Interactions of Metal Ions with Polynucleotides and Related Compounds. XI. The Reversible Unwinding and Rewinding of Deoxyribonucleic Acid by Zinc(II) Ions through Temperature Manipulation*

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ABSTRACT: It has been previously shown that, in the presence of copper(II) ions, the double-stranded form of deoxyribonucleic acid (DNA) is destroyed by heating at low ionic strength and restored after cooling at high ionic strength. We have now discovered that zinc ions can be used to reversibly unwind and rewind double-helical DNA by heating and cooling, respectively, and that this process can be carried out repeatedly, resulting always, during the rewinding step, in the renaturation of the DNA. We can explain this behavior of zinc by its ability to hold the two chains in close proximity by binding to the bases during denaturation, as does copper (though zinc binds the bases less strongly), but that zinc, unlike copper, binds phosphate strongly enough that at low temperature the zinc itself produces the same effect as high ionic strength in the copper reaction. This reaction makes it possible for the first time to go back and forth between native and denatured DNA, without external interference, simply by changing the temperature of the medium.

The conditions under which the DNA double helix is unwound into single coils and the rewinding of the latter into a double helix have been the subject of many investigations (Marmur *et al.*, 1963), since the equilibrium between the double- and single-stranded forms of DNA is of some importance in biological replication and transcription. Partially heat-denatured bacterial or viral DNA has been readily renatured by careful cooling (Marmur and Doty, 1961). Reversibility has also been achieved by the treatment of DNA with alcohol or perchlorate (Geiduschek and Herskovits, 1961; Hamaguchi and Geiduschek, 1962; Geiduschek, 1962). Totally reversible denaturation has been obtained by the reaction of DNA with cross-linking agents such as sodium nitrite and nitrogen mustard (Geiduschek, 1961; Kohn *et al.*, 1966). It has recently been found that copper ions can bring about the total reversibility of the denaturation of a very heterogeneous DNA preparation such as that obtained from calf thymus (Eichhorn and Clark, 1965; Hiai, 1965).

DNA can be unwound at low ionic strength by heating with copper(II) and subsequently rewound by cooling and then adding solid electrolyte. This effect is explained by copper ions binding to a few of the bases and holding the chains close together during unwinding so that the bases can readily re-form complementary pairs and rewinding can occur when the conditions are again favorable for the latter.

In their reactions with polyribonucleotides zinc(II) ions have been noted for their ability to break phosphodiester linkages as a consequence of binding to phosphate (Butzow and Eichhorn, 1965). On the other hand, polydeoxyribonucleotides are not degraded in this way. The present communication reveals that zinc(II) ions also bind to the nucleoside bases, and as a consequence of this binding produce a remarkable reversible unwinding–rewinding reaction with DNA, dependent solely on changes in the temperature of the solution.

Results and Discussion

Heating and Cooling Curves of Zinc(II)–DNA Solutions. Figure 1A represents the well-known heating and cooling curves of DNA at low ionic strength. The melting temperature (T_m) is 63°, and the transition is relatively noncooperative. Cooling of the denatured DNA produces a slight decrease in absorbance due to randomized restacking.

Figure 1F shows what happens to DNA under the same conditions in the presence of 2 moles of zinc(II)/mole of DNA(P). The original T_m is increased to 68.5°, and the transition is much more cooperative. The most remarkable feature of Figure 1F is that on cooling the absorbance virtually returns to the absorbance prior to heating, and reheating the cooled solution results in a heating curve very similar to the heating curve of the original solution.

The implication of Figure 1F is that the DNA is unwound during the heating process, and practically completely rewound during the cooling process. Presumably zinc ions hold the DNA chains close together in the unwound form in the same manner as copper ions. Thus zinc ions must also bind to the bases. The

