crystallization from hexane and treatment with Norit-A gave a pure product, m.p. 61–62°.

The DL-amino acid derivative was resolved with α-chymotrypsin. Solution of 7.7 g. of the acylated amino acid ester in 1500 ml. of water and addition of 0.05 g. of α-chymotrypsin resulted in hydrolysis. Standardized sodium hydroxide solution was added, keeping the pH between 7 and 8. After 45 min. sodium hydroxide corresponding to 49% hydrolysis had been added. The reaction mixture was extracted with three 200-ml. portions of chloroform. The cloroform extract was dried over magnesium sulfate and evaporated. The resulting oil was recrystallized from petroleum ether (b.p. 30–60°) to yield N-acetyl-D-2-aminoheptanoic acid methyl ester, m.p. 27.5–28.5°, $[\alpha]^{25}D$ −23.6° (*c* 1.5, MeOH).

Anal. Calcd. for $C_{10}H_{19}NO_3$ (201.2): C, 59.7; H, 9.5; N, 7.0. Found: C, 60.1; H, 9.1; N, 7.1.

The aqueous layer from the resolution was acidified with HCl and evaporated *in vacuo*. The organic residue was dissolved in ethyl acetate and extracted into aqueous sodium bicarbonate. The resulting aqueous layer was again acidified and extracted with ethyl acetate. Evaporation of the organic solvent yielded 1.4 g. of white powder. This was recrystallized from water to give 1.05 g. of white needles, m.p. 112° (lit.[36] m.p. 108°).

The L-acid was re-esterified as described above. The product was recrystallized from hexane and ethyl acetate to give white needles, m.p. 26–27°, $[\alpha]^{25}D$ 22.6 ± .5° (*c* 2, MeOH).

Anal. Found: C, 59.6; H, 9.2; N, 6.9; ash, 0.44.

N-Acetyl-α-aminocaprylic Acid Methyl Ester. Commercial α-aminocaprylic acid (Calbiochem) was esterified and acetylated as described above to give the racemic product, m.p. 74–75.5°. The DL-ester was resolved with α-chymotrypsin as indicated for 2-aminoheptanoic acid and the L-acid was re-esterified. The D-ester was obtained after recrystallization from hexane, m.p. 57–58.5°, $[\alpha]^{25}D$ −20.8 ± 1.0° (*c* 2, MeOH). The L-ester was also recrystallized from methanol, m.p. 55.5–57°, $[\alpha]^{25}D$ −18.5 ± 1.0° (*c* 2, MeOH).

Anal. Calcd. for $C_{11}H_{21}NO_3$ (215.3): C, 61.4; H, 9.8; N, 6.5. Found: C, 61.5; H, 9.6; N, 6.5.

Kinetic Studies. The procedure was identical with that described previously.[10,37] All experiments were conducted in aqueous solutions at 25.0°, pH 7.90 ± 0.10, and 0.10 *M* with respect to sodium chloride. The enzyme preparation was crystalline, bovine, salt-free α-chymotrypsin, Armour Lot No. T-97207. The primary data were analyzed with a Datatron 220 digital computer, programmed as described earlier.[38]

Acknowledgment. The authors wish to thank Dr. Werner A. Mukatis for his assistance in the preparation of this manuscript.

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The Effect of Magnesium Ion on the Secondary Structure of Deoxyribonucleic Acid

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The effect of magnesium ion (Mg^{2+}) on the thermal and phase stability of deoxyribonucleic acid (DNA) has been studied by means of spectrophotometric and nuclear magnetic resonance techniques with the following conclusions: (1) Mg^{2+} interacts with DNA at the phosphate sites only. (2) Whereas aqueous solutions of the pure magnesium salt of DNA are relatively resistant to thermal denaturation, their thermal stability is reduced in the presence of added $MgCl_2$. (3) The proposed mechanism for the precipitation of DNA with an excess of Mg^{2+} is that site-bound magnesium forms ionic links between separated DNA strands through –P–O–Mg–O–P complexes leading to the exposure of the bases to solvent. This is followed by hydrophobic base–base interaction leading to large aggregates and, finally, phase separation.

