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# Millimolar concentrations of zinc and other metal cations cause sedimentation of DNA

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## ABSTRACT

**We demonstrate that DNA sediments in the presence of millimolar concentrations of zinc or related metal cations and that EDTA entirely dissolves the sediment. The sedimentation is promoted by alkaline pH but the pH dependence is abolished by submillimolar concentrations of phosphate anions. We suspect that the metal cations generate sedimenting particles of insoluble hydroxides or phosphates for which DNA has a strong affinity. The events involved in DNA–metal phosphate co-sedimentation are similar to the processes that enable calcium phosphate-assisted transfection. Hence, work with even submillimolar concentrations of zinc and most other metal cations, which many DNA-binding proteins need for their activities, requires care to avoid the sedimentation of DNA. Literature reporting about zinc effects on DNA is discussed from the point of view of the present results.**

## INTRODUCTION

DNA is a polyanion under physiological conditions whose behavior crucially depends on the presence of positively charged species (monovalent and divalent cations, polyamines, histones and other basic polypeptides) in solution. For example, they modify B-form DNA (1,2), induce or enhance DNA bending (3,4), generate $K^+$-dependent barriers to replication (5,6), switch dinucleotide repeat DNAs into non-B conformers (7–10) and induce various other effects (11,12) which can influence the replication, transcription, recombination and other nucleoprotein machineries operating in the cell nuclei.

Zinc cations belong among the smallest, strongest and most universal inducers of DNA conformational changes. They influence denaturation and renaturation of DNA by stabilizing the DNA duplex (13) as well as by their ability to facilitate rewinding of heat-denatured DNA, probably through the formation of crosslinks between the complementary strands (14,15). Interaction of zinc cations with DNA is sequence-selective (16). Zinc is the smallest ligand that causes a perturbation of specific DNA sequence (17). It promotes sequence-directed bending of kinetoplast DNA (4), induces a bend within the TFIIIA-binding region (18) and causes kinks in strained DNA minicircles, containing tandemly repeating $d(A)_5$ and $d(G_3C_{2-3})$ sequences (19,20). The $d(GA.CT)_{22}$ sequence forms an intramolecular triplex consisting of one pyrimidine and two purine strands in negatively supercoiled DNA in the presence of zinc at neutral pH (21,22). Zinc cations also promote formation of unusual duplexes, including parallel-stranded or antiparallel-stranded homoduplexes of a $d(GA)_{20}$ DNA sequence (23) and a d(GA.GA) hairpin (H* hairpin) (24). Some complexes of zinc switch DNA into the left-handed Z-form (25,26). In addition, many DNA binding proteins contain zinc ions as structural and/or catalytic components (27).

Here we describe experiments showing that zinc and some other metal cations cause sedimentation of DNA. This observation has practical consequences for biochemical and biophysical studies of DNA in the presence of metal cations. In addition, this phenomenon has recently provided a basis for the development of a simple method of DNA extraction with zinc (28). We were contacted by 49 laboratories regarding this method. The present article was in part motivated by their questions concerning the mechanism of the sedimentation and the importance of factors participating in this phenomenon. Zinc has also been used to precipitate bacteriophages (29,30).

## MATERIALS AND METHODS

### Chemicals

Plasmid pUC19 DNA and λ phage DNA digested with *Eco*RI + *Hin*dIII (MBI Fermentas), zinc chloride (Aldrich), zinc nitrate (Sigma), cobalt chloride (Kodak), nickel chloride (Lachema), manganese chloride (Lachema), barium chloride (Lachema), magnesium chloride (Merck), calcium chloride (Lachema), Tris (Sigma), hydrochloric acid (Lachema), boric acid (Lachema), acetic acid (Lachema), Na phosphate buffer (Lachema), $Na_2EDTA$ (Sigma), agarose (Serva) and ethidium bromide (Boehringer Mannheim) were all used as supplied by the producers. All solutions were made using deionized water (EASYpure RF; Barnstead) whose resistivity was 18 MΩ/cm or higher.