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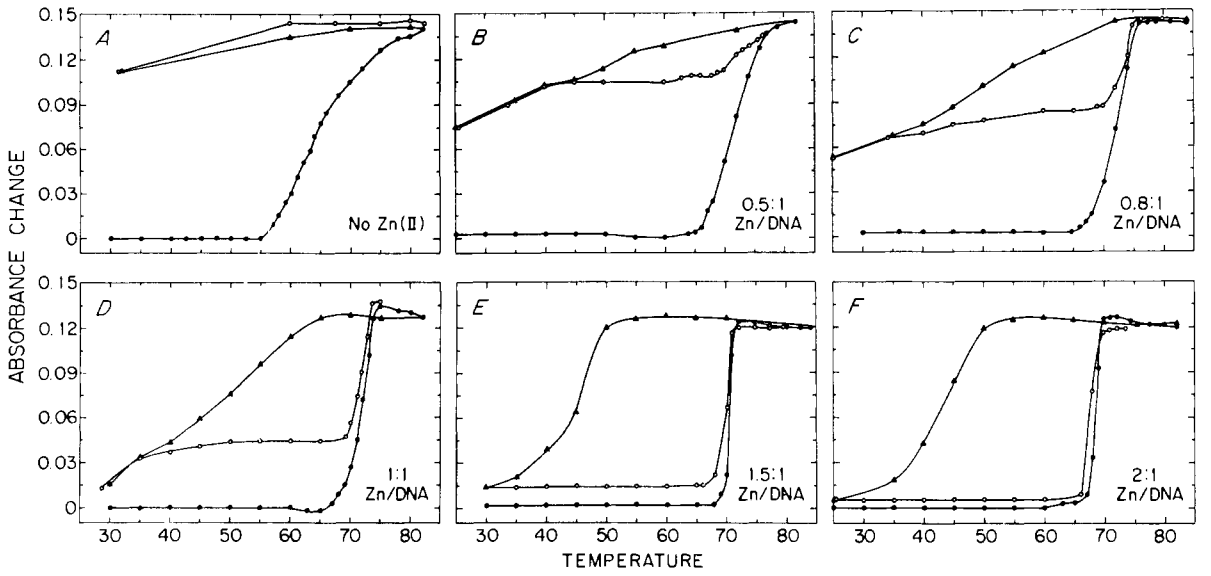

FIGURE 1: Melting behavior of zinc(II)–DNA solutions. (●) First heading, (▲) cooling, and (○) reheating. All solutions contained 5×10^{-5} M (P)DNA, 5×10^{-3} M sodium nitrate, and zinc(II) nitrate in the mole ratios to DNA shown. Absorbances measured at 260 mμ. Initial absorbances at 25° prior to heating were (A) 0.367, (B) 0.363, (C) 0.367, (D) 0.367, (E) 0.366, and (F) 0.368.

rewinding of the DNA strands on cooling is in great contrast to the total lack of any hypochromicity in the cooling of copper–DNA solutions. The zinc–nucleoside bonds are presumably much weaker than the copper–nucleoside bonds, allowing the zinc ions to be displaced by cooling.

Additional insight into the reversibility of the zinc–DNA reaction can be gained by a comparison of the curves obtained at various zinc:DNA ratios, as shown in Figure 1A–F, and in Table I. It will be noted that the T_m of DNA in the presence of zinc is always greater than in its absence. This increase in T_m is related to the stabilization of DNA by zinc ions binding to phosphate groups. However, T_m does not continue to rise with increasing increments of zinc. In fact, the maximum T_m is reached at 1:1 zinc:DNA(P) ratio. The existence of such a maximum T_m indicates that zinc binds to DNA in two different ways. Low concentrations of zinc bind primarily to phosphate, raising T_m. High concentrations of zinc permit a second reaction with the nucleoside bases. It should be noted that even at the highest zinc(II) concentrations used, the T_m is still higher than the T_m of DNA in the absence of zinc. Copper ions greatly decrease the T_m of DNA. Thus, whereas both copper and zinc ions bind both to phosphate and nucleoside bases, the ratio of affinities for phosphate to base is much greater for zinc than for copper.

TABLE I: Melting Behavior of DNA and Zinc (II).[a]

Mole Ratio Zn:DNA (P)	T_m (°C)	ΔT (°C)[b]	$\frac{-\Delta A \text{ (cooling)}}{\Delta A \text{ (heating)}}$
0	63.0	23	0.28
0.5	71.5	12.5	0.48
0.8	72.0	10	0.64
1.0	72.0	9	0.90
1.5	70.5	4	0.96
2.0	68.5	4	0.96
3.0	66.3	3.5	0.97
4.0	64.5	4	1.00

[a] All absorbances measured at 260 mμ. [b] Temperature span between initial rise and final leveling of the absorbance.

