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Interaction of Metal Ions with Polynucleotides and Related Compounds. XV. Nuclear Magnetic Resonance Studies of the Binding of Copper(II) to Nucleotides and Polynucleotides*

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ABSTRACT: Proton magnetic resonance studies comparing the broadening effect of Cu(II) on the peaks of nucleotides and polyribonucleotides reveal that generally binding to monomer and polymer occurs preferentially at the same site. In poly(C), as in CMP, broadening of the H-5 resonance, but not the NH_2 proton peaks, indicates binding to N-3. Poly(U) and UMP, as well as uridine, also appear to bind Cu(II) at N-3; but the metal also exhibits an affinity for the hydroxyl groups of the ribose in uridine. Cu(II) broadens the H-2 and H-8 peaks of IMP simultaneously, while broadening H-8 in preference to H-2 in poly I; the former presumably involves chelation to N-7 and O-6, whereas in poly(I) the O-6 atom is occupied in H bonding, thus restricting binding to N-7. The unwinding of the poly(A)·poly(U) double helix by heating can be followed by proton magnetic resonance, since the broadened peaks of the double helix are sharpened on unwinding.

In the presence of Cu(II) the broadening effects of the paramagnetic ion on the separate polymer strands are noted as soon as unwinding is complete, providing further evidence to support the possibility of bridging of polynucleotide strands by metal ions.

Metal ions have profound effects on the structure and function of nucleic acids and mono- and polynucleotides. They alter the coding specificity of polynucleotides acting as templates for protein synthesis (Szer and Ochoa, 1964), and are required for stabilizing the structure of tRNA (Fresco *et al.*, 1966). Their role in unwinding and rewinding of the double helix (Eichhorn and Clark, 1965; Shin and Eichhorn, 1968) and in degradation of polynucleotides (Butzow and Eichhorn, 1965) has been demonstrated. It is therefore of interest to determine the loci of interaction of metal ions with these substances.

Previously, nuclear magnetic resonance has revealed some of the binding sites of metal ions on the mononucleotides (Cohn and Hughes, 1962; Sternlicht *et al.*, 1965; Eichhorn *et al.*, 1966). The nuclear magnetic resonance technique of water proton relaxation enhancement has been used to study the binding of metal ions to DNA (Eisinger *et al.*, 1962) and to polyribonucleotides and tRNA (Cohn *et al.*, 1969). To determine if metal ions bind to the heterocyclic bases of the polynucleotides and to identify the binding sites, we investigated the broadening effects of Cu(II) on the base proton resonances of the polymers as in the monomers.

This paper correlates the binding sites of Cu(II) on polynucleotides with those on the mononucleotides. Frequently the binding sites are the same in monomer and polymer; an interesting exception is the difference in binding observed for IMP and poly(I).

Because of the fact that proton magnetic resonance data can pinpoint reaction sites on the nucleotide bases, this technique is used to study the unwinding of the base-paired complex poly(A)·poly(U) in the presence of Cu(II) ions.

Methods

Uridine and 5′-UMP were from Sigma Chemical Co., 5′-IMP was from P-L Biochemicals, and homopolyribonucleotides were from Miles Laboratories. Nucleosides were dissolved directly in Me_2SO-d_6 and nucleotides in D_2O. Polynucleotides were dissolved in D_2O by gentle rotary shaking at 4°. Aqueous solutions were adjusted to pH 7.5 and molarity was determined as in the preceding paper. The base-paired complex poly(A)·poly(U) was prepared both by dissolving each homopolymer separately and then mixing or by dissolving them together. Similar spectra were obtained for complexes prepared by either method.

Nuclear magnetic resonance spectra were obtained with the Varian spectrometer, A60D, using the same calibrations and techniques indicated in the pre-

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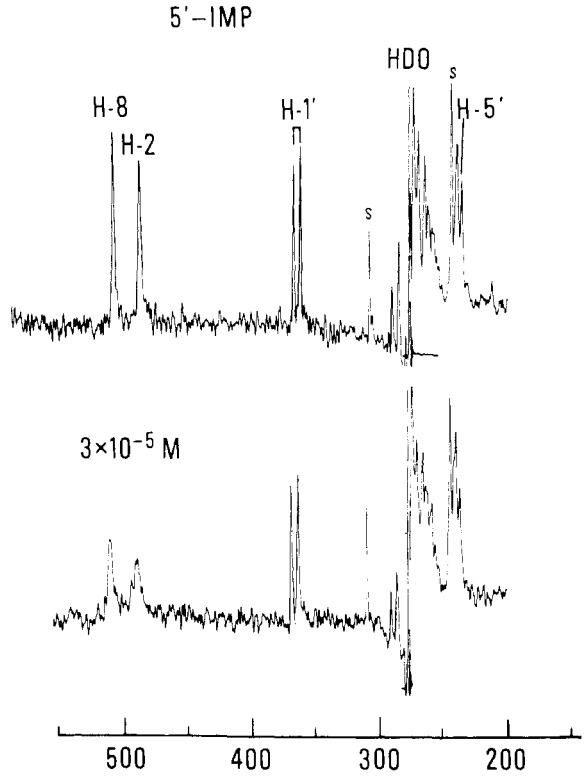

FIGURE 1: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.1 M 5'-IMP (pH 7.5) in D_2O. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Spinning side bands of water are labeled s. The other peaks on either side of the water are the ribose H-2', -3', and -4'. Abscissa is in cycles per second downfield from DSS as the internal standard.

vious paper. The technique of incremental additions of Cu(II) to the same solution was again used to study progressive paramagnetic ion induced broadening at 40°. The spectra in Figure 5 were obtained with the Varian HR 220-MHz nuclear magnetic resonance spectrometer at the normal operating probe temperature of 21°. When the same solution of nucleotide was heated repeatedly following each increment of Cu(II), it was difficult to distinguish between metal-induced broadening and deuterium replacement of some base protons. Therefore, in any experiment requiring heating, individual solutions of polynucleotide were used for each concentration of copper studied.

