (mol wt >100,000) (to obtain the excluded volume) and adenosine 5′-monophosphate (for obtaining the included volume). The hydrolyzed polymers were then fractionated and the molecular weights (chain lengths) of the eluted hydrolyzed polymer fractions were determined by

$$\text{mol wt} = 300 - 1.16 \times 10^4 \log K \qquad (1)$$

K is given by

$$K = (V - V_{ex})/(V_{inc} - V_{ex}) \qquad (2)$$

where V is the elution volume of the polynucleotide fraction; V_{ex} is the excluded volume; and V_{inc} is the included volume (Stanley, 1967). In order to demonstrate that the fractionation pattern for the partially hydrolyzed polynucleotides represented a sequence of resolved polynucleotides, individual fractions and mixtures of several fractions were rechromatographed. The fractionation pattern obtained on rechromatographing a mixture of every fifth fraction showed the fractions as well-resolved peaks which appeared again in their proper elution positions.

The polynucleotide fractions were desalted using a 10-ml Bio-Rad fiber dialyzer. Samples were dialyzed against several liters of distilled and deionized H_2O for about 3 hr. These samples were then lyophilized to dryness.

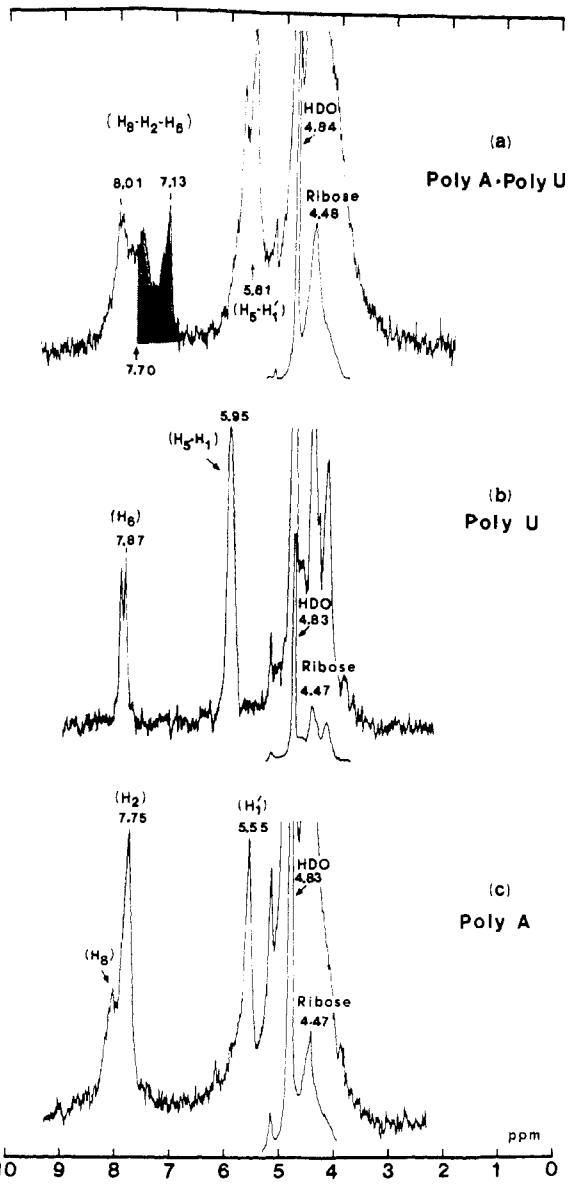

FIGURE 2: The 100-MHz proton nmr spectra of: (a) poly(A)·poly(U) (20–24 nucleotides in chain length); (b) single-stranded poly(U) (20–24); and (c) poly(A) (20–24). The concentration for each of the polymers was ~0.085 M (nucleotide concentration), NaCl concentration was ~0.08 M for the single-stranded polymers and ~0.16 M for the double-stranded polymers. The pH was ~6.4 for all three samples. The spectra were run at 20° in D_2O. The shaded area in (a) starting at 7.70 ppm represents the new resonance area formed by poly(A)·poly(U) that is at higher field than would be expected from superimposing the spectra of the individual polymers.

Nmr spectra were recorded on a JEOL JNM-MH-100 spectrometer using external nmr lock. Chemical shifts were measured in relation to the internal HDO resonance. However, before and after each group of nmr runs, or when the probe temperature was changed, a standard sample was used to calibrate the spectrometer. The standard sample contained 2,2-dimethylsilapentane-5-sulfonate and D_2O. The 2,2-dimethylsilapentane-5-sulfonate resonance was set at zero ppm and the position of the HDO resonance recorded (usually ~4.84 ppm at 20°). The position of the HDO resonance in almost all samples studied was found to be within ±0.03 ppm of that of the standard sample. Thus, this method allows chemical shift determination without adding internal reference compounds which might possibly interfere with the inter-

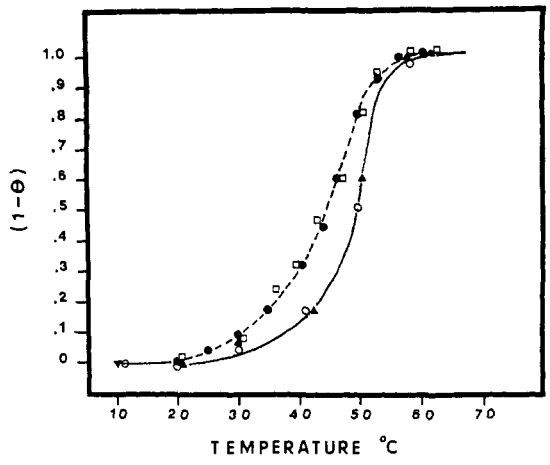

FIGURE 3: Melting curves for poly(A)·poly(U) (20–25) determined from nmr spectral changes and uv absorbancy changes at 260 nm. The (▲) $1-\theta$ values were calculated from amplitude changes in the 7.1–7.3-ppm region, the (○) $1-\theta$ values were calculated from area changes in the 7.70–7.00-ppm region. The nmr experiments were done at a poly(A)·poly(U) concentration of ~0.08 M, and pD of 6.4. The (□,●) $1-\theta$ values represent two experiments done by uv at 260 nm with a poly(A)·poly(U) concentration of $\sim 3.0 \times 10^{-5}$ M and with 0.025 M Na_2PO_4 and 0.175 M NaCl, at pH 6.8. The T_m values obtained are ~45° from the uv curve (□,●), and ~49° from the nmr curve (▲,○).

