Some Helical Interactions of Poly(N^6-[Δ^2-isopentenyl]adenylic acid)†

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ABSTRACT: The spectrum of magnesium poly(N^6-[Δ^2-isopentenyl]adenylate) was hypochromic and red shifted compared to the sodium polynucleotide and monomer between 2 and 12.5 mM $MgCl_2$. The spectrum of magnesium poly(adenylate) is also red shifted (but hyperchromic) relative to monomer and sodium salt but only at [$MgCl_2$] >10 mM. Equivalent ethylenediaminetetraacetate completely reversed the magnesium polyadenylate spectral change but excess chelating agent was required for complete reversal of the magnesium poly(N^6-[Δ^2-isopentenyl]adenylate) spectral change. This indicates a stronger binding of magnesium ion to the alkylated poly(adenylate) than to the parent polynucleotide. Absorbance–temperature profiles of the sodium and magnesium salts of the polynucleotides show that sodium poly(adenylate) is more structured and is more stable to thermal denaturation than sodium poly(N^6-[Δ^2-isopentenyl]adenylate) while the reverse is true comparing the magnesium salts. Characteristic thermal transitions of the magnesium salt of the alkylated poly(adenylate) occur at [$MgCl_2$] as low as 2 mM but poly(adenylate) melts like its sodium salt at 8 mM $MgCl_2$ and below. There is no measurable interaction of the sodium salts of poly(N^6-[Δ^2-isopentenyl]adenylate) and poly(uridylate) at 4° or above (pH 7.4, ionic strength 0.11) using the criterion of hypochromicity. However, the two magnesium salts interact to form a duplex helix (pH 7.4, ionic strength 0.06, 12.5 mM $MgCl_2$) giving thermal transitions with melting temperatures of 8, 45, and near 90°. The Mg^{2+} dependence of the spectral change and the melting profile of poly(N^6-[Δ^2-isopentenyl]adenylate) suggest that magnesium ion interacts with the base leading to a stable helical structure throughout the physiological range of magnesium concentration. The base pairing between poly(N^6-[Δ^2-isopentenyl]adenylate) and poly(uridylate) is also Mg^{2+} dependent and may also be caused by interaction of cation and base. The increased base stacking and pairing in the presence of magnesium suggest the possibility that site binding of magnesium may also occur with the single hypermodified base in certain transfer ribonucleic acids increasing the potential for base stacking and pairing involving this residue and possibly contributing to imposition of a biologically active tertiary structure.

Homopolynucleotides usually exist as single strands in solvents simulating physiological conditions (Steiner and Beers, 1957; Warner, 1957; Felsenfeld and Rich, 1957a) though poly(G) is an exception (Fresco and Massoulie, 1963). However, the homopolyribonucleotides can be induced to self-interact under conditions of increased ionic strength, decreased temperature, and changed pH (Fresco and Doty, 1957; Rich, 1958; Fresco and Klemperer, 1959; Steiner and Beers, 1959; Lipsett, 1960). The self-interaction results in formation of single or multi-stranded helices maintained by ionic bonds, by hydrogen bonds, and by stacking of bases promoted through reduction of intrastrand phosphodiester repulsion with hydrophobic interactions between stacked bases; these types of helix stabilizing forces in homopolyribonucleotides are demonstrated by studies of poly(A) (Fresco and Klemperer, 1959; Rich *et al.*, 1961; Leng and Felsenfeld, 1966).

Alkylation of purine or pyrimidine residues might interfere with the stacking interactions giving rise to hypochromicity by sterically preventing sufficiently close association of the aromatic rings. On the other hand, such nonpolar groups might increase hydrophobic interactions between the aromatic rings thus promoting increased stacking. However, increased helix stability was not found with poly(N^6-MeA)[1] (Griffin *et al.*, 1964) or poly(N^6-HEA) (Van Holde *et al.*, 1965).

Effects of alkyl substituents on base pairing in homopolyribonucleotides have also been studied. Poly(5-methyl-U) (or polyribothymidylate) forms a more stable helix with poly(A) than poly(A)·poly(U) (Shugar and Szer, 1962) but the poly(N^6-MeA)·poly(U) helix is less stable than poly(A)·poly(U) (Griffin *et al.*, 1964) and poly(N^6-HEA) does not interact with poly(U) (Van Holde *et al.*, 1965). Alkylation of a hydrogen bond donor group such as adenosylyl-N^6H_2 might hinder normal base pairing capacity dependent on the steric orientation of the alkyl group relative to the pairing bases but additional bonding capability may be gained through hydrophobic interactions as seen in bases (Helmkamp and Kondo, 1968; Leonard *et al.*, 1969). In the cases of ApiA and iApA, how-

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[1] Abbreviations used are: poly(IPA), poly(N^6-[Δ^2-isopentenyl]-adenylate); poly(N^6-MeA), poly(N^6-methyladenylate); poly(N^6-HEA), poly(N^6-hydroxyethyladenylate); poly(A), poly(adenylate); poly(U), poly(uridylate); IPA, N^6-(Δ^2-isopentenyl)adenylate residue in polynucleotide; IPAdo-5′-P, N^6-(Δ^2-isopentenyl)adenosine 5′-phosphate; 2MeS-IPA, 2-methylthio- derivative of IPA; Na_2EDTA, disodium salt of ethylenediaminetetraacetic acid. Other mono- and polynucleotide abbreviations follow the 1970 IUPAC-IUB rules (*Biochemistry 9*, 4022).

ever, the isopentenyl group prevents such close base stacking as found in ApA (Schweizer *et al.*, 1971) presumably due to a perpendicular orientation of the bulky isopentenyl group relative to the adenine ring analogous to 2-methylthio-N^6-(Δ^2-isopentenyl)adenine (McMullan and Sundaralingam, 1971).