Results

Chemical Shifts of the Ligands. The nuclear magnetic resonance spectra of the polynucleotides resemble those of the mononucleotides. Characteristic narrow proton line widths were obtained for the protons of the heterocyclic base and the H-1' ribose of the polymers. Other ribose protons could not be as clearly identified in these polymers as they were in the monomers. The chemical shifts of the nucleoside, nucleotides, and polynucleotides observed are presented in Table I. The shifts of 5'-AMP and poly(A) from the previous paper are listed for comparison with the other polynucleotides.

With the exception of UMP and poly(U), peak assignments of the mono- and polynucleotides are those established previously (Jardetzky, 1960, 1964; Jardetzky and Jardetzky, 1960), and are in agreement with the general area assignment for the polynucleotides (McDonald *et al.*, 1964; McTague *et al.*, 1964).[1] Heating in D_2O resulted in deuterium replacement of the most downfield peaks of poly(A) and poly(I), confirming their assignment as H-8 in the polymers as previously demonstrated in the monomers by Bullock and Jardetzky (1964). Assignments for uridine in Me_2SO are those of Gatlin and Davis (1962). The H-5 doublet and the H-1' doublet are clearly separated in solutions of uridine in Me_2SO, but are overlapped in aqueous solutions of the nucleotide. With the finer resolution available at 100 MHz, Bangartner and Chan (1968) clearly separated and identified the upfield doublet as the H-5 resonance and the low-field doublet as the H-1' of poly(U). At 60 MHz the upfield half of the H-5 doublet of UMP (Figure 4) and poly(U) (Figure 6) can be identified; however, the low-field half is obscured by overlapping with the H-1'. The chemical shift and paramagnetic broadening of the H-5 resonance of UMP and poly(U) were determined from the upfield half of the doublet. Clear resolution of the overlapped peaks and confirmation of the specific broadening of the H-5 resonance of UMP was obtained by Dr. E. D. Becker and Mr. R. B. Bradley who examined our samples at 220 MHz (Figure 5).

The upfield polymerization shift, designated by Jardetzky (1964), was observed between the nucleotides and the corresponding homopolynucleotides. A much greater shift was observed from AMP to poly(A) than from UMP to poly(U) or from IMP to poly(I). Analysis of the polymerization shift

TABLE I: Proton Chemical Shifts (60 MHz).

		Pyrimidine[a]			
	Concn (M)	H-3	H-6	H-5	H-1'
Nucleoside in Me_2SO-d_6 at 37–40°					
Uridine	0.1	674	473	338	347
			Purine[b]		
			H-8	H-2	H-1'
Nucleotides in D_2O at 37–40°, pH 7.5					
5'-AMP	0.1		514	491	367
Poly(A)	0.038		478	469	340
	0.067				
5'-IMP	0.1		512	492	367
Poly(I)	.04		496	485	357
			Pyrimidine[b]		
			H-6	H-5	H-1'
5'-UMP	0.1		489	357	362
Poly(U)	0.1		472	352	357
Poly(C)	0.09		471	353	343

[a] Cycles per second using Me_4Si as the internal standard. [b] Cycles per second using dimethylsilapentanesulfonate (DSS) as the internal standard.

[1] In this paper, the purines and pyrimidines are numbered according to the IUPAC system now in general use. For comparison to an earlier paper in this series (Eichhorn *et al.*, 1966) it should be pointed out that the pyrimidine ring was numbered in the same manner as the six-membered ring of the purine. To convert the numbers in the preceding paper into the IUPAC system, the 1-pyrimidine position becomes the 3 position, the 4 becomes 6, and the 6 becomes 4. The numbering of the purines is identical in both systems.

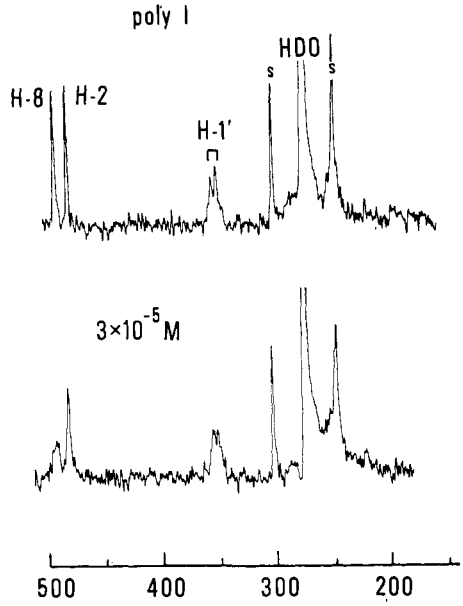

FIGURE 2: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.04 M poly(I) (pH 7.5) in D_2O. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Spinning side bands are labeled s. Abscissa is in cycles per second downfield from DSS as the internal standard.