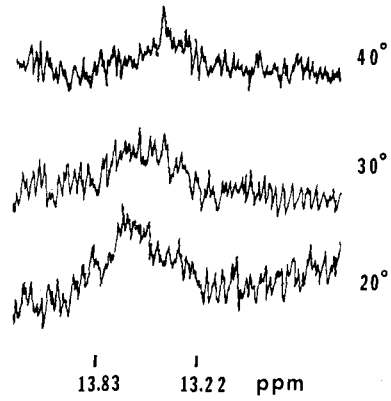

FIGURE 4: The low-field nmr spectra of poly(A)·poly(U) (20–24) in "H_2O," resonances due to hydrogen bonded N–H protons, showing the effect of increasing temperature. The poly(A)·poly(U) concentration was 0.08 M, the pH was ~6.4.

actions being studied. All chemical shifts (resonance positions) are reported in ppm, with respect to 2,2-dimethylsilapentane-5-sulfonate.

All polymer concentrations were determined spectrophotometrically by uv at 260 nm in 0.1 M Na_2HPO_4 buffer (pH 7.0) at 20°. Polymer concentrations were calculated using molar extinction coefficients (a_m values)[1] of 1.0×10^4 for poly(U) and 1.1×10^4 for poly(A), and are reported in the text as nucleotide concentration, or nucleotide concentration per strand in the case of double-stranded polymers. All drug and dye solutions were prepared by careful weighing and volumetric measurements. Various amounts of drug or dye were then aliquoted into solutions of polymer of known concentration. In cases where the number of drug molecules is compared with the number of nucleotides or base pairs, integration of nmr resonance peaks was used to determine the ratio of drug to polymer. This method served as a check on the results obtained from determining concentrations by other methods.

Sodium chloride was added to all polymer solutions at a concentration equal to the nucleotide concentration. The pD was adjusted using NaOD or DCl, and was checked both before and after each sample was used. All polymer samples were lyophilized several times from D_2O, and the nmr samples were made up by adding 0.5 ml of 100% D_2O.

Melting curves are shown as plots of $(1-\theta)$ *vs.* temperature. $(1-\theta)$ is the transition fraction, where θ is equal to the observed change in a given parameter divided by the maximal change. Parameters used for obtaining melting curve data were nmr resonance amplitude changes, nmr resonance area changes, and OD changes at 260 nm.

Results

Nmr Study of Poly(A)·Poly(U). When equimolar amounts of poly(A) (20–24 nucleotides in length) and poly(U) (20–24) were mixed together, the resulting nmr spectrum was changed significantly from the superposition of the spectra of single-stranded poly(A) and poly(U), as shown in Figure 2. The poly(A)·poly(U) spectrum at 20° (Figure 2a) shows a substantial redistribution of the H_8–H_2–H_6 resonance pattern, with a new amplitude maximum at ~7.13 ppm. This new resonance position at ~7.13 ppm is at 0.70 ppm higher field than the center position (~7.83 ppm) of the pattern that is obtained when the H_8–H_2 resonance of poly(A) (Figure 2c) and the H_6 resonance of poly(U) (Figure 2b) are superimposed. Also, 45–50% of the area of the H_8–H_2–H_6 resonance of poly(A)·poly(U) (see shaded area Figure 2a) is at higher field than would be expected from overlapping the individual poly(A) and poly(U) spectra.

The combined H_5–H_1' resonance of poly(A)·poly(U) was found to shift 0.20 ppm to higher field. The position of the maximum of the resonance envelope (~4.48 ppm) for the remaining ribose protons of poly(A)·poly(U) was not shifted significantly in relation to the spectrum of single-stranded poly(A) and poly(U).

The high-field portion (7.70–7.00 ppm) of the H_8–H_2–H_6 resonance envelope of the nmr spectrum of poly(A)·poly(U) was found to decrease in both amplitude and integrated intensity at higher temperatures. At 60° it was completely gone and the resulting spectrum was the same as would be expected from superimposing spectra of poly(A) and poly(U). These changes in the nmr spectra reflect the melting of the poly(A)·poly(U) helix. Both the amplitude change in the 7.10–7.30-ppm region and the decrease in resonance area from 7.70 to 7.00 ppm were used to calculate $(1-\theta)$ values (Cross and Crothers, 1971) for the melting curve shown in Figure 3. For comparison, Figure 3 also shows the melting curve of poly(A)·poly(U) obtained by uv analysis at 260 nm.

The nmr spectra of poly(A)·poly(U) (20–24) in H_2O show a broad resonance at very low field, ~13.60 ppm, due to hydrogen bonding between the base pairs (Figure 4). As the temperature was raised this resonance decreased in amplitude and was not detectable above 50°.

Nmr Study of Miracil D with Poly(A) and Poly(U). The spectrum of Miracil D is shown in Figure 5a. The resonance pattern at ~3.30 ppm is due to the eight protons from the four methylene groups in the diethylaminoethylamino chain (see Figure 1 for Miracil structure). The singlet at ~1.92 ppm is due to the lone methyl group on the thiaxanthenone ring and the triplet at ~1.24 ppm is due to the two methyl groups of the diethylaminoethylamino chain. The resonances from 8.00

[1] These approximate values are given in the Miles Laboratories, Research Catalog D, 1971, p 6, for characteristics of polynucleotides.

TABLE I: Miracil D Resonance Positions for Interaction with Poly(A), Poly(U), and for the Drug Alone.

