Interaction of Copper(II) with Deoxyribonucleic Acid below 30°*

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ABSTRACT: The techniques of gel filtration, difference spectroscopy, viscosity, and ultracentrifugation have been used to study interactions of salmon sperm deoxyribonucleic acid (DNA) with Cu(II) below 30° at 5 mM $NaNO_3$ and at different pH values. The binding of Cu(II) to DNA was sensitive to changes in Cu(II) concentration and was found to be time dependent at 25 ± 5°. The binding was constant between pH 5.6 and 6.6. Other evidence is presented to confirm Cu(II)–DNA interactions in the room temperature range. Cu(II) and DNA also produce spectral changes sensitive to Cu(II) concentration, temperature, and reaction time. By gel filtration, the average molar ratio of DNA nucleotide bound to Cu(II) was 2:1. The binding constant of 2×10^4 at pH 6.1 ± 0.5 and 26 ± 1° was calculated by substituting values from binding experiments into the equilibrium expression: $K_a = [(BdRP)_{n/2} - Cu(II)]/[(BdRP)_{n/2}][Cu(II)]$. This constant is in good agreement with the constant evaluated from a Scatchard plot of the same data and the constant determined from difference spectra.

One possible explanation of the data is to assume that only the purine nucleotide units bind to Cu(II) in a 1:1 complex.

The well-known fact that metal ions interact with the nucleic acids (Frieden and Alles, 1958; Neuberg and Roberts, 1949; Wacker and Vallee, 1959) is now receiving great attention in the modern context of our expanded knowledge of the chemistry of DNA and RNA (Hiai, 1965; Eichhorn, 1962; Eichhorn and Clark, 1965; Coats *et al.*, 1965; Eichhorn *et al.*, 1966; Venner and Zimmer, 1966). In particular, 10^{-4} M Cu(II) has been shown to decrease the transition temperatures of DNA solutions of low ionic strength and to produce a hyperchromic effect in the ultraviolet region, with a bathochromic shift (Hiai, 1965; Coats *et al.*, 1965; Eichhorn *et al.*, 1966). Thus, there is agreement that the evidence strongly indicates binding of Cu(II) to heated DNA.[1] It has been proposed that the bases of the DNA helix become available for Cu–DNA binding only after heat denaturation of DNA, while the phosphate groups are the only accessible sites in DNA prior to heating (Hiai, 1965; Coats *et al.*, 1965; Eichhorn *et al.*, 1966). Moreover, it has been contended that the interaction of Cu(II) with the nitrogen bases of DNA does not exist in the room temperature range (25 ± 5°) as suggested earlier by the work of Frieden and Alles (1958).

To study this question further, we have used the techniques of gel filtration, viscosity, ultracentrifugation, and ultraviolet difference spectra. Evidence is presented which suggests that 10^{-4} M Cu(II) interacts with DNA below 30°. In addition, stoichiometry and binding constants for the Cu–DNA complexes have been evaluated by molecular sieve chromatography and by difference spectrophotometry.

Experimental Procedures

Salmon sperm DNA and calf thymus DNA were purchased from Calbiochem Corp. and Worthington Biochemical Corp., respectively. All other chemicals were reagent grade and all solutions were prepared with water which was redistilled, twice deionized, and chelexed (100). Stock solutions of DNA were prepared by dissolving 0.20 mg/ml in 5 mM $NaNO_3$ at 4° over a period of 5–7 days with occasional mixing. Unless otherwise stated, an aliquot of this stock solution was passed through Sephadex G-25, previously equilibrated with 5 mM $NaNO_3$, before further use. DNA was analyzed by the method of Fredericq *et al.* (1961) and Cu(II) was analyzed by the neocuproine method as adapted by Frieden *et al.* (1962).

The salmon sperm DNA used in these experiments was subjected to a number of different analyses. No protein could be detected by the Biuret and Folin–Ciocalteau tests. The ratio of absorbances at 260 and 280 mμ was 1.68. The maximum hyperchromism after exposure to 80° as measured by the absorbances at 25°

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[1] Unless otherwise stated, the term DNA refers to gel-filtered salmon sperm deoxyribonucleic acid in 5 mM $NaNO_3$ with the properties of melting (T_m 65° in 5 mM salt and T_m 83° in 0.15 M salt) and of chromatography (methylated albumin) which have been used to describe "native" DNA (Sueoka and Cheng, 1962); however, to avoid semantic confusion, we have omitted the word "native." This DNA heated at 80° for 20 min and quenched in ice is referred to as "heated" DNA. The conditions of temperature and time used in heating DNA in 5 mM $NaNO_3$ have differed widely among other investigators. We assume 80° to be adequate, since it is 15° above the T_m.

at 260 mμ was 1.38. T_m was 83° in 0.15 M $NaNO_3$ and 65° in 5 mM $NaNO_3$. The s_{20} was 12.6 ± 1.0 S in 5 mM $NaNO_3$ (pH 6.8) at a DNA concentration (A_{254} 0.80). Intrinsic viscosity in 5 mM $NaNO_3$ and 14 mM phosphate as determined in a Beckman low-shear viscometer was 310 dl/g. The supplier (Calbiochem Corp. lot no. 50208) reported 14.2% nitrogen, 8.2% phosphorus, and <0.1% arginine and chloride.

To further characterize the preparation after gel filtration, salmon sperm DNA was chromatographed on a methylated albumin column (Sueoka and Cheng, 1962), using stepwise elution (increments of 0.1 M) from 0.10 to 1.00 M in unbuffered salt. The first and largest peak appeared with 0.70 M NaCl and smaller peaks were eluted with 0.80 and 1.00 M salt. More than 70% (by peak heights) was eluted in the 0.70 and 0.80 M salt fractions which have been described as "native" by Sueoka and Cheng (1962).

Sephadex G-25 and Sephadex G-200 columns (8 mm × 40 cm and 14 mm × 10 cm, respectively) were equilibrated at 25 ± 2° by washing with 100–150 ml of the appropriate eluting salt solution. Essentially the same procedure was followed in gel filtration binding experiments, as was reported by Fairclough and Fruton (1966) while this investigation was in process. Our studies entailed the analysis of individual fractions associated with the elution peak of the Cu–DNA complex, and the areas under these peaks were measured with a planimeter.

All spectra were determined at 25 ± 2° with a Cary 15 recording spectrophotometer, equipped with 0.0–0.1 and 0.0–1.0 full-scale absorbance. Four 1-ml cuvets (1-cm path) were used to determine difference spectra as follows. A zero base line was obtained by placing an aliquot of DNA and an aliquot of solvent (usually 5 mM $NaNO_3$) into each of two cells placed in the sample and the reference compartment. An experiment was initiated by adding microliter quantities of copper nitrate to the sample DNA and to the solvent in the reference compartments, while solvent was added to the DNA housed in the reference compartments.

Melting curves were obtained on a Gilford Model 2000 absorbance recorder used in association with a Beckman DU monochromator; samples were heated in 3-ml Teflon or glass-stoppered quartz cuvets and the temperature was controlled by a constant-temperature circulator.

A rotating cylinder viscometer as described by Zimm and Crothers (1962) was used to determine viscosity. Measurements were made under conditions with shear rates below 0.1 sec^{-1}.

The sedimentation coefficients were determined in the Beckman analytical ultracentrifuge in a standard 4° cell at 25° using the ultraviolet optical system. The s_{25}'s are calculated from the radial position of the midpoint of the densitometer tracing obtained with the Beckman Analytrol and represent the slope of the least-squares straight line of the data. Since the difference between the viscosity of the solvent containing the highest concentration of Cu(II) and that without Cu(II) was within the experimental error in the determination of the sedimentation coefficients, the observed values were not corrected to water at 20° as a solvent. The concentration of DNA used in all runs was 1.39 × 10^{-4} M in phosphate and the samples were introduced into the disassembled cell with a large bore glass tube to avoid shearing the DNA. A few runs were made at different speeds with the DNA samples containing no copper, and the results showed that the sedimentation coefficients were independent of speed.

Results

Properties of DNA Affected by Gel Filtration. The transition temperature for DNA in 5 mM $NaNO_3$ was 58° before and 65 ± 2.5° after gel filtration, while the T_m of DNA in higher salt solutions (0.15 M $NaNO_3$) was essentially the same (83 ± 1°) in both cases. If T_m values are assumed to be a reflection of secondary structure stability, it would appear that subjection of DNA to gel filtration results in the removal of destabilizing contaminants, possibly heavy metal ions (see Discussion).

Hyperchromism at 260 mμ can be demonstrated in DNA before and after gel filtration (Table I), but approximately four times the ratio of Cu(II)/DNA-(P) is required to give a relative absorbancy of 1.22 if the material has not passed through Sephadex G-25. In our hands, the spectra of salmon sperm DNA prior to gel filtration (with and without Cu(II)) were extremely variable (values in Table I are average values for four to five readings), whereas the gel-filtered material was quite stable and the spectra were reproducible within ±0.01 OD unit on 0–1.0 full-scale absorbancy.

Binding Studies by Gel Fitration. A sample of DNA applied to a Sephadex G-25 column will be eluted sharply

TABLE I: Effect of Cu(II) on Absorbance of Sephadexed and Non-Sephadexed DNA.[a]

Cu(II) (M × 10^4)	Rel Absorbance[b]	Approx Cu(II)/DNA-P
	Non-Sephadexed	
No Cu(II)	1.00	
0.7	0.96	2
1.5	1.06	4
3.4	1.22	10
	Sephadexed (G-25)	
No Cu(II)	1.00	
0.25	1.00	0.5
0.48	1.07	1
0.90	1.10	2
1.10	1.22	2.5
No Cu(II)[c]	1.00	
3.6[c]	1.34	6

[a] Salmon sperm. [b] A(observed)/$A_{(no\ Cu)}$ at 260 mμ and at 26 ± 1°. [c] Different experiment.

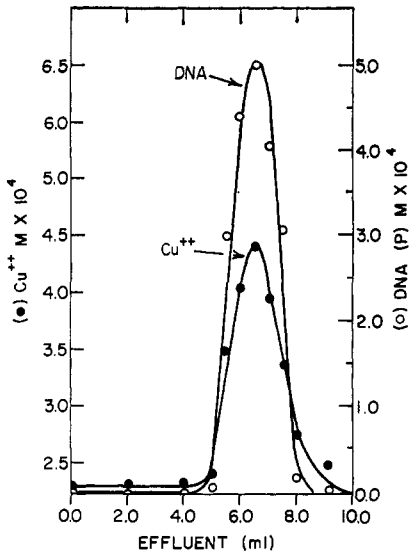


FIGURE 1: Representative elution profile of DNA (salmon sperm) on Sephadex G-25 previously equilibrated in 2.3 × 10^{-4} M $Cu(NO_3)_2$ in 5 mM $NaNO_3$ (pH 6.4). A 2-ml aliquot stock DNA-P (5 × 10^{-4} M) was applied to the column and eluted with the equilibrating buffer.

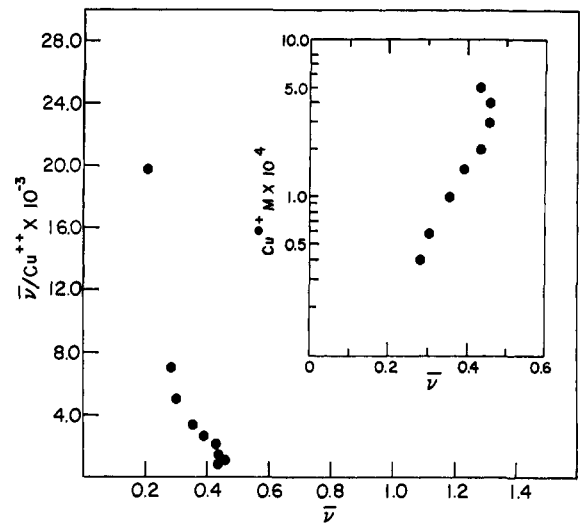


FIGURE 2: DNA–Cu binding data from gel filtration experiments. A Scatchard plot is shown in the main graph and a Weber plot of the same data is given in the insert. $\bar{\nu}$ is the mean number of moles of copper bound per DNA nucleotide.

after the void volume. If the column has been previously equilibrated with $Cu(NO_3)_2$, Cu(II) will bind to DNA as indicated by the elution profile given in Figure 1 and moles of Cu(II) bound per mole of DNA nucleotide can be calculated from the areas under the respective peaks. A 2-ml aliquot of stock DNA-P (5 × 10^{-4} M) is eluted (essentially 100%) between 4.5 and 8.5 ml; thus, fractions (0.5 ml) were collected over this elution region and analyzed for Cu(II). The area under the Cu(II) peak is a function of concentration of the equilibrating-eluting $Cu(NO_3)_2$ solution for a given DNA nucleotide concentration and was used to evaluate $\bar{\nu}$, the mean number of moles of Cu(II) bound per DNA nucleotide. From a Scatchard plot (Edsall and Wyman, 1958) of $\bar{\nu}$/Cu(II) against $\bar{\nu}$ (Figure 2), intercepts give K_a (as $\bar{\nu}$ approaches zero) and n (as $\bar{\nu}$/Cu(II) approaches zero), where $K_{association}$ (K_a) is the apparent association constant for a set of binding sites. A line, fitted by least squares, drawn through the points in Figure 2, intercepts the axis at 0.43. If one point is omitted ($\bar{\nu}$/Cu(II), 20; $\bar{\nu}$, 0.2 representing 1 × 10^{-5} M Cu(II) and 5 × 10^{-4} M DNA-P) the calculated intercept becomes 0.50. Weber and Anderson (1965) have shown that data from determination of binding constants are valid only when outside stoichiometric ranges (a plot as suggested by Weber is given in the insert in Figure 2). Thus, in saturating concentrations of Cu(II), the average molar ratio of DNA nucleotide to Cu(II) is 2:1 and K_a is 2.5 × 10^4 by graphical analysis (Figure 2).

The gel filtration method was used primarily to evaluate *n*. The data in Figure 2 provides only a rough approximation of K_a since many factors including Donnan effects can produce variations in $\bar{\nu}$. However, Fairclough and Fruton (1966) have shown that this method can be used for the determination of apparent binding constants which appear to be as reliable as those estimated from equilibrium dialysis experiments. The constant is reported here for comparison with K_a from difference spectroscopy.

Effect of pH. Both buffered (pH 6.5) and unbuffered (pH approximately 5.8–6.4) equilibrating salt solutions were used in the gel filtration binding experiments. A buffer system (triethylamine–nitrate) was prepared by adding an aliquot (2 ml/l.) of amine to the equilibrating-eluting Cu(II) solutions and adjusting the pH to 6.5 with

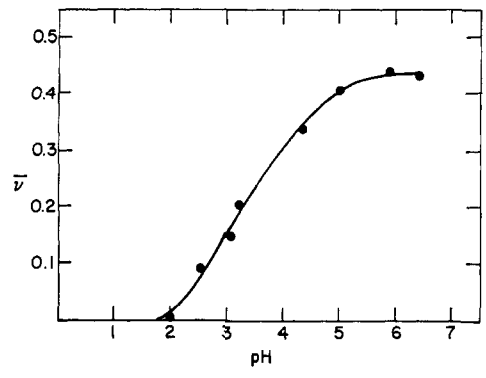


FIGURE 3: pH dependence of DNA–Cu binding. The equilibrating-eluting salt solutions were 2.0 × 10^{-4} $Cu(NO_3)_2$ in 5 mM $NaNO_3$ and adjusted to the appropiate pH as described in the text. The DNA sample in each case was the same as Figure 1.

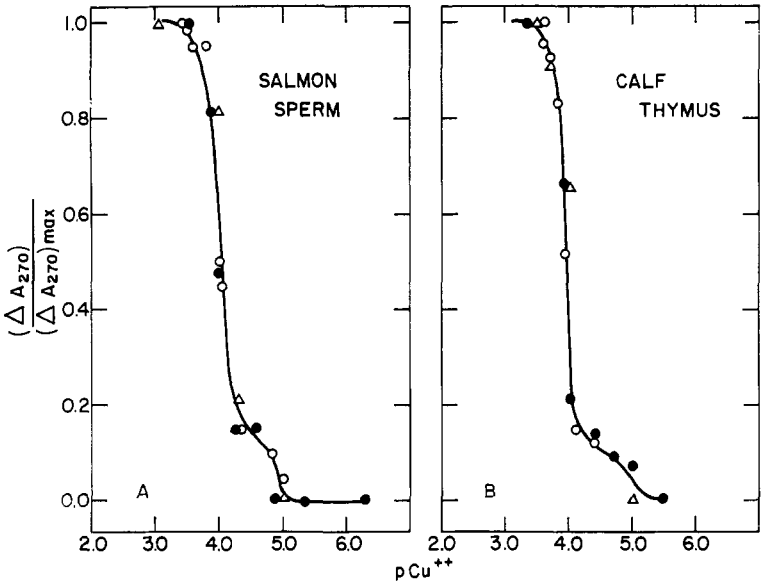


FIGURE 4: pCu^{2+}-dependent titrations of DNA at 270 mμ. All solutions were 5 mM $NaNO_3$ and DNA-P concentrations as indicated: (A) salmon sperm (△) 4.8×10^{-5} M, (○) 7.0×10^{-5} M, and (●) 5.6×10^{-5} M; (B) calf thymus (△) 3.6×10^{-5} M, (○) 4.5×10^{-5} M, and (●) 4.4×10^{-5} M.

TABLE II: Binding Constants for DNA–Cu Complexes.[a]

DNA Source	Method	K_{assoc}	Binding Constants (range $\times 10^{-4}$)
Salmon sperm	Gel filtration (graph)[b]	2.0×10^4	(1.5–2.5)
Salmon sperm	Gel filtration (calculated)	1.9×10^4	(0.3–3.9)
Salmon sperm	Difference Spectra (graph)[c]	1.0×10^4	(1.0–1.2)
Calf thymus	Difference Spectra (graph)[c]	0.9×10^4	(0.7–0.9)

[a] K_{assoc} for heat-denatured salmon sperm was 1×10^5. [b] See Figure 2. [c] See Figure 4.

nitric acid. However, Cu(II) binding essentially was found to be the same as that observed in unbuffered solutions. The effect of pH on $\bar{\nu}$ is given in Figure 3. In this experiment, the equilibrating-eluting salt solutions were adjusted to the appropriate pH with nitric acid or sodium hydroxide in the absence of a buffering agent. The pH of each fraction (of Cu–DNA peak) was checked and was found to remain within ±0.2 pH unit of the initial pH. Binding of Cu(II) appeared to be constant between pH 5.6 and 6.6. Since the pH of unbuffered Cu–DNA solutions studied fall into this range, most of the experiments in this study were in the unbuffered systems.

Binding Studies by Difference Spectroscopy. Using the technique of difference spectrophotometry described by Suelter *et al.* (1963, 1966), changes in absorbancy were found to be related to reaction time and Cu(II) concentrations at sub-T_m temperatures. If the molar ratio of Cu(II)/DNA-P is approximately one, the difference spectrum was found to remain constant over a period of 90 min. However, if the ratio is increased to approximately 3:1, the absorbancy increases with time until equilibrium is reached, as indicated by a constant spectra. The wavelength of greatest difference changed from 275 to 270 mμ. Difference spectra at varying levels of Cu(II), taken after samples had stood at 26 ± 1° for 7 hr, were used to determine $\alpha = ((\Delta A_{270})/(\Delta A_{270})_{max}) =$ fraction of Cu(II)–DNA complex. Since $pK = pCu(II) + \log \alpha/(1 - \alpha)$, the dissociation constant for Cu(II)–DNA complexes can be determined from a plot of α against pCu(II) (Figure 4). Binding constants from both gel filtration (K_a) and difference spectra (converted to K_a) are summarized in Table II.

DNA, denatured with Cu(II), can be renatured by making the solution 0.2 M in NaCl. The renaturation process was found to be time dependent at low concentrations of NaCl and could be followed spectrophotometrically.

Effect of High Concentrations of Cu(II) on Cu(II)–DNA Binding. In both gel filtration and difference spectroscopy experiments, the affinity of Cu(II) for DNA

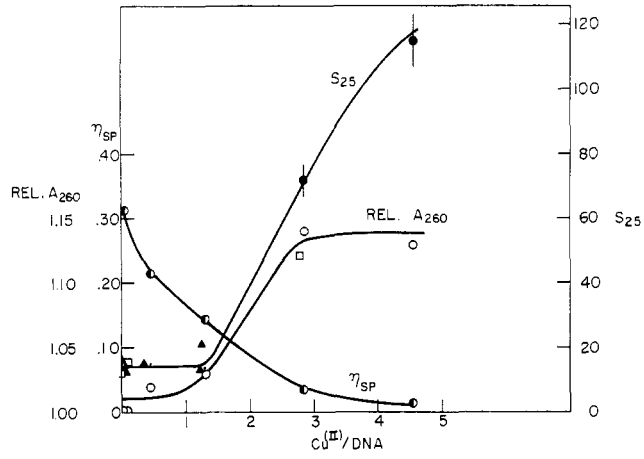


FIGURE 5: Effect of total Cu(II)/DNA-P on the specific viscosity (η), sedimentation coefficients (s_{25}), and relative absorbance at 260 mμ in 5 mM $NaNO_3$ at 25°. s_{25} values for 1.38×10^{-4} M (●) salmon sperm DNA-P on the same samples used for viscosity and absorbance measurements.

appeared to be reduced at higher levels of Cu(II). For example, moles of Cu(II) bound per DNA nucleotide (Figure 2) and the hyperchromic effect at lower concentrations decreased when Cu(II) exceeded 4×10^{-4} M.

Viscosity and Sedimentation Studies. Changes in viscosity (s_{25}) and relative absorbancy as a function of the ratio of Cu(II)/DNA-P are summarized in Figure 5. The ratio of 1:1 where there is little or no change in hyperchromicity or in s_{25} even though the viscosity has decreased is the ratio of total Cu(II)/DNA in the "peak" fraction shown in Figure 1 (see Discussion for possible explanation).

Discussion

Previous studies (Hiai, 1965; Coats *et al.*, 1965; Eichhorn *et al.*, 1966; Venner and Zimmer, 1966) suggest that destabilization of the secondary structure of DNA is due to Cu(II) combining with helical nitrogen bases in DNA. Conditions under which such Cu(II)–DNA interactions have been demonstrated are relatively unphysiological (*e.g.*, by heat denaturation or with high levels of Cu(II)). Furthermore, some of the parameters used to evaluate Cu(II)–DNA interactions may be affected by methods employed in sample preparation. One might explain the differences in T_m and hyperchromicity before and after gel filtration (Table I) by assuming that low molecular weight anions, retained by Sephadex, could react preferentially with Cu(II). Thus, the ratio of Cu(II)/DNA-P needed to produce hyperchromism is less in the absence of these contaminating ions. The slight increase in T_m (in 5 mM $NaNO_3$) after gel filtration treatment might be due to trace levels of stabilizing cations (Schildkraut and Lifson, 1965) bound to DNA firmly enough to pass through Sephadex, whereas an over-all slightly destabilizing effect is produced if other low molecular weight contaminants (anions and cations) are not removed.

Heterogeneity of the DNA sample may also be an important factor to consider in evaluating Cu(II) interactions. Methylated albumin chromatography and ultracentrifugation studies indicate that salmon sperm DNA after gel filtration is somewhat heterogenous. Further fractionation of this material, now in progress, may show other interactions not yet demonstrated and such phenomena as the "time effect" may only be a reflection of the heterogenous nature of the sample. However, time-dependent Cu(II) induced conformational changes in another macromolecule, metmyoglobin, have been reported.[2]

The fact that the ionic strength of DNA solutions will influence Cu–DNA interactions has been well recognized (Eichhorn *et al.*, 1966; Venner *et al.*, 1966). Other variables equally important but receiving little or no attention include time of exposure to Cu(II) and the ratio of Cu(II) to DNA at temperatures below 30°. Hiai (1965) has shown the effect of Cu/DNA on relative absorbance at 260 mμ. However, he does not emphasize that these absorbance changes are especially sensitive to the 25–30° temperature range, nor does he report any time effects comparable to those observed in the present work.

Binding studies at 26 ± 2° on DNA are reported. The Scatchard plot intercept (Figure 2) gives n (maximum number of occupied sites per DNA-P) equal to 0.5 ± 0.1 and suggests a 2:1 complex such as (DNA-P–Cu-P-DNA) previously reported (Ropars and Viovy, 1964). This type of complex, however, is not compatible with other binding data. Apparent association constants evaluated by graphical analysis (Table II) should correspond to a constant calculated from binding data. Substituting values from binding experiments into an equilibrium expression as proposed by Ropars and Viovy (1964) in which two DNA-P nucleotides bind to each

[2] Paper 882 presented at the 50th Annual Meeting of the Federation of American Societies for Experimental Biology by Gillespie *et al.* (1966).

Cu(II) gives K_a in the order of 10^7. The simplest equilibrium which fits the observed stoichiometry is presented in reaction 1 and represented by eq 2, where $(BdRP)_{n/2}$ represents one-half of the nucleotide units of DNA. The calculated K_a is in good agreement with the experimental K_a shown in Table II.

$$(BdRP)_{n/2} + Cu(II) \rightleftharpoons [(BdRP)_{n/2}\text{–}Cu(II)] \quad (1)$$

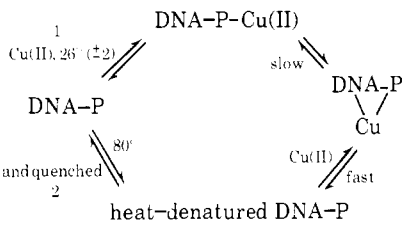
$$K_a = \frac{[(BdRP)_{n/2}\text{–}Cu(II)]}{[(BdRP)_{n/2}][Cu(II)]} \quad (2)$$

We do not believe that charge neutralization arising from Cu^{2+} and a single negative charge per nucleotide unit can account for the stoichiometry because of the strong binding constant. A more attractive hypothesis is to assume that only one-half of the nucleotides (*e.g.*, the purines) bind to Cu(II) in a 1:1 complex. The data in Figure 3 could support a Cu–purine-type complex in view of the fact that the midpoint in this pH-dependence curve (pH 3.4) is in the range of the apparent pK_a values reported for purine deoxynucleotides (Martin, 1964). The nuclear magnetic resonance studies of Eichhorn *et al.* (1966) on free deoxyribonucleotides are in support of this type of complex. Recent studies on the effect of GC composition of T_m of DNA are of interest here. Hiai (1965) concluded that T_m in the presence of Cu(II) is independent of GC content while Venner and Zimmer (1966) have reported that Cu(II), at appropriate concentrations, produces a decrease in T_m dependent on base composition, but that the GC dependence becomes zero around a molar ratio of $(Cu(II)/(BdRP)_{n/2}$ of 0.6 to 0.8. We find in this concentration range [Cu(II)/DNA-P of 1.2–3.0] maximum binding by both gel filtration and difference spectroscopy. Thus, the initial Cu(II)–DNA complex formed at 26° is probably not dependent on the base composition of the DNA. This does not preclude that under harsher conditions (*e.g.*, elevated temperatures and a higher Cu(II) concentration) other complexes form which might be a reflection of the GC content of the DNA.

The possible unique roles of purines in Cu(II)–nucleic acid interactions were pointed out earlier by Frieden and Alles (1958) in connection with studies in which large differences were observed between the effectiveness of purine compounds as compared to pyrimidine derivatives in the inhibition of the Cu(II)-catalyzed oxidation of ascorbate. This earlier work is in firm support of and in full agreement with our current thesis that Cu(II)–nitrogen–base interactions do occur in the room temperature range and that such interactions suggest a purine specificity.

The mechanism whereby such a binding process occurs is not yet known. The dissociation curves (Figure 4) and viscosity data (Figure 5) suggest that two binding sites are involved and that binding is of a cooperative nature. One could assume that the lower portion of the dissociation curve [10^{-5} M Cu(II)] corresponds to Cu(II)–phosphate binding. Increasing Cu(II) concentrations produces reaction conditions such that Cu(II) ions gain access to helical bases and conformation changes occur and can be followed spectrally. However, if the DNA is heat denatured, nitrogen bases are more accessible for binding and maximum hyperchromism is produced by much lower Cu(II) concentrations. Thus, while different conditions (reaction time and concentration) are required, maximum spectral changes produced by Cu(II) were found to be identical in nonheated and heat-denatured DNA. A possible sequence of reactions for this model might be described in Scheme I.

SCHEME I



Further evidence, from viscosity and ultracentrifugation data for Cu(II)–DNA interactions at room temperature, is presented in Figure 5 and is compatible with the above mechanism. The fact that viscosity decreases before hyperchromism and sedimentation changes occur could be explained if one assumed that formation of the initial species, DNA-P–Cu(II), produces loosening in the coiling of the molecule, prior to major changes in shape. The second species

(at a higher ratio of Cu(II)/DNA-P), produces changes in shape as shown by the increased s_{25}. The decreased viscosity is a result of interactions with the nitrogen bases suggested by the hyperchromic effect. We do not yet know if the separation of double strands accompanies complex formation. It could well be that the Cu(II) hyperchromic effect is only a reflection of a distortion induced in the secondary structure of the molecule, perhaps by interfering with the stacking of the bases, and thus does not necessarily bring about either unwinding of the helices or strand separation. The very high s_5 values at the higher Cu(II)/DNA ratios also suggest aggregation in addition to shape changes as proposed by Hiai (1965) and others.

The numerous questions raised in this and in earlier papers relative to the physiological significance of Cu(II)–DNA complexes remain to be answered, but we emphasize that these complexes can be demonstrated below 37°.

Finally, there seems to be a lack of appreciation of all of the known important factors which influence the ability of Cu(II) and other metal ions to complex with DNA and to produce changes in its conformation. Therefore, we think that it is useful to enumerate these key parameters as follows.

Temperature. Cu(II) not only reduces T_m more than

25° but also causes the time-dependent reactions described in this paper below 30°.

Time. In the 25–30° range, the time of exposure to Cu(II) to reach equilibrium may be critical.

Ionic Strength. The effects of Cu(II) at 0.005 M $NaNO_3$ can be reversed at higher ionic strength (*e.g.*, 0.2 M).

Cu/DNA. Ratios of greater than 2:1 in the 10^{-4} range are required for effects below 30°.

pH. The binding is relatively constant in the pH 5.0–7.0 range, but is greatly reduced below pH 5.0.

Purity of DNA. Contaminating ions may affect the T_m significantly.

Composition of DNA. The proportion of purine nucleotides should be a crucial factor, since these nucleotides may favor Cu(II) binding.

Acknowledgments

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Added in Proof

After the acceptance of this work for publication, a closely related paper by Yatsimirskii *et al.* (1966), came to our attention. Their estimated value for the formation constants for copper–DNA complexes from difference spectra was 1.3×10^4, in good agreement with values in Table II. (No temperature was given; so, it must be assumed to be room temperature.) These authors also postulated the formation of copper–DNA complexes in several steps involving both phosphate groups and nitrogen bases.

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