The alkylated bases are of particular interest because of their prevalence in tRNAs. The large isopentenyl residue (or its 2-methylthio derivative in *Escherichia coli*) is on N^6 of adenine adjacent to the 3′-end of the anticodon of yeast $tRNA^{Ser}_{I \text{ and } II}$ (Zachau *et al.*, 1966), yeast $tRNA^{Tyr}$ (Madison and Kung, 1967), rat liver $tRNA^{Ser}$ (Staehelin *et al.*, 1968), *E. coli* $tRNA^{Tyr}_{SuIII}$ (Gefter and Russell, 1969), and *E. coli* $tRNA^{Trp}$ (Hirsh, 1970) and probably will be found in this position in other tRNAs which respond to codons beginning with uridylate as they are sequenced (Armstrong *et al.*, 1969a,b). This hypermodified nucleoside (Schweizer *et al.*, 1969) has been reported to affect the binding of such tRNAs to the mRNA–ribosome complex (Fittler and Hall, 1966; Gefter and Russell, 1969; Furuichi *et al.*, 1970; Gefter and Bikoff, 1971) but other specific functions of this alkylated adenosine cannot be eliminated. Since only a limited set of tRNAs contain IPA or 2MeS-IPA, the poor binding cannot be due to lack of recognition by a specific ribosomal binding site but may reflect a requirement for the hypermodified nucleotide in recognizing the Up. . . initiated codon on mRNA (Gefter and Bikoff, 1971). However, rather than a direct effect on codon recognition, it seems possible that the uniquely located isopentenyl group may impart some particular structure to the tRNAs in which it occurs making them biologically competent. Such an indirect effect could operate at the level of secondary or tertiary structure of these tRNAs (Lindahl *et al.*, 1966; Adams *et al.*, 1967).

We have synthesized poly(N^6-[Δ^2-isopentenyl]adenylic acid) [poly(IPA)] (Thedford and Straus, 1972) in order to study the effect of such hypermodified nucleosides on polynucleotide structure, particularly in regard to possible functions of the isopentenyl residues in tRNAs. We report here our findings concerning the self-interaction of poly(IPA) and its interaction with poly(U).

Materials and Methods

The poly(IPA) was synthesized as previously described (Thedford and Straus, 1972). Poly(A) and poly(U) were obtained from Miles Laboratories and used without further purification. Sodium cacodylate was from British Drug House. Sodium chloride and $MgCl_2$ were reagent grade materials. The Na_2EDTA was the Fisher Certified reagent.

Ultraviolet absorbance measurements were made using a Zeiss PMQII spectrophotometer and spectra were determined with a Hitachi-Coleman 124 recording spectrophotometer. Concentrations of poly(IPA), poly(A), and poly(U) were determined from their established extinction coefficients per mole of P at 260 nm (ϵ_P, 260 nm) in water (pH 5.5–7), 16.2×10^3 for poly(IPA) (Thedford and Straus, 1972), 11.4×10^3 for poly(A) (Felsenfeld and Huang, 1960), and 8.9×10^3 for poly(U) (Miles Laboratories). Changes in spectra and ϵ_P accompanying changes in ionic strength or cation were determined by first recording the spectrum of the sodium salt of the polynucleotide in water, then adding measured volumes of buffered NaCl or $MgCl_2$, and the spectrum was redetermined; the new ϵ_P's resulting from solvent changes were then calculated correcting for dilution. The spectrum of Mg^{2+}-poly(IPA) changed slowly at ionic strengths above 0.02 (see Results) and these samples were sealed and equilibrated 2 hr at 25° (when spectra were stable) before spectra determinations. The spectra of Mg^{2+}-poly(A) in 12.5 mM $MgCl_2$ did not become stable, due to precipitation (Eisinger *et al.*, 1963), so spectra and absorbance–temperature profiles of this polynucleotide salt were determined as rapidly as possible after addition of $MgCl_2$. Precipitation of Mg^{2+}-poly(IPA) also occurred on much longer standing, 24–48 hr (see Results). No precipitation of Mg^{2+}-poly(IPA)·poly(U) was observed.

The stoichiometry of polynucleotide interaction was studied by the method of continuous variation (Job, 1928; Felsenfeld and Rich, 1957b). Solutions of poly(IPA) and poly(U) were made up to identical concentrations (monomer basis) and mixtures of varying volumes of the two polynucleotide solutions to the same final volume were made. Absorbances of the mixtures were determined at several wavelengths after equilibrating 2 hr at 25°.