### Experimental procedure

Unless stated otherwise, supercoiled pUC19 DNA or *Eco*RI + *Hin*dIII restriction fragments of λ phage DNA (concentrations indicated in figure captions) were dissolved in Tris–HCl (concentrations and pH values in figure captions) and incubated with Na phosphate buffer ($NaH_2PO_4 + Na_2HPO_4$, pH 7.0) and the metal chlorides (concentrations given in figures or figure

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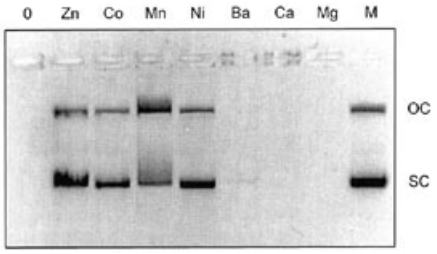


**Figure 1.** An agarose gel showing sediments of pUC19 DNA [a common mixture of supercoiled (SC) and open circle (OC) forms, concentration 4 μg/ml, total amount 400 ng) generated by incubation with 10 mM metal chlorides in 10 mM Tris–HCl, pH 9.0. 0, sediment obtained with the control sample to which no metal cation was added; M, the DNA amount marker (400 ng).

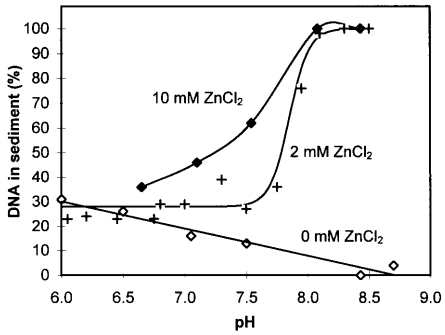


**Figure 2.** pH dependencies of the sedimentation of pUC19 DNA (concentration 4 μg/ml, total amount 400 ng) incubated with 0, 2 or 10 mM $ZnCl_2$ in 100 mM Tris–HCl.

captions) for 20 min at room temperature. Following incubation, the samples were centrifuged at 7800 *g* for 5 min, the supernatant was carefully removed and the sediment was resuspended in 20 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.2) for 30 min at 4°C. STOP buffer (6× concentrated, 0.25% bromphenol blue, 30% glycerol, 50 mM EDTA, pH 8.0) was then added and the samples were electrophoresed in 1.0 or 1.5% agarose gels using TBE buffer. Gels were stained with ethidium bromide (0.5 μg/ml), photographed and quantified using Personal Densitometer SI model 375A and the ImageQuant software (Molecular Dynamics). The percentage of sedimented DNA was calculated using the DNA amount marker, i.e. the original sample that was not exposed to the metals and centrifugation.

## RESULTS

Numerous molecules of DNA (supercoiled as well as linearized pUC19 and pBR322, their restriction fragments and restriction fragments of the λ phage DNA) were incubated with various concentrations of chloride or nitrate of zinc and chlorides of six other metal cations. The samples were centrifuged, the supernatants discarded, the sediments dissolved in an appropriate buffer and the dissolved sediments were electrophoresed in agarose gels to determine the percentage of DNA in the sediment in each particular case. A representative result of these experiments, where circular pUC19 DNA was used [containing a common mixture of supercoiled (SC) and open circle (OC) forms], showed that no detectable amount of DNA was found in the sediment in the absence of metal cations (Fig. 1). Similarly, no detectable amounts of DNA were found in the sediment of samples to which 10 mM chlorides of barium, calcium or magnesium were added (Fig. 1). In contrast, zinc, cobalt, manganese and nickel caused strong sedimentation of DNA under the same conditions. The presence of divalent cations resulted in DNA nicking ($Mn^{2+}$ especially converted a portion of SC molecules into relaxed OC ones), probably by an activation of contaminating nucleases, and the sharpness of bands was compromised by traces of divalent cations that remained in the sediment, but this was not important in this work because we only measured the total amount of DNA in each lane. It follows from Figure 1 that >90% of DNA was found in the sediment in the case of zinc and manganese. The same results were obtained with all tested DNA molecules.