In a recent report[2a] dealing with the properties of the magnesium salt of deoxyribonucleic acid (MgDNA) we showed that, in the absence of added simple salt, MgDNA is much more resistant to thermal denaturation than is NaDNA. We also reported that when $MgCl_2$ is added to previously heat-treated and quenched (denatured) MgDNA a precipitate forms, whereas no precipitate is observed with native MgDNA. Moreover, if native MgDNA is heated in the presence of $MgCl_2$ a precipitate appears at a temperature lower than the melting temperature, T_m, of the pure MgDNA solution. In experiments in which the activity coefficient of magnesium ion was measured in MgDNA–$MgCl_2$ mixtures,[2b] we have found that magnesium ion is bound to an extent greater than 1 mole of Mg^{2+} to 2 moles of P(DNA); *i.e.*, some magnesium ion is ren-

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(2) (a) J. W. Lyons and L. Kotin, *J. Am. Chem. Soc.*, **86**, 3634 (1964); (b) J. W. Lyons and L. Kotin, *ibid.*, **87**, 1670 (1965).

dered inactive either by binding by other portions of the DNA than the phosphate site or by ion pairs at the phosphate sites with a 1:1 mole ratio. The latter form of binding we postulate as a likely explanation: it would lead to charge reversal on segments of the DNA polyion producing areas with the properties of a polycation with univalent counterions (Cl^-). Charge reversal could lead to a destabilizing effect on the double helix of DNA. It is the purpose of this paper to pursue further the study of the precipitation phenomenon, to locate the site of the additional binding of magnesium ion by DNA, and to relate these results to destabilization of the double helix through charge reversal.

Other multivalent cations have been shown to precipitate DNA. Indium(III), for example, will precipitate denatured DNA leaving native material in solution.[3] Lead(II) has a similar effect.[4] In the experiments with lead(II) it was found there is a difference between heating the DNA solution, cooling, and adding lead ion *vs.* adding the lead before heating. In the former procedure no precipitate formed until the temperature approached T_m for lead-free solution; in the latter procedure a precipitate formed some 30° below T_m. This seems to indicate that (1) lead(II) only precipitates denatured DNA, and (2) lead(II), if present during the heating cycle, destabilizes the double helix.

An informative set of experiments on the effect of magnesium ion on polynucleotides has been carried out by Eisinger and his co-workers.[5] In this work no heat was used and sodium ion was also present. Polyadenylic acid (poly-A) and polyinosinic acid (poly-I) are largely precipitated by magnesium ion; polycytidylic (poly-C) and polyuridylic (poly-U) acids are not precipitated. Poly-A and poly-I contain purine bases; poly-C and poly-U contain pyrimidines. Studies[6] of the n.m.r. spectra of these polynucleotides show that poly-A and poly-I have ordered structures in solution while poly-U and poly-C do not.[7] It has been also reported that thermally denatured NaDNA aggregates in the presence of NaCl but no precipitate forms.[8] Thus it appears from the foregoing paragraphs that (1) the precipitate with magnesium is formed from nonhelical strands of DNA, (2) the nature of the bases is important, and (3) that the divalent magnesium ion brings about precipitation under conditions where sodium ion does not.

Experimental

Materials. The preparation and properties of the MgDNA (from calf thymus) have been fully described.[1] The model compounds, cytidine and deoxyadenosine, were obtained from the Sigma Chemical Company, St. Louis, Mo. Both compounds were lyophilized in D_2O and solutions prepared in D_2O to enhance sensitivity in n.m.r. measurements. Concentrations of these materials were determined by optical density at 260 mμ or by careful weighing of the dry material. The $MgCl_2$ used in the magnetic resonance work was also lyophilized in D_2O. Downward pH adjustments were made with DCl.

Procedures. T_m was determined in dilute MgDNA solution ($\approx 2 \times 10^{-4}$ *m* in DNA phosphorus) by the ultraviolet spectral method using a Cary Model 14 spectrophotometer as previously described.[1] Visual detection of the onset of precipitation in MgDNA solutions was accomplished by immersing in a beaker of heated water the Cary 10-mm. cell with a glass-shielded thermocouple inside, and slowly raising the bath temperature and recording the temperature of the cell contents and the visual appearance. The apparatus was such that the temperature could be increased smoothly or in a stepwise manner.