Sample	pD	Temp (°C)	HDO[c]	Polymer–Drug Concn (M)	Miracil D Resonances (ppm)			Relative[a] Amplitude
					Chain $(-CH_2-)_4$	Ring $(-CH_3)$	Chain $(-CH_3)_2$	Ring $(-CH_3)$ (%)
Poly(A)[b] + Miracil D	6.4	20	4.88	0.08–0.004	3.32	1.57	1.32	34
	6.4	30	4.77	0.08–0.004	3.30	1.62	1.28	47
	6.4	40	4.69	0.08–0.004	3.32	1.65	1.31	46
Poly(U)[b] + Miracil D	6.4	20	4.88	0.09–0.004	3.31	1.86	1.31	28
	6.4	30	4.77	0.009–0.004	3.34	1.86	1.32	50
	6.4	40	4.69	0.09–0.004	3.34	1.90	1.32	50
Poly(A) + Miracil D	6.8	20	4.83	0.05–0.0025	3.31	1.63	1.25	40
	6.8	30	4.71	0.05–0.0025	3.33	1.66	1.25	50
	6.8	40	4.61	0.05–0.0025	3.30	1.69	1.23	60
Poly(U) + Miracil D	6.8	20	4.83	0.05–0.0025	3.31	1.74	1.28	40
	6.8	30	4.71	0.05–0.0025	3.31	1.74	1.27	50
	6.8	40	4.63	0.05–0.0025	3.33	1.82	1.27	55
Miracil D	6.3	20	4.84	0.005	3.33	1.92	1.23	100
Miracil D	6.3	20	4.84	0.010	3.32	1.86	1.25	100
Miracil D	6.3	20	4.88	0.013	3.30	1.78	1.27	100
Miracil D	6.3	40	4.68	0.013	3.34	1.88	1.29	100

[a] Represents the signal amplitude (reduced by broadening) of the thiaxanthenone ring methyl group observed upon interaction of Miracil with polymers; reported as per cent of amplitude for this group in the absence of polymers. [b] (27–34) nucleotide units. [c] HDO resonance position of the standard sample was 4.84 ppm at 20°.

to 6.00 ppm belong to the various protons on the thiaxanthenone ring and were not assigned. Integration results of the spectrum were consistent with the assignments made. The thiaxanthenone ring methyl resonance was found to move to higher field as the concentration increased and as temperature decreased (Table I).

TABLE II: High-Field Shifts of H_8–H_2–H_6 Resonance of Poly(A)·Poly(U) with and without Miracil D Present.

Sample	Temp (°C)	High-Field Percentage[a] (7.70–6.80 ppm) (%)
Poly(A)·poly(U)[b] + 0.005 M Miracil D	20	50
Poly(A)·poly(U) + 0.01 M Miracil D	20	60
Poly(A)·poly(U) + 0.015 M Miracil D	20	66
Poly(A)·poly(U) + 0.015 M Miracil D	30	63
Poly(A)·poly(U) + 0.015 M Miracil D	40	60
Poly(A)·poly(U)	20	50
Poly(A)·poly(U)	30	48
Poly(A)·poly(U)	40	41
Poly(A)·poly(U)	50	24

[a] Percentage of the area of the H_8–H_2–H_6 resonance pattern occurring at higher field than 7.70 ppm, where 7.70 ppm is the high-field limit for superimposing the individual H_8–H_2 resonances of poly(A) and the H_6 resonance of poly(U). [b] (20–25) chain length, pD ~6.4, polymer concentration ~0.07 M.

The effect of the interaction of Miracil D with poly(A) (27–34) and with poly(U) (27–34) on the nmr spectrum of Miracil D is also shown in Figure 5. The results in Figure 5 and Table I show that at 20° the resonance of the thiaxanthenone methyl group is significantly shifted. Some smaller shifts in the resonances of the methyl and methylene groups of the chain are observed, and the resonances of these groups are found to broaden slightly in the poly(U) or poly(A) systems. However, broadening of the thiaxanthenone ring methyl resonance is pronounced, the amplitude being reduced in both the poly(A) and poly(U) systems to ~33–40% of its value in the absence of polymer.

At concentrations of 0.05–0.08 M poly(A) or poly(U) the highest Miracil concentrations employed were 0.0025–0.004 M. Any additional Miracil D added to poly(A) or poly(U) formed a precipitate.

Nmr Study of Miracil D with Poly(A)·Poly(U). The effect on the nmr spectrum of the interaction of Miracil D with poly(A)·poly(U) (20–25) is shown in Figure 6. It can be seen that the resonance of the thiaxanthenone ring methyl group is shifted to higher field and broadened more than those of methylene and methyl groups of the chain portion of Miracil D, and that this effect becomes more pronounced at higher Miracil D concentrations. Figure 6 also shows that the H_8–H_2–H_6 resonance envelope (8.00–7.00 ppm) of poly(A)·poly(U) further broadens and shifts upfield as the Miracil D concentration increases. Although some broadening appears in the spectra at higher Miracil D concentrations, which is manifested in the combined H_5–H_1' resonances and the ribose envelope, these patterns appear not to be shifted nearly as much as the H_8–H_2–H_6 envelope.

Table II shows the variation of signal areas with temperature change for the high-field portion (7.70–6.80 ppm) of the H_8–H_2–H_6 resonance envelope of poly(A)·poly(U) with and without Miracil D. Table II also shows the variation in area for