In order to understand the origin of sedimentation, we analyzed how it depends on various factors. The most significant factor was found to be the value of pH. It was 9.0 in the experiments described above and hence we measured the sedimentation pH dependencies. In the control experiments, the sediment contained no DNA in the absence of metals at pH 8.5, but it was very surprising to see that a proportion of the DNA sedimented at lower pH values (Fig. 2). The sediment contained more than a quarter of the DNA amount in the sample at pH 6.0. We did not analyze this phenomenon further because it had nothing in common with the metal cations, but it was reproducible so that the choice of Tris buffers having a pH of ~8.5 is very much justified to avoid the non-negligible sedimentation of DNA occurring at neutral and slightly acidic pH values.

However, the choice of pH 8.5 is obviously problematic in the presence of, for example, 2 mM $ZnCl_2$, because this concentration of zinc totally sedimented all of the DNA present in the sample at this alkaline pH value. The sedimentation cooperatively depended on the pH value (Fig. 2), being promoted at alkaline values. At higher zinc concentrations (10 mM), a significant part of the DNA sedimented even at neutral pH. In addition, Figure 2 shows that there is essentially no value of pH where a negligible amount of DNA sediments in the presence of millimolar concentrations of zinc.

The pH dependence inspired a notion that insoluble hydroxides of zinc or the other metal cations stood behind the sedimentation because: (i) metal cations form hydroxides at alkaline pH values; (ii) the hydroxides are insoluble in water and hence sediment; (iii) the solubility is known to be much higher with the hydroxides of magnesium and calcium as compared with the hydroxides of zinc, cobalt, nickel or manganese (31), which exactly conforms to what we observed. Soluble metal hydroxides do not sediment while the insoluble metal hydroxides sediment in line with the data shown in Figure 1.

These ideas inspired us to study the effects of phosphate anions, which are also known to generate insoluble compounds with metal cations. Indeed, addition of 10 mM $ZnCl_2$, $CoCl_2$, $MnCl_2$ or $CaCl_2$ to the samples containing 1 mM Na phosphate resulted in strong DNA sedimentation even at neutral pH (Fig. 3). Especially enhanced was the sedimentation with calcium, which caused no sedimentation in the absence of phosphate. This is in good agreement with the fact that calcium generates compounds with the phosphate that are useful in DNA transfection of

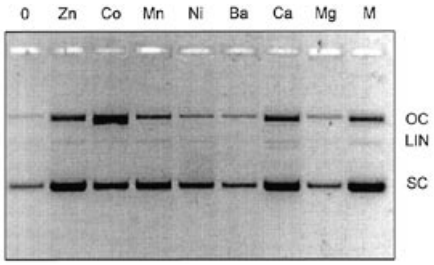


**Figure 3.** An agarose gel showing sediments of pUC19 DNA (concentration 4 μg/ml, total amount 400 ng) that was incubated with 10 mM metal chlorides and 1 mM Na phosphate in 10 mM Tris–HCl, pH 7.0. 0, the control sample to which no metal was added; M, the DNA amount marker (400 ng).

mammalian cells (32–35) and in the preparation of hydroxyapatite, which strongly adsorbs DNA (reviewed in 36).

As expected, the sedimentation of DNA by metals was pH-independent in the presence of phosphate anions in the tested range pH 6.5–9.0 (not shown). On the other hand, the sedimentation depended both on the metal and phosphate concentrations. Sedimentation increased with the metal concentration (Fig. 4), but the extent of sedimentation was different with the particular metals, being higher, for example, for zinc than calcium. At least 1 mM $ZnCl_2$, but 5 mM $CaCl_2$, was needed to cause total DNA sedimentation under the conditions of the experiment shown in Figure 4.