The unwinding of DNA by copper (and heat) cannot be reversed by cooling, but requires the addition of added salt. The unwinding of DNA by zinc can be reversed by cooling without addition, presumably because the zinc ions binding to phosphate sites can perform the same function as added electrolyte in the copper reaction.

Observation of Figure 1B–E shows that lower quantities of zinc than in Figure 1F result in partial rewinding of DNA on cooling, the extent of the rewinding being related, as expected, to the zinc concentration. Table I shows that the rewinding, as measured by hypochromicity, becomes complete at a zinc:DNA(P) ratio of 4:1. It will be observed also that the cooperativeness of the melting transition increases with increasing zinc concentration, as shown in the ΔT column of Table I.

Figure 1C,D is particularly instructive in demonstrating partial reversibility of the unwinding in the presence of less than optimal quantities of zinc. When the solutions are cooled after the initial heating, the absorbance decreases considerably more than in the absence of zinc, but it does not reach the absorbance of native DNA. When the cooled solution is reheated, the reheating curve follows the cooling curve for a short temperature range, then reaches a plateau that is maintained until the temperature reaches approximately the

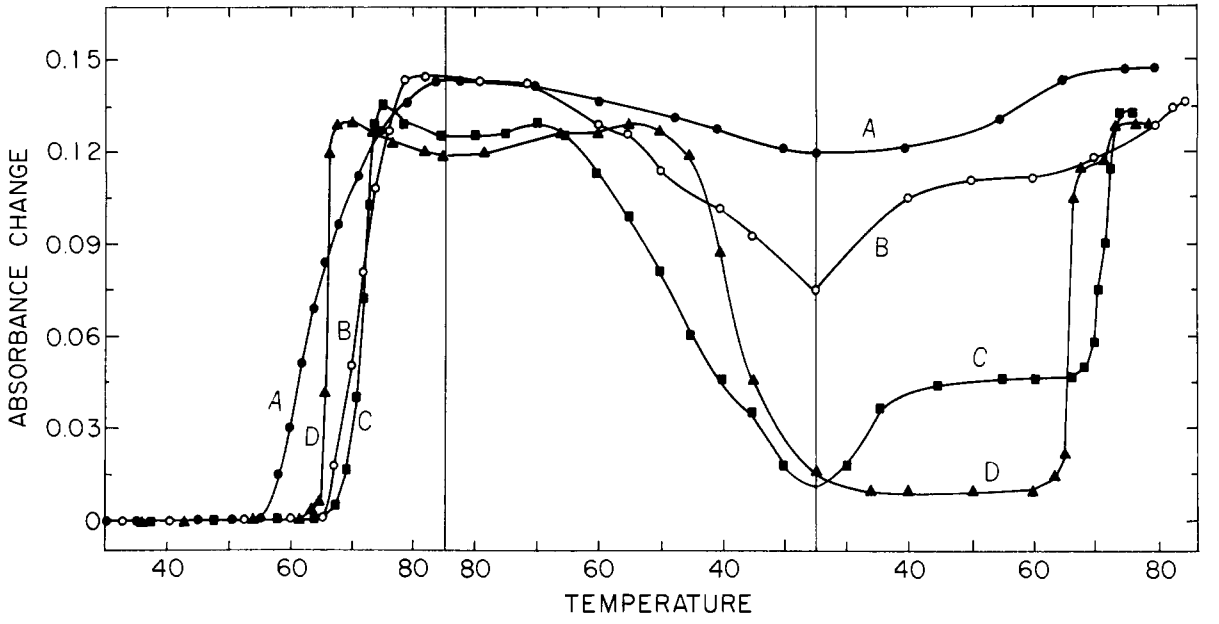

FIGURE 2: Heating–cooling–reheating profile of zinc(II)–DNA solutions, containing 5×10^{-5} M (P)DNA, 5×10^{-3} M sodium nitrate, and zinc(II) nitrate in the following mole ratios to DNA: A (●) 0, B (○) 0.5, C (■) 1, and D (▲) 2. This figure is constructed from the same data as Figure 1.

T_m value, and then the absorbance rises again to the upper limit. The two-step reheating curve can be explained as follows. The initial rise in absorbance is due to the dissociation of randomly formed stacks of nucleotides that are not part of a double helix. After these randomly formed stacks have been melted out, the absorbance remains constant until the re-formed double helix is no longer stable. In Figure 1E,F practically all of the DNA has regenerated the double helix on cooling, so that the first step in Figure 1C,D (as well as in Figure 1B) is no longer observed. In Figure 1A, of course, only this first step, but no second step, can be seen.