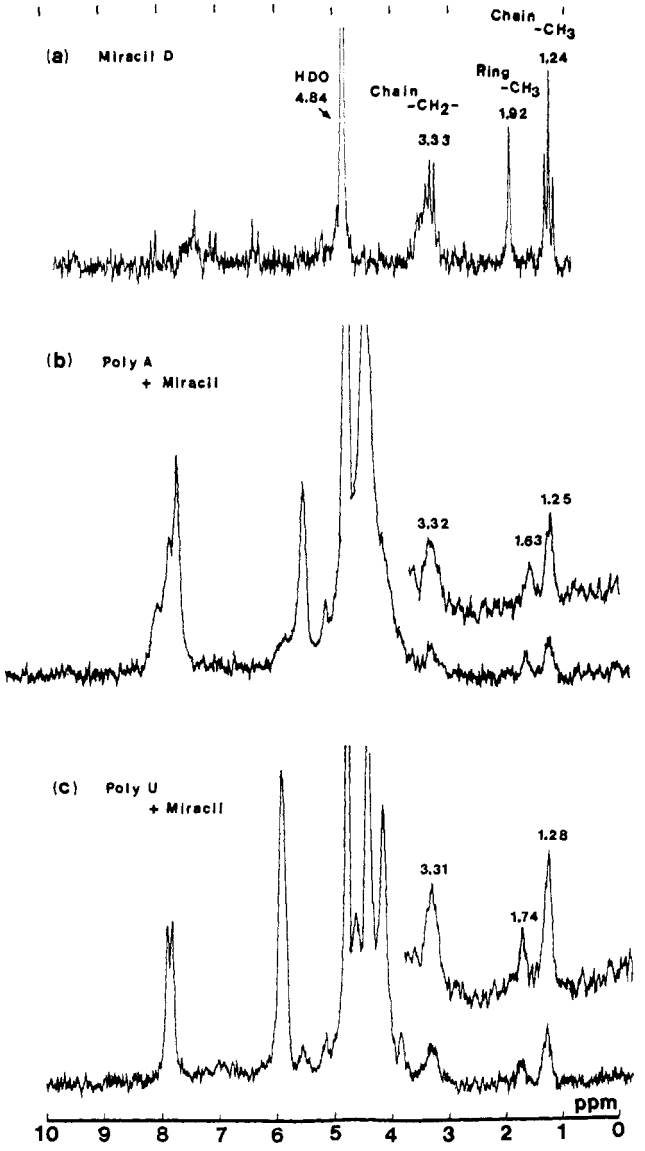

FIGURE 5: The 100-MHz proton nmr spectra of: (a) Miracil D at 0.05 M; (b) a sample with poly(A) (27–34) (0.05 M) and Miracil D (0.0025 M); and (c) a sample with poly(U) (27–34) (0.05 M) and Miracil D (0.0025 M). The pD of the Miracil D sample was 6.3 and pD of polymer–drug mixtures was 6.8. All spectra were run at 20°. Spectra (b) and (c) show the Miracil D resonances of the polymer–drug mixture return at higher amplitude.

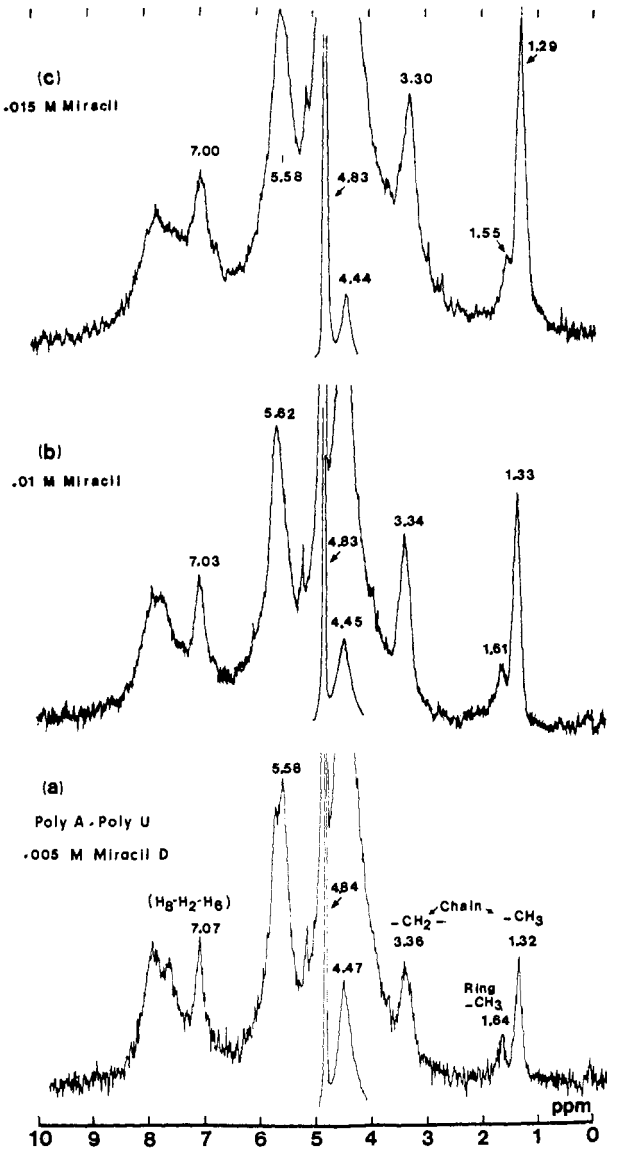

FIGURE 6: The 100-MHz nmr spectra of samples of poly(A)·poly(U) with increasing Miracil D concentration: (a) 0.005 M; (b) 0.01 M; and (c) 0.015 M. The polymer concentration was 0.07 M, and the pD was 6.2–6.4 in all samples studied. The spectra were run at 20°.

the H_8–H_2–H_6 resonance envelope at different Miracil concentrations and fixed temperature.

Table III compares the shift values and the maximum amplitudes of the resonance of the thiaxanthenone ring methyl group of Miracil D for solutions of the drug with poly(A), poly(U), and poly(A)·poly(U) at various temperatures and Miracil D concentrations.

Studies of the Miracil D–poly(A)·poly(U) interaction done at different pD values, ranging from 6.0 to 8.0, showed no clear dependency of the shifts or line widths of the Miracil D resonances or of the polymer resonances on pD.

With poly(A)·poly(U) concentrations of ~0.07 M (nucleotide concentration of each strand), it was possible to obtain drug concentrations as high as 0.015 M. Poly(A)·poly(U) was thus found to solubilize much more drug than would normally be soluble in the same pD and temperature ranges for the drug in the absence of polymers. From known constituent concentrations and integration of nmr resonance signals,[2] it appears that there is one drug molecule interaction for every eight nucleotides (or four base pairs) of poly(A)·poly(U).

Nmr Study of Related Interactions. The effects of the interactions of triethylamine (as triethylammonium ion) and Acridine Orange with poly(A)·poly(U) (25–35) at 20° are shown in Figure 7.

Nmr studies of the interaction of Miracil D with adenosine 5-monophosphate showed that the H_8–H_2 and H_1' resonances of AMP-5′ and the ring methyl resonance of Miracil D were all shifted about 0.10 ppm to higher field. The ribose resonances of AMP-5′ and the chain methyl and methylene resonances of Miracil D were not shifted from their normal positions.