Phosphate anions stimulated sedimentation at low concentrations but higher (>10 mM) concentrations exerted an inhibitory effect (Fig. 5). The phosphate concentration causing the strongest metal-induced sedimentation of DNA depended on the metal cations. For example, it was ~0.1 mM phosphate with 1–10 mM zinc but 1.0 mM phosphate with 1–10 mM calcium (Fig. 5). Other anions (chloride, borate or acetate) used in Tris buffer did not influence the sedimentation. Neither did replacement of $ZnCl_2$ by $Zn(NO_3)_2$ have any significant effect.

We also examined the role of other factors that were likely to influence sedimentation. The role of DNA concentration was tested in the range 0.1–80 μg/ml with various λ phage DNA restriction fragments. The sedimentation proved not to be significantly dependent on the concentration of DNA up to ~16 μg/ml, but higher concentrations of DNA led to decreased sedimentation in 0.5 mM $ZnCl_2$ and 0.1 mM Na phosphate at neutral pH (Fig. 6). This is a remarkable aspect because, for example, sedimentation of DNA caused by ethanol increases with the concentration of DNA. This demonstrates that the mechanisms are different for these two kinds of sedimentation. Obviously, the capacity of insoluble zinc phosphates or hydroxides to sediment DNA is limited. In contrast, DNA is what primarily sediments in ethanol and, therefore, ethanol-induced sedimentation increases with DNA concentration. Our experiments demonstrate that owing to the different mechanism, zinc makes possible DNA extraction from large volumes (volumes up to 8 ml were tested) of dilute solutions (0.1 μg/ml), where ethanol precipitation especially is not very efficient.

Another factor was DNA length. This factor was tested with λ *Hin*dIII, λ *Hin*dIII + *Eco*RI and pUC19/*Dra*I restriction fragments covering lengths from 564 to 23 130 bp, but no significant dependence of zinc-induced sedimentation on DNA length was observed in this range (not shown).

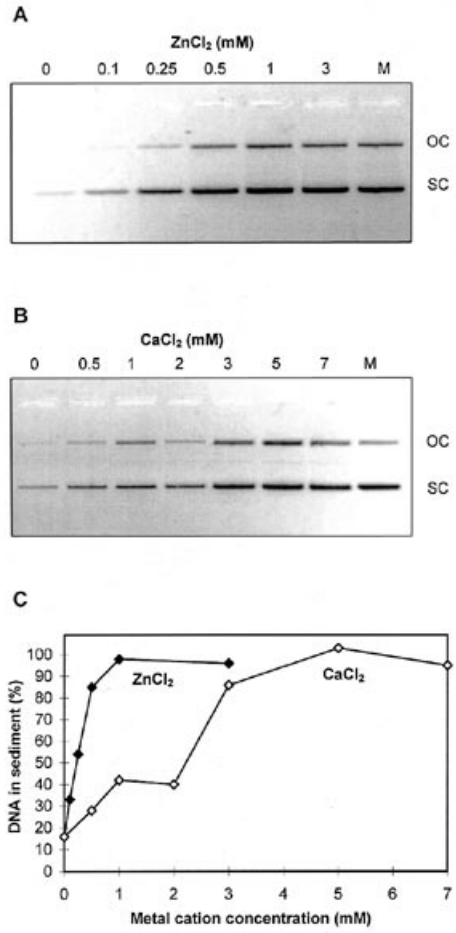


**Figure 4.** Agarose gels showing sediments of pUC19 DNA (concentration 4 μg/ml, total amount 400 ng) caused by incubation with an increasing concentration (0–3 mM) of $ZnCl_2$ in the presence of 0.1 mM Na phosphate (**A**) and (0–7 mM) $CaCl_2$ in the presence of 1 mM Na phosphate (**B**) at neutral pH (10 mM Tris–HCl, pH 7.0). M, the DNA amount marker (400 ng). (**C**) Quantitation of the gels presented in (A) and (B).

The sedimentation was completely avoided, or totally reversed, after addition of an appropriate concentration of EDTA that solubilized the insoluble particles and hence released DNA into solution. The EDTA effective concentration increased with increasing concentration of the metal in solution (Fig. 7) so that metal chelation by EDTA probably stood behind the dissolution effect. Hence metal binding was relatively weak in insoluble phosphates.