It is in fact this phenomenon of going through a two-step reheating process in Figure 1B–D which makes the single-step conversion in Figure 1E,F important evidence for the rewinding of DNA on cooling in the presence of excess zinc. The reversion to practically the initial absorbance, as in Figure 1D, does not prove total re-formation of double helix, but indicates only the re-formation of stacked molecules which can have a variety of shapes. The increase in absorbance in Figure 1D between 30 and 35° indicates, indeed, that the portion of DNA characterized by this absorbance change is not double helical. The T_m of the remainder of the DNA indicates that this remainder *is* double helical. When all of the DNA melts out at approximately the same T_m in the second heating as in the first, it can be assumed that essentially all of the DNA must have assumed a double-helical configuration during the cooling process.

Figure 2 demonstrates in a striking manner how the addition of zinc ions affects the heating–cooling–reheating cycle of DNA. In the absence of zinc (Figure 2A) the DNA absorbance rises in the first heating cycle, and stays at an elevated level through the succeeding cycles, with only a minor absorbance decrease on cooling. This relative constancy of the absorbance during cooling and reheating may be contrasted with the absorbance decrease on cooling and the increases on heating as a consequence of the addition of zinc (especially in Figure 2C,D).

In order for the renaturation to occur, zinc ions must be present prior to the heating step. If the DNA solution is heated to 85° without zinc, and zinc (in 1:1, 1:2, or 1:3 DNA(P):Zn ratio) is then added at the elevated temperature and the solution subsequently cooled, the reheating curve is a broad curve (like that in Figure 1A, except that the absorbance after the initial cooling is lower). This behavior is exactly in line with what one would expect if the renaturation does indeed result from zinc ions holding DNA chains together in a type of cross-linking phenomenon.

Spectra and Sedimentation Velocities of Zinc(II)–DNA Reaction Mixtures. The addition of zinc has no effect on the spectrum of DNA at room temperature. The absorbance maximum at 258 mμ as well as the extinction coefficient remain undisturbed. Heating DNA in the absence of divalent metal (Figure 3C) gives hyperchromicity with no effect on the absorption maximum. The presence of zinc during the heating process shifts the absorption maximum of heated DNA approximately 3 mμ (Figure 3B). The ultraviolet absorption spectra thus confirm the ability of zinc to react with the DNA bases. The spectrum of denatured DNA in the presence of zinc(II) (Figure 3B) resembles that in the presence of copper(II) (Eichhorn *et al.*, 1966), indicating that zinc perhaps binds DNA in a manner similar to that of copper.

Just as heating DNA with zinc results in a displacement of the absorption maximum, the cooling of this

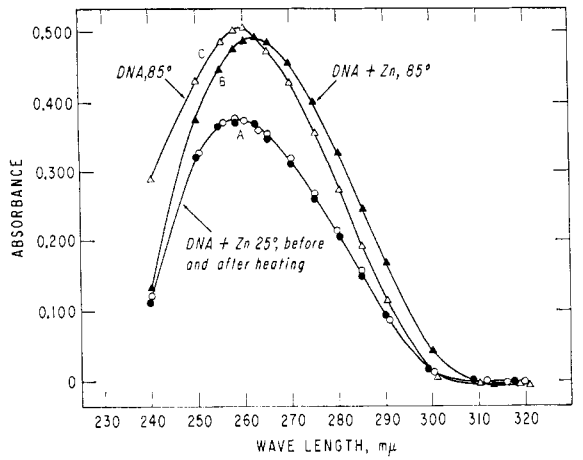

FIGURE 3: Spectra of zinc(II)–DNA solutions, containing 5×10^{-5} M (P)DNA and 5×10^{-3} M sodium nitrate. A (○), with 1.5×10^{-4} M zinc(II) nitrate, 25°, before heating; (●) same as (○), after heating and cooling; B (▲), same at 85°; C (△), without zinc(II), 85°.