[2] Integration of nmr resonance areas were done as a second check on the drug-to-polymer ratios. Comparison were made using the poly(A)·poly(U) H_8–H_2–H_6 resonance area and the ring methyl and chain methyl resonances of Miracil D.

TABLE III: Chemical Shifts and Signal Amplitudes for the Resonance of the Thiaxanthenone Ring Methyl Group of Miracil D with Poly(A)·Poly(U), Poly(A), and Poly(U).

Sample	Temp (°C)	Chemical Shift (ppm)	Relative[a] Amplitude (%)
Poly(A)·poly(U)[b] + 0.005 M Miracil D	20	1.63	30
Poly(A)·poly(U) + 0.01 M Miracil D	20	1.61	22
Poly(A)·poly(U) + 0.015 M Miracil D	20	1.55	21
	30	1.55	28
	40	1.56	32
Poly(A)[c] + Miracil D	20	1.57	34
	30	1.62	47
	40	1.65	46
Poly(U)[c] + Miracil D	20	1.86	28
	30	1.86	50
	40	1.90	50

[a] Represents the signal amplitude (reduced by broadening) of the thiaxanthenone ring methyl group observed upon interaction of Miracil with polymers; reported as per cent of amplitude for this group in the absence of polymers. [b] (20–25) chain length, pD 6.4, polymer concentration ~0.07 M. [c] (27–35) chain length, pD 6.8, polymer concentration ~0.05 M, Miracil D concentration ~0.0025 M.

Discussion

The double-helical structures formed from polynucleotides in the range of 20–40 nucleotides in chain length appear to be of ideal size for proton magnetic resonance studies. This is due to the fact that polymers of this size do not experience the adverse broadening effects that are usually encountered in nmr studies of rigid macromolecules. The reason for this broadening in rigid macromolecules is that nmr line widths, or transverse relaxation times, are sensitive functions of molecular motions, which in turn are determined in part by the size and rigidity of the molecule, as well as by the viscosity of the medium (Bovey, 1969; Farrar and Becker, 1971; Bloembergen *et al.*, 1948). The reason for the dependence on molecular motions is the occurrence of strong, slowly changing, dipole–dipole interactions among the nuclear magnetic moments in systems of low molecular mobility. The multitude of diverse local magnetic fields associated with these dipole–dipole interactions are responsible for the broad nmr lines usually obtained in solid samples; in nonviscous liquids rapid tumbling motions of small molecules effectively average these local fields to zero, and narrow lines are observed. For large macromolecules, especially if structural rigidity inhibits segmental motion, these dipolar effects can be quite strong, and broad lines are the result, even though the solution itself may be relatively nonviscous. One finds, then, because of the high molecular weight and rigidity of DNA, dipolar relaxation effects are very strong, and there is no observable proton nmr spectrum for DNA in the double helix form (McDonald *et al.*, 1964, 1967), nor is there an observable spectrum for high molecular weight poly(A)·poly(U) below its thermal transition point (Chan *et al.*, 1966; Rich and Davies, 1956; Warner, 1956). Concerning the interactions of small molecules with macromolecules, we found no observable proton nmr resonances for Miracil D when it was added to high molecular weight poly(A)·poly(U) (>250 nucleotides in chain length).

Polymers of 20–40 nucleotides in length represent a more ideal size for nmr studies, since the dipolar broadening is not serious, stable double helices can be formed at room temperatures, and the interaction with drugs can be observed. In addition, the availability of polyribonucleotides and the simple methods for obtaining smaller chain lengths make them suitable for model studies. However, it must be remembered that the polyribonucleotide double helix geometry is different from that of the polydeoxyribonucleotide double helix. The differences are believed to be due to the fact that the ribose sugar molecule has a 2′-OH group, while the deoxyribose sugar has 2′-H, which results in a slightly different stacking arrangement of bases for the two polymers (Cross and Crothers, 1971).

Several criteria satisfactorily demonstrate the formation of a double helix upon mixing poly(A) and poly(U) in the present work. First, a large portion of the H_8–H_2–H_6 resonance pattern of poly(A)·poly(U) is shifted 0.70 ppm to higher field. These high-field shifts are due to the positioning or stacking of the aromatic adenine rings which, because of their ring currents, increase the magnetic shielding of the protons in the bases immediately above and below. The magnitude of the high-field shift due to stacking interactions in purine compounds is found to increase as one goes from monomers (nucleosides and nucleotides), to dinucleotides, to polynucleotides (Bovey, 1972; Yamane 1971). The high-field shifts of the base resonances also observed by Cross and Crothers (1971) for the interaction of complementary strands of deoxyribopentanucleotides at 0–10° are believed to indicate double helix formation. The observation of large high-field shifts (0.70 ppm) in our study of poly(A)·poly(U) is interpreted as an indication of a tightly stacked arrangement for the bases, and indicates that a high degree of cooperativity exists between the complementary strands, as would be expected of double helix formation. The high-field region of the resonances of the H_8–H_2–H_6 protons of the poly(A)·poly(U) system (7.70–7.00 ppm) contains 50% of the total H_8–H_2–H_6 resonance area (Figure 2). This constitutes the shifting of a considerable amount of resonance area and is presumably due primarily to the adenine bases, since the uracil bases are not aromatic. The other resonances of poly(A)·poly(U) were found to be less affected with the H_5–H_1' envelope being shifted only ~0.20 ppm to higher field and only a small change in the position of the ribose resonance envelope. Here, by looking at chemical shifts, one can see the contrast between those portions of the double helix involved in strong interactions (*i.e.*, base stacking) and the ribose sugar portion, which is less affected by double helix formation. The H_8–H_2–H_6 resonance envelope of the poly(A)·poly(U) system is observed to broaden more in relation to the other resonances upon formation of the double helix (Figure 2a). It is difficult to determine whether this broadening is due to a relative restriction of motion for the tightly stacked bases (which would shorten T_2 relaxation times and cause broadening), or to nonequivalence of resonance positions. The former effect would be consistent with helix formation, but both effects most likely contribute to the observed broadening. The fact that the total resonance area of the poly(A)·poly(U) (20–25) spectrum compares reasonably well with the resonance area for an equal amount of single-stranded polymer indicates that the formation of poly(A)·poly(U) polymers of limited chain length does not broaden nmr resonances beyond detection.