Absorbance–temperature profiles were determined using the Zeiss PMQII spectrophotometer. Temperatures were maintained using a refrigerated, thermostated bath (Tamson) circulating 50% ethylene glycol through the Zeiss cell carrier. Temperatures were determined with a thermistor inserted into the blank cell through its Teflon stopper and a microammeter (Yellow Springs Instrument Co.). The Zeiss cell compartment was continually flushed with dry nitrogen to prevent condensation on the cells when operating at temperatures <15°. Temperature increments of 5° were used except in transition regions when such increments were about 3°; samples were allowed to equilibrate 15–20 min at each temperature. All absorbances were corrected for solvent expansion (Mandel and Marmur, 1968).

Thermal denaturation of poly(IPA) and poly(A) in 0.01–0.4 M NaCl was carried out using 0.01 M Na cacodylate–5×10^{-4} M Na_2EDTA (pH 7.4) solvent; in 2–12.5 mM $MgCl_2$, the solvent was 0.01 M NaCl–0.01 M Na cacodylate (pH 7.4). The mixtures of Na^+-poly(IPA) and Na^+-poly(U) were examined in 0.1 M NaCl–0.01 M Na cacodylate–5×10^{-4} M Na_2EDTA (pH 7.4) solvent and the mixtures of Mg^{2+} salts over the 2–12.5 mM range of $MgCl_2$ in 0.01 M NaCl–0.01 M Na cacodylate (pH 7.4). With Mg^{2+}-poly(IPA)·poly(U), the helical complex was first heated to 31° and then cooled to 2° over several hours before thermal denaturation.

Many of the optical transitions of Na^+- and Mg^{2+}-poly(IPA) and poly(A) and the poly(IPA)·poly(U) helical duplex determined in the absorbance–temperature profiles were quite broad making precise estimation of T_m's difficult. The procedure used was to estimate the region of the profile where rate of change of absorbance with temperature was high and to record the temperature incident to one half of the total absorbance increase in such a region as the T_m. Such estimates of T_m are reasonably precise for transitions occurring over a temperature range of <20° but less precise for broader transitions which vary by as much as ±2° in replicate experiments. In the case of hypochromic transitions the same procedure was used to estimate transition midpoints (not T_m in the usual sense).

Results

Magnesium Salts of Poly(A) and Poly(IPA). Direct comparison of Mg^{2+}-polynucleotide spectra taken without equilibration (poly(A), Figure 1) and after 2 hr (poly(IPA), Figure 2) may only reflect differing kinetics of aggregation. However, Mg^{2+}-poly(IPA) in 0.01 M NaCl–0.01 M Na cacodylate–0.0125 M $MgCl_2$ (pH 7.4) immediately after adding $MgCl_2$ (at 10° where the spectrum is more stable) has λ_{max} 261 nm;

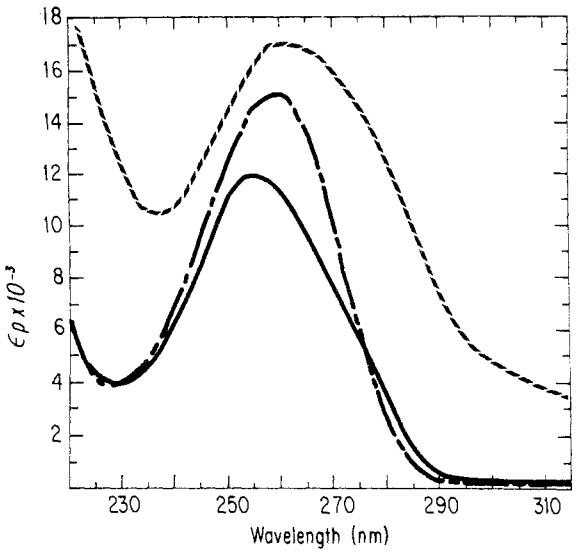

FIGURE 1: Spectra of Na^+ and Mg^{2+} salts of poly(A) and Ado-5'-P. Solvents were: 0.01 M NaCl–0.01 M Na cacodylate–5 × 10^{-4} M Na_2EDTA (pH 7.4) (—) and 0.0125 M $MgCl_2$–0.01 M NaCl–0.01 M Na cacodylate (pH 7.4) (----) for poly(A); 4.4 × 10^{-5} M P in each case. Both solvents were used for Ado-5'- (— — —) which gave identical spectra in each; 5.0 × 10^{-5} M P. Spectra were measured at 25° immediately after mixing with $MgCl_2$.