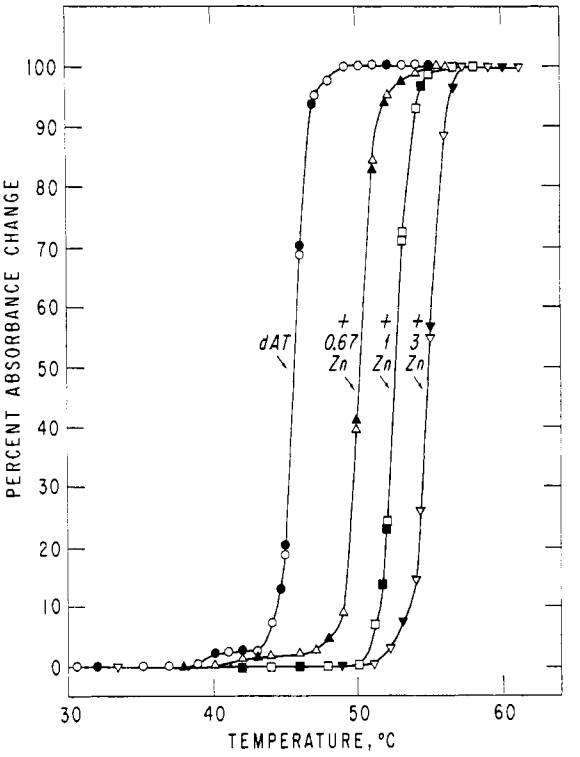

FIGURE 4: Melting behavior of zinc(II)–dAT copolymer. All solutions contain 2.5×10^{-5} M (P)dAT, 5×10^{-3} M $NaNO_3$, and (●○) 0, (△▲) 0.67, (□■) 1, and (▽▼) 3 moles of zinc per mole of dAT(P). Open symbols represent heating curves and closed symbols represent cooling curves.

solution returns the spectrum to what it was before the heating process. This is what is to be expected if the cooled solution contains DNA that is essentially similar in structure to the DNA prior to heating. The spectra thus are in line with the conclusion reached from the melting experiments.

Similarly, the sedimentation velocity of the zinc(II) DNA solution remains virtually unchanged when the data are compared before and after heating (as in the experiments of Figure 1). s_{20} before heating gave 19.8 and 20.2 S; s_{20} after heating gave 21.0 and 19.9 S.

Heating and Cooling Curves of Zinc(II) dAT Solutions. dAT is a synthetic polymer resembling double-stranded DNA in structure, but containing only adenine and thymine, and no guanine and cytosine. Unlike DNA, it has a regular sequence of alternating bases. Thus the cooling curve of dAT is superimposable upon the heating curve (Inman and Baldwin, 1962), in the absence of added metal ions, since the regular sequence enables the re-formation of the hydrogen bonds between complementary bases simply by random recombination, without the necessity of being properly lined up as is required by the irregular base sequence of DNA. This is shown in Figure 4. The addition of zinc to dAT increases its melting temperature, just as in the case of DNA, but the T_m of dAT, unlike that of DNA, continues to rise with increasing zinc concentration; *i.e.*, no maximum T_m is observed at a 1:1 ratio of zinc to dAT. Such an effect is to be expected from zinc ions binding to phosphate, and there is therefore no evidence from the melting experiments that zinc binds to the bases in dAT. A comparison of the effect of zinc concentration on T_m of DNA and dAT could lead to one of two possible conclusions. The first is that zinc(II) binds to the bases primarily in the form of "sandwich" type complexes by π bonding to two adjacent purines (or two adjacent pyrimidines) in the same chain. DNA contains such adjacent purines (and pyrimidines), whereas dAT copolymer does not. Alternatively, zinc ions could prefer binding to either guanine or cytosine, or both. Possibly these two phenomena contribute to the difference in the behavior of DNA and dAT. The "sandwich" effect alone is unlikely to account for this difference, since there is no particular reason why such an effect could not be produced by purine–zinc–pyrimidine sandwiches. Also, sandwiching alone could not account for the renaturation of DNA in the presence of zinc; some "cross-linking" type of base binding must occur to bring about the renaturation. Experiments in our laboratory have shown that zinc has a rather low tendency to cleave guanylic acid bonds in the degradation of RNA molecules by zinc, indicating preferential binding to guanine bases (G. L. Eichhorn, J. J. Butzow, and E. Tarien, unpublished data). We have also shown that the metal ions of the first transition series exhibit qualitatively similar behavior in their interactions with DNA (G. L. Eichhorn and Y. A. Shin, unpublished data), and evidence has been obtained that copper(II) binds preferentially to GC pairs rather than AT pairs (Hiai, 1965). Copper(II) binds guanosine, adenosine, and cytidine, in that order, and does not bind to thymidine (Fiskin and Beer, 1965; Eichhorn *et al.*, 1966). For all of these reasons we believe that the difference between the reaction of zinc with dAT and DNA is due at least in part to the presence of guanosine in the latter and not the former.