The second criterion concerns the low-field resonance at

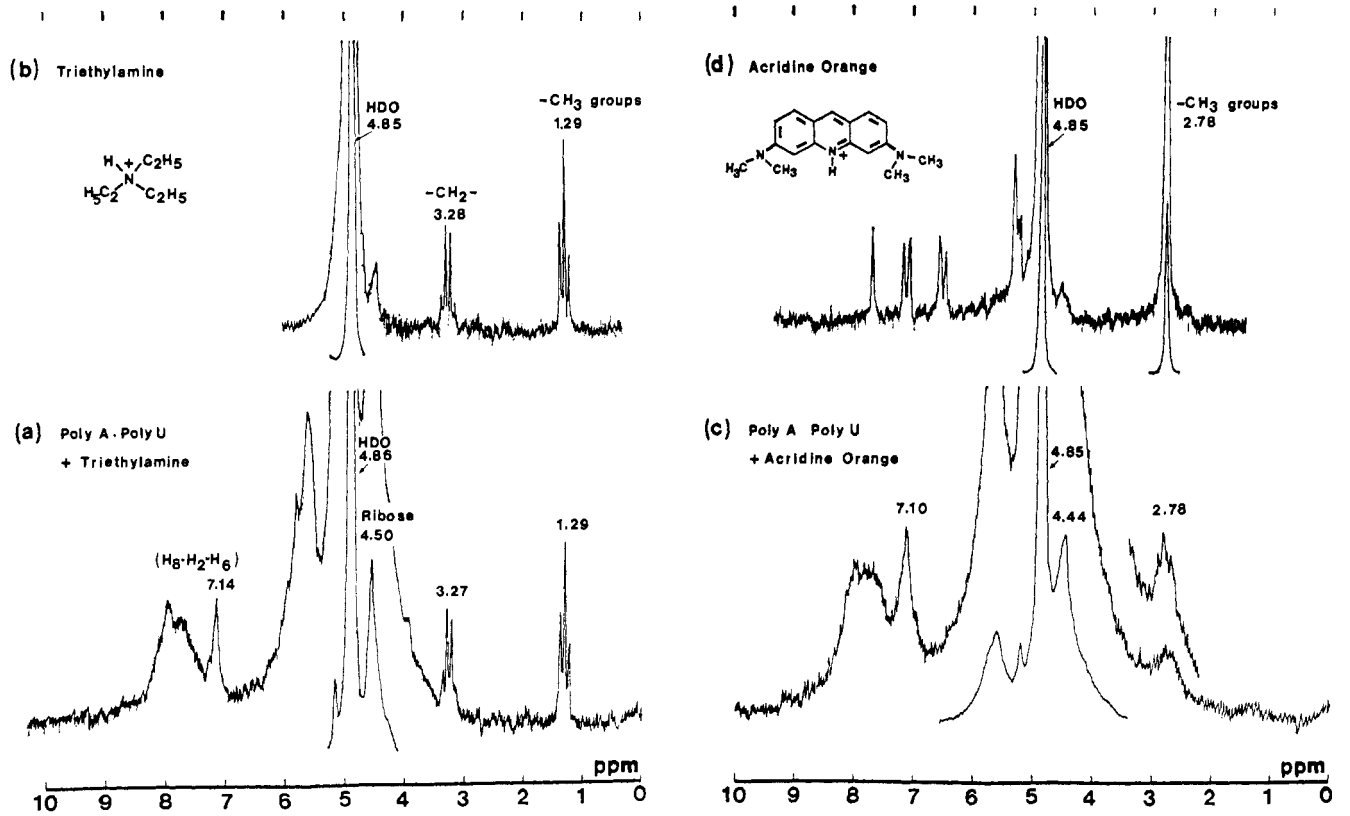

FIGURE 7: The 100-MHz nmr spectra of: (a) triethylamine (0.005 M) (as the cation) with poly(A)·poly(U) at a pD of 7.2; (b) triethylamine (0.005 M) (as the cation) alone at pD 7.0; (c) a sample of Acridine Orange (0.007 M) with poly(A)·poly(U) at pD 6.8; and (d) Acridine Orange alone at pD 6.8. The poly(A)·poly(U) concentration was ~0.70 M, and all spectra were run at 20°.

~13.60 ppm observed for poly(A)·poly(U) in H_2O. This 13.60-ppm resonance is a good indication of hydrogen bonding between the complementary bases of poly(A) and poly(U) strands. Such low-field resonances, which are associated with the large reduction in shielding of the N–H protons due to hydrogen bonding, have also been observed by Kearns *et al.* (1971) for phenylalanine tRNA, using 220-MHz nmr. From the comparison of the integrated area of the 13.60-ppm resonance peak, due to two –NH protons, with the area of the H_8–H_2–H_6 resonance pattern, we find that most of the bases appear to be involved in hydrogen bonding. Thus, most of the poly(A)·poly(U) molecule probably exists as a true double helix structure, with hydrogen-bonded base pairs, and with little single-stranded region. Since the expected amount of hydrogen bonding is observed, the poly(A)·poly(U) spectrum (Figure 3) probably represents mainly double-stranded polymer, with little contribution from single-stranded regions.

The third criterion for double helix formation is the melting behavior observed for the poly(A)·poly(U) molecules studied. Melting curves for poly(A)·poly(U) (20–25) obtained by both the nmr method and the uv method (Figure 3) show the typical behavior commonly seen for high molecular weight double-stranded nucleic acids. The transition temperature (T_m) obtained by nmr was ~49°, and by uv was ~45°. The temperature-variation study done for the poly(A)·poly(U) resonance at ~13.60 ppm (Figure 4) shows the characteristics of melting, and is found to be completely absent above 50°, which is consistent with the other melting data presented.