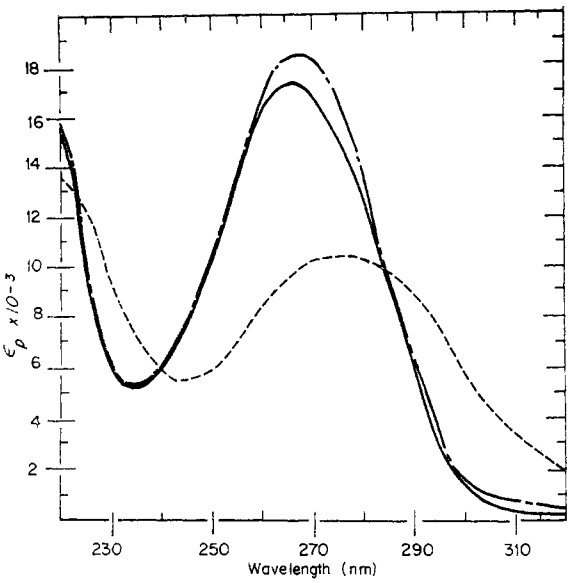

FIGURE 2: Spectra of Na^+ and Mg^{2+} salts of poly(IPA) and IPAdo-5'-P. Solvents were: 0.01 M NaCl–0.01 M Na cacodylate–5 × 10^{-4} M Na_2EDTA (pH 7.4) (—) and 0.0125 M $MgCl_2$–0.01 M NaCl–0.01 M Na cacodylate (pH 7.4) (-----) for poly(IPA); 3.1 × 10^{-5} M P. Both solvents were used for IPAdo-5'-P (— — —) which gave identical spectra in each; 4.6 × 10^{-5} M P. Spectra were measured at 25°.

λ_{min} 230 nm; $\epsilon_{P,261\ nm}$ 14.1 × 10^3; $A_{\lambda_{max}}/A_{\lambda_{min}}$ = 4.4; and A_{290}/A_{260} = 0.25. The Na^+-poly(IPA) in 0.01 M NaCl–0.01 M Na cacodylate (pH 7.4) at 10° shows λ_{max} 261 nm; λ_{min} 231 nm; $\epsilon_{P,261\ nm}$ 15.9 × 10^3; $A_{\lambda_{max}}/A_{\lambda_{min}}$ = 4.8; and A_{290}/A_{260} = 0.19. The hypochromicity of the Mg^{2+}-poly(IPA) relative to the Na^+ salt is opposite to the hyperchromicity observed in comparing Mg^{2+}- and Na^+-poly(A) (Figure 1). The red shift, apparent immediately with Mg^{2+}-poly(A), requires an equilibration period with Mg^{2+}-poly(IPA) (Figure 2). Similar spectral changes have been found for interaction of Hg^{2+} (Nandi *et al.*, 1965), Ag^+ (Eichorn *et al.*, 1967), and Zn^{2+} (Rifkind and Eichorn, 1972) with the base in mono- and polynucleotides. Such red shifts and absorbance changes may be characteristic of cation interaction with base ligands in polynucleotides.

The addition of 1 equiv of Na_2EDTA (12.5 mM) to the Mg^{2+}-poly(A) completely reversed the spectral change giving the spectrum of the sodium salt. However, the Mg^{2+}-poly(IPA) spectral change was not completely reversed under these conditions, such reversal occurring between 12.5 and 15 mM Na_2EDTA. It is estimated that the *maximum* deviation of either Mg^{2+}- or Na_2EDTA from equivalence under the experimental conditions would be 0.2 mM. These results suggest that Mg^{2+} interacts both with adenine and N^6-(Δ^2-isopentenyl)-adenine residues in the polynucleotides but is more strongly bound to the hypermodified base. Hypochromicity caused by Mg^{2+} also occurs with poly(N^6-MeA) compared with its sodium salt but information regarding shift in λ_{max} is not available (Griffin *et al.*, 1964). The Mg^{2+} salt of poly(A) has also been examined previously but at much lower Mg^{2+} concentrations (10^{-3}–5 × 10^{-5} M) where *hypo*chromicity is observed (Felsenfeld and Huang, 1960) but without the changes in λ_{max} and spectral shape (Stevens and Felsenfeld, 1964) observed in this work using 12.5 mM $MgCl_2$. The hypochromicity and spectral changes due to Mg^{2+} in poly(IPA) persist at least down to 2 mM $MgCl_2$ at which concentration the spectrum of poly(A) resembles the Na^+ salt (Stevens and Felsenfeld, 1964) (as it does at 8 mM $MgCl_2$) supporting the conclusion that Mg^{2+} is more strongly bound to the base in poly(IPA) than in poly(A).

The extensive precipitation of poly(A) in >5 mM $MgCl_2$ after 24-hr equilibration has been reported (Eisinger *et al.*, 1963; Sander and Ts'o, 1971). This precipitation complicated interpretation of Mg^{2+} dependent spectral changes and absorbance–temperature profiles of Mg^{2+}-poly(A) using 12.5 mM $MgCl_2$. The precipitation occurs slowly but, unlike Mg^{2+}-poly(IPA), there does not appear to be a lengthy period where Mg^{2+}-poly(A) is completely dissolved exhibiting a stable spectrum above 5 mM $MgCl_2$. Turbidity due to Mg^{2+}-poly(A) is observed after 15 min at 25° in 12.5 mM $MgCl_2$ and more rapidly at higher temperatures up to about 65° though such solutions remain water clear for up to 60 min at 10° exhibiting progressive red shift and hypochromicity consistent with intermolecular aggregation (D. B. Straus, unpublished experiments). Solutions of Mg^{2+}-poly(IPA) become turbid only after more than 5 hr at 25°. Changes in Mg^{2+}-poly(IPA) spectra consistent with intermolecular aggregation occur over about 2 hr but the spectra are then stable for at least 3 hr without precipitation. As with Mg^{2+}-poly(A), the rate of aggregation and precipitation of Mg^{2+}-poly(IPA) was directly related to temperature. Precipitation of Mg^{2+}-poly(IPA) appears complete in 20–24 hr at 25°. Both Mg^{2+}-polynucleotide precipitates are soluble at temperatures above 65°.