Spectra of Zinc(II) dAT Solutions. Figure 5 shows that a heated solution of Zinc(II) dAT gives hyperchromicity, but unlike zinc(II) DNA, no shift in absorption maximum. We therefore attribute the absorp-

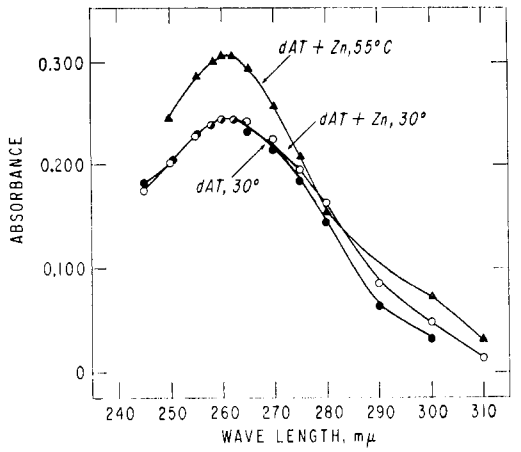

FIGURE 5: Spectra of zinc(II)–dAT solutions, containing 2.5×10^{-5} M (P)dAT and 5×10^{-3} M $NaNO_3$. (●) No zinc, 30°; (○) with 7.5×10^{-5} M zinc nitrate, 30°; (▲) with 7.5×10^{-5} M zinc nitrate, 55°.

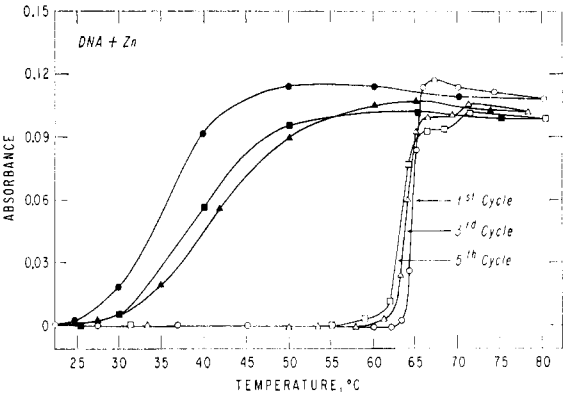

FIGURE 6: Repeated heating and cooling of a solution containing 5×10^{-5} M (P)DNA, 2×10^{-4} M zinc nitrate, and 5×10^{-3} M $NaNO_3$. (○) First heating, (●) first cooling, (△) third heating, (▲) third cooling, (□) fifth heating, and (■) fifth cooling. The second and fourth cycles were omitted for clarity.

tion maximum exhibited in Figure 3 at least in part to binding of zinc to guanosine. It should be pointed out, however, that in the absence of the considerations of the preceding section, this maximum shift could also be explained on the basis of purine–purine (or pyrimidine–pyrimidine) sequences that do not exist in the dAT copolymer.

Continuously Reversible Unwinding and Rewinding. The results cited up to this point show that zinc ions do indeed bring about reversible unwinding and rewinding of DNA by variation in the temperature of the solution. It is of some interest to know whether it is possible to go back and forth between unwound and rewound states indefinitely. Figure 6 exhibits the results of heating and cooling successively for five cycles, and it is apparent that the reversibility is still present in the fifth cycle. We therefore conclude that unwinding and rewinding can be prolonged through probably numerous cycles, making the zinc–DNA system a useful one for the investigations requiring such a simple reversible system.

Subtleties in the Heating and Cooling Curves of Zinc(II)–DNA. So far in this presentation we have concerned ourselves with the obvious characteristics of the repeated heating and cooling of zinc(II)–DNA solutions, indicating a reversible unwinding and rewinding phenomenon. Close inspection of the various heating and cooling curves reveals that more than just two species are involved in the process. Returning to Figure 6, we can see that, even though T_m is about the same in each heating cycle, it decreases slightly (but reproducibly) from cycle to cycle. Moreover, the behavior of the heating curves at the elevated temperatures, *i.e.*, after the unwinding, also varies from cycle to cycle.