Miracil D is a most suitable drug for a model system study of the type undertaken in this work, for at least three reasons. First, there is a great deal of information in the literature which shows that it exerts its biological effect as an inhibitor of nucleic acid synthesis, through direct noncovalent interactions with nucleic acids (Weinstein and Hirschberg, 1971). Second, Miracil D inhibits RNA polymerase activity when polyribonucleotides or polydeoxyribonucleotides are used as templates, and has a more pronounced effect when the polyribonucleotides contain adenine residues (Weinstein and Hirschberg, 1971). Third, the drug has a relatively simple structure in comparison, for example, to actinomycin. Its most important structural attribute from the point of view of nmr measurements is that the thiaxanthenone ring contains a methyl group (Figure 1). This ring methyl group is important since its resonance position is at high field, at a position that is not blocked out by the resonances of the polynucleotide. The resonances of most of the thiaxanthenone ring protons are found to be obscured by the polymer's resonances and, therefore, give no information about the ring. The fact that the resonance of the ring methyl group of Miracil is also resolved from the resonances of the methylene and methyl groups on the chain portion allows one to observe the differences in environments between the chain and chromophore portion of the drug upon binding to polymers.

One can see from the results presented in Figure 5 and Table I that Miracil D interacts with both single-stranded poly(A) and single-stranded poly(U) and that the thiaxanthenone ring methyl group is most affected. In the case of poly(A), the resonance of the ring methyl group of Miracil D is shifted quite significantly upfield ~0.3 ppm, while the upfield shift for the poly(U) case is about ~0.1 ppm. The large high-field shift of the thiaxanthenone methyl resonance observed for the Miracil D–poly(A) interaction is consistent with intercalation of the thiaxanthenone ring between the adenine rings; the adenine rings are known to exert a strong shielding influence. The uracil rings of poly(U) are not aromatic; therefore, the shifting of the ring methyl resonance is less

pronounced for Miracil D with poly(U). In both the poly(A) and poly(U) systems, the thiaxanthenone ring methyl group resonance is broadened in comparison to the resonance of the chain methyl groups. This type of broadening has been observed by Chan and Kreishman (1970) for purine interaction with poly(U). They proposed that the purine protons experience a strong dipolar field when intercalated between the adjacent bases of poly(U), and that the resonances of these protons are broadened by nuclear spin relaxation induced by fluctuations of these local fields. The fact that the thiaxanthenone ring methyl resonance is broadened as well as shifted (particularly for poly(A)) is, then, a rather clear indication of the intercalation of the ring within the stacked bases of poly(A) and poly(U).

The spectral changes observed for the interaction of Miracil D, at high concentration, with poly(A)·poly(U) are more pronounced than those observed for the interactions with either single-stranded polymer. When poly(A)·poly(U) is present, the resonance of the thiaxanthenone ring methyl group is shifted 0.30 ppm to higher field and broadened to about 20% of its normal amplitude (Figure 6, Table III). As was seen with samples containing the single-stranded polymers, the resonances of the chain methyl and methylene groups are much less affected by the interaction with the double-stranded polymer than that of the thiaxanthenone ring methyl group. Again, the high-field shifts and broadening are strong evidence that the ring portion of Miracil D is intercalated within the stacked base pairs of the poly(A)·poly(U) molecule. Concerning the relative strengths of the interactions, the temperature variation studies (Table III) show that as the temperature increases the thiaxanthenone ring methyl resonance both shifts less dramatically back to lower field and experiences a less marked increase in amplitude (remains broader) for the poly(A)·poly(U) interaction, than for the interaction with either of the single-stranded polymers. This indicates that the interaction of Miracil D with poly(A)·poly(U) is stronger than the interaction of the drug with either of the single-stranded polymers. In line with this evidence for a stronger interaction is the fact that the double-stranded polymer is able to solubilize two to three times as much Miracil D as is either single-stranded polymer of equal nucleotide concentration. Thus, for equivalent amounts of total polymer, the double-stranded poly(A)·poly(U) interacts with one drug molecule for every four to five base pairs, while the single-stranded polymers interact with one drug molecule for every 20–25 nucleotides. Even at a pD as high as 7.5, where the drug solubility is normally much less than 0.001 M in the absence of polymers, drug concentrations as high as 0.01 M could be reached with poly(A)·poly(U) solutions. Thus, it appears that the double-stranded polymer is a more favorable structure for binding Miracil D than single-stranded materials.

Some particularly interesting findings for the interaction of the drug with poly(A)·poly(U) are: (1) both the magnitude of the shielding and the amplitude reduction of the Miracil D ring methyl resonance become larger as the concentration of the drug increases (Table III, Figure 6a–c); (2) the H_8–H_2–H_6 resonance envelope of poly(A)·poly(U) is found to move to higher field (~0.15 ppm), as drug concentration increases (Table II); (3) temperature variation studies show that the area of the high-field portion of the H_8–H_2–H_6 resonance pattern of poly(A)·poly(U) with high Miracil concentration (0.015 M) is reduced less markedly as temperature increases than for samples of the double-stranded polymer without drug (Table II). These results suggest a cooperative effect for Miracil D binding and that, as concentration of the drug increases, the poly(A)·poly(U) double-stranded structure is actually stabilized. This stabilization is reflected by the increased resistance of the poly(A)·poly(U)–Miracil D complex to melting or denaturation.

The studies of the separate interactions of Acridine Orange and triethylamine (which are structurally analogous to the ring and chain portions of Miracil D, respectively) with poly(A)·poly(U) provide some interesting results (Figure 7). First, the methyl resonance (2.78 ppm) of Acridine Orange is found to broaden (~20% of normal amplitude), but not shift in the interaction with poly(A)·poly(U). The broadening is a good indication that the planar acridine ring is intercalated between the base pairs. The absence of an upfield shift might be due to the fact that the methyl groups are separated from the acridine ring by a nitrogen atom, which could put these groups just outside the shielding field of the base pairs; and, thus, there would be no upfield shifts observed. Acridine Orange has a pK_a of ~10.5, which means that at a pD of ~6.8 it exists in cationic form, and we would also expect electrostatic interaction of this molecule with poly(A)·poly(U). From data collected by other physical–chemical methods alternate types of binding processes have been proposed for the Acridine Orange–nucleic acid interaction; these are intercalation of the Acridine Orange within the stacked bases, and external binding and stacking of the Acridine Orange by electrostatic interactions (Peacocke, 1973). The broadening of the Acridine Orange methyl resonance observed in our studies could also be due to electrostatic interactions; however, these electrostatic interactions would have to be very strong in order to produce the very large broadening effect that was observed. This might be true of Acridine Orange, when it is in its dicationic form. Experiments on the triethylamine interaction with poly(A)·poly(U) show no significant changes in either the polymer resonance or the triethylamine resonance. Since the pK_a of triethylammonium is high (~10.6), we would expect electrostatic binding of this cationic molecule to the anionic phosphate groups of poly(A)·poly(U) at a pD of 6–7. This type of interaction probably occurs, but it has no large effect on the nmr resonances of the triethylammonium ion. In relation to this point, the tertiary amine group of Miracil D has a pK_a of ~6.1–6.3, and in most of the binding studies reported here we would expect some electrostatic interactions. Although no pronounced effects are observed for the methyl or methylene groups on the chain portion of Miracil D upon interaction with poly(A)·poly(U), there do seem to be some small shifts in the resonances of the chain methyl groups upon interaction with the polymers. A more careful investigation might reveal more information about electrostatic interactions. The results on Acridine Orange and triethylamine show rather clearly, then, the contrasts that are observed for the binding of a cationic planar molecule and a cationic nonplanar molecule to double-stranded poly(A)·poly(U).