Melting of Poly(A) and Poly(IPA) Salts. Absorbance–temperature profiles of Na^+- and Mg^{2+}-poly(A) are given in Figure 3. The Na^+-poly(A) at ionic strength 0.02 shows a profile similar to those already reported (*e.g.*, Fresco and Klemperer, 1959; Leng and Felsenfeld, 1966), a relatively noncooperative hyperchromic transition with a T_m of approximately 33°. The Mg^{2+}-poly(A) at ionic strength 0.06 (12.5 mM $MgCl_2$) shows a broad temperature-dependent hypochromic transition from 15 to 55° followed by a more cooperative hyperchromic transition with T_m about 74°. The hypochromic transition is coincident with the temperature range where Mg^{2+}-poly(A) precipitates most rapidly and the hypochromicity probably reflects removal of the precipitated poly-

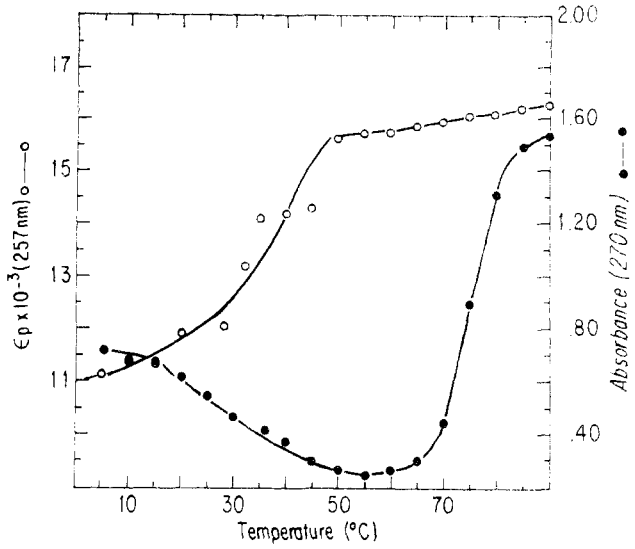

FIGURE 3: Absorbance–temperature profiles of poly(A) salts. For Na^+-poly(A) (5.8×10^{-5} M P), the solvent was 0.01 M NaCl–0.01 M Na cacodylate–5×10^{-4} M Na_2EDTA (pH 7.4) with denaturation followed at 257 nm (○). For Mg^{2+}-poly(A) (4.4×10^{-5} M P), the solvent was 0.0125 M $MgCl_2$–0.01 M NaCl–0.01 M Na cacodylate (pH 7.4) followed at 270 nm (●). Note the different ordinates for the two profiles; absorbance rather than ϵ_P was recorded for Mg^{2+}-poly(A) due to precipitation visible between 20 and 65° (see text).

nucleotide for the most part. Observable turbidity in Mg^{2+}-poly(A) solutions disappears at the beginning of the final hyperchromic transition so the latter reflects both increased concentration of Mg^{2+}-poly(A) and formation of random coils. The absorbance–temperature profiles of poly(A) in 2 and 8 mM $MgCl_2$ are similar to the Na^+-poly(A) (Table II).

The melting of Na^+-poly(IPA) (Figure 4) shows a small hyperchromic transition between about 8 and 20° (T_m = 13°) followed by an equal hyperchromic change between 20 and 65° above which temperature poly(IPA) extinction coefficient is constant. The cooperative low temperature transition was not greatly affected by increased solvent NaCl (Table I), the T_m being reduced to about 10° at ionic strength 0.41. However, the noncooperative transition occurred at higher temperatures and was better separated from the low-temperature transition with increasing ionic strength and became hypochromic at ionic strength 0.41. The greatly reduced hyperchromicity of the low temperature transition of Na^+-poly(IPA) (Figure 4) compared to Na^+-poly(A) (Figure 3) indicates a much lower degree of stacked base structure in the poly(IPA). The broad high temperature hyperchromic transitions of both polynucleotides may result from similar structural changes.

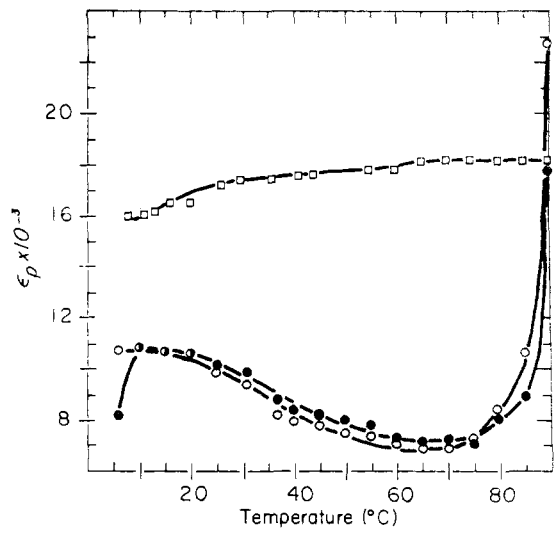

FIGURE 4: Absorbance–temperature profiles of poly(IPA) salts. The solvent was 0.01 M NaCl–0.01 M Na cacodylate–5×10^{-4} M Na_2-EDTA (pH 7.4) for Na^+-poly(IPA) (3.8×10^{-5} M P) followed at 267 nm (□). For Mg^{2+}-poly(IPA) (6.2×10^{-5} M P), the solvent was 0.0125 M $MgCl_2$–0.01 M NaCl–0.01 M Na cacodylate (pH 7.4) followed at 267 nm (○) and at 280 nm (●). Faint turbidity was observed in Mg^{2+}-poly(IPA), 40–60°, and so ϵ_P's in this temperature range are slightly inaccurate.