These variations can be most readily examined with reference to Figure 2D. During the initial heating the absorbance reaches a maximum at 67° and then gradually decreases again on continued heating to 85°. Obviously there must be at least two species of unwound DNA in the presence of zinc at elevated temperature. On cooling from 85° to room temperature the absorbance first goes back up, reaching another maximum at 50°, and then returns to the absorbance of the DNA prior to heating. During the second heating the plateau at 67° is followed by another plateau upon continued heating.

It is not possible, of course, to determine from this evidence the nature of the intermediates produced, and it would be difficult, and possibly of questionable value, to obtain definitive evidence for the structure of the intermediates. The complexity of the reaction does, however, provide some clues to its mechanism and it is worth examining these clues for whatever indications they may provide.

Since the heating of RNA and other polyribonucleotides with zinc(II) and other metal ions results in the degradation of the polynucleotide chains, it should be emphasized that DNA is not so affected. This difference between DNA and RNA has been reported for the reaction with lanthanum(III) ions (Bamann *et al.*, 1954; Eichhorn and Butzow, 1965), and the implications have been frequently discussed (*e.g.*, Eichhorn, 1966). Similar studies were carried out specifically with zinc(II) ions, but have not been previously reported (Butzow and Eichhorn, 1965). We have heated a solution containing 10^{-4} M (P)DNA and 2×10^{-4} M zinc(II) nitrate, in 1×10^{-2} M sodium nitrate, for 100 hr at 64°, with no formation of uranyltrichloroacetic acid soluble fragments whatever. Thus, these absorbance changes are not explained by complicating depolymerization reactions, as the sedimentation studies also indicate. Degradation could not really account for these absorbance changes anyway; for example, it would not result in the observed lowering in absorbance between 67 and 85° in Figure 2D.

Speculations on the Nature of the Intermediates. The absorbance changes that can be traced in Figure 2D could indicate the presence of seven different species, characterized as follows: (I) low initial absorbance, (II) maximum at 67° during first heating, (III) minimum at 85°, (IV) maximum at 50° during cooling, (V) minimum at room temperature on cooling, (VI) plateau at

67° on reheating, and (VII) final plateau above 70° on reheating.

Species I is of course native double-helical DNA. Species II and III are unwound DNA held together by some zinc(II) ions (they must be, otherwise rewinding could not occur). We do not know the nature of the zinc(II)–DNA complexes II and III, except that they differ. Perhaps II contains zinc bound to electron donor atoms on the bases and III has zinc bound to π electrons between bases (a sandwich-type complex). Perhaps IV is the same as II (the absorbance indicates this). The absorbance of V is identical with that of I, indicating re-formation of native DNA. However, V cannot be *exactly* the same as I, because heating V produces a somewhat different pattern than heating I. We suggest that a small amount of zinc remains incorporated in species V, not enough to affect the spectrum, but enough to affect the course of events on reheating. The presence of such a small amount of zinc in the rewound DNA could account for the slight decrease in T_m during the reheating. Plateau VI may result from the unwinding of all the DNA that did *not* contain zinc, and VII may be similar to III (as its absorbance would indicate). According to this scheme the differences between the various heating curves in the succeeding cycles of Figure 6 (progressively slightly lower T_m, lower absorbance of plateau VI) can be accounted for by increasing amounts of zinc retained in the regenerated double helix after each cooling cycle.

Although the speculations in this section are not proved, they do explain the observations. In our view the only difference in the rewound DNAs produced in the various cycles of Figure 6 is the retention of different quantities of zinc, but never enough to affect the DNA spectrum. The incorporation of such small quantities of zinc is in line with the idea that the presence of the zinc holds a few bases "in register" in the unwound form (Geiduschek, 1962), thus preventing random coil formation and making the rewinding possible.

Conclusions. The ability of zinc(II) ions to promote the temperature-dependent unwinding and rewinding of DNA can be attributed to the unique binding characteristics of this metal ion to coordinating sites on the DNA molecule. The zinc ions bind quite weakly to the bases, but strongly enough nevertheless to keep the two DNA strands together in the denatured state. Zinc ions bind strongly enough to phosphate to stabilize the double helix at the lower temperatures.

It is of interest to consider the question as to whether the complementary bases in the zinc denatured DNA are "in register." We have refrained from using this term throughout most of this paper (on the advice of a referee), since there appears to be some confusion as to just what it means. It is frequently used in the sense that, if complementary bases are "in register" in denatured DNA, they will re-form all possible hydrogen bonds when the conditions for double-helix formation are more favorable. In this sense the bases in zinc denatured DNA are in register. On the other hand, the term is also used to imply that a cross-linking agent holds together at least one pair of complementary bases in the denatured state. Thus, favorable conditions for the double helix will bring about a zipping up in both directions from the cross-link (Geiduschek, 1962).

It is conceivable that the bases cross-linked by zinc ions are not complementary bases. The zinc ion bound to a base on one chain may be bound to a base some distance apart from its complement on the other chain; *i.e.*, the two chains may have slipped during the cross-linking process. If the slippage is not too great, it is apparent that the slippage can be readily reversed and result in total re-formation of native DNA. The function of the zinc ions would then be to hold the chains together, although the bases would not be in register according to the more strict definition of the term. There is, in fact, a good possibility that the zinc ions would tend to find the bases on both strands to which they are most readily bound; we would suppose that guanine–zinc–guanine links could be highly favored. The fact that the cooling curves and the heating curves are not superimposable may be given as evidence for slippage, and possibly extensive slippage, but this nonsuperimposability can also be explained by the variety of zinc complexes that appear to form during the course of this reaction. Thus we do not know whether or not the bases are in register, in the denatured DNA, according to the restricted definition of the term.

One important difference between the zinc cross-linked DNA and the "reversible" DNA of Geiduschek (1961) and others, which contains small organic molecules as the cross-linking agent, is that the latter cross-links are much more stable than the former. Whereas one cross-link may be all that is required to keep two chains together with an organic cross-link, the stability of the zinc–DNA complex is so low that a number of metal ions are undoubtedly required. The equilibrium between the zinc(II)–DNA complex and its components would provide for continuous dissociation and re-association of the complex.

The ability of zinc to degrade RNA but not DNA at neutral pH has already received application. The ability of zinc to reversibly unwind and rewind DNA should find application in systems in which such reversibility is desired.

Experimental Section

The DNA was Sigma type I calf thymus sodium salt. The dAT was obtained from Biopolymers, Inc. as a solution in Tris buffer. Other chemicals were reagent grade.

The DNA stock solution was prepared as previously, by gentle shaking over a 4-day period (Eichhorn and Clark, 1965). The dAT stock solution was prepared from the commercial material by dialyzing for 3 days *vs.* 5×10^{-3} M $NaNO_3$ to replace the buffer (undesirable in the experiments with metals) with the desired electrolyte concentration.

Heating and cooling curves were obtained with the Gilford Model 2000 recording spectrophotometer equipped for automatically timed measurement of absorbance and temperature, a synchronous motor

for regulated temperature change manufactured by Hurst, and a Haake Model F circulating water bath. The appropriate amounts of DNA and metal ion solutions were mixed immediately before placement into glass-stoppered cuvets fitted with vacuum grease.

Sodium nitrate solution (5×10^{-3} M) was used as a blank. The samples were heated at a constant rate of 2.5 min/deg and cooled at 1 min/deg. (The slow cooling rate was not a requirement in the reversibility phenomenon; the "renaturation" was also observed after reheating quenched zinc(II)–DNA solutions.)

The spectra were observed by manual manipulation of the Gilford spectrophotometer, so that they could be readily compared at various temperatures. Room temperature spectra were checked against spectra obtained with a Cary Model 14 spectrophotometer.

Sedimentation velocities were carried out using solutions containing 5×10^{-5} M (P)DNA, 10^{-4} M zinc nitrate, and 5×10^{-3} M sodium nitrate, and heated and cooled in the Gilford spectrophotometer, as above. The experiments were performed in a Spinco Model E ultracentrifuge using ultraviolet optics and cells with KEL-F center pieces.

The pH of the solutions used in all melting and spectrophotometric experiments was obtained with a Radiometer Model 25 pH meter. In all cases the pH was between 6.3 and 6.5.

Acknowledgments

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