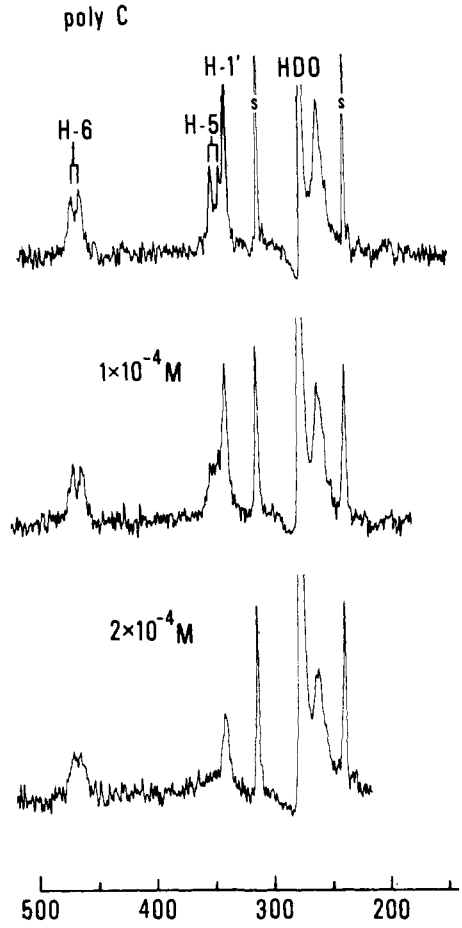

FIGURE 3: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.09 M poly(C) (pH 7.5) in D_2O. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Spinning side bands of water are labeled s. The peak between water and the high field side band is due to ribose protons. Abscissa is in cycles per second downfield from DSS as the internal standard.

from 5′-AMP to poly(A) indicated that the shift of the H-2 resonance was accounted for by base stacking, whereas the larger H-8 shift was due to both the increase in base stacking and the removal of the deshielding influence of the 5′-phosphate (Berger and Eichhorn, 1971). When comparing the 5′-purine nucleotide to the polynucleotide, the shift of the H-2 resonance provides the most direct assesment of stacking in the polymer. The major deshielding effect of the 5′-phosphate has been localized to the H-6 resonance of the pyrimidine nucleotides (Schweizer *et al.*, 1968). Like the purine H-2 resonance, the polymerization shift of the H-5 pyrimidine resonance, which only exhibits a small deshielding effect from the phosphate, is the best indicator of stacking in the pyrimidine polynucleotides.

Subtraction of the upfield polymerization shift of the poly(U) H-5 resonance from the shift of the H-6 resonance leaves 12 cps which is presumably due to removal of the phosphate deshielding effect from the H-6. This is similar to the 13-cps shift produced by removing the 5′-phosphate deshielding effect from the H-8 resonance of poly(A). Comparison of the 22-cps upfield shift of the H-2 resonance of poly(A) to the 5-cps upfield shift of the H-5 resonance of poly(U) demonstrates the much greater stacking tendency of the bases in poly(A) than of those in poly(U). This is in agreement with the random coil structure of poly(U) at room temperature (Richards *et al.*, 1963; Michelson and Monny, 1966). Inosine has been demonstrated to have less of a stacking tendency than adenosine (Broom *et al.*, 1967; Jardetzky, 1964). This is reflected in the small upfield polymerization shift of 4 cps for the H-2 resonance from 5′-IMP to poly(I), which seems to indicate a random coil structure in poly(I) as in poly(U). However, poly(I) was the most viscous of all polymer solutions examined in this study, indicating the presence of a highly ordered structure. This presents a paradoxical situation in which the slight upfield shift of the base proton resonance is insufficient to account for this apparent degree of order by base stacking alone.

Line Broadening by Copper(II). Incremental addition of Cu(II) to 5′-IMP (Figure 1) broadened the H-8 and H-2 resonances simultaneously, indicating binding of Cu(II) near both protons. This could be explained by chelation to N-7 and O-6 as suggested by Tu and Friederich (1968); an alternate explanation is binding of copper to nitrogen atoms on both rings, and finally a combination of these effects could account for the observed simultaneous broadening. In poly(I), (Figure 2) Cu(II) produced broadening of the H-8 proton resonance in advance of the H-2. This effect is similar to that found in poly(A) and demonstrates coordination of Cu(II) to N-7 in poly(I) as in poly(A). The changes that occur in converting 5′-IMP into poly(I) apparently alter the affinity for Cu(II) so that binding is preferentially to N-7 in the polymer.

On examining Cu(II) binding to the pyrimidine homopolymers, the nuclear magnetic resonance spectra demonstrate that the H-5 resonance of poly(C) is preferentially broadened (Figure 3) just as previously demonstrated with 5′-CMP and 5′-dCMP (Eichhorn, *et al.*, 1966). This indicates that coordination of Cu(II) to poly(C) is at the N-3 position, as in the monomer. Addition of Cu(II) to 5′-UMP (Figures 4 and 5) and poly(U) (Figure 6) also produced an effect on the H-5 resonance indicating the binding of Cu(II) near N-3 in 5′-UMP and poly(U) as in 5′-CMP and poly(C).