TABLE I: Effect of Ionic Strength on Thermal Transitions of Na^+-Poly(IPA).

[NaCl][a] (M)	Ionic Strength	Approximate Cooperative Transition T_m (deg)	Uncooperative Transition Breadth (deg)
0.01[b]	0.02	13	20–65
0.10	0.11	12	45–85
0.15	0.16	12	55–>95[c]
0.20	0.21	12	65–>95[c]
0.40	0.41	10	40–90[d]

[a] The solvent for thermal denaturation was 0.01 M Na cacodylate–5×10^{-4} M Na_2EDTA (pH 7.4) (except for 0.10 M NaCl where pH was 7.0) with the indicated NaCl concentration. [b] The melting profile at this [NaCl] presented in Figure 4. [c] Absorbance increased with temperature to the highest temperature used. [d] This transition was hypochromic.

The melting of Mg^{2+}-poly(IPA) is similar in form to Mg^{2+}-poly(A) with an added structural transition at low temperature evident from the reproducible change in A_{280}/A_{267} from 0.78 at 6° to 1.00 at 10°. There is a broad hypochromic change between 20 and 65° with A_{280}/A_{267} remaining close to 1.0 and then a highly cooperative hyperchromic transition between 80 and 90° (incomplete at 90°) with change in A_{280}/A_{267} to 0.78 at 90°; the spectral ratio might become even lower on completion of the transition at a slightly higher temperature not tested. The hypochromic transition may in part reflect precipitation of Mg^{2+}-poly(IPA) since turbidity was observed in such solutions between 40 and 60° under conditions used in Figure 4.

Compared with Mg^{2+}-poly(A), both the hypochromic and hyperchromic transitions of Mg^{2+}-poly(IPA) are at higher temperatures and the high temperature transition is more cooperative and much more hyperchromic. The ϵ_P's at both 280 and 267 nm for Mg^{2+}-poly(IPA) at 90° are well above those for either the Na^+ salt at 90° (Figure 4) or IPAdo-5′-P at 25° (Figure 2) suggesting that Mg^{2+} binding to the base chromophore may persist at this temperature.

The variation in Mg^{2+}-poly(IPA) transitions with [$MgCl_2$] was also studied (Table II). The low-temperature change in A_{280}/A_{267} (transition at 280 nm) was not affected by [Mg^{2+}] between 2 and 12.5 mM. The T_m of the high-temperature transition was similarly unaffected above 8 mM Mg^{2+} (and possibly lower concentrations) but was markedly decreased at 2 mM $MgCl_2$. The hypochromic transition had midpoint and breadth inversely related to Mg^{2+} concentration.

Stoichiometry of Poly(IPA)–Poly(U) Interaction. The variation of absorbance with composition of Na^+-poly(IPA) and

TABLE II: Mg^{2+} Dependence of the Thermal Transitions of Mg^{2+}-Poly(A) and -Poly(IPA).

		Approximate Transition Midpoint[b] (deg)				
		Poly(A)		Poly(IPA)		
[Mg^{2+}][a] (mM)	Ionic Strength	Hypochromic	Helix → Coil	280 nm	Hypochromic	Helix → Coil
2	0.03	Absent	43[d]	10	>85[c]	56
8	0.04	Absent	56[d]	10	62	>87[c]
12.5	0.06	36	74	9	42	>87[c]

[a] Solvent was 0.01 M NaCl–0.01 M Na cacodylate (pH 7.4) with the indicated concentrations of $MgCl_2$. [b] For Mg^{2+}-poly(A) at 12.5 mM Mg^{2+}, two transitions were observed between 0 and 95° at 270 nm; one hypochromic and the second hyperchromic (*i.e.*, Figure 3). For Mg^{2+}-poly(IPA), three transitions were observed over the same temperature range at 267 and 280 nm; the first at 280 nm but not at 267 nm; a second hypochromic transition; and a third hyperchromic transition considered to be the helix → coil transition (*i.e.*, Figure 4) (see text). For 2 and 8 mM Mg^{2+}, these different transitions for each polynucleotide were changed as indicated. [c] Transitions incomplete at 95°, the highest temperature used. It is estimated that the midpoints would be only a few degrees higher than values given if the transitions could be completed. [d] These hyperchromic transitions were uncooperative and resembled the melting of the Na^+-poly(A) (see Figure 3).