Proton magnetic resonance experiments were carried out using a Varian A-60 spectrometer at room temperature (60 Mc./sec., 14,093 gauss) and chemical shifts were referenced to the tetramethylammonium (TMA) proton line as an internal standard.[9] For this purpose, TMACl was added to the sample solution.

Results

At an ionic strength of 0.2 *m* (0.062 *m* $MgCl_2$, 0.005 *m* $Mg(OAc)_2$) "native" MgDNA is soluble, but thermally "denatured" and quenched MgDNA forms a precipitate on addition of $MgCl_2$ at room temperature. In this experiment the MgDNA concentration was 10^{-2} *m* in phosphorus. On heating the "native" MgDNA in the presence of $MgCl_2$ a precipitate forms. The temperature at which the precipitate can first be observed visually is designated as T_{ppt}. Careful stepwise increases in the temperature above T_{ppt} demonstrated that more and more material precipitates with each increment in temperature.

With the Cary spectrophotometer set at 258 mμ experiments were carried out to obtain a measure of the influence of the ionic strength of $MgCl_2$ at low MgDNA concentration ($\approx 2 \times 10^{-4}$ *m* in phosphorus). The results are presented in Table I. We were able to obtain what appeared to be ordinary melting curves at all but the highest ionic strength. T_m is not notably affected by the presence of the $MgCl_2$ except at $I = 0.2\,m$. In the last instance the melting curve reached the halfway mark (compared with the normal curves), then became erratic, and finally dropped sharply as the precipitate coalesced and settled. We report the temperature on the curve at half-height as T_m in the table, even for the last entry.

Visual observation of the effect of heating MgDNA at various concentrations in the presence of 0.2 *I* in $MgCl_2$ showed that the onset of precipitate formation coincides with the beginning of the erratic behavior in the ultraviolet absorbance study. Table II indicates that increasing the MgDNA concentration at a fixed simple salt level lowers T_{ppt}. The results of Tables I and II demonstrate that precipitate formation is

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Table I. Melting Temperature of MgDNA at Various Levels of $MgCl_2$[a]

Ionic strength of $MgCl_2$	T_m, °C.	Appearance at end
0	82	Clear
7×10^{-4}	82	Clear
1.5×10^{-2}	77.5	Clear
3×10^{-2}	81	Very slightly turbid
2×10^{-1}	74.5	Precipitate

[a] DNA concentration: $\sim 2 \times 10^{-4}$ *m* in P.

Table II. Observation of Precipitation in Solutions of MgDNA Heated with $MgCl_2$[a]

MgDNA concn., *m* of P	Onset of turbidity T_{ppt}, °C.
2×10^{-4}	73
4.6×10^{-4}	74
3.7×10^{-3}	68
7.4×10^{-3}	62 ± 5[b]

[a] $MgCl_2$ concentration: 0.067 *m* (ionic strength = 0.2). [b] Estimated from spectrophotometric measure of absorption at 320 mμ where DNA normally does not absorb at all.

dependent on both the concentrations of salt and MgDNA. Hence, the mechanism by which precipitation is brought about probably involves a mass action, interaction (*vide infra*). Comparison of the temperatures reported in the last entry of Table I and the first entry of Table II (both of which correspond to identical conditions) reveals that within experimental error T_m and T_{ppt} coincide. In view of the evidence that the precipitated material is denatured, it is necessary that $T_{ppt} \geqslant T_m$; the fact that these two temperatures are identical in the example cited above may be accidental. However for the purposes of the following discussion we need only assume that T_{ppt} and T_m exhibit similar trends.

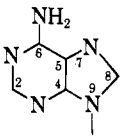
Discussion

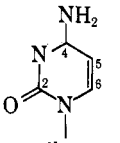
There are two clearly distinct phenomena occurring: (1) denaturation with some destabilization or lowering of T_m, and (2) precipitation of the denatured strands. Magnesium ion is involved in both interactions as shown by its effect on T_m and T_{ppt}, and by the fact that precipitation does not occur in the absence of magnesium ion. We therefore will discuss, in order, the most probable site of the interaction of the excess magnesium ions, the mechanism of destabilization, and, finally, the mechanism of precipitation.