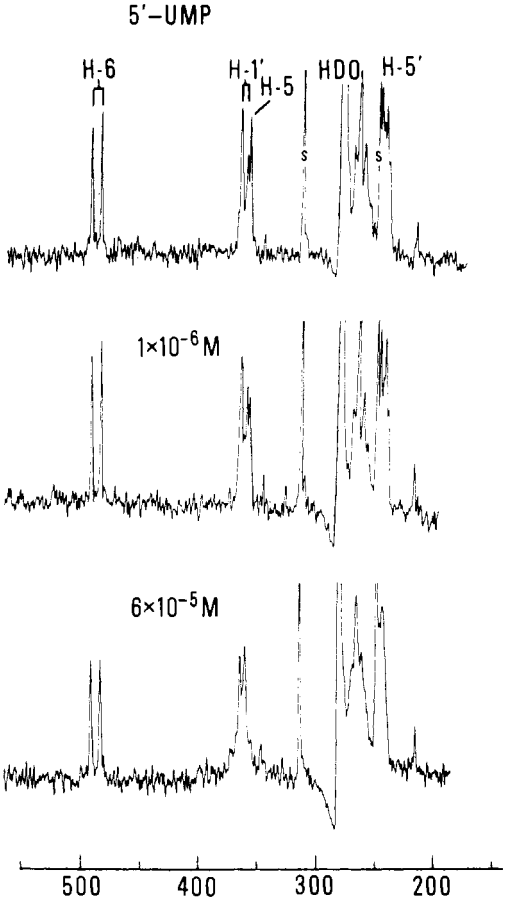

FIGURE 4: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.1 M 5'-UMP (pH 7.5) in D_2O. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Spinning side bands of water are labeled s. The peaks between water and the high field side band are due to ribose protons. Abscissa is in cycles per second downfield from DSS as the internal standard.

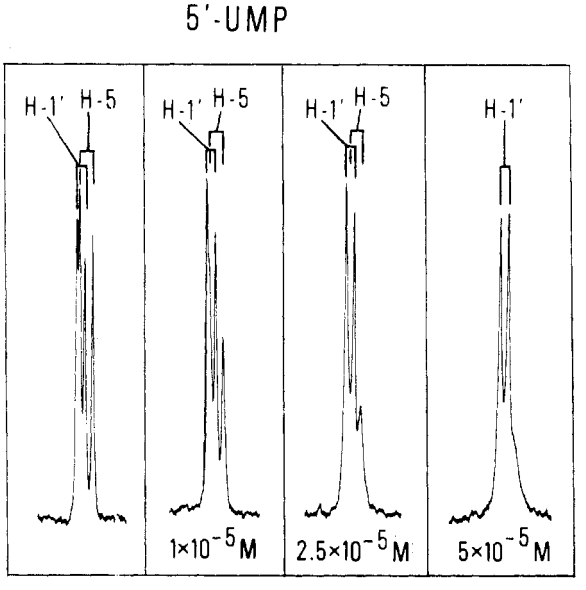

FIGURE 5: Effect of Cu(II) on the overlapped H-1', H-5 area of the proton magnetic resonance spectra at 220 MHz of 0.1 M 5'-UMP (pH 7.5) in D_2O. The spectrum at the left is the metal-free solution; the Cu(II) concentration is indicated beneath the others.

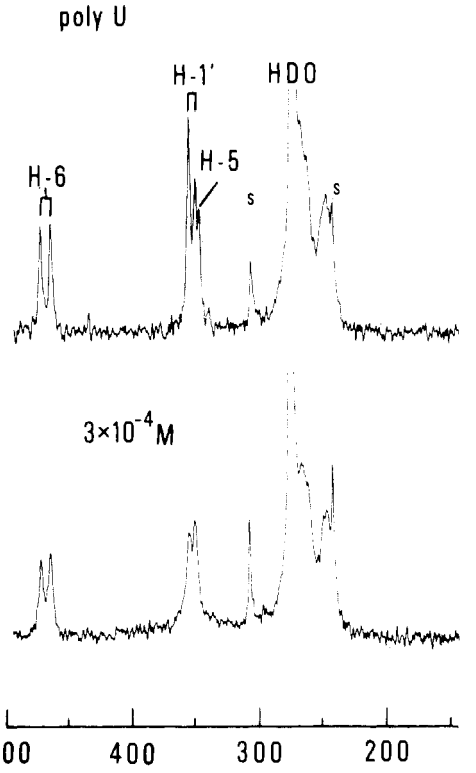

FIGURE 6: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.1 M poly(U) (pH 7.5) in D_2O. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others, labeled as in Figure 4. Abscissa is in cycles per second downfield from DSS as the internal standard.

The question arises as to whether base broadening, specifically, the 5-pyrimidine proton resonance, could be caused by the proximity of the Cu(II) bound to the 5'-phosphate. Prestegard and Chan (1969) showed that alteration of solvent structure, binding of magnesium(II) ions to phosphate, and protonation of phosphate all produce profound effects at the H-6 resonance of UMP. In contrast, the Cu(II)-induced effects occur at the H-5 resonance with only a negligible effect at the H-6, suggesting that the observed broadening is unrelated to phosphate binding.

Binding of Cu(II) to uridine (Figure 7) provides an assessment of metal binding in the absence of phosphate. Since uridine is protonated at the N-3 position, the opportunity to observe the hypothetical base binding site directly by nuclear magnetic resonance is also provided. Copper added to uridine in Me_2SO-d_6 produces broadening of the H-5, as in the nucleotide. As the concentration of Cu(II) is increased, the ribose hydroxyl protons and residual water proton resonances are drastically broadened, while the H-3 and H-5 resonances are also altered. As the hydroxyl and water protons are completely broadened, continuing increments of Cu(II) produce more apparent broadening of both the H-3 and H-5 protons.