Na^+-poly(U) are presented in Figure 5. At 280 nm, no interaction is discernible while at 267 nm there is a suggestion of an inflection corresponding to a 1:1 complex. Other information was sought to confirm the possibility of helical association of the Na^+ polynucleotides. Difference spectra were constructed using the measured absorbances of an equimolar mixture of the Na^+ polynucleotides and the summed absorbances of the noninteracted Na^+-poly(IPA) and Na^+-poly(U) between 240 and 300 nm at several temperatures. There was no significant deviation of the difference spectra from zero (maximum ΔA was -0.007) between 4 and 25°. Thus, the suggested inflection in the mixing curve could not be confirmed and it appears that the sodium salts of poly(IPA) and poly(U) do not form a helix at ionic strength 0.11 using the criterion of hypochromicity. Mixing curves run at 4° and ionic strength 0.11 and at lower ionic strengths also indicated lack of helical interaction. Omission of the 5×10^{-4} M Na_2EDTA in the polynucleotide solvent had no effect on the mixing curves.

In contrast to the lack of hypochromic helical interaction of the sodium salts, the Mg^{2+} salts of poly(IPA) and poly(U) show a clear inflection in the mixing curve where the composition of the mixture is equimolar in each polynucleotide indicating formation of a duplex poly(IPA)·poly(U) helix (Figure 6).

Absorbance–Temperature Profiles of Equimolar Poly(IPA)–Poly(U). The heat denaturation of equimolar mixtures of the sodium polynucleotides at ionic strengths 0.02 and 0.11 resemble those for Na^+-poly(IPA) alone (Figure 4) at the same ionic strengths between 6 and 95°. This result supports the previous conclusion that there is no base pairing or other optically observable interaction between the sodium homopolyribonucleotides. However, the Mg^{2+}-poly(IPA)·poly(U)

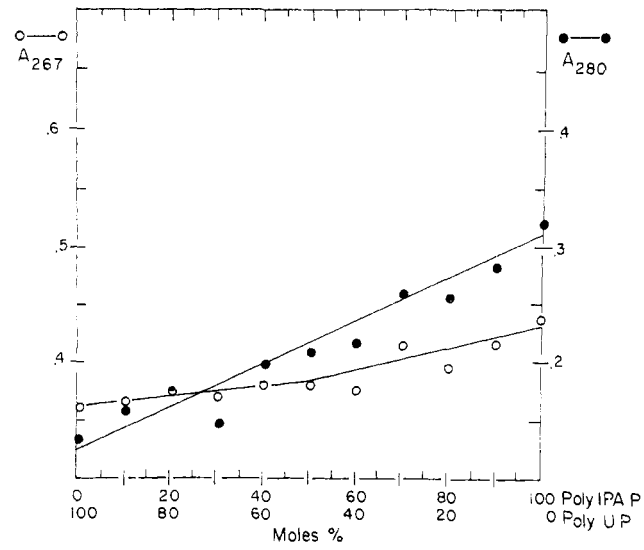

FIGURE 5: Interaction of the sodium salts of poly(IPA) and poly(U) by the method of continuous variation at 25°. Solvent was 0.1 M NaCl–0.01 M Na cacodylate–5×10^{-4} M Na_2EDTA (pH 6.95): (○) for 267 nm; (●) for 280 nm. Polynucleotide concentration 5.0×10^{-5} M P.

helical complex gave a distinctive melting profile (Figure 7) indicating base pairing between the two polynucleotides even to high temperatures.

The melting of Mg^{2+}-poly(IPA)·poly(U) shows an initial hyperchromic transition observed at 267 nm but not at 280 nm with a T_m of 8° followed by a hypochromic transition with no change in A_{280}/A_{267} between 25 and 65° and, finally, a large hyperchromic transition with a decrease in A_{280}/A_{267} incomplete at the highest temperature used, 95°. This melting profile of the duplex helix (Figure 7) appears similar to that of Mg^{2+}-poly(IPA) (Figure 4) but the differences in T_m's, the differing breadths of the transitions, the different spectral changes in the hyperchromic transitions exemplified by the A_{280}/A_{267} ratio, and the widely different degree of hyperchromicity in the final transitions demonstrates that a different

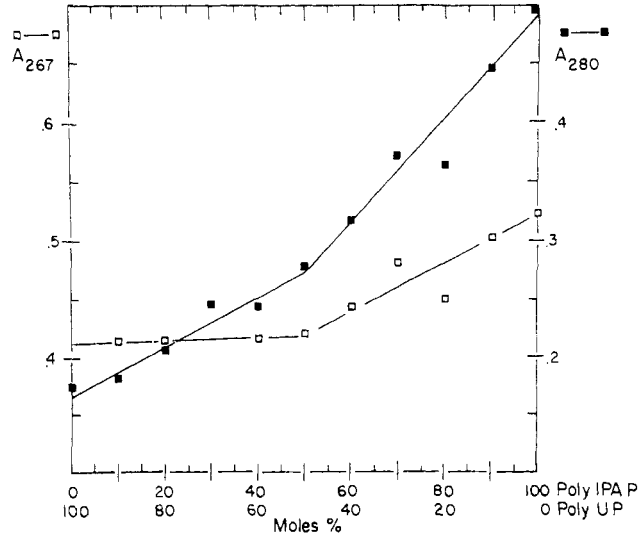

FIGURE 6: Interaction of the magnesium salts of poly(IPA) and poly(U) by the method of continuous variation at 25°. Solvent was 0.0125 M $MgCl_2$–0.01 M NaCl–0.01 M Na cacodylate (pH 7.4): (□) for 267 nm; (■) for 280 nm. Polynucleotide concentration 5.0×10^{-5} M P.