Site of the Interaction. There are only two likely places where magnesium ion might interact with the DNA polyion, the phosphates, or the bases. Binding by the bases is a possibility when excess magnesium ion is present since a number of multivalent metals are known to be bound at these sites. We have investigated this possibility in two ways. In the first method we observed the maximum in the ultraviolet spectrum of MgDNA and noted no shift from that of NaDNA (258 mμ). A spectral shift in the ultraviolet would be a definite indication of interaction with the bases, and such a shift has been reported for a system in which strong binding by the base is expected, namely, mercury(II) and DNA.[10]

A second method is by proton magnetic resonance. It has been shown that, in adenosine triphosphate (ATP), zinc(II) causes a downfield shift of 0.25 p.p.m. at H-8.[9] This shift is believed due to binding of the zinc ion at N-7 of the adenine ring and perhaps also at the 6-amino group. In order to avoid the complication of binding by the triphosphate segment of this type of molecule we selected deoxyadenosine and cytidine for study. Deoxyadenosine was selected because it contains the same base as ATP.

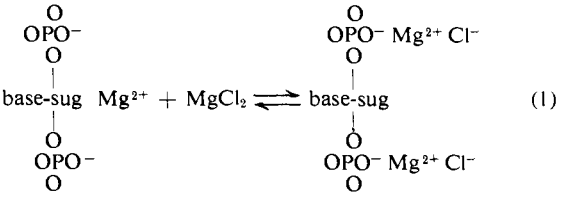
In this base, adenine, there are two nitrogens in the same relation to each other as those in dipyridyl or *o*-phenanthroline. The latter compounds are excellent complexing agents for multivalent cations. It was anticipated, therefore, that if any binding of magnesium occurs it would most likely be detected in a nucleoside containing adenine. We also studied binding by cytidine in which there are two nitrogens in a slightly less favorable but similar relationship.





The assignment of the various resonance lines has been made for these compounds.[11] The results of experiments with $MgCl_2$ in D_2O are shown in Table III. Even with a sevenfold excess of magnesium ion no significant chemical shifts can be detected. We therefore believe there is no strong interaction between the DNA bases and magnesium ion.

Mechanism of Destabilization. The excess $MgCl_2$ must be interacting with the phosphate sites, perhaps as



(1)

The postulated site binding indicated on the right hand side of eq. 1 has been discussed in another communication[2b] and will not be discussed in detail here. The result is reversal of charge on the segment. Considered from the point of view of the thermodynamic activity coefficients γ_i and values of T_m reported for NaDNA and MgDNA in the native states, we can estimate roughly how much charge reversal occurs in the case of MgDNA in $MgCl_2$. Native NaDNA has an effective charge about 0.3 of the total possible; *i.e.*, $\gamma_{Na^+} \approx 0.3$; and for MgDNA, $\gamma_{Mg^{2+}} \approx 0.1$.[2a] NaDNA in the absence of added electrolyte has T_m close to room temperature at, say, 10^{-2} *m* in P,[12] whereas MgDNA has a T_m of 82°.[2a] Assuming that electrostatic repulsion[13] between phosphate groups on adjacent strands is a major influence on helix instability in these systems, it appears that a reduction in effective charge from 0.3 to 0.1 results in an increase in T_m of some 60°.