Miracil D was found to interact with adenosine 5′-monophosphate. Small high-field shifts were observed mainly for the proton resonances of the adenine ring and the thiaxanthenone ring methyl group, with no significant broadening occurring for any of the observed resonances. This study shows that the Miracil D ring system is probably interacting (stacking) with the adenine ring of AMP-5′, as it does in the polymer systems studied, but to a lesser degree, as would be expected.

The nmr data support the postulate of a model for the interaction of Miracil D with poly(A)·poly(U) in which the thiaxanthenone ring is intercalated between the stacked base

pairs of the double-stranded polynucleotides. The cationic chain portion of Miracil D extends out from the stacked region, most likely interacting with the anionic phosphate groups of the polymer. The results of the nmr studies further suggest that the thiaxanthenone ring methyl group of Miracil D is probably located between the adenine bases of poly(A)·poly(U), since the shielding of this methyl group is quite high. Thus, the 1,2,3,4 end of the thiaxanthenone ring is probably under the adenine bases and the 5,6,7,8 end is under the uracil bases of poly(A)·poly(U) (see Figure 1). From studies of CPK space-filling atomic models for both double-helical poly(A)·poly(U) and poly(dA)·poly(dT), it appears that Miracil D has a better fit into the double-helical structure if the thiaxanthenone ring is inserted between the base pairs such that the 1-diethylaminoethylamino chain portion extends from the major grove side of the double helix, with the 4-methyl group pointing out the minor grove side. This orientation allows the complete intercalation of the thiaxanthenone ring system by the stacked base pairs and also allows the distal nitrogen on the Miracil D chain to reach near the phosphate oxygen atoms of the polymer, making electrostatic interaction possible. Another possible orientation, in which the Miracil D chain portion extends out from the minor grove side of the double helix, is unfavorable in both poly(A)·poly(U) and poly(dA)·poly(dT), since it does not allow the close proximation of the chain to phosphate oxygen atoms. This orientation is particularly unfavorable in the poly(A)·poly(U) helix, due to the steric interference of the 2′-OH group on the ribose sugar of poly(A)·poly(U). This interference causes a larger separation of the Miracil D chain from the phosphate oxygen atoms of poly(A)·poly(U) than in the case of poly(dA)·poly(dT). Figure 8 show a model for the Miracil D interaction with poly(A)·poly(U) based on the above description. Other results of the nmr studies described above indicate that Miracil D stablizes the double helix structure of poly(A)·poly(U) to heat denaturation. All of these results are consistent with similar conclusions on related systems based upon a large amount of data collected by various other physical–chemical techniques (see Weinstein and Hirschberg, 1971).

Alternate types of binding processes are possible for Miracil D. One type involves external binding and stacking of the drug to the polymer structure. The results of our study appear not to be consistent with this type of process, for two reasons. First, the broadening and shifting effects observed for the Miracil D ring methyl resonance reflect very strongly the effects observed for the bases of poly(A) and poly(U) upon double helix formation. Second, upon interaction with single-stranded poly(A) and poly(U), the Miracil D ring methyl resonance shifts to higher field with poly(A), but not with poly(U). Since poly(U) is not aromatic, but the Miracil D ring is, one would expect high-field shifts to occur if Miracil D were stacking with itself in the Miracil D poly(U)–interaction. Thus, even though external binding may occur to some extent, the results of this study are more consistent with intercalation of Miracil D.

On the basis of this and other work, we believe that a particularly informative approach to studying the interaction of various compounds, like Miracil D, to nucleic acids would be to use fully deuterated drugs with small molecular weight nucleic acids in the protium (H) form. Then, both deuterium and proton nmr could be used on the same samples, and there would be no interference between the resonances of the drug and those of the polymer. With this technique, much more detailed information could be obtained about interactions between drugs and nucleic acids. Such experiments are under way.

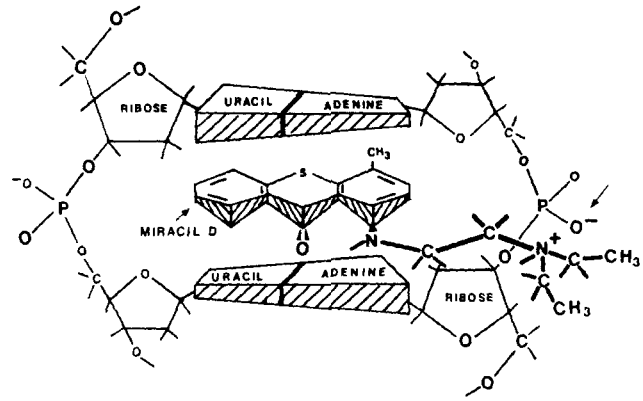

FIGURE 8: The proposed structure for the poly(A)·poly(U)–Miracil D complex. The diethylaminoethylamine chain portion of Miracil D extends out from the major groove of the poly(A)·poly(U) double helix and reaches the phosphate oxygen group of poly(A)·poly(U), thus making electrostatic interaction possible. The thiaxanthenone ring of Miracil D is intercalated completely by the base pairs of poly(A)·poly(U).

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