This marked broadening in Me_2SO-d_6 indicates that Cu(II) binds to the ribose hydroxyls of uridine, and as these sites are being occupied, binding is also apparent on the uracil ring in the vicinity of N-3 and C-5. Observation of H-3 and H-5 broadening in the nucleoside clearly demonstrates the occurrence of base binding in the absence of phosphate. Similar broadening of the H-5 resonance in the mono- and polynucleotide demonstrates that in uridine and UMP, as well as in poly(U), Cu(II) is coordinated to the base in a similar fashion.

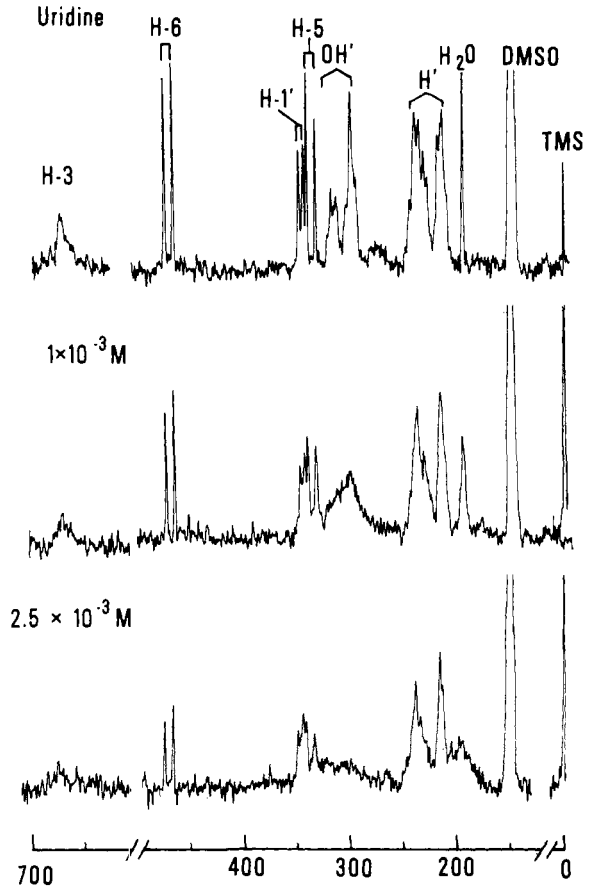

FIGURE 7: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.1 M uridine–Me_2SO-d_6. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Protons on the base and ribose H-1′ are specifically labeled. The ribose hydroxyl protons are designated collectively as OH′ and the remaining ribeos protons as H′. Abscissa is in cycles per second downfield from Me_4Si (TMS) as the internal standard.

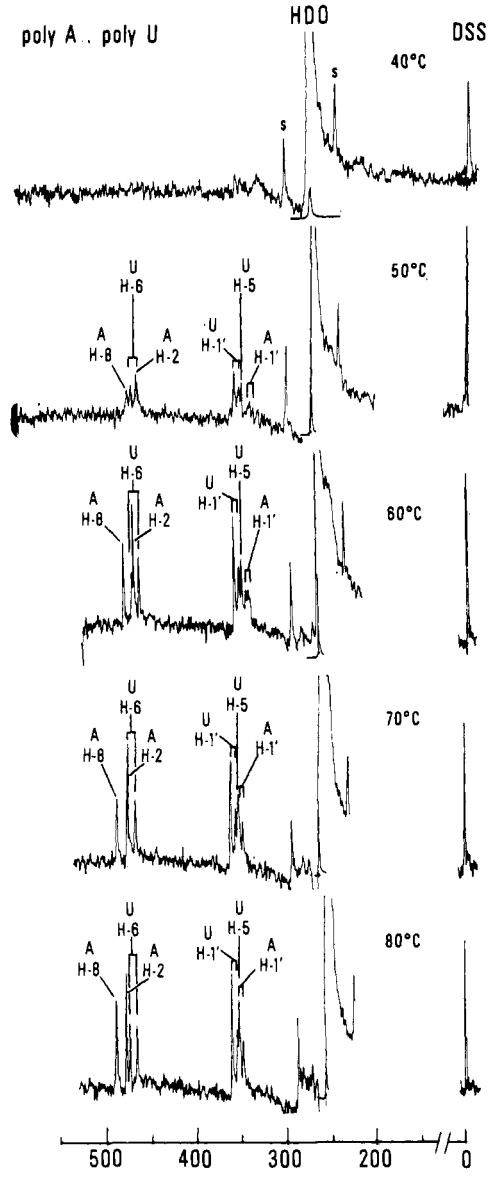

FIGURE 8: Temperature dependence of proton magnetic resonance spectra (60 MHz), poly(A)·poly(U), 0.05 M each in D_2O (pH 7.5). All spectra obtained in the absence of metal. Spinning side bands of water are labeled s. Temperature is indicated for each spectrum and the poly(A) protons (AH-8, AH-2, AH-1′) and poly(U) protons (UH-6, UH-5, and UH-1′) are indicated at each temperature. Abscissa is in cycles per second downfield from DSS as the internal standard.

Interaction of Poly(A)·Poly(U) with Copper(II). The nuclear magnetic resonance spectrum produced by an equimolar mixture of 5′-AMP and 5′-UMP in aqueous solution represents the combined spectrum of each individual nucleotide component. The lack of either broadening or position shift demonstrates that no change in stacking or hydrogen bonding occurs.

In contrast to the mononucleotides, an equimolar mixture of poly(A) and poly(U) produces a nuclear magnetic resonance spectrum broadened beyond resolution (Figure 8). The total broadening of the characteristic polynucleotide resonances indicates the formation of the highly ordered, base-paired, double helix and the restriction of the mobility necessary to observe proton resonance (McDonald *et al.*, 1964; McTague *et al.*, 1964).

