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Dependence of 4'-(Hydroxymethyl)-4,5',8-trimethylpsoralen Photoaddition on the Conformation of Ribonucleic Acid[†]

John F. Thompson, Jean-Pierre Bachellerie,[‡] Kathi Hall, and John E. Hearst*

ABSTRACT: The photoaddition of 4'-(hydroxymethyl)-4,5',8-trimethylpsoralen (HMT) to different conformational states of RNA was studied. Poly(U), poly(A,U) (random copolymer), poly(A-U) (alternating copolymer), poly(A)-poly(U) (double stranded), and poly(U)-poly(A)-poly(U) (triple stranded) were reacted with HMT at different temperatures and salt concentrations. The conformation of the polymers

was monitored by UV absorption and circular dichroism. It was found that the rate of HMT photoaddition changed dramatically at structural transitions in the RNA. The alternating copolymer poly(A-U) was found to have the highest rate of addition. Low salt and temperature produced maximal incorporation.

Psoralen and its derivatives have been used increasingly to study the structure of nucleic acids. Psoralen is a planar, aromatic molecule that can intercalate into the double helix and then photoreact with the 5,6 double bond of a pyrimidine.

We have used a derivative of psoralen, 4'-(hydroxymethyl)-4,5',8-trimethylpsoralen (HMT), which is both more reactive and more water soluble than the parent compound (Isaacs et al., 1977). HMT has two reactive sites that, with two photons, can cross-link opposite and adjacent bases to covalently link two strands. These cross-links can then be localized to provide information about the secondary and tertiary structure of a molecule. Electron microscopy has been used to map cross-links in 16S ribosomal RNA (Wollenzien et al., 1979) and fd virus (Shen et al., 1979), while more recently, monoadducts (Bachellerie & Hearst, 1982) and cross-links (Thompson et al., 1981; Rabin & Crothers, 1979) have been mapped to sequence resolution in smaller RNAs.

In order to take full advantage of this structural probe, it is necessary to find both the specificity and the optimal conditions for the reaction. It has been apparent for some time

[†] From the Department of Chemistry (J.F.T., J.-P.B., and J.E.H.), University of California, Berkeley, California 94720, and the Divisions of Chemical Biodynamics (J.F.T. and J.E.H.) and Biology and Medicine (K.H.), Lawrence Berkeley Laboratory, Berkeley, California 94720. Received May 18, 1981. This work was supported in part by National Institutes of Health Grant GM 11180, by an NIH Fogarty Fellowship to J.-P.B., by National Institutes of Health Grant GM 25936-01 to K.H., and by the Assistant Secretary for Environment, Office of Environmental Research and Development, Biomedical and Environmental Research Division of the U.S. Department of Energy under Contract No. W-7405-ENG-48.

[‡] Present address: Centre de Recherche de Biochimie et Génétique Cellulaires du CNRS, 31062 Toulouse Cedex, France.

that the most reactive base is uracil (Krauch et al., 1967; Pathak et al., 1974; Ou & Song, 1978; Bachellerie et al., 1981). Only more recently has it become known that adjacent uracils in a weak helix in natural RNA are a particularly strong site for addition (Bachellerie & Hearst, 1982; Thompson et al., 1981; Youvan & Hearst, 1982). In DNA, it has been suggested that an A-T site is preferred (Chandra et al., 1973; Dall'Acqua et al., 1978b). Optimal conditions for psoralen reaction with 5S RNA (Thompson et al., 1981) and Col E1 DNA (Hyde & Hearst, 1978) have already been described. Studies with synthetic DNA polymers have determined relative reactivities but only in a narrow range of conditions (Dall'Acqua et al., 1979). Other studies have examined even more heterogeneous systems (Dall'Acqua et al., 1969).

We have chosen to study simpler systems in which a more homogeneous, controllable structure can be obtained. We have examined a wide range of salt concentrations, temperatures, and conformations for their effect on HMT incorporation. We have used circular dichroism and UV absorption spectroscopy to correlate the structure of the polymers with HMT addition. This has been done with poly(U), poly(A,U) (random), poly(A-U) (alternating), and poly(A)-poly(U).

The synthetic polymers of adenine and uridine are worthwhile to study because they can adopt a variety of conformations, and these have been well characterized by a variety of techniques (Stevens & Felsenfeld, 1964; Brahms, 1965; Massoulié, 1968; Steiner & Millar, 1970; Arnott & Bond, 1973). The transitions between different conformations are sharp and easily monitored.