We also studied the time dependence of sedimentation. Our results suggested that the DNA–Zn-P complexes were formed very fast because even a 1 min incubation was sufficient to have almost 100% of the DNA sedimented after a brief centrifugation (not shown). The DNA–Zn-P complexes sedimented even without centrifugation. For example, >50% of DNA was in the sediment after a 2 h incubation with 5 mM $ZnCl_2$ and 0.1 mM Na phosphate (data not shown). Sedimentation did not depend on whether the experiments were done in Eppendorf or glass tubes. These experiments were motivated by a report claiming that the properties of some DNA molecules are influenced by the polypropylene walls of Eppendorf tubes (37).

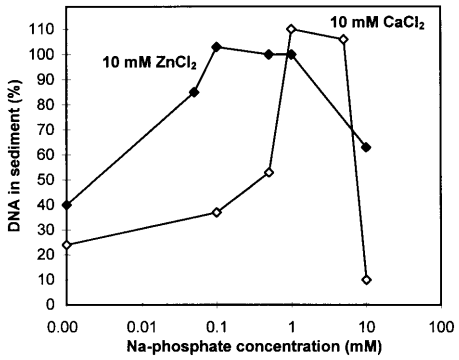


**Figure 5.** Sedimentation of pUC19 DNA (concentration 4 μg/ml, total amount 400 ng) caused by incubation with 10 mM $ZnCl_2$ or $CaCl_2$ in the presence of an increasing concentration of Na phosphate at neutral pH (10 mM Tris–HCl, pH 7.0).

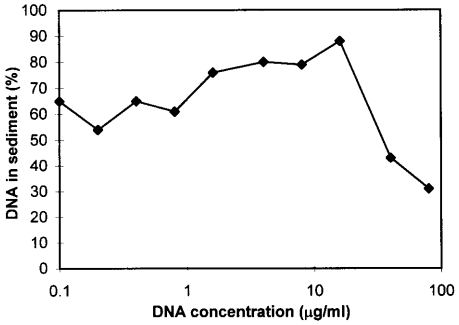


**Figure 6.** Sedimentation of *Hin*dIII + *Eco*RI restriction fragments of λ phage DNA caused by incubation with 0.5 mM $ZnCl_2$ and 0.1 mM Na phosphate at neutral pH (10 mM Tris–HCl, pH 7.0) at an increasing concentration of DNA (0.1–80 μg/ml).

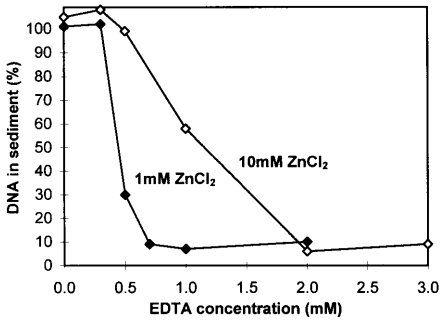


**Figure 7.** Sedimentation of pUC19 DNA (concentration 0.4 μg/ml, total amount 400 ng) caused by 1 or 10 mM $ZnCl_2$ and 0.1 mM Na phosphate in the presence of an increasing concentration of EDTA at neutral pH (10 mM Tris–HCl, pH 7.0).

## DISCUSSION

This article reports on a simple, but important and, to the best of our knowledge, novel phenomenon of DNA sedimentation in the presence of zinc and other metal cations. We have found no description of this phenomenon in the literature although there are many dozens of papers reporting on the properties of DNA in solutions containing divalent metal cations (3,4,8,10,13–26). We noticed the sedimentation by mere chance when our experiments seemed to indicate that zinc unbelievably protected DNA against damage by UV light. However, control experiments revealed that the 'protection' originated from the fact that we irradiated free buffer because DNA was sedimented at the bottom of the Eppendorf tube.