Induction of strand dissociation by increasing temperature reveals peaks which are still broadened but clearly identifiable at 50°, and at 60° they have attained their characteristic narrow line width. Thus, heating from 40 to 50° produces partial separation of poly(A)·poly(U) and complete melting of the double helix at 60°. Heating to higher temperatures produces a continuous downfield shift of poly(A) resonances but no shift of poly(U) resonances occurs (Figure 9). The constancy of poly(U) resonances on continued heating indicates that poly(U) acquires its characteristic random coil conformation as soon as it is released from the base-paired double helix. In contrast the downfield shift of poly(A) resonances are similar to those produced when the polymer is heated in solution alone (Berger and Eichhorn, 1971). This continuous downfield shift of poly(A) resonances demonstrates that it is released from the base-paired double helix as a partially ordered structure and continues to undergo transition to a random coil until approximately 90°.

Addition of Cu(II) to an equimolar mixture of 5′-AMP and 5′-UMP produces a slight broadening of the uracil H-5 resonance and a drastic broadening of the adenine H-8 resonance followed by H-2 broadening. This demonstrates the binding of Cu(II) to each of the nucleotide bases in this mixture, but preferentially to AMP. Addition of Cu(II) to the poly(A)·poly(U) helix does not affect the nuclear magnetic resonance spectrum at room temperature or the chemical shift during

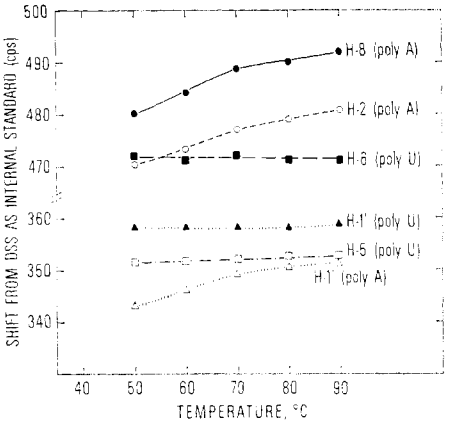

FIGURE 9: Temperature dependence of proton magnetic resonance shifts (60 MHz), poly(A)·poly(U), 0.05 M each, in D_2O (pH 7.5). Shifts are downfield from DSS as the internal standard.

heating. As melting occurs in the presence of Cu(II) (Figure 10) the poly(U) H-5 resonance is slightly broadened, the poly(A) H-2 broadened more, and the poly(A) H-8 broadened most. Melting in the presence of increasing concentrations of Cu(II) produces greater broadening of the resonances of poly(A) than poly(U), indicating that the metal is predominantly associated with poly(A) when the double strand is unwound.

Discussion

Paramagnetic metal ion induced broadening of characteristic proton resonances has been used to locate binding sites of Cu(II) on nucleotides. Cohn *et al.* (1969) demonstrated binding of paramagnetic metal ions to polynucleotide phosphates. To determine if copper also binds directly to the base, we performed direct observation of base proton resonance broadening in the polynucleotides as in the mononucleotides. The observed paramagnetic metal ion induced broadening of the polymer base protons clearly establishes that the polynucleotide bases bind copper and localizes the binding sites.

Copper(II) Binding to IMP and Poly(I). Cu(II) broadens the H-8 and H-2 resonance of 5'-IMP at a concentration of metal approximately ten times less than it does in 5'-AMP. Inosine has been demonstrated to have the least stacking tendency of 14 substituted purine nucleosides (Broom *et al.*, 1967). This low degree of association makes both rings readily available to metal ions and Cu(II) binding could occur at either the N-1 or N-3 atoms on the six-membered ring or the N-7 atom on the five-membered ring. Perhaps more important, the oxygen substituent at the 6-purine position and the N-7 form a potential chelating group which would make a stronger complex with copper than simple coordination to a nitrogen atom. Since this chelate structure involves both the six-membered and five-membered rings, it might produce broadening of the proton resonances of each. The combination of the chelate structure and the exposure of all potential ligand atoms account for the simultaneous broadening of the H-8 and H-2 resonance of IMP and its occurrence at low concentrations of copper.

Solutions of poly(I) contained highly ordered structures which could not be accounted for by base stacking alone. This is in agreement with the proposal that poly(I) exists as a triple helix in which hydrogen bonding links the N-1 of each

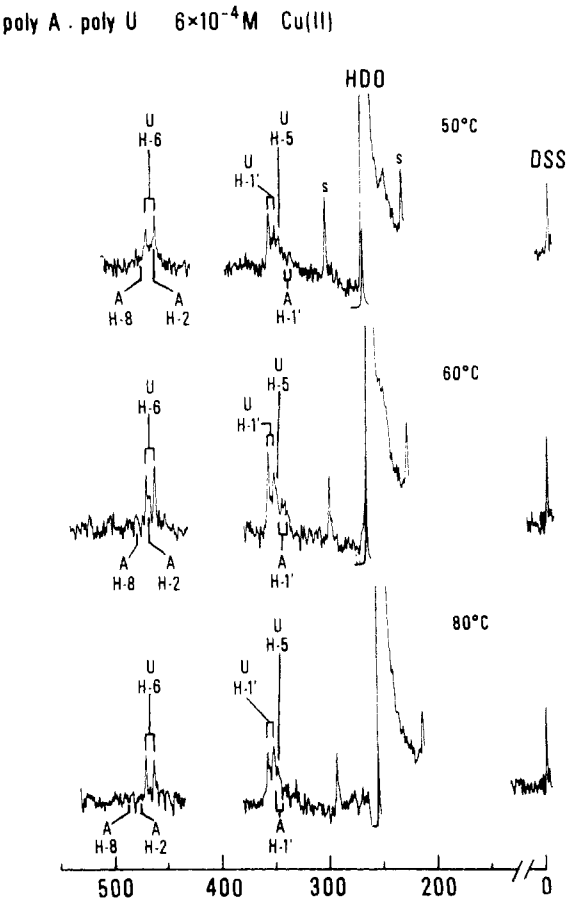

FIGURE 10: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz), poly(A)·poly(U), 0.05 M each, in D_2O (pH 7.5). Cu(II) concentration constant during temperature variation, labeled as in Figure 8. Broadened poly(A) peaks are designated at their location in the absence of copper. Abscissa is in cycles per second downfield from DSS as the internal standard.

base to the oxygen at the 6 position of the next base (Rich, 1958). In this configuration the N-7 atom, which is not involved in hydrogen bonding, is sterically the most accessible potential binding atom on the inosine base and therefore the preferential site for Cu(II) coordination.

Copper(II) Binding to CMP and Poly(C). The pyrimidine ring has only two available nitrogen atoms to act as ligands for copper, and one is eliminated by involvement in the glycosidic linkage to ribose. Stacking interactions are weaker in the pyrimidines than in the purines (Ts'o *et al.*, 1963; Schweizer *et al.*, 1965) and probably have little or no effect on the one remaining potential pyrimidine binding site.

Crystallographic studies have demonstrated that copper coordinates to the N-3 atom of cytosine (Carrabine and Sundaralingam, 1968), indicating that in the base alone N-3 is the preferred binding site. Formation of the glycosidic bond at the N-1 position of the base completely eliminates the possibility of its binding copper. The 6-amino group of adenine has little potential to bind copper (Schneider *et al.*, 1964) and similar behavior can be anticipated for the amino group at the 4-pyrimidine position in cytidine. During the Cu(II)-induced broadening of the H-5 resonance of the cytidine nucleoside the 4-amino group retains its characteristic resonance demonstrating that the amino group is not involved in base binding of Cu(II) (Eichhorn *et al.*, 1966). The N-3 atom, which is the preferred ligand in the base alone, has a

negative charge density similar to the densities present on the adenine ring nitrogens (Jordan and Pullman, 1968; Pullman, 1968, 1969). Only negligible variation in the N-3 charge is produced on conversion from cytosine into cytidine and then the N-3 is presumably the preferred binding site on the pyrimidine ring of CMP and poly(C), although binding to oxygen is not ruled out. Broadening of the H-5 resonance before the H-6 resonance in CMP and poly(C) is in line with binding at the N-3 atom, rather than at the oxygen.

Copper(II) Binding to UMP and Poly(U). Copper is coordinated to the pyrimidine base of uridine, UMP, and poly(U) in a similar fashion. Previous spectrophotometric and potentiometric studies were unable to detect any interaction of Cu(II) with uridine or UMP (Fiskin and Beer, 1965; Tu and Friederich, 1968). When Me_2SO is used as solvent to observe uridine by nuclear magnetic resonance, the protons on the ribose hydroxyls are readily apparent. They cannot be seen in D_2O where they exchange rapidly with the solvent. Copper broadens the ribose hydroxyl protons of uridine while the H-6 and H-1 proton resonances are unaffected. This is in contrast to the effects of copper on the spectrum of adenosine in Me_2SO where base broadening occurred while all ribose peaks retained their narrow line width (Berger and Eichhorn, 1971). The protons at N-3 and C-5 atoms of the uridine base also broaden well in advance of those at the C-6 and H-1′ positions. This selective broadening indicates that in uridine, Cu(II) coordinates to ribose hydroxyls but that base interaction also occurs. This weak base interaction which is not apparent by conductometric or spectrophotometric techniques is well demonstrated by the paramagnetic induced broadening of the proton magnetic resonance of uridine, UMP, and poly(U).

The negative charge density at the N-3 atom of uracil is three times less than the charge at either the N-3 of cytidine or the ring nitrogens of adenine (Pullman, 1968, 1969). This weakly negative N-3 of uridine may account for the weak interaction of copper with this ligand. It is clear, however, that copper does react with uridine in the vicinity of N-3 and C-5. Because of the comparatively low negative charge at the N-3 and the location of binding being somewhere near N-3 and C-5, the possibility of binding to the oxygen at C-4 must also be considered. The similarity of broadening and the concentrations required to produce it in the polymer and monomer indicate that coordination of Cu(II) to poly(U), where stacking is negligible, occurs exactly as in UMP.

Copper(II) and the Unwinding of Poly(A)·Poly(U). When the poly(A)·poly(U) complex was heated from 40°, where the spectrum is flat, to 50°, the emergence of broad resonance peaks at chemical shifts characteristic of the individual polymers demonstrates partial separation of the strands. Complete strand separation is indicated by the narrow line widths attained at 60°. The addition of copper(II) ions to this complex did not affect the nuclear magnetic resonance spectrum at 40°. Copper lowers the T_m of double helices in solution when present at a stochiometric ratio of metal to nucleotide (Eichhorn and Clark, 1965). At that ratio the nuclear magnetic resonance spectrum is completely broadened by the paramagnetic effect of Cu(II), whereas the binding sites are determined at low ratios of copper to nucleotide in the range of 10^{-4}–10^{-3}. At these low concentrations of Cu(II) the observed melting range and shifts are the same as in the absence of metal ions. As the Cu(II) concentration is increased, and the strands are dissociated by heating, the poly(A) resonances are broadened in preference to the poly(U) resonance with a slight tendency for the poly(A) H-8 to broaden before the H-2. The H-5 resonance of poly(U) was also broadened. Although the resonance signals of the double helix are broadened beyond resolution, it is clearly demonstrated that copper can be found coordinated to preferential nucleotide base sites as soon as they emerge from the double helix. These experiments indicate that the ability of Cu(II) to lower the T_m of double helices is, as expected, due to Cu(II) coordinated to electron donor sites on the bases. They also indicate that bridges formed by copper between the strands of unwound polynucleotide involve copper binding to bases, as had been anticipated from the ability of copper to reversibly rewind single-stranded polynucleotides into multiple helices (Eichhorn and Tarien, 1967).

Acknowledgments

We thank Dr. E. D. Becker for helpful discussions and also Mr. R. B. Bradley for the 220-MHz spectra in Figure 5.

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Characterization of Mitochondrial and Nuclear Satellite Deoxyribonucleic Acids of Five Species of Crustacea*

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ABSTRACT: DNAs localized in nuclei purified from ovaries or midgut glands of five different crustacean species have been characterized. In addition to the main band, three or four species of crab had the poly[d(A-T)] satellite while the fourth had a (dA + dT)-rich satellite with the same buoyant density in CsCl but with other physical characteristics differing from poly[d(A-T)]. The lobster and two of the crabs had distinct (dG + dC)-rich satellites. DNA isolated from the mitochondria of each of the five species has a density of 1.688 g/cm^3, and mitochondrial DNA from at least one species separates into two distinct bands in alkaline CsCl gradients. The reassociation characteristics of mitochondrial DNA from all species are similar to those of vertebrate mitochondrial DNAs.

Satellite DNAs have been described in many species of plants and animals. The poly[d(A-T)][1] DNA found in various crustacea is one of the most remarkable of these in that more than 90% of it is alternating adenylate and thymidylate residues (Swartz *et al.*, 1962; Skinner, 1967). Among the true crabs, this satellite is more widespread than was originally suspected; various publications describe its isolation from a total of at least seven species of *Cancer* (Sueoka, 1961; Smith, 1963, 1964; Pochon *et al.*, 1966) and two other crab genera (Skinner, 1967; Skinner *et al.*, 1970).

To our knowledge poly[d(A-T)] has not been described in any group of animals other than the crustaceans, although the (dA + dT)-rich satellite DNA of *Drosophila melanogaster* (Fansler *et al.*, 1970) and the mtDNA of a strain of petite yeast (Bernardi *et al.*, 1968, 1970) have a number of physical properties similar to those of poly[d(A-T)]. In contrast to the mitochondrial localization of the yeast d(A-T)-like satellite, crab poly[d(A-T)] has been reported to be localized in the nuclei of testicular cells of *Cancer productus* (Astell *et al.*, 1969). Since the mitochondria of crab spermatocytes either degenerate or are incorporated into the nuclear membrane during maturation (Langreth, 1969), it was possible that DNA from nuclei of such cells might contain mtDNA as well. It seemed desirable to determine the subcellular localization of the satellite in tissues containing large numbers of intact mitochondria so that nuclei and mitochondria could be separated and their DNAs compared.

To that end, we have developed methods for the purification of crustacean nuclei and mitochondria and have characterized the DNAs from these organelles isolated from midgut glands (hepatopancreas) and ovaries of five crustacean species. Three of them have the poly[d(A-T)] satellite, and in each case it is localized in the nucleus, confirming the conclusion of Astell *et al.* (1969). In addition we find the (dA + dT)-rich satellite of *Callinectes* as well as three (dG + dC)-rich satellites from several other animal species are also localized in the nucleus. By various manipulations, we can enrich the amount of satellite in relation to main-band DNA in a manner which suggests that the ratio of satellite to main band is not the same for all nuclei. The mitochondria of all five crustacean species contain a DNA of ρ = 1.688 g/cm^3. In at least one species mtDNA separates into two distinct bands in alkali. Its reassociation characteristics are similar to those of the mtDNAs of rat liver (Leffler *et al.*, 1969, 1970) and several amphibia as well as chicken and yeast (Dawid and Wolstenholme, 1967, 1968).

Materials and Methods

Animals. Specimens of the Bermuda land crab, *Gecarcinus lateralis*, were obtained and maintained in the laboratory as previously described (Skinner, 1962). The marine crabs—

* From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. *Received November 13, 1970.* Research sponsored by the U. S. Atomic Energy Commission under contract with the Union Carbide Corp. M. S. K. was a University of Tennessee Postdoctoral Trainee, National Institute of Child Health and Human Development (Grant No. 1T01HD00296-01). The Oak Ridge National Laboratory is operated by Union Carbide Corp. for the U. S. Atomic Energy Commission.

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[1] Abbreviations used are: poly[d(A-T)], naturally occurring satellite DNA composed of more than 90% alternating adenylate and thymidylate residues and some guanylate and cytidylate residues. Some thymidylate and adenylate residues are also present in nonalternating sequences (Skinner, 1967). mtDNA, mitochondrial DNA; SSC/10, 0.01 M NaCl–0.0015 M sodium citrate (pH 7).