Materials and Methods

Poly(U), poly(A), and poly(A,U) were purchased from P-L Biochemicals. Poly(A-U) was synthesized by a modification of the method described by Chamberlin et al. (1963). The reaction (1.0 mL) was done with 50 mM Tris-HCl¹ (pH 8.0), 1 mM MnCl₂, 5 mM MgCl₂, 10 mM β -mercaptoethanol, 10 mM ATP, 10 mM UTP, 10 mM KCl, 10 μ g/mL poly(dA-dT) (P-L Biochemicals), 1 μ M 3'-O-methyl-ATP, and 0.25 mg/mL *Escherichia coli* RNA polymerase (a gift of Professors Robert Woody and A. Young M. Woody of Colorado State University) for 2 h at 37 °C. After reaction, the solution was phenol extracted 3 times, ethanol precipitated 3 times, and dialyzed against 50 mM Tris, pH 7.5, and 10 mM EDTA for 6 h, followed by 6 h with 1 mM Tris, pH 7.5, and 0.1 mM EDTA. After ethanol precipitation, the sample was run through a Sephadex G-100 column, and the void fractions were combined and precipitated. The yield was 800 μ g. Aliquots of the reaction contained [α -³²P]UTP or [α -³²P]ATP (Amersham). Nearest-neighbor analysis was done by digestion with RNase T₂ (Sigma), followed by paper electrophoresis at pH 3.5 (Bachellerie et al., 1981). The average length of the product was determined by electrophoresis on a 12% polyacrylamide gel (Thompson et al., 1981).

[³H]HMT with a specific activity of 4.0×10^7 cpm/ μ g was synthesized by S. Isaacs of this laboratory (Isaacs et al., 1982). In some experiments, 5'-³²P-labeled RNA was used. Poly(U) (1 mg) was dephosphorylated with 1 unit of calf alkaline phosphatase (Boehringer-Mannheim) in 50 mM Tris, pH 8.8, at 37 °C for 1 h. The reaction mixture was then phenol extracted twice, ether extracted, and ethanol precipitated. The polymer was redissolved in 50 mM Tris, pH 8.5, 10 mM

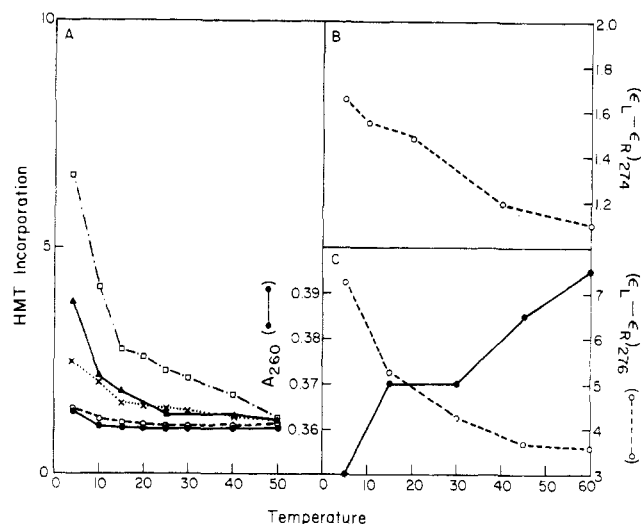


FIGURE 1: (A) Temperature dependence of HMT addition to poly(U) at 0 mM NaCl (●), 5 mM NaCl (○), 100 mM NaCl (×), 5 mM MgCl₂ and 5 mM NaCl (□), and 50 mM MgCl₂ and 500 mM NaCl (▲). (B) $(\epsilon_L - \epsilon_R)_{274}$ for poly(U) at 5 mM MgCl₂ and 500 mM NaCl (▲). (C) $(\epsilon_L - \epsilon_R)_{276}$ for poly(U) at 50 mM MgCl₂ and 500 mM NaCl (○) and absorption at 260 nm (●).

MgCl₂, and 5 mM DTT. [γ -³²P]ATP (50 μ Ci) (Amersham; 3000 Ci/mmol) and 1 unit of T₄ polynucleotide kinase (Boehringer-Mannheim) were added and incubated for 30 min at 37 °C. The mixture was phenol extracted, ethanol precipitated, and run through a Sephadex G-100 column.