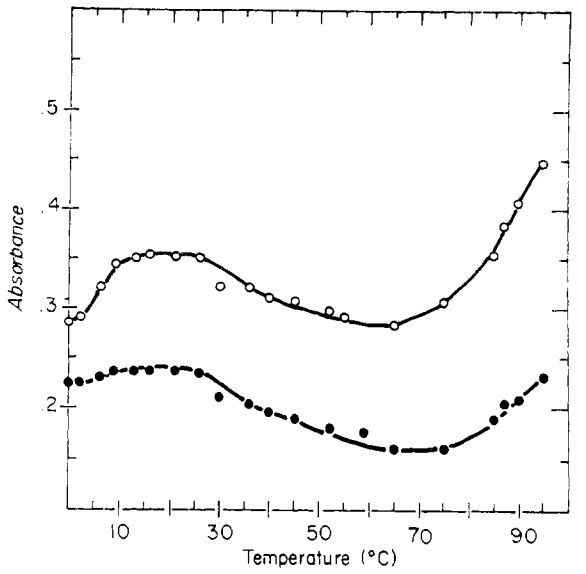

FIGURE 7: Absorbance–temperature profiles of Mg^{2+}-poly(IPA)·poly(U). Solvent was 0.0125 M $MgCl_2$–0.01 M NaCl–0.01 M Na cacodylate (pH 7.4.) Sample heated to 31° and cooled to 0° before melting: (○) for 267 nm; (●) for 280 nm. Polynucleotide concentration 5.0×10^{-5} M P.

structure is involved in the denaturation of Mg^{2+}-poly(IPA)·poly(U) than in Mg^{2+}-poly(IPA) throughout the temperature range studied.

Discussion

Interaction of Mg^{2+} with Poly(A) and Poly(IPA). The spectral changes and the qualitatively similar changes in absorbance–temperature profiles of the Mg^{2+} salts of poly(A) and poly(IPA) compared to their Na^+ salts (Figures 1–4) indicate that the divalent cation (12.5 mM) is causing a major change in the conformation of each homopolyribonucleotide. The question remains whether such conformational change is due to indirect effects of Mg^{2+} acting by more effective neutralization of phosphodiester repulsive force through site binding (reviewed by Felsenfeld and Miles, 1967) and changing solvent structure (Dix and Straus, 1972) or whether a direct interaction between Mg^{2+} and purine base causes the observed change.

The strong interaction of Mg^{2+} with poly(A) results in virtual stoichiometric binding of 1 Mg^{2+} per 2 P as determined conductometrically at low poly(A) concentration (Felsenfeld and Huang, 1959) and by specific Mg^{2+} activity determination at higher poly(A) concentration (Sander and Ts'o, 1971). Optical properties of poly(A) at the equivalence point remain essentially constant at least to a ratio of Mg^{2+}: P of 25 (Felsenfeld and Huang, 1960; Stevens and Felsenfeld, 1964) and only a single type of binding (presumably to phosphodiester) could be identified using the Mg^{2+}-selective electrode (Sander and Ts'o, 1971). The finding of characteristic changes in poly(A) and poly(IPA) spectra and melting profiles at 12.5 mM Mg^{2+} (Mg^{2+}: P of 200), much above the stoichiometric concentration where all phosphodiester sites are occupied, strongly suggests site binding of the divalent cation to the purine bases. This possibility is given further support by the observation of different sensitivities of the optical and melting properties of the two polynucleotides to [Mg^{2+}] (Table II); such differences in sensitivity to Mg^{2+} must be attributed to differences in the base ligands since these are the only structural elements in the two polynucleotides which differ.

The hypochromicity characteristic of base stacking in polynucleotides (Holcomb and Tinoco, 1965) is accompanied by small changes in λ_{max} and spectral shape (*e.g.*, Figure 1 where Na^+-poly(A) λ_{max} is blue shifted 3 nm compared to Ado-5′-P and Figure 2 where Na^+-poly(IPA) λ_{max} is blue shifted 2 nm compared to IPAdo-5′-P). It does not appear possible to explain the red shifts in λ_{max} of the Mg^{2+} salts of poly(A) and poly(IPA) compared to their monomers on the basis of a different type or higher degree of base stacking since the spectral change is both larger and in the opposite direction to that found comparing Na^+ salts and monomers. Rather, the change in poly(A) and poly(IPA) spectra caused by 12.5 mM Mg^{2+} is most likely due to changed electron distribution in the chromophores caused by direct interaction of Mg^{2+} ion with base ligands. The qualitatively similar spectral changes caused by other cations proven to associate directly with specific polynucleotide bases by a variety of methods (Nandi *et al.*, 1965; Eichorn *et al.*, 1967; Rifkind and Eichorn, 1972) support the conclusion that Mg^{2+} ion interacts directly with adenine and N^6-(Δ^2-isopentenyl)adenine moieties in poly(A) and poly(IPA).

Since Mg^{2+} ion appