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*INTERACTIONS OF METAL IONS WITH POLYNUCLEOTIDES AND RELATED COMPOUNDS, V. THE UNWINDING AND REWINDING OF DNA STRANDS UNDER THE INFLUENCE OF COPPER (II) IONS**

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The conditions under which the polynucleotides display ordered structures, on the one hand, and random orientations, on the other, have been a subject of interest since the discovery of the macromolecular nature of the naturally occurring nucleic acids. The influence of metal ions upon the conformation of DNA was realized early, when it became apparent that metal ions are involved in the stabilization of the Watson-Crick double helix.^{1, 2}

It has now been amply demonstrated that different metal ions, under a variety of conditions, are capable of producing quite different effects upon the structure of polynucleotides.³⁻⁷ Metal ions can degrade the ribose phosphate backbones by scission of the phosphate bonds.⁸⁻¹³ Mercury (II) ions can bind to the nucleoside bases in such a manner that the two chains become cross-linked;¹⁴⁻¹⁷ native double-stranded DNA can be regenerated from this structure by removal of mercury with complexing anions.^{15, 16}

A striking demonstration of the differences in the influence of various metal ions upon DNA is the discovery that different metal ions exert opposite effects on the T_m of DNA. Whereas most metal ions increase T_m , some of them decrease it, and among the latter category copper (II) is exceedingly effective.¹⁸ The increased T_m has been attributed to a stabilization of the DNA structure by metal binding to phosphate, and the decrease in T_m has been considered as resulting from coordination of the metal to electron donor groups on the nucleosides; such coordination would displace the hydrogen bonds that hold together the two strands of the DNA helix.^{18, 19}

The present investigations have shown that copper (II) causes the unwinding of the DNA helical structure, and that the unwound strands can be rewound into the native double-helical configuration; the evidence indicates that this recombination is total.

Materials and Methods.—Stock solutions of DNA, purchased from Sigma Chemical Company, were prepared by placing a weighed quantity of the material into an Erlenmeyer flask, and adding aliquots of 0.01 *M* NaNO₃ (prepared from sterilized water) at regular intervals with gentle shaking at 4°C. Solution was generally complete after five days. The stock solution was analyzed for DNA content by the method of Fredericq *et al.*²⁰

The melting curves were obtained by reading the absorbance and an appropriate blank at 260 μ at the ambient temperature on a model 2400 Beckman DU spectrophotometer with a model 2985 hydrogen lamp power supply. A Haacke Thermostat F (Gebrüder Haacke, K. G., Berlin) was connected to a pair of thermospacers adjacent to the DU cuvette compartment in order to control its temperature, which was determined by a thermometer whose bulb was immersed in a cuvette in the cell compartment. The optical rotation readings were taken at room temperature on a Rudolph photoelectric spectropolarimeter model 200S/80Q/650 manufactured by O. C. Rudolph & Sons, Inc., Caldwell, N. J. Sedimentation experiments were carried out in a Spinco model E ultracentrifuge at 42,040 rpm at 25°C.

Results and Discussion.—*Effect of concentration of copper (II) upon the T_m of DNA:* In the absence of copper (II), the T_m of the DNA under the experimental conditions used is 59°C.²¹ The addition of small amounts of copper (II)—less than 0.4 mole per mole of nucleotide—produces a slight increase in the value of T_m . It can be seen from Figure 1 that the T_m of DNA is drastically lowered by increasing concentrations of copper. There is a suggestion of a break at a molar ratio of 1.5 copper/nucleotide. This can be interpreted as resulting from a reaction of copper (II) with specific sites on the DNA molecule. The observed ratio of 1.5 is puzzling at first, but may reflect the ability of copper to bind both to phosphate and nucleoside bases. One copper bound to each phosphate and one additional copper bound to two nucleosides that were hydrogen-bonded would account for the apparent stoichiometry of the process. In view of the fact that some of the copper may not be bound at all, however, no definite conclusion about the stoichiometry of the reaction can be reached.

The fact that low copper (II) concentrations increase T_m probably means that the stabilization due to phosphate binding overcomes the destabilization due to base binding. At concentrations higher than 0.4 mole per mole of nucleotide the destabilization effect predominates, and it is with this effect that we shall be concerned during the remainder of this study.

Effect of electrolyte concentration: The experiments of Figure 1 were all conducted in the presence of 5×10^{-3} *M* sodium nitrate. This concentration of electrolyte is sufficient to stabilize the double-helical DNA structure.

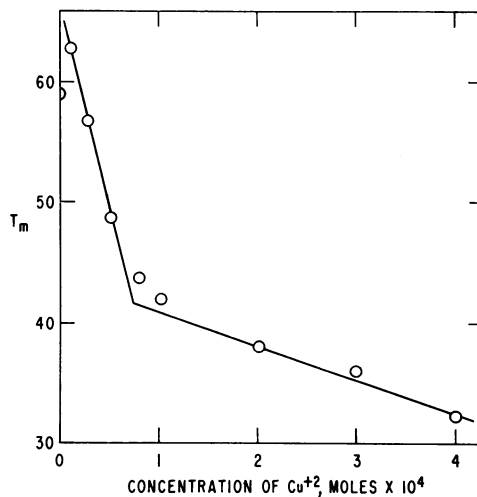


FIG. 1.—Variation of T_m with copper (II) nitrate concentration for 5×10^{-5} *M* (P) DNA in 5×10^{-3} *M* NaNO₃.

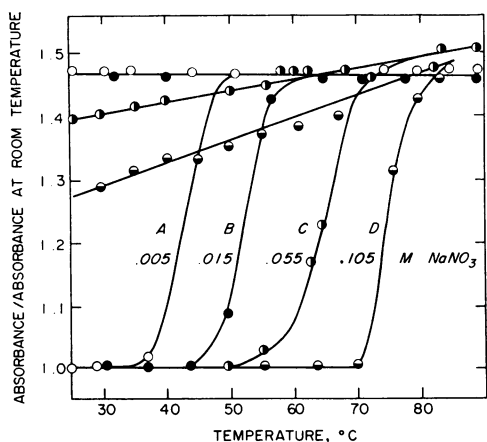


FIG. 2.—Dependence of the copper effect (T_m decrease) on electrolyte concentration. The samples contain $5 \times 10^{-5} M$ (P) DNA, $10^{-4} M$ $\text{Cu}(\text{NO}_3)_2$, and a sodium nitrate concentration of: \circ (A), $5 \times 10^{-3} M$; \bullet (B), $1.5 \times 10^{-2} M$; \circ (C), $5.5 \times 10^{-2} M$; \bullet (D), $0.105 M$.

Values of T_m of solutions similar to these, but containing no copper, are: A, 59° ; B, 67° ; C, 73° ; D, 79° .

of DNA strands that cannot be reversed by cooling.

The presence of 0.055 and 0.105 M sodium nitrate (in curves C and D, respectively) results in further increase in T_m . Moreover, at these electrolyte concentrations, a partial decrease in absorbance does occur on cooling and, in fact, the extent of this decrease increases with ionic strength (greater in D than in C). It is therefore apparent that an increase in the ionic strength of the solution counteracts the effect of the copper on T_m .

Reversal of the copper denaturation by addition of electrolyte: When a solution containing $10^{-4} M$ copper (II), $5 \times 10^{-3} M$ sodium nitrate, and $5 \times 10^{-5} M$ (P) DNA is heated to 50° and then cooled, the change in absorbance can be followed in Figure 2, curve A. If then enough solid sodium nitrate is added to the cooled solution to increase the NaNO_3 concentration to 0.105 M , the absorbance gradually decreases and reaches the value exhibited prior to heating, in 5 hr. If the solution with the added nitrate is then reheated and recooled, the absorbance changes now follow those recorded in Figure 2, curve D. Therefore, in spite of the fact that the copper binding cannot be reversed by cooling, the addition of sufficient electrolyte to the cooled solution apparently does result in the displacement of the copper, as manifested in the regeneration of a solution with the original absorbance and, presumably, the original native conformation.

This reversal is evaluated in greater detail in Figure 3. All of the solutions used for the data of Figure 3 were originally made up as in Figure 2, curve A, and taken through the heating and cooling cycle, again as in curve A. They were then treated with varying quantities of solid sodium nitrate so that the total NaNO_3 concentration reached the values indicated by the abscissa. The absorbance was then recorded again at intervals of 5 min, 1, 3, 5, and 24 hr after the addition. The reversal of the absorbance increase comes to completion in a period of 5 hr. The midpoint of the curve relating absorbance versus final NaNO_3 concentration occurs at a

The use of much higher concentrations of electrolyte tends to offset the effect of copper, as can be seen in Figure 2.²²

Curves A and B in the presence of only 0.005 and 0.015 M sodium nitrate, respectively, show that T_m is increased at the higher electrolyte concentration. Nevertheless, B still resembles A in that no decrease in absorbance is observed on cooling the solution to room temperature. The lack of any observable diminution in optical density upon cooling is in line with the interpretation that the copper (II) ions have displaced hydrogen bonds by the formation of coordination bonds, and that the latter prevent the reformation of the hydrogen bonds. Copper ions at low electrolyte concentration thus appear to bring about an unwinding

$3.5 \times 10^{-2} M$ concentration of electrolyte, and is taken as representing the point of equilibrium between denatured and renatured DNA, in the same manner as T_m represents such an equilibrium (obtained by going in the opposite direction). This electrolyte concentration can be called c_f , or freezing concentration, in analogy with the term T_m for melting temperature.

These results indicating that complete regeneration of double-helical native DNA can be made to follow the complete unwinding of the strands can be contrasted with other studies using denaturation agents different from Cu (II) in which complete renaturation cannot be accomplished with a heterogeneous preparation like calf thymus DNA. For example, when calf thymus and *Diplococcus pneumoniae* DNA were heat-denatured and then slowly cooled, both types of DNA regained 60–70 per cent of their original hyperchromicity.^{23, 24} However, when carried through the heating cycle again, neither sample exhibited the sharp transition in the melting curve characteristic of the native DNA of each type. An annealing procedure made possible a partial renaturation of the *D. pneumoniae* DNA, but was not successful with calf thymus DNA. The finding that denaturation of calf thymus DNA by copper (II) ions can be totally reversed is thus contrary to usual experience, and can be explained, perhaps, by DNA single strands that are held in register by the copper ions.

Evidence that the reversal reaction regenerates native DNA: The fact that the addition of a high concentration of electrolyte to a previously heated solution of DNA brings back the original ultraviolet absorption is an indication, but no proof, that the native configuration of DNA has been regenerated. The sharp transition in the melting curve of the "renatured" DNA on reheating probably constitutes a good confirmation of this hypothesis. Since it is important to know whether the "renatured" DNA is indeed totally renatured, other properties of the DNA were compared before and after denaturation as well as after renaturation.

Table 1 contains the results of the comparisons of absorption at $260 m\mu$, optical rotation at $436 m\mu$,²⁵ and the sedimentation velocity. It can be observed that all of these properties have changed drastically when native DNA has been heated in the presence of copper and recooled, and that all of the properties revert to their values in native DNA after the addition of solid sodium nitrate. The optical rotation values, though of a magnitude corresponding to the lower limit of precision of the instrument, are such that it is clear that, within the error range, the final rotation is the same as the initial rotation and quite different from the rotation after heating and before the addition of electrolyte. The sedimentation velocity of the copper-denatured DNA has a value so high as to suggest very extensive aggregation of the molecules. Such aggregation is consistent with a picture of copper not only

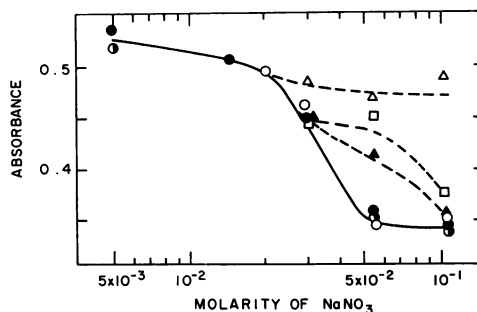


FIG. 3.—Reversal of copper effect (T_m decrease) with excess electrolyte. The initial samples, containing $5 \times 10^{-5} M$ (P) DNA, $10^{-4} M$ $\text{Cu}(\text{NO}_3)_2$, and $5 \times 10^{-3} M$ NaNO_3 , were heated to 50°C at a rate of 1° per min and allowed to cool to room temperature. Solid sodium nitrate was then added to give the concentrations indicated on the abscissa. The absorbance was measured after: Δ 5 min; \square 1 hr; \blacktriangle 3 hr; \circ 5 hr; \bullet 24 hr. The initial absorbance, as shown in Table 1, was 0.355.

binding the bases between complementary strands, but also holding together phosphate groups of different DNA chains in a possibly random fashion. The original sedimentation velocity is regained upon "renaturation," again in line with the hypothesis that native DNA is indeed reformed.

TABLE 1
PHYSICAL PROPERTIES OF THE NATIVE, DENATURED, AND RENATURED DNA

	Absorption	Optical Rotation (436)		Sedimentation velocity
Native DNA*	0.355	0.018	0.024	29.3
DNA, heated† and recooled	0.53	0.003	0.007	175, 182
DNA heated,‡ recooled, and electrolyte added	0.363	0.025	0.022	29.9, 22.9

* 5×10^{-5} M (P) DNA, 10^{-4} M $\text{Cu}(\text{NO}_3)_2$, 5×10^{-3} M NaNO_3 in water solution.

† Above, heated to 67°C at the rate of 1°C per min and recooled at the same rate.

‡ Above, with solid NaNO_3 added to make a final NaNO_3 concentration of 0.105 M, upon standing 5 hr.

Finally, the native and "renatured" DNA were subjected to density gradient sedimentation in cesium chloride and compared with heat-denatured DNA, as shown in Figure 4. No such experiment was possible with the copper-denatured intermediate, since contact with a high concentration of cesium chloride would, of course, bring about renaturation. The "renatured" DNA sedimented at the same density as the native DNA, and, when sedimented along with native DNA, produced one peak, with no trace of a peak corresponding to denatured DNA.

The evidence appears therefore quite good that the two strands of DNA are completely unwound by heating with copper (II), and that the strands are totally rewound upon the addition of electrolyte to the denatured solution.

The effect of pH: The experiments reported here were conducted generally in the absence of buffer, because it was desired to avoid the complications that buffers could introduce into the system. The question may be raised, however, whether some of the effects attributed to copper (II) are not due to the protons that are released through the hydrolysis of the copper ions.

That such is not the case can be seen from the following considerations.

The initial pH of the solution used in Figure 2, curve A, is 5.7. Its pH after heating and cooling is 6.1 (the increase may be due to loss of CO_2) and remains at this level during the renaturation. The pH of a DNA solution similar to that of Figure 2, curve A, but containing no copper, is initially 6.1 and rises to 6.8 after heating and cooling. The work of Cavalieri *et al.*²⁶ would suggest that the pH difference between the solutions with and without copper would account for very little change in the conformation of DNA at room temperature. In order to discover whether such a change might occur at elevated temperature, a solution of DNA similar to that of Curve A, but containing no copper, was adjusted to pH 5.7. The T_m of this solution decreased 0.5° (instead of the 17° of the solution in curve A), and the lack of absorption decrease on cooling, noted in the experiment with copper, was not observed. The pH change introduced by the copper ions thus does not account for the copper denaturation.

Studies were also carried out in the presence of cacodylate buffer, which maintained the solutions at pH 6.2. The same difference in T_m between the solutions with and without copper was observed as in the unbuffered solutions. There was, however, a 5 per cent (of the maximum possible) decrease in absorbance on cooling

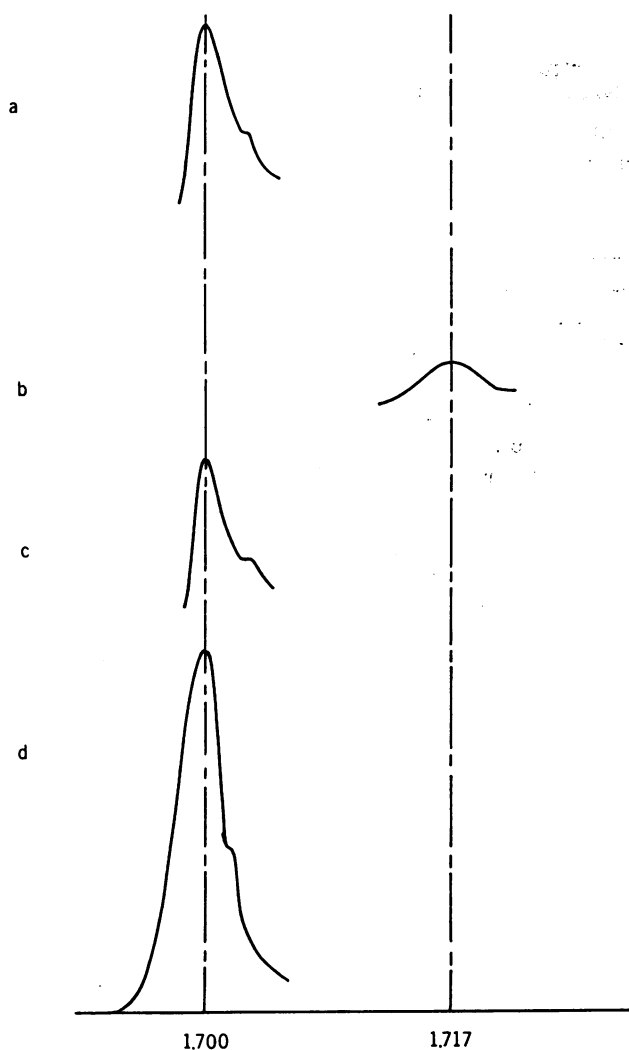


FIG. 4.—Density gradient patterns of $5 \times 10^{-5} M$ (P) calf thymus DNA in $5 \times 10^{-3} M$ sodium nitrate. The gradient was produced by adding cesium chloride to give an approximate density of 1.7. DNA from *Pseudomonas aeruginosa*, density = 1.727 (not shown in figs.), was used as a standard. (a) Native DNA in the presence of $10^{-4} M$ $\text{Cu}(\text{NO}_3)_2$ (vs. standard); (b) DNA denatured by slow heating to 85°C , after rapid cooling to room temperature (vs. standard); (c) DNA in $10^{-4} M$ $\text{Cu}(\text{NO}_3)_2$ heated to 60°C slowly, kept at this temperature for 10 min, cooled to room temperature, and made $0.105 M$ in NaNO_3 (vs. standard); (d) a mixture of (c) and native DNA (no standard). Curve b is not on the same density scale as the other three curves; the shoulder in curves a, b, and c occurs at a density of 1.713.

and a 10 per cent loss in the regeneration of the initial absorbance upon renaturation with sodium nitrate. Density gradient runs carried out as in Figure 4 nevertheless gave values of 1.701 for both native and renatured DNA, and no second peak was observed along with the renatured peak.

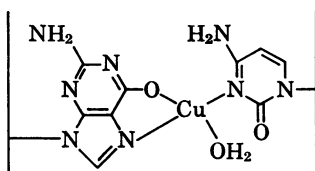
We cannot explain the minor discrepancies observed in the presence of cacodylate, but assume that interaction with either copper or DNA is involved. At any rate,

the experiments are qualitatively the same as in the unbuffered solutions. The fact that DNA adjusted to the same pH as that of the copper—DNA solution shows none of the behavior exhibited in the presence of copper—would seem to prove that copper ions, and not protons, are responsible for the observations made on the unbuffered solutions.

Conclusions.—These results can be explained if the copper (II) nucleoside coordination bonds are of the same order of stability as the DNA double helix. Evidently the stability of the copper (II) nucleoside bond is not great enough to overcome the attractive forces between the two DNA strands at room temperature. When the temperature is raised, however, and the DNA structure is weakened, the copper complex becomes relatively strong enough to compete effectively. At low ionic strength the stability of the double helix is not sufficiently greater than that of the copper complex to allow reformation of native DNA at an appreciable rate. A large increase in ionic strength, however, increases the stability of the double helix relative to the copper complex to such an extent that the native structure is reformed.

The structure of the copper (II)-denatured DNA is a matter of some importance and, unfortunately, cannot be completely understood on the basis of the present investigations. It has been concluded from a consideration of the absorbance and the optical rotation of the denatured product that the two strands have been unwound. On the other hand, the sedimentation data indicate that the denatured DNA is extremely highly aggregated. The apparently complete renaturation accompanying the addition of electrolyte is best explained by assuming that the copper ions bound to the bases somehow keep the bases in register, even in the denatured state.

The picture of the copper-denatured state that emerges from all of these considerations is one of copper (II) ions interposed between complementary strands by coordination to the bases in such a manner that all of the hydrogen bonds are broken and the secondary structure of each strand is completely destroyed. Perhaps the structure involves copper (II) bound as follows:



Similar structures have been proposed in the past for the copper complexes of nucleotides.²⁷

A structure of this type represents a separation of the complementary strands of DNA, but the term "strand separation" is frequently based upon the criterion that the individual strands are randomly distributed through the solution.²⁸ In order to avoid semantic confusion, we have referred to the copper-denatured state as one in which the helices are unwound, rather than one in which the strands have been separated.

The unwinding and rewinding of the DNA double helix is a process of presumed importance during the replication of DNA in a living system. In the absence of

any evidence, however, that copper is involved in this replicative process, the applicability of the present studies to the natural phenomenon remains in question.

The reaction of mercury (II) with DNA bears considerable similarity to the copper (II) DNA system, but much of the secondary structure remains in the denatured form. The mechanism of the reversal must be quite different, however, since mercury complex is formed in the presence of a high electrolyte concentration and decomposed by competing ligands without change in the ionic strength.^{15,16}

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