Unless otherwise noted, all irradiations were performed with 200 μ g/mL of each polymer, 10 μ g/mL [³H]HMT, 1 mM Tris, pH 7.5, and 0.1 mM EDTA for 30 s or less. Incorporation was linear with time in this range. Irradiations involving poly(A,U) or poly(A-U) were done with 400 μ g/mL polymer in order to maintain the same amount of uridine in all cases. Samples containing both poly(A) and poly(U) were incubated at 50 °C for 1 h and then slow cooled to the temperature of interest. Each incubation took place for a minimum of 18 h. Irradiations were done as previously described (Thompson et al., 1981).

After irradiation, samples were extracted twice with 2 volumes of chloroform/isoamyl alcohol (24:1 v/v) and then ethanol precipitated twice. The precipitate was resuspended and counted in a solution of toluene/Triton X-100/water (6:3:1 v/v/v) with 3.92 g/L PPO and 0.08 g/L bis-MSB.

CD spectra were measured with a Cary 60 spectropolarimeter equipped with a 6003 unit. The computerized data collection system has been described previously (Brunner & Maestre, 1975). Data are presented as $\epsilon_L - \epsilon_R$ per mole of monomer, where ϵ_L and ϵ_R are the extinction coefficients for left and right circularly polarized light, respectively. Ultraviolet absorption spectra were measured with a Cary 14 spectrophotometer. In both instruments, the temperature of the sample was maintained with a circulating water bath. Melting curves for poly(A-U) were done with a Gilford Model 250 spectrophotometer with a Model 2527 thermoelectric temperature programmer. These were interfaced to a Commodore PET Model 2001 microcomputer.

Results

Poly(U). The incorporation of [³H]HMT into poly(U) as a function of temperature at different salt concentrations is shown in Figure 1A. The unit of HMT incorporation on the vertical axis is the same for Figures 1, 2, and 4–7 and is based on the reactivity of poly(U) in Tris-EDTA at 25 °C (equal to 1). The CD and absorption spectra of poly(U) were taken

¹ Abbreviations: Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5'-triphosphate; UTP, uridine 5'-triphosphate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PPO, 2,5-diphenyloxazole; CD, circular dichroism; bis-MSB, *p*-bis(*o*-methylstyryl)benzene.

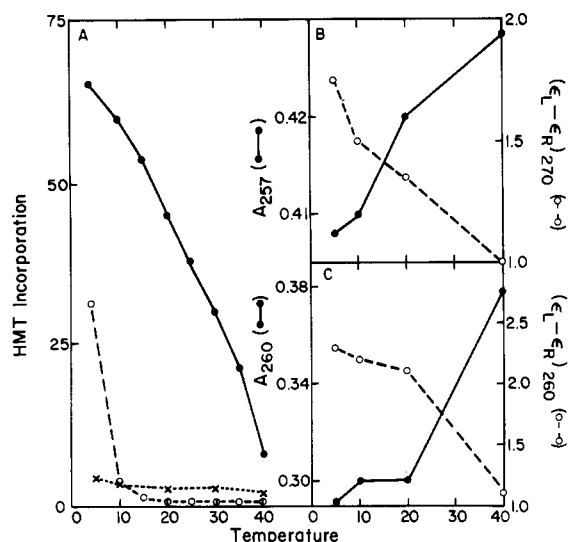


FIGURE 2: (A) Temperature dependence of HMT addition to poly(A,U) at 0 mM NaCl (O), 100 mM NaCl (●), and 5 mM NaCl and 5 mM MgCl_2 (X). (B) $(\epsilon_L - \epsilon_R)_{270}$ for poly(A,U) at 0 mM NaCl (O) and absorption at 260 nm (●). (C) $(\epsilon_L - \epsilon_R)_{260}$ for poly(A,U) at 100 mM NaCl (O) and absorption at 260 nm (●).

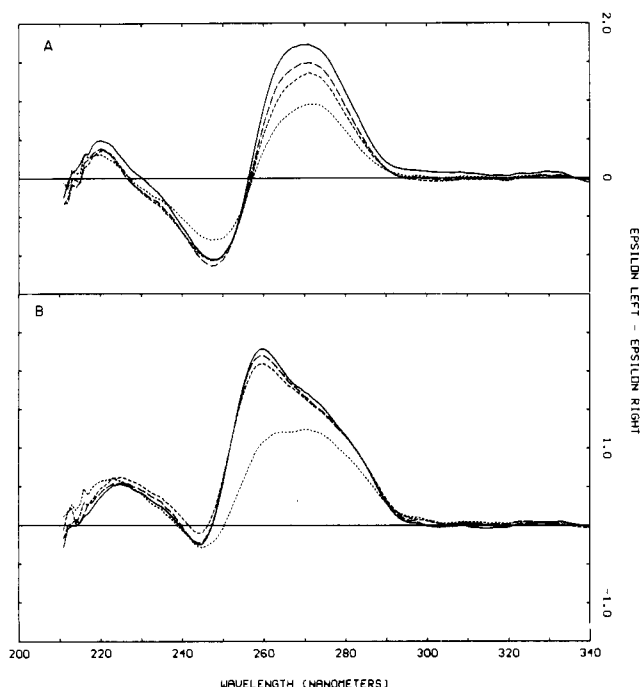


FIGURE 3: (A) Circular dichroism spectra of poly(A,U) with 0 mM NaCl at 5 (—), 10 (---), 20 (---), and 40 °C (---). (B) Circular dichroism spectra of poly(A,U) with 100 mM NaCl at 5 (—), 10 (---), 20 (---), and 40 °C (---).

under identical conditions (Figure 1).

Random Poly(A,U). Figure 2 shows the incorporation of HMT into the random copolymer poly(A,U) in Tris-EDTA and in 0.1 M NaCl. Figure 3 shows the temperature dependence of the CD spectra for these two conditions.

Alternating Poly(A-U). Before HMT incorporation studies were done on the alternating copolymer poly(A-U), its properties were determined in order to assure its authenticity. Nearest-neighbor analysis using α -labeled triphosphates showed that the polymer was over 99% correct. The UV absorption spectra of both the coil and helix were identical with published spectra (Chamberlin et al., 1963) with isosbestic points at 279 and 295 nm. Carefully annealed polymer showed a hyperchromism of 67.2% at 260 nm, which is slightly larger than previously observed (65%). Fast-cooled polymer showed

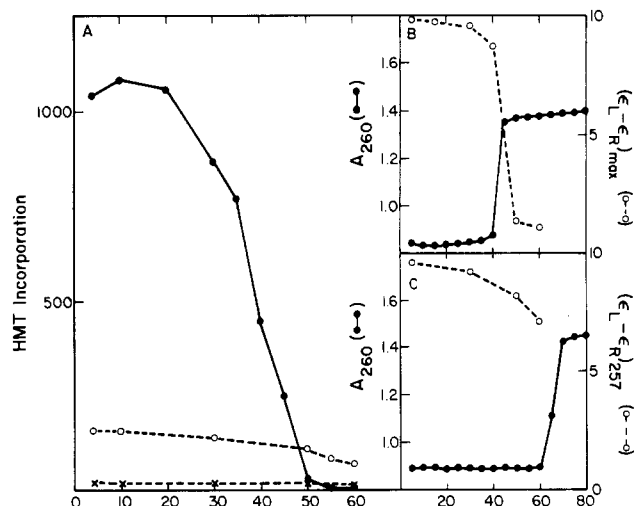


FIGURE 4: (A) Temperature dependence of HMT addition to poly(A-U) at 5 mM NaCl (●), 100 mM NaCl (O), and 5 mM NaCl and 5 mM MgCl_2 (X). (B) $(\epsilon_L - \epsilon_R)_{\text{max}}$ for poly(A-U) at 5 mM NaCl (O) and absorption at 260 nm (●). (C) $(\epsilon_L - \epsilon_R)_{257}$ for poly(A-U) at 100 mM NaCl (O) and absorption at 260 nm (●).

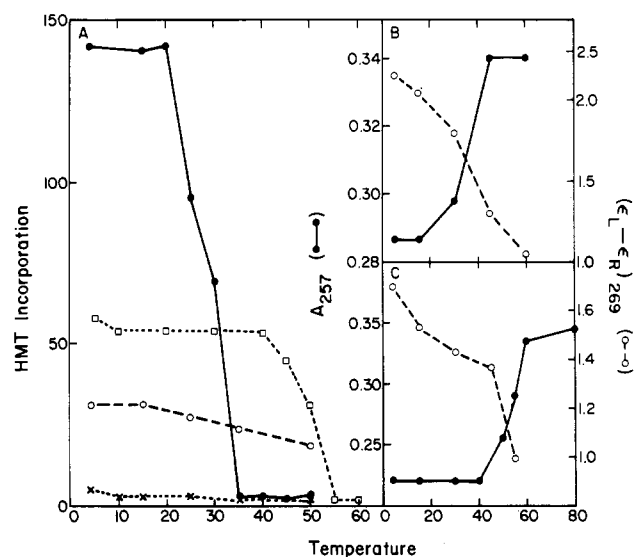


FIGURE 5: (A) Temperature dependence of HMT addition to poly(A)·poly(U) (equal mole amounts of each polymer) at 5 mM NaCl (●), 50 mM NaCl (□), 100 mM NaCl (O), and 5 mM NaCl and 5 mM MgCl_2 (X). (B) $(\epsilon_L - \epsilon_R)_{269}$ for poly(A)·poly(U) at 5 mM NaCl (O) and absorption at 257 nm (●). (C) $(\epsilon_L - \epsilon_R)_{270}$ for poly(A)·poly(U) at 50 mM NaCl (O) and absorption at 257 nm (●).

a hyperchromism at 260 nm of 63.4%. These measurements were done with 5 mM NaCl between 20 and 80 °C. The melting points with 5 mM NaCl (42 °C) and 100 mM NaCl (65 °C) were sharp. Circular dichroism spectra of the helix and coil forms were identical with those previously published (Gray et al., 1972). The polymer was between 500 and 2000 bases long. The temperature dependence of HMT incorporation, UV absorption, and CD is shown in Figure 4.

Double-Stranded Poly(A)·Poly(U) and Triple-Stranded Poly(A)·2Poly(U). By proper choice of salt condition and molar ratio of homopolymers, it is possible to form either a double or triple helical structure (Stevens & Felsenfeld, 1964). The HMT reactivity of double-stranded poly(U)·poly(A) was examined in a wide range of temperature and salt concentrations. After heating and slow cooling to the temperature of interest, an equimolar mixture of poly(U) and poly(A) was photoreacted with HMT (Figure 5A). The temperature dependence of the CD and UV absorption is also shown in Figure 5.

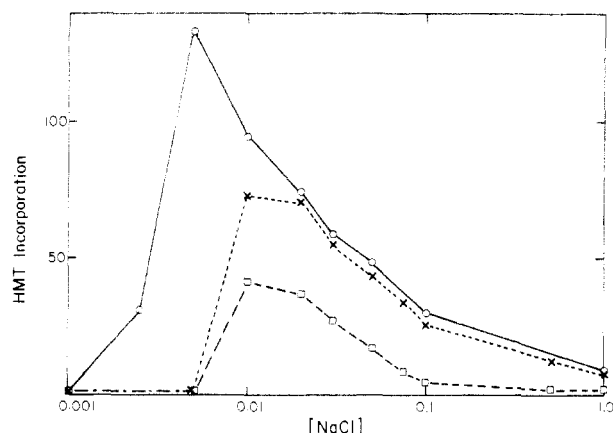


FIGURE 6: The concentration of NaCl was varied for poly(A) (200 $\mu\text{g}/\text{mL}$) and poly(U) (200 $\mu\text{g}/\text{mL}$) at 4 $^{\circ}\text{C}$ (O) and 37 $^{\circ}\text{C}$ (X) and for poly(A) (100 $\mu\text{g}/\text{mL}$) and poly(U) (200 $\mu\text{g}/\text{mL}$) at 37 $^{\circ}\text{C}$ (□).

The range of HMT incorporation into poly(A)·poly(U) was examined over a wide range of salt concentrations as shown in Figure 6. At 4 $^{\circ}\text{C}$, the amount of incorporation fell logarithmically between 5 and 100 mM NaCl and then tailed off more slowly. The salt dependence at 37 $^{\circ}\text{C}$ was monitored at the poly(A) to poly(U) ratios, 1:1 and 1:2. The equimolar mixture curve is virtually identical with that at 4 $^{\circ}\text{C}$ except that incorporation does not increase until 10 mM NaCl (the lowest salt concentration at which the double-stranded complex is stable). The salt dependence of the mixture, which contains only half as much poly(A), has the same shape at low salt concentration but drops off more quickly at higher salt concentrations.

In order to determine the rate of HMT addition to the triple-stranded poly(A)·2poly(U), we varied the amount of poly(A) while keeping the amount of poly(U) constant. This is shown in Figure 7. In conditions where the triple-stranded complex cannot form (25 $^{\circ}\text{C}$, 10 mM NaCl) (Stevens & Felsenfeld, 1964; Massoulié, 1968), a linear increase in the rate of HMT addition with added poly(A) is seen. The amount of HMT incorporated reaches a plateau at a molar ratio of slightly more than 1:1 (A:U). In conditions where both the triple- and double-stranded complexes can exist (25 $^{\circ}\text{C}$, 10 mM NaCl), the amount of HMT incorporated increases only slightly with increasing poly(A) until a ratio of 1:2 is reached. At this point, the amount of HMT added increases quickly until an A:U ratio of 1:1 is reached. This experiment was repeated with the same salt conditions but at 4 $^{\circ}\text{C}$. At 100 mM NaCl, the incorporation profiles are identical at the two temperatures. At 10 mM NaCl, the rate of addition is lower than would be predicted for a double-stranded complex below a 1:2 ratio of A:U. This effect is not nearly as dramatic as at 100 mM NaCl.

Discussion

The conditions used to photoreact HMT with RNA in this study were chosen to be as gentle as possible. The irradiation was short in order to prevent cross-linking. Quantification of cross-links by paper electrophoresis of hydrolysates (Bachelier et al., 1981) showed there was a negligible amount. The binding constant of HMT is sufficiently low (Isaacs et al., 1977) that the noncovalently bound HMT has a minimal impact on the polymer structure. This is not true for intercalators with a higher binding constant such as ethidium bromide. Ethidium bromide can unfold the tertiary structure of tRNA but stabilizes other polymers (Urbanke et al., 1973). HMT does not have as large an effect because not as many molecules are bound.

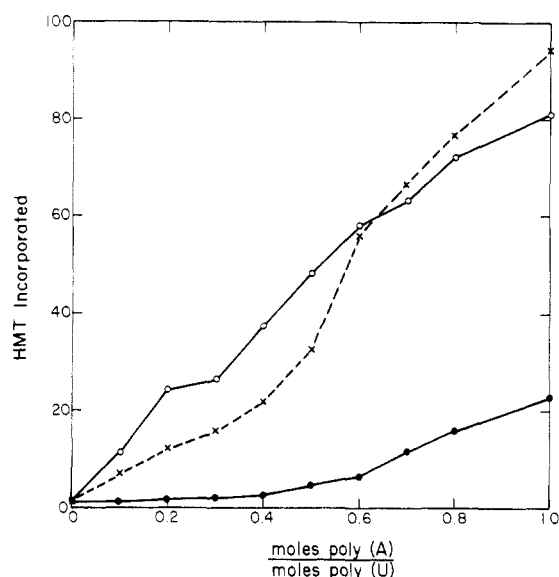


FIGURE 7: Poly(U) (200 $\mu\text{g}/\text{mL}$) was mixed with varying amounts of poly(A) at 25 $^{\circ}\text{C}$ and 10 mM NaCl (O), 4 $^{\circ}\text{C}$ and 10 mM NaCl (X), and 4 and 25 $^{\circ}\text{C}$ and 100 mM NaCl (●).

Ultraviolet absorption and circular dichroism are useful probes for studying the structures of our RNA systems because they monitor different aspects of the geometry. UV absorption measures mainly the stacking between bases and hence is a good method of observing secondary structure, but it is relatively insensitive to higher order structure. CD, on the other hand, arises principally from the interaction between optical transitions of the bases. The theory for predicting the structural changes that are occurring when the CD changes is not well advanced yet, but progress is being made (Greve et al., 1978).

Poly(U). In low salt concentration, poly(U) has no secondary structure. At 0.1 M NaCl, poly(U) is optically identical with the polymer at low salt concentration, but the level of HMT addition is slightly elevated, particularly at low temperature. This could be caused by a small amount of structure that is not discernible by CD.

When 5 mM MgCl_2 is present, CD indicates that poly(U) has secondary structure. This is not apparent from the UV absorption, however. The latter is a result of the poor base stacking properties of uridine. Thrierr et al. (1971) have shown that poly(U) folds back on itself at high salt concentration to form a double-stranded structure. Even though the base pairing has been shown to be much different than the classical Watson-Crick type (Young & Kallenbach, 1978), HMT can intercalate and react with the uridine. The CD spectrum suggests that the helix is an A form, similar to normal double-stranded RNA. It is not clear whether the intercalation site in the poly(U) is essentially the same as in other polymer structures. At very high salt concentrations (500 mM NaCl and 50 mM MgCl_2), both absorption and CD spectra indicate a stable structure exists. In fact, the structure in the poly(U) is stabilized so much that intercalation is made difficult. Because intercalation is accompanied by an unwinding of the helix (Wieschahn & Hearst, 1978) and a lengthening of the RNA, anything that stabilizes the helix and condenses the RNA will inhibit intercalation. Although the rate of HMT addition falls at these high salt concentrations, it is still higher than in the randomly coiled poly(U).