Zinc forms complexes with many ligands. The complexes include zinc hydroxides, whose solubility in water is very low. It follows from our calculations that formation of the zinc hydroxides starts at ~pH 7.5 and that zinc exists exclusively in the form of the insoluble zinc hydroxides between pH 8.5 and 9.0. The sedimentation of DNA caused by zinc approximately follows the same pH dependence (Fig. 2). The inclination to insoluble hydroxide formation decreases in the sequence Zn, Co, Ni, Mn, Mg, Ca (31). For example, magnesium starts precipitating only above pH 10, in line with the present observation that it causes no DNA sedimentation under the conditions tested in this work.

The coordination geometry of zinc is tetrahedral (27) and the phosphate group is a possible ligand. Low concentrations of inorganic phosphate are expected to promote the sedimentation of DNA because it is difficult for DNA itself to saturate all of the tetrahedral sites, which is probably necessary for formation of the sedimenting particles. However, if the inorganic phosphate concentration is much higher than the concentration of the DNA backbone phosphates, then sedimenting particles form without including the DNA, whose sedimentation is then decreased. This is exactly what we observed (Fig. 5), which supports the above notion of the mechanism of DNA sedimentation.

Co-precipitation of DNA with calcium phosphate has been used for transfection of mammalian cells for >20 years (32) and the method has recently been improved (33–35). In addition, calcium phosphate (CaP) or hydroxyapatite has been used in chromatography of nucleic acids since the 1960s (36). DNA is also adsorbed on calcium phosphate microcrystals in hydroxyl radical or DNase I studies of DNA structure (38–40). Work in these directions revealed the following facts which are either in line with, or relevant to, the present article. First of all, even the most recent papers state that the precise function of CaP is still not known and that the direct components participating in CaP–DNA complex formation are undefined (41), although large metal colloids bound to DNA, which are likely to sediment, have recently been observed by electron microscopy (42). In line with our results, the transfection work revealed that the co-precipitation of DNA with CaP is fast (34,35) and that DNA participates in the precipitate but high concentrations of DNA inhibit precipitation (34).

Besides the metal hydroxide solubility mentioned above, there are further interesting correlations between the metal-induced sedimentation of DNA and properties of the metals. The correlations include their relative affinity for bases of DNA as compared with the backbone phosphates, which decreases in a similar sequence $Cu^{2+} > Cd^{2+} > Zn^{2+} > Mn^{2+} > Ni^{2+} > Co^{2+} > Mg^{2+}$ (14) or $Cu^{2+} > Cd^{2+} > Zn^{2+}$, $Mn^{2+} > Co^{2+}$, $Ni^{2+}$, $Fe^{2+} > Ca^{2+} > Ba^{2+}$, $Mg^{2+}$ (43), like their efficiency in causing DNA sedimentation. It is also interesting that the sequence of the tendency to form insoluble metal hydroxides, as well as the efficiency of various metals in inducing sedimentation of DNA, is very similar to the sequence of the magnitudes of the metal-induced DNA bending revealed by electron microscopy:

$Zn^{2+}$ > $Co^{2+}$ > $Ni^{2+}$, $Mn^{2+}$ > $Ba^{2+}$ > $Ca^{2+}$, $Mg^{2+}$ (4). The sedimentation coefficients of a bacterial DNA complexed with metals also decreases in the sequence $Zn^{2+}$, $Ni^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Mg^{2+}$ (43). Conditions of some published studies of DNA in the presence of zinc, nickel and other transition metal cations are indeed at the very edge of the situation when DNA starts sedimenting, although the choice of a different buffer or other conditions could help DNA not becoming a part of the sedimenting particles. The present work demonstrates that studies of DNA in the presence of zinc and other transition metal cations require maximum care to avoid the sedimention which can substantially influence the results of quantitative studies. This not only concerns free DNA, but also complexes of DNA with proteins containing zinc fingers or requiring millimolar concentrations of divalent metal cations for their enzymatic activities.

## ACKNOWLEDGEMENTS

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