Interactions of Metal Ions with Polynucleotides and Related Compounds. VII. The Binding of Copper(II) to Nucleosides, Nucleotides, and Deoxyribonucleic Acids*

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ABSTRACT: The ultraviolet spectrum of native deoxyribonucleic acid (DNA) is unaffected by copper(II) ions, but the spectrum of denatured DNA is shifted and enhanced, in line with previous studies indicating that copper reacts with the bases in DNA. Increasing concentrations of copper bring about increased spectral intensity up to a ratio of between 1 and 2 copper ions/nucleotide base. The presence of any of the four nucleosides has no effect on the potentiometric titration of copper, indicating that binding of copper to the bases proceeds without removal of protons. The presence of adenosine does, however, eliminate the hysteresis effect in the titration of copper, and the binding of the nucleoside to copper is further indicated by the ability of the latter to shift the visible absorption of copper to lower wavelengths. Nuclear magnetic resonance studies on the deoxyribonucleotides show that copper(II) broadens the H^2 and H^8 peaks in deoxyadenosine monophosphate, H^4 and H^5 peaks of deoxycytidine monophosphate, and the H^8 peak of deoxyguanosine monophosphate; deoxythymidine monophosphate is unaffected. Nuclear magnetic resonance studies in dimethyl sulfoxide reveal further that the protons on the amino groups and the heterocyclic nitrogen atoms are not broadened by copper ions. It is concluded that copper binds to the N_7 positions of adenosine and guanosine and to N_1 of cytidine, but does not bind thymidine. The phosphate resonance in deoxyadenosine monophosphate and deoxythymidine monophosphate is broadened by copper ions, showing that copper also binds phosphate, which is also demonstrated by potentiometric titration of copper adenosine monophosphate and copper DNA solutions. The ability of copper ions to cleave the phosphodiester bonds in polynucleotides as well as to bring about the denaturation of DNA is thus explained by the demonstration that copper can bind to both the phosphate and base moieties.

Recent investigations have shown that copper(II) ions have very remarkable and yet very different effects upon the polyribonucleotides and upon deoxyribonucleic acid (DNA). The polyribonucleotides, when heated with copper ions, are degraded into small oligonucleotides by the cleavage of phosphate bonds (Butzow and Eichhorn, 1965). In this behavior copper does not differ from many other divalent and trivalent ions. When DNA, however, is heated with copper(II), the phosphodiester linkages are unaffected because the degradation reaction requires chelation to phosphate and the 2′-hydroxy group, and the latter is not present in DNA (Bamann *et al.*, 1954; Eichhorn and Butzow, 1965). Instead, the T_m of DNA is drastically lowered by the presence of the copper ions at low ionic strength, indicating that copper ions influence the severing of the bonds between the double helix (Eichhorn, 1962; Eichhorn and Clark, 1965). Native DNA can apparently be regenerated by the addition of electrolyte to the copper-denatured DNA (Eichhorn and Clark, 1965; Hiai, 1965).

The effect of copper on DNA leads logically to the interpretation that copper(II) must bind to electron donor groups on the nucleoside bases. The effect of copper on the polyribonucleotides, on the other hand, similarly appears to implicate binding of the phosphate moieties. It is obvious that both of these results can be explained only if copper(II) can bind both to the nucleotide bases and to the phosphate group. There have been various copper-binding studies in the past that have implicated either the bases or phosphate groups (Harkins and Freiser, 1958; Albert, 1953; Frieden and Alles, 1957; Zubay and Doty, 1958; Schneider and Brintzinger, 1964; Moll *et al.*, 1964; Brintzinger, 1963). Several studies have indicated that both groups are involved (Cohn and Hughes, 1962; Eisinger *et al.*, 1962). These investigations have generally been conducted with one nucleoside or nucleotide. Fiskin and Beer (1965) have just communicated a potentiometric study of the interaction of copper(II) with the four nucleoside bases. Because copper(II) ions are capable of producing such interesting and drastic changes in

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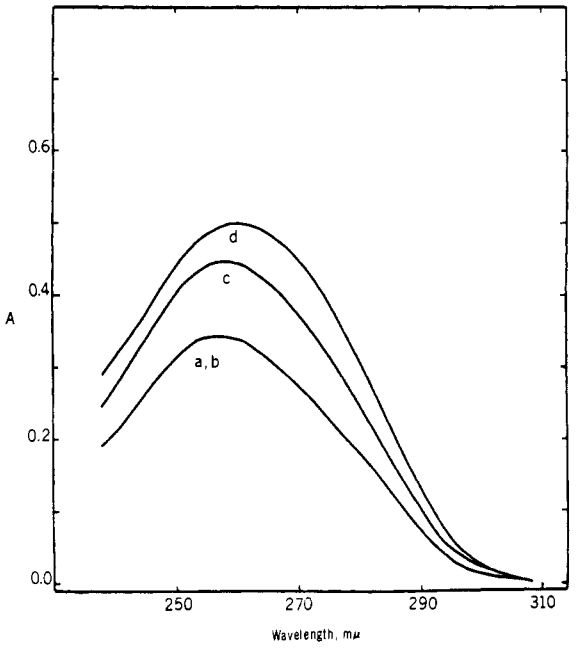


FIGURE 1: Effect of copper(II) on the spectrum of DNA. All solutions contain 5×10^{-5} M (P) DNA and 5×10^{-3} M sodium nitrate. (a) Native DNA; (b) 10^{-4} M copper-(II) nitrate added to native DNA; (c) DNA heated 5 min at 95° and quenched; (d) 10^{-4} M copper(II) nitrate added to DNA treated as in (c).

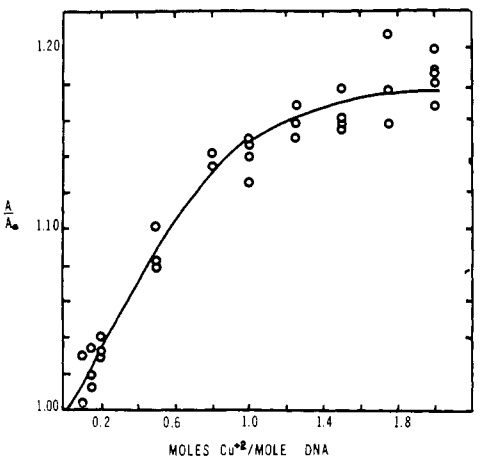


FIGURE 2: Spectrophotometric titration of DNA denatured as in Figure 1c with copper(II) nitrate. Solutions contain 5×10^{-5} M (P) DNA, 5×10^{-3} M sodium nitrate, and copper(II) nitrate as shown in abscissa. A_0 is absorbance at 262 mμ of the denatured DNA solution, and A is the absorbance of the solution after addition of the appropriate increment of $Cu(NO_3)_2$.

nucleic acid structure, it is of some importance to confirm the ability of copper(II) to interact with both phosphate and nucleoside bases, and particularly to determine to which nucleosides and nucleotides the copper ions become attached. At the same time it is desirable to localize the sites of attachment as much as possible. The objective of the present study has been to carry out systematic studies toward these ends, and, by a correlation of these studies with those in the literature, to come to an understanding of the copper–nucleotide interactions.

Reaction of Copper with the Bases. SPECTROPHOTOMETRIC AND POTENTIOMETRIC INVESTIGATIONS. Since the ultraviolet spectrum of DNA results from transitions of the nucleoside bases, it should be expected to be affected by the presence of copper ions if the latter react with the bases. Figure 1 shows that copper(II) added to native DNA has no effect whatever upon the DNA spectrum. The apparent inability of copper(II) to react with the bases of native DNA at room temperature (Eichhorn, 1962; Eichhorn and Clark, 1965; Hiai, 1965) is thus confirmed. When the DNA is denatured by heat, and copper ions are added to the denatured DNA, on the other hand, the absorbance of the denatured DNA is enhanced and a bathochromic shift of 4 mμ is observed. This effect confirms the ability of copper ions to react with the bases in the DNA molecule when they lie exposed after the hydrogen bonds present in the intact molecule have been severed.

Figure 2 represents a spectrophotometric titration of denatured DNA with copper(II) ions. The data are in line with the interpretation of Figure 1 in paper V (Eichhorn and Clark, 1965) that copper(II) reacts stoichiometrically with DNA. They indicate that between 1 and 2 moles of copper(II) react per DNA nucleotide; they do not permit a more precise establishment of the reaction stoichiometry.

If copper binds to the bases in the DNA molecule, it is to be expected that such binding should occur with at least some of the monomeric constituents of DNA as well. There is in the literature, however, a study by Harkins and Freiser (1958) that would tend to indicate a failure of copper(II) to react with adenosine. Having demonstrated by means of potentiometric titration that adenine complexes with copper(II), they discovered that the titration curve of copper adenosine is virtually the same as that of hydrated copper. We have confirmed this observation for all four nucleosides; the data are shown in Figure 3. The failure to observe a shift in the potentiometric titration of a potential ligand in the presence of a metal ion can be explained in one of two ways. Either there is no complex formation or the formation of the complex proceeds without the removal of protons from the ligand molecule. Since other experiments leave no doubt that complexes are formed, the second of these explanations must be correct, and the data are very useful in that they eliminate competition of copper ions with protons in the reaction with any of the four nucleosides.

It is nevertheless possible to utilize potentiometric titration techniques to demonstrate copper(II) nucleoside complex formation. Figure 4A and 4B represent the titration of an acid solution of copper(II) with base and

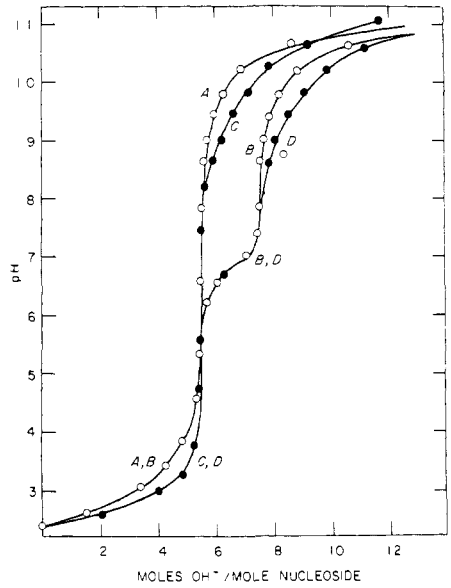


FIGURE 3: Comparison of titration curves for the nucleosides and their copper(II) complexes. All of the solutions contained 0.975 × 10^{-3} M nucleoside. Enough HNO_3 was added to each solution to make it 5.35 × 10^{-3} M in HNO_3 before titration with 0.0103 M NaOH under nitrogen at 25°. (A) O, adenosine or cytidine; (B) O, 0.975 × 10^{-3} M copper(II) nitrate added to (A); (C), ●, guanosine or uridine; (D) ●, 0.975 × 10^{-3} M copper(II) nitrate added to (C).

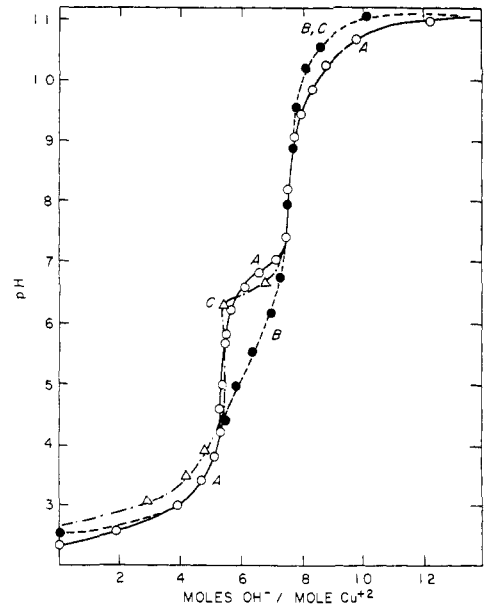


FIGURE 4: Effect of adenosine on back titration of copper(II). All solutions contained 0.975 × 10^{-3} M copper(II) nitrate, and the titrations were carried out under nitrogen at 25°. Solution A (O) was 4.88 × 10^{-3} M in HNO_3 and was titrated with 9.95 × 10^{-3} M NaOH. Solution B (●) was 4.6 × 10^{-3} M in NaOH and was titrated with 1.05 × 10^{-2} M HNO_3. Solution C (Δ) contained 0.975 × 10^{-3} M adenosine, and was otherwise similar to solution B.

the titration of a basic solution of copper(II) with acid, respectively. During the titration represented by curve 4A, the inflection region around neutral pH is accompanied by the precipitation of copper hydroxide, which of course remains out of solution on continued titration to alkalinity. The back titration follows path 4B unless the titration is prolonged for several days. The reason for the nonsuperimposability ofcurve 4B on curve 4A lies in the fact that the copper(II) hydroxide precipitate consists of a giant molecule in which hydroxide groups serve as bridges to the copper atoms (Figure 5A) (Gimblett, 1963). Protons react very rapidly with OH^- groups on a monomeric metal complex, but their rate of penetration into a bridged olated precipitate is of necessity much slower. (At a point on curve 4B corresponding to a pH of 4.4 the solution was allowed to come to equilibrium, and it can be seen that the pH of the solution eventually approaches that of curve 5A.)

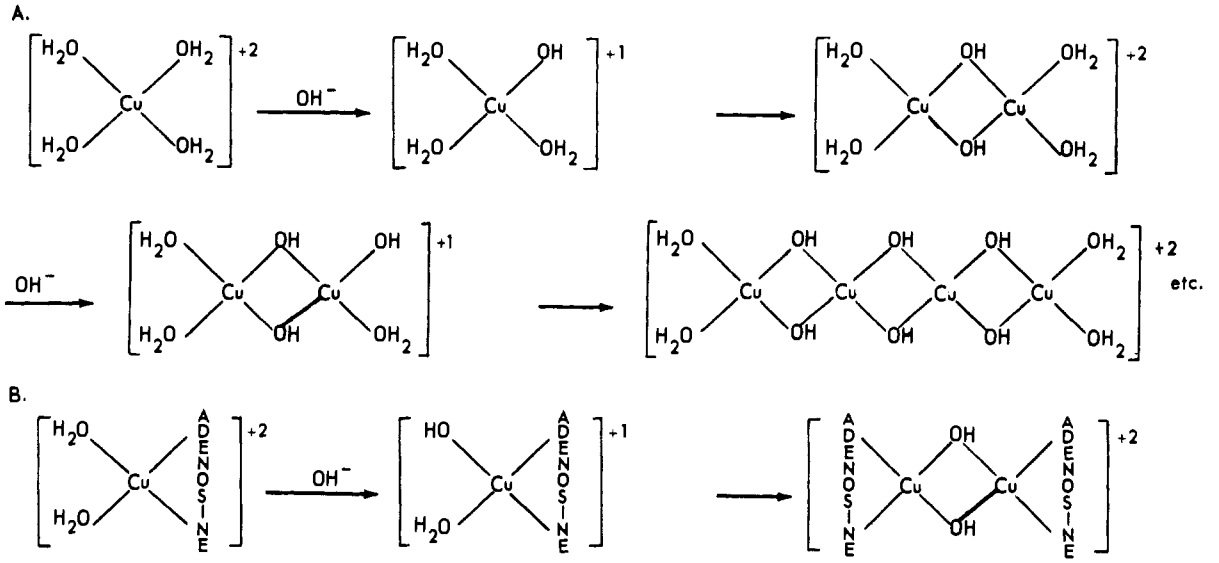
The back titration of copper(II) adenosine, on the other hand, follows curve 4C, with the inflection occurring at the same location as in curve 4A. The fact that the copper(II) adenosine back titration does not duplicate curve 4B is explained by the prevention of OH^- bridge formation beyond the dimeric state by complexation of copper with adenosine. Dimerization is the limit of alkaline polymerization of the 1:1 complex (Gustafson and Martell, 1960; Courtney *et al.*, 1959), as can be seen in Figure 5B. Precipitation does occur at neutral pH, but the precipitate is completely dissolved at high pH.

The lack of polymerization in alkaline solution and the consequent solubility of the copper nucleoside complexes indeed make it possible to obtain visible spectra of these complexes. Figure 6 contains a comparison of the spectrum of a 1:2 mixture of copper(II) with guanosine at pH 5 and 11. The curve at pH 5 is the characteristic spectrum of $[Cu(H_2O)_4]^{2+}$ with a maximum at 800 mμ, whereas the curve at pH 11 has a much more intense peak at 680 mμ. The shift of the 800 mμ peak of copper(II) to lower wavelengths is characteristic of nitrogen-bonded copper complexes.

It is thus clear that nucleosides, which do not contain phosphate, are capable of binding to copper(II). Two previous investigations had led to this conclusion, and these will be discussed presently.

Previous Studies Indicating that Copper(II) Binds to the Bases. The first evidence that nucleosides can react with copper was reported by Albert (1953), who made potentiometric measurements of the stability of a copper guanosine complex. (This evidence appears to be in conflict with the results shown in Figure 3.) Frieden and Alles (1957) later carried out a series of investigations that revealed that nucleosides, as well as nucleotides and nucleoside bases, will inhibit the catalysis by

FIGURE 5: Reactions of copper(II). (A) Hydrolysis and polymerization of the copper(II) ion. (B) Hydrolysis and dimerization of the copper(II)–adenosine complex.



copper(II) of the enzymatic oxidation of ascorbate. The inhibition was attributed to a complex formation of copper with the inhibitors, and the wide variation in the extent of inhibition by, *e.g.*, the various nucleosides was given as evidence that the copper is bound to the base component of the nucleoside. The recent work of Fiskin and Beer (1965) on copper–nucleoside interaction will be discussed in the Conclusion.

No other studies have been reported on the copper binding of nucleosides, but several other investigations have indicated copper binding to bases. Cohn and Hughes (1962) have shown that copper(II) ions broaden the peak due to H^8 of AMP,[1,2] and Schneider and Brintzinger (1964) and Moll *et al.* (1964) have carried out spectrophotometric and kinetic experiments that implicate copper bound to the base of ATP. (These workers (Schneider and Brintzinger, 1964; Moll *et al.*, 1964) present evidence that copper is bound simultaneously to the phosphate and base of the same ATP molecule, but no such evidence exists for the binding of copper to AMP or any other nucleoside monophosphate.) Eisinger *et al.* (1962) have interpreted nmr relaxation studies to indicate that copper ions may be bound to the bases of DNA.

The excellent studies of Hiai (1965) on the reaction of copper(II) with DNA came to our attention when this paper was substantially completed. We note that his Figures 2 and 3 resemble Figures 1 and 2, respectively, of the present paper. We are presenting our data, nevertheless, since they represent somewhat different conditions from those of Hiai. In Figure 1 we have determined that copper does not affect the spectrum of native DNA; he apparently did not perform such an experiment. We studied the effect of copper on the spectrum of denatured DNA by adding copper ions to DNA that had been previously denatured; he looked at the copper–DNA spectrum of DNA denatured in the presence of copper. The end result is the same, as might be expected.

Hiai's spectrophotometric titrations were carried out with DNA that was either partly denatured or

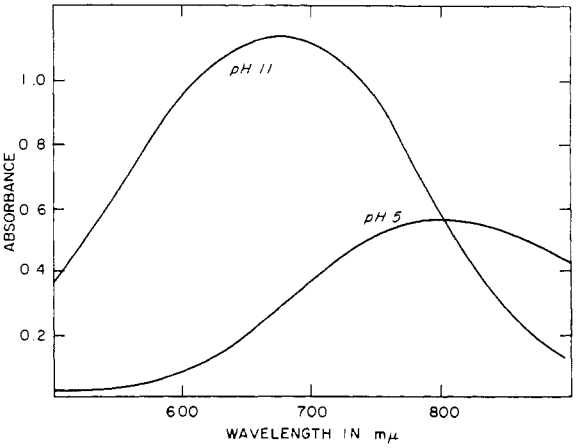


FIGURE 6: Spectra of a solution of 0.025 M copper(II) nitrate and 0.050 M guanosine at pH 5 and 11.

[1] Since two different numbering systems are in use for the pyrimidines, it should be pointed out that in this paper the pyrimidines are numbered in the same manner as the six-membered ring of the purines; H^2 refers to the hydrogen atom on the 2-carbon atom.

[2] Abbreviations used in this work: AMP, CMP, GMP, TMP, adenosine, cytidine, guanosine, and thymidine monophosphates; dAMP, etc., the 2-deoxy counterpart of AMP; nmr, nuclear magnetic resonance; ADP and ATP, di- and triphosphates of adenosine.

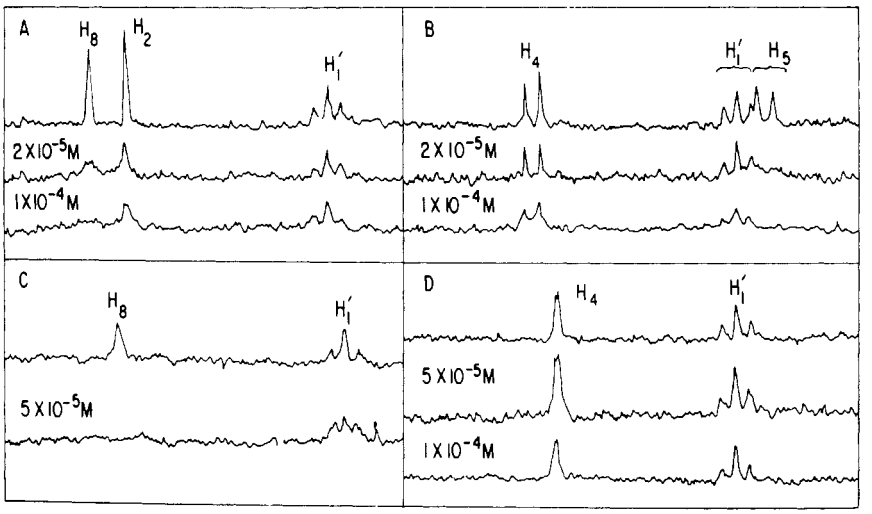


FIGURE 7: Proton nmr spectra (60 Mc) of (A) dAMP, (B) dCMP, (C) dGMP, and (D) dTMP. All are 0.1 M solutions in D_2O with pD $\approx$ 7. For each set of spectra the top curve is the metal-free solution and the copper concentration is indicated for the others.

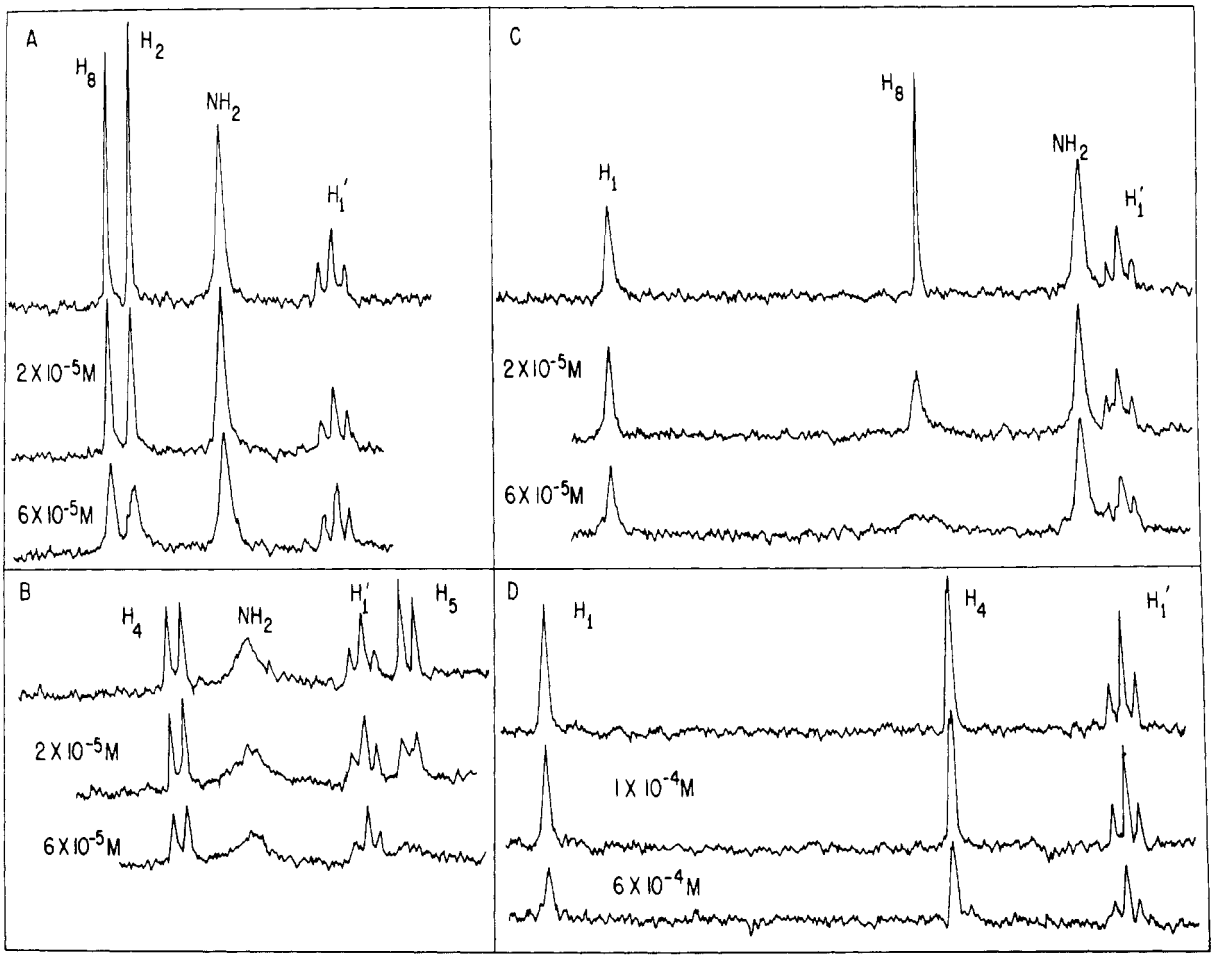


FIGURE 8. Proton nmr spectra (60 Mc) of (A) deoxyadenosine, (B) deoxycytidine, (C) deoxyguanosine, and (D) deoxythymidine. All are 0.2 M solutions in dimethyl sulfoxide-d_6. Legend as for Figure 7.

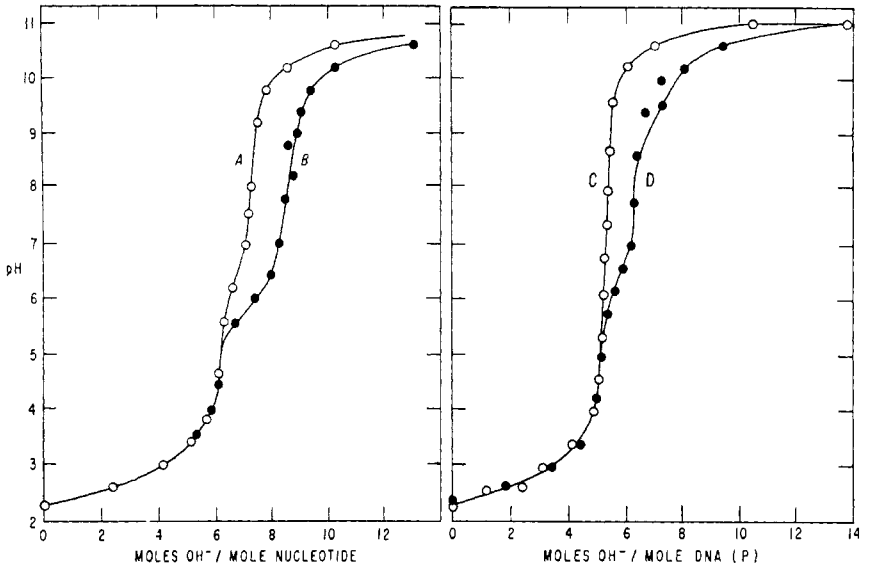


FIGURE 9: Comparison of titration curves of AMP and DNA and their copper(II) complexes. All solutions were titrated under nitrogen at 25°. (A) ○, 0.975×10^{-3} M AMP in 5.3×10^{-3} M HNO_3 titrated with 0.995×10^{-2} M NaOH. (B) ●, same as (A), but contains 0.975×10^{-3} M copper(II) nitrate. (C) ○, 0.975×10^{-3} M (P) DNA in 4.34×10^{-3} M sodium nitrate and 5.3×10^{-3} M HNO_3 titrated with 1.04×10^{-2} M NaOH. (D) ●, 0.975×10^{-3} M (P) DNA, with 0.975×10^{-3} M copper(II) nitrate in 4.45×10^{-3} M $NaNO_3$ and 5.3×10^{-3} M HNO_3, titrated with 0.995×10^{-2} M NaOH.

partly renatured. Figure 2 of the present paper is a titration of completely denatured DNA with copper, and the details of the titration curve differ from those of either of the curves in Hiai's Figure 3.

Proton Nuclear Magnetic Resonance Studies on the Reaction of Copper with the Deoxyribonucleotides. The most direct evidence for the binding of copper to the bases, along with information about which bases and what sites on the bases are involved, has been obtained from proton nuclear magnetic resonance. It has been shown previously that when Cu(II) binds to a ligand the protons near the binding site are relaxed rapidly by the paramagnetic ions and their nmr lines are thus broadened (Li *et al.*, 1962). In these experiments the concentration of Cu(II) is much less than that of the ligand (*ca.* 1:10,000), and the rapid exchange of ligands causes all molecules to be affected equally. Deoxyribonucleotides were selected for most of the comparative studies since these compounds are more soluble in water than either ribonucleotides or nucleosides. The solubility of compounds in the cytidine series was such that spectra could be obtained not only for dCMP, but also for CMP as well as cytidine and deoxycytidine. Results were the same with all four compounds. Deoxythymidine was also studied, and results similar to those with dTMP were obtained. Thus, as might be expected, the conclusions about copper binding drawn from the studies with the deoxyribonucleotides are generally applicable to compounds with or without the 2′-hydroxy group or the phosphate.

Figure 7 shows portions of the proton nmr spectra containing lines due to the base protons as well as the ribose $H^{1'}$. These spectra were obtained in D_2O, and in this solvent only the protons on the carbon atoms can be seen. All other protons (on amino groups, hydroxyl groups, and heterocyclic nitrogen atoms) do not appear. The assignments of the nmr lines given in the figure have been established previously (Jardetzky and Jardetzky, 1960; Bullock and Jardetzky, 1964).

Figure 7A reveals that the peaks of H^2 and H^8 in dAMP are both broadened,[1] but that H^8 is considerably more affected than H^2. For dAMP as well as the other nucleotides the sugar proton lines are influenced very little by addition of Cu(II). The primary attachment of the copper to N_7, postulated by Cohn and Hughes (1962) as well as Schneider and Brintzinger (1964) and Moll *et al.* (1964), is thus confirmed. The effect on H^2 can be explained by weaker binding to N_1 or N_3 or possibly by involvement of the amino group in a chelate ring with N_7. The latter alternative is eliminated by an experiment described below.

The presence of copper(II) broadens both H^4 and H^5 of dCMP (Figure 7B), but H^5 is much more strongly affected. Binding to N_1 or to the amino group could explain this behavior.

In dGMP (Figure 7C) there is only one base proton, H^8, that gives rise to a resonance peak in D_2O, and this peak is broadened by copper ions. It is concluded that copper is bound to N_7. The question arises whether the oxygen atom on carbon 6 participates in the formation of a chelate ring. The oxygen atom is generally in the ketonic form, with a hydrogen atom on N_1; nevertheless, chelation could bring about a loss of the proton and a displacement of the negative charge to the oxygen

atom. Figure 3C, however, shows that the presence of copper does not cause the removal of this proton at neutral pH, thus contraindicating chelation. Chelation without loss of the N_1 proton is, however, possible.

The only deoxyribonucleotide nmr spectrum not affected in any way by the presence of copper ions is that of dTMP (Figure 7D), and it appears, therefore, that copper is not bound to thymidine.

The foregoing studies provide no direct evidence regarding the exchangeable hydrogens and cause some ambiguity of interpretation. It is conceivable, for instance, that copper could bind to N_1 of the thymine moiety and not affect H^4 because it is so far from the binding site. We therefore carried out experiments in dimethyl sulfoxide-d_6; all base protons may be identified in this solvent. The low-field portions of the nmr spectra of the deoxyribonucleosides, showing all the ring protons and $H^{1'}$, are given in Figure 8. The deoxyribonucleosides were chosen for these experiments because they are more soluble in dimethyl sulfoxide-d_6 than the deoxyribonucleotides. (However, data were obtained also with AMP and dAMP; the results were similar to those obtained with deoxyadenosine.)

The peaks corresponding to the carbon-bound protons appear in the same spectral region in dimethyl sulfoxide-d_6 as in D_2O. The NH and NH_2 peaks are readily identified by their areas and widths and by their absence in D_2O. Addition of D_2O to a dimethyl sulfoxide-d_6 solution of a nucleoside wipes out the NH and NH_2 peaks, leaving the carbon-bound protons unaffected.

The effect of copper(II) on the carbon-bound protons is generally the same with the deoxyribonucleosides and the deoxyribonucleotides, confirming the expected similar complexing abilities of the two types of compounds. This similarity is evident in spite of the fact that the former are studied in dimethyl sulfoxide and the latter in water. This fact may be important in discounting the possibility that metal binding to nucleosides in dimethyl sulfoxide may differ from the binding behavior in water. That the solvents should not produce different metal complexes was anticipated from a knowledge of the fact that dimethyl sulfoxide and water form metal complexes of similar nature and stability.

The NH and NH_2 lines are relatively unaffected by addition of copper(II) ion in all four of the deoxyribonucleotides. Thus, in deoxyadenosine copper is not chelated to the amino group, in agreement with the proposal of Schneider and Brintzinger (1964) and Moll *et al.* (1964). In deoxycytidine the amino group is ruled out as a principal binding site, leaving N_1 as the location of copper binding. In deoxyguanosine both the amino group and N_1 are eliminated as possible sites, and the broadening at H^8 leaves little doubt that binding occurs at N_7. In deoxythymidine there is no evidence of copper binding to N_1, and it appears therefore that this base is indeed not complexed in any appreciable degree to copper ions.

Binding of Copper to Nucleotide Phosphate. Examination of Figure 3 has revealed that the presence of nucleosides does not affect (except additively) the titration curve corresponding to the deprotonation of two of the water molecules bound to the copper(II) ion. The presence of phosphate in a nucleotide or polynucleotide appears to modify this titration quite significantly, as can be seen in Figure 9.

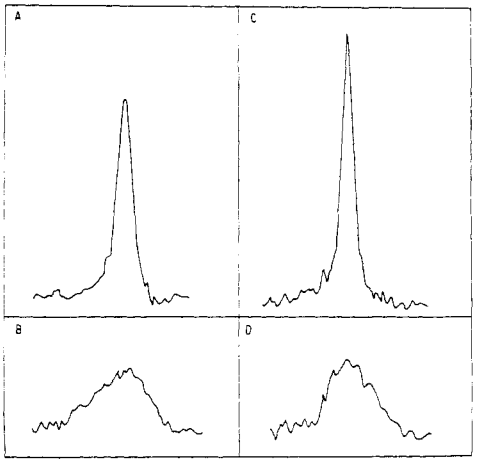


FIGURE 10: ^{31}P nmr spectra (24.3 Mc) of (A) dAMP, (B) dAMP + 2×10^{-5} M Cu(II), (C) dTMP, (D) dTMP + 4×10^{-5} M Cu(II). All are aqueous solutions and are about 1.0 M in the nucleotide.

Comparison of the titration curves of AMP and copper(II) AMP (Figure 9A) shows that fewer than 2 moles of base are used per mole of copper ion in the metal hydrolytic region; the number is, in fact, approximately 1.5 moles. The interpretation of this result is as follows. Every copper ion bound to a negatively charged phosphate group will lose only one proton from its coordinated water, whereas every copper ion bound to a nucleotide base as well as every unbound copper ion will lose two protons from its hydration sphere. The loss of between one and two protons per copper ion, therefore, indicates that some of the copper ions are bound to phosphate, while others are unbound or bound to the bases.

The situation is somewhat less complicated for the titration of copper(II) DNA. Figure 9B demonstrates that approximately 1 mole of base is used per mole of copper ion. These data suggest that most of the copper is bound to phosphate.

Cohn and Hughes (1962) have demonstrated copper binding to phosphate in AMP as well as in ADP and ATP by nuclear magnetic resonance. Brintzinger (1963) has verified this type of bonding in ADP and ATP by nmr and infrared spectroscopy. We have determined that copper(II) ions broaden the phosphate resonance of the deoxyribonucleotides of adenine and thymine as shown in Figure 10. The lower solubility of the cytosine and guanine deoxyribonucleotides precluded similar experiments with these substances, but there is every reason to believe that the results would have been the same.

Conclusion

From the investigations that have been reported here and elsewhere it is apparent that copper(II) binds to the phosphate groups of nucleotides and deoxynucleotides as well as to adenine, cytosine, and guanine of the base moieties in nucleosides, nucleotides, and the corresponding deoxy compounds. Bonding to the adenine and guanine bases occurs at the N_7 positions, and to the cytosine base at the N_1 position. The thymine base apparently is unaffected by copper ions.[3]

These results are in line with the ability of copper ions to break the ribose–phosphate bonds in polyribonucleotides and to destabilize the Watson–Crick structure of DNA. The lack of reaction with the thymine base leads to the expectation that copper ions are more effective in reacting with a guanosine–cytidine base pair than with an adenosine–thymidine base pair. This expectation has just been realized in the experiment of Hiai (1965) in which it was shown that dAT copolymer has a considerably higher melting temperature in the presence of copper than in its absence. Other manifestations of the lack of reactivity of thymine base will be reported in later papers.

Fiskin and Beer (1965) have just reported a potentiometric study of copper(II) nucleoside complexes. They were able to determine relative stabilities of the complexes by observing proton displacement in acid solution. This study is relevant to the present one for two reasons. First, their inability to find proton displacement with thymidine, in contrast to adenosine, cytidine, and guanosine, is in line with our result that copper(II) uniquely does not broaden the nmr lines of thymidine. Second, there is an apparent discrepancy between some of our interpretations and those of Fiskin and Beer. We have taken the fact that the titration curves of copper nucleoside and hydrated copper ion are essentially identical as evidence that the complex formation occurs without replacement of a proton. This appears to be in conflict with the determination of relative stabilities based on proton displacement. Actually, there is no discrepancy. Fiskin and Beer studied proton competition with copper ions in the acidic region. Copper complex formation at some site simply competes with protons somewhere on the molecule, not necessarily on the complexing site. Our interpretation of our results is that copper does not displace protons from the complexing sites that are protonated in the pH range of maximum complexing ability. For example, the proton that is titrated in uridine or guanosine around pH 9 is titrated at the same pH in the presence of copper. If complexing occurred in competition with this proton, it would be titrated at a lower pH in the presence of copper. Such behavior is indeed observed with the mercury(II) nucleoside complexes (Eichhorn and Clark, 1963).

[3] It is important to note that binding to the bases in nucleosides, nucleotides, etc., is very different from binding to the purines and pyrimidines unattached to any ribose, since the position at which the ribose is attached to all the bases becomes a site for metal binding in the absence of the riboside bond.

Experimental Section

The nucleosides and nucleotides, as well as the DNA, were obtained from Sigma, and the DNA was put into solution as described previously (Eichhorn and Clark, 1965). All inorganic chemicals were reagent grade, and the copper concentrations were determined iodometrically.

D_2O and dimethyl sulfoxide-d_6 were obtained from Merck, Ltd., and were 99–99.7% enriched in deuterium. Since only very low concentrations of copper(II) were needed for the nmr experiments, an aqueous stock solution of $CuSO_4$ was used. A separate experiment showed that the small quantity of water introduced into the dimethyl sulfoxide-d_6 solutions did not affect the nmr spectra of the nucleosides.

Spectra were obtained by means of a Cary Model 14 recording spectrophotometer, and potentiometric titrations were carried out using a Radiometer Model TTT1 Titragraph. Proton and ^{31}P nmr studies were carried out with, respectively, Varian A-60 and HR-60 spectrometers, both using a 12-in. magnet.

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Sequential Periodate Oxidation of the α_1-Acid Glycoprotein of Human Plasma*

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ABSTRACT: The sequential periodate oxidation of sialic acid free α_1-acid glycoprotein of human plasma and of a derivative obtained by treatment with *Diplococcus pneumoniae* neuraminidase and β-galactosidase is described. After three degradations of both products only 2-acetamido-2-deoxyglucose units remain attached to the polypeptide chain, implicating these monosaccharide units in the linkage between carbohydrate and protein moieties. From the content of 2-acetamido-2-deoxyglucose, a minimal value of 5–6 carbohydrate–protein linkages was estimated. The sequential periodate oxidation indicated also for each molecule of glycoprotein the presence of 8–9 chains containing *O*-β-D-galactopyranosyl-(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2- or -6)-D-mannopyranosyl residues, five chains containing *O*-β-D-galactopyranosyl-(1→6)-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2- or -6)-D-mannosyl residues, with 30 to 40% of the chains possessing branch points at C-3 or C-4 of the mannosyl residues, as well as the presence of 2-acetamido-2-deoxyglucosyl-2-acetamido-2-deoxyglucosyl residues.

The α_1-acid glycoprotein (orosomucoid) of human plasma (Weimer *et al.*, 1950; Schmid, 1950, 1953) has a content of approximately 45% of carbohydrate which is linked to a polypeptide moiety. Previous studies undertaken in this laboratory have been concerned with the number and the structure of the carbohydrate chains and with the mode of linkage between the carbohydrate and protein moieties of the macromolecule (Eylar and Jeanloz, 1962a,b; Jeanloz and Closse, 1963; Hughes and Jeanloz, 1964a,b). Present evidence suggests that the α_1-acid glycoprotein contains a carbohydrate moiety consisting of a total of 16 chains. Each chain possesses a *N*-acetylneuraminic acid residue as nonreducing and terminal group with the exception of two chains, where L-fucose replaces *N*-acetylneuraminic acid. Some of the chains are terminated by the sequence *O*-(*N*-acetylneuraminyl)-(2→4)-*O*-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose. The inner core of the polysaccharide moiety is of unknown structure and contains several D-mannose and 2-acetamido-2-deoxy-D-glucose units.

In order to obtain information on the chemical structure of the core, and on the linkage between the carbohydrate and protein moieties, the α_1-acid glycoprotein and two derivatives have been subjected to sequential periodate oxidation, a method based on the observations of Smith and co-workers (Smith and Unrau, 1959; Goldstein *et al.*, 1959; see also Whelan, 1960). Periodate oxidation of a polysaccharide results in the cleavage of available glycol groups and the production of a polyaldehydic structure. Subsequent reduction of the aldehyde groups with sodium borohydride produces a polyalcohol containing acetal linkages, which are easily hydrolyzed under conditions which do not cause accompanying cleavage of the remaining glycosidic bonds. Cleavage of the acetal linkages exposes new monosaccharide units which may be susceptible to further oxidation. Analysis of the monosaccharide units destroyed after each application of the reaction sequence provides a basis for deducing a partial structure for the intact polysaccharide.

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