Random Poly(A,U). As determined by CD, the structure of the random copolymer poly(A,U) is substantially different in no NaCl and 100 mM NaCl. In Tris-EDTA, the CD spectrum is characteristic of a B-form structure with a positive

and then a negative band of approximately equal strength (a conservative spectrum) (Tinoco et al., 1980). This type of CD spectrum is very uncharacteristic of double-stranded RNA. Since the ionic strength is low and would not shield the phosphate charges effectively upon double-strand formation, it is uncertain that any base pairing is present. The spectrum is more like that observed with adenylate oligomers (Brahms et al., 1966). As observed with these oligomers, the peaks decrease in intensity and are red shifted with increasing temperature. Since short runs of adenines are present in the polymer, it seems likely that this type of interaction could be occurring. Neither the CD spectrum nor the UV absorption spectrum predicts the temperature dependence of the HMT incorporation. It is possible that, even in the absence of base pairing, the stacking of adenines is sufficient to create local order in the uridine residues at very low temperatures. This ordered structure would allow intercalation and reaction. Because uridine does not stack well, its contribution to the change in the CD and UV absorption spectra would be small.

The CD spectrum of poly(A,U) in 0.1 M NaCl is more complex. It is much more like the normal A-form RNA spectrum with a large positive band and a small negative band (nonconservative spectrum). There are clearly at least two components in the positive band. It seems likely that normal Watson-Crick base pairing is present, although the type of structure postulated for the low-salt poly(A,U) could also be present. The large change in optical properties occurring between 20 and 40 °C is probably caused by a loss of base pairing. The drop in HMT incorporation is not as sharp but falls almost linearly between 5 and 40 °C.

Alternating Poly(A-U). Although the shape of the CD spectrum for poly(A-U) suggests an A-form helix, there are differences between it and the normal RNA spectrum (Gray et al., 1972). Below the melting temperature, UV absorption is essentially constant while HMT incorporation and CD decrease somewhat with increasing temperature. Of the synthetic polymers studied, poly(A-U) has the highest rate of HMT reaction, with over 1000 times more HMT incorporated than poly(U). In DNA, it was also observed that the alternating purine-pyrimidine polymers had higher reactivities than the homopolymers (Dall'Acqua et al., 1979).

Double-Stranded Poly(A)·Poly(U) and Triple-Stranded Poly(A)·2Poly(U). When poly(A) and poly(U) are mixed in equal proportions in the range 5–100 mM NaCl, the CD spectrum indicates that the polymers adopt an A-form geometry. The CD spectra are more complex than normally associated with this geometry but contain many A-form features. In both 5 and 50 mM NaCl, HMT incorporation falls off at temperatures slightly below the optical melting temperatures.

Poly(A) and poly(U) are also capable of forming triple helices. Depending on the salt, temperature, and molar ratios, single-, double-, or triple-stranded complexes may be present in solution. We have examined conditions in which the double-stranded complex is the most stable over all molar ratios of A:U and conditions in which either the double- or triple-stranded complex can form, depending on the ratio in which the polymers are mixed. Although conditions exist in which only the triple helix is stable, very high salt concentration and temperature are necessary. This produces very low levels of HMT incorporation, and thus changes are not easily measured.

At 10 mM NaCl and 25 °C, only the double-stranded complex is stable. As expected, HMT incorporation increases linearly with poly(A) added (Figure 7). Once all of the poly(U) has been base paired to poly(A), the incorporation levels off. In conditions where both double- and triple-stranded

complexes can form (100 mM NaCl at 4 and 25 °C, 10 mM NaCl at 4 °C), HMT incorporation is not a simple function of poly(A) added. In all cases, the amount of HMT reacted is substantially lower with the triple helix than would have been predicted for a double helix. When sufficient poly(A) has been added to start formation of double-stranded molecules, the level of HMT incorporation rises dramatically.

At 10 mM NaCl and 4 °C, the incorporation curve can be approximated by two straight lines. If the line connecting the points up to a poly(A) to poly(U) molar ratio of 0.5 is extrapolated, it predicts that the plateau value for HMT incorporation would be one-half of what it actually turns out to be. One interpretation of this is that the strand of poly(U) that is involved in Watson-Crick base pairing reacts normally while the strand that is in the major groove running parallel to the poly(A) (Arnott & Bond, 1973) does not react to an appreciable extent. At 100 mM NaCl, the third strand could be so tightly bound that it inhibits reaction of both strands of poly(U). Alternatively, the structure of the strands that are involved in Watson-Crick base pairing might be perturbed sufficiently that the photoreactive bonds are not aligned correctly after intercalation occurs.

Evidence for reduced incorporation after triple-strand formation may also be seen in Figure 6. At 37 °C and a 1:1 ratio of polymers, double-stranded complexes form at all salt concentrations above 10 mM. At a 1:2 (A:U) ratio, the situation is more complex. At 1 and 5 mM NaCl, only single strands are present. Only single- and double-stranded molecules should be present from 10 to 30 mM NaCl. As expected, the lower amount of poly(A) leads to reduced incorporation of HMT (50–60% of the 1:1 ratio). Above 50 mM NaCl, both double strands and triple strands should be present. The amount of HMT incorporation relative to the 1:1 ratio drops. At 50 mM NaCl, the level of incorporation is 40% of the 1:1 mixture, 25% at 75 mM NaCl, and less than 15% at 100 mM NaCl and above.

The fact that the triple-stranded poly(A)·2poly(U) is much less reactive to HMT than the double-stranded complex is not surprising. X-ray diffraction studies have shown that the triple helix has virtually the same radius and pitch as the double helix (Arnott & Bond, 1973). It is thus much more condensed and difficult, if not impossible, to unwind. It seems probable that a marginally stable triple helix does allow some intercalation, possibly with the third strand looping out. Another possibility is that imperfections in the helix arise which allow double-stranded regions to exist.

In the tremendous variety of conditions used in these experiments, we never found the amount of HMT incorporated into poly(U) to be lower than that found in the random coil. This implies two types of reaction. The preferred mode of reaction is preceded by intercalation (Dall'Acqua et al., 1978a; Tjernelund et al., 1979). This allows π -bond stacking, which stabilizes the structure such that the reactive bonds of the HMT and uridine are optimally situated for photoreaction. This can lead to a high rate of incorporation as seen in poly(A)·poly(U) at low salt concentration and temperature. Clearly, this type of interaction cannot take place when poly(U) is in a random coil or a very tightly packed helix. It is unlikely that any type of overlap between the base and the HMT could occur in a tight helix, so the low level of reactivity must arise from either random encounters from the solution or some type of weak, outside binding. Analyzing the stereoisomers of the monoadducts produced with each compound should prove useful in determining the type of interactions taking place.

From this study, it is obvious that certain types of helical structure accelerate the reaction of HMT with RNA. In general, low salt concentration and low temperature favor HMT reaction. These conditions lead to a relatively weak helix, which can be easily unwound. When salt is added, it becomes difficult for the drug to intercalate between the more tightly packed base pairs. Magnesium has a particularly strong inhibitory effect. The fact that HMT reactivity is intimately linked to RNA structure is further exemplified by the same type of logarithmic dependence on salt concentration as is observed for melting temperature (Dove & Davidson, 1962). At higher temperatures, the thermal fluctuations in the RNA shorten the lifetime of the intercalation complex and thus lower the reaction rate. Temperature has a greater effect on the dissociation constant than on the association constant (Hyde & Hearst, 1978). In more complex systems, these generalities do not always hold true. For tRNA, an increase in HMT reactivity with increasing temperature was observed at one salt concentration (Bachelier & Hearst, 1982).

Changes in the rate of HMT incorporation could not be perfectly predicted by either CD or UV absorption transitions. These three probes of RNA conformation are influenced by different aspects of structure, so it is not surprising that they yield slightly different results. It is not trivial to extrapolate the structure-reactivity relationships found in synthetic polymers to the more complex RNAs of biological interest. Also, there are many structures found in natural RNAs that cannot be duplicated with synthetic polymers. Despite these complications, study of these model compounds does provide insight into what types of factors are likely to influence HMT reactivity. Combining the knowledge of what conformations are most reactive to HMT with the known base specificity (Bachelier & Hearst, 1982; Thompson et al., 1981; Youvan & Hearst, 1982) should allow workers to choose systems and conditions that are most likely to yield worthwhile results.

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