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Table III. Effect of Mg and pD on Chemical Shifts on Deoxyadenosine and Cytidine in D_2O

Compd.	Base, *M*	Mole ratio Mg/ base	pD	Chemical shift,[a] p.p.m. H'-1[b]	H-2	H-8
Deoxyadenosine	0.060	0	7.2	−3.15	−4.83	−4.96
			6.9	−3.16	−4.86	−4.99
	0.060	1	8.4	−3.14	−4.80	−4.95
			6.5	−3.15	−4.83	−4.96
	0.060	2	9.0	−3.15	−4.81	−4.97
			6.4	−3.14	−4.82	−4.96
	0.060	4	9.3	−3.13	−4.81	−4.97
			5.8	−3.15	−4.80	−4.96
	0.060	8	9.2	−3.14	−4.80	−4.97
			5.0	−3.14	−4.81	−4.98
					H-5	H-6
Cytidine	0.060	0	7.0	−2.65	−2.82	−4.55
	0.060	1	7.1	−2.67	−2.80	−4.55
	0.060	8	7.1	−2.66	−2.82	−4.55

[a] Tetramethylammonium ion internal reference; added as TMACl; see ref. 9. [b] H'-1 refers to the proton on the sugar carbon which is attached to the base. For the numbering system of the ring protons see text.

If the effective charge of MgDNA is reduced to zero by some charge reversal, we would expect T_m to increase; if the charge is further reversed producing a polycation with 0.1 of the total possible positive charges, T_m should again be 82°. Further reversal to the extent of 0.3 of the total possible would produce a material scarcely stable at room temperature. These statements assume that the equilibrium constant for eq. 1 strongly favors the product. If, however, the equilibrium constant is relatively small, then much of the added $MgCl_2$ will not be involved in the product and will be available for electrostatic screening of the phosphate sites. Thus, even if the charge on the polycationic product were 0.3 of the total possible, T_m would be lowered considerably but not to the level of NaDNA due to reduction of electrorepulsion by the screening effect.[13] The observed decrease in T_m and T_{ppt} is consistent with the charge-reversal hypothesis and with the conclusion that K for eq. 1 is not large. The data in Tables I and II qualitatively follow the mass action law giving more plausibility to eq. 1 as the destabilizing mechanism.

We do not rule out completely the possible effect of $MgCl_2$ on the hydrophobic bonds between the bases. However, it has been shown that chloride ion has little or no effect on these forces,[14] and we assume that the cation has relatively little effect as compared with its known effect on the phosphates.

Mechanism of Precipitate Formation. We have seen that both a specific type of base and magnesium are required for precipitate formation. The evidence seems conclusive that the precipitate is formed from denatured portions of the DNA molecules, hence the importance of the bases. The n.m.r. result rules out extensive bridging between bases by magnesium ion. The charge-reversal hypothesis explains the observed destabilization but the data indicate that the reaction is not quantitative and the site-bound magnesium ions might be able to enter into other types of interaction. We propose that precipitation occurs in three steps: (1) disruption of the double helix by electrorepulsion brought about by charge reversal; (2) interchain bridging between phosphate sites by $PO^-Mg^{2+}PO^-$ linkages; (3) base–base interactions to build up larger structures which eventually form a separate phase. The interchain bridging by magnesium essentially removes from the solvent the hydrophilic phosphate groups and their associated deoxyribose units. The result is a micelle-like structure with phosphate, magnesium, and sugar groups on the inside and nitrogenous bases on the outside. In the third step these bases interact with each other in preference to the solvent, and the precipitate is then formed from clusters of these micelles.

The foregoing, though largely speculative, explains the observed facts. Charge reversal is certainly plausible; it has been invoked many times to explain changes in sign of electrophoretic mobilities of charged particles.[15] The bridging of phosphates by magnesium ion involves no new ideas; in fact, one might ask why it does not happen in native MgDNA. Although viscosity data do not indicate that this occurs,[2a] it is possible. However, in native MgDNA the bases are not exposed and the third step could not occur.

The base–base interactions causing the final precipitation in step 3 also seem quite reasonable. The aggregation in denatured NaDNA–NaCl[8] is attributed by us to this type of interaction. The relative strength of the base–base *vs.* base–solvent interactions will govern whether or not the micelles will cluster into insoluble particles. Thus the purine bases evidently interact with other purines or with pyrimidines to form preferential base–base linkages.

Pyrimidines evidently do not form strong linkages with other pyrimidines, thus explaining the failure to observe precipitates with poly-C and poly-U.[5] These observations are consistent with the relatively lower degree of order found in poly-C and poly-U in solution *vs.* poly-A and poly-I as shown by n.m.r.,[6] which also reflects the strength of base–base interactions in solutions of the pure polynucleotides.

We conclude, then, that both magnesium and the bases are involved, that the magnesium has its effect through interactions at the phosphates, and that the

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nature of the bases is critically important in producing precipitates. Although there may be other explanations of the phenomena which also fit all the data, the present postulates seem to us to be the most reasonable in light of the presently available evidence.

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Conversion of Mono- and Oligodeoxyribonucleotides to 5′-Triphosphates[1]

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Contribution from the Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico.
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Reaction of the phosphorimidazolidate formed from a nucleotide and 1,1′-carbonyldiimidazole with inorganic pyrophosphate provides the nucleoside triphosphate in good yield. The method is convenient, generally applicable, and particularly suitable for microscale syntheses from mono- or oligonucleotides.

The immediate precursors in the replication of DNA are the deoxyribonucleoside 5′-triphosphates.[2] An interesting question is the possible ability of oligonucleotides bearing 5′-triphosphate end groups to function as substrates in enzymatic DNA and polynucleotide syntheses. Despite a negative result recently reported,[3] the question warrants further investigation in view of preliminary indications in this laboratory that certain dinucleotide derivatives are incorporated, without degradation, into DNA synthesized by DNA polymerase from *E. coli*.[4] Adequate inquiry into this and related questions requires the availability of suitable substrates. A method for the synthesis of 5′-triphosphates of mono- and oligonucleotides, adaptable to a microscale and suitable for radioactively labeled materials, is thus highly desirable.

Chemical methods, as opposed to enzyme-catalyzed synthesis,[5] would appear to be more uniformly applicable for this purpose. The method which has been most widely employed,[6] however, is not readily adaptable to small-scale preparations. An approach involving condensation of an activated nucleotide derivative, *e.g.*, a nucleoside 5′-phosphoromorpholidate,[7] with inorganic pyrophosphate[8] offers greater versatility and a minimum of side reactions, although preparation of the intermediate in optimal yield on a small scale is difficult owing to the requirement of slow addition of a reagent to a reaction mixture. Cramer and co-workers[9] have demonstrated that phosphorimidazolidates are useful intermediates for synthesis of pyrophosphate bonds, and that these amides can be readily prepared under very mild conditions from a phosphomonoester and 1,1′-carbonyldiimidazole.

The series of reactions illustrated in Figure 1 have been successfully applied[10] to microscale preparations of 5′-triphosphates of various deoxyribonucleotides. Formation of the imidazolidate (III) from a nucleotide (I) and excess 1,1′-carbonyldiimidazole (II) is complete in about 1 hr. at room temperature. Unreacted II is decomposed with methanol, before inorganic pyrophosphate (IV) is added, to eliminate formation and subsequent necessity for separation of inorganic polyphosphates. Owing to the precipitation of imidazolium pyrophosphate, an excess of IV is used. The phosphorylation is essentially complete within 24 hr.,

Table I. Distribution of Products in Triphosphate Synthesis

Parent nucleotide (I)[a]	Monophosphate (I)	Imidazolidate (III)	Diphosphate (VI)	Triphosphate (V)	Other[b]	Isolated salt of triphosphate
pT	8	3	8	66	2	50
d-pC	7[c]	0	7	56	4	45
d-pA	1	6	4	73[d]	5	36[e]
d-pG	1	3	8[d]	70[f]	3	59[f]
pTpT	11	..	8[f]	76	4	60
d-pTpC	3	..	8[f]	51[g]	3	20[e]
pTpTpT	3[f]	..	6[f]	81	2	70
d-pApT	17	2	19	50[f]	4	..

[a] Abbreviations conform to *J. Biol. Chem.*, **238**, 6 (1963). [b] Includes all ultraviolet-absorbing material eluted after triphosphate peak. [c] P^1,P^2-dinucleoside pyrophosphate (VII) also detected by paper chromatography. [d] Incompletely resolved from an impurity having parent nucleotide spectrum. [e] From homogeneous portion of peak only. [f] A second substance with parent nucleotide spectrum detectable by paper chromatography. [g] Incompletely resolved from material with altered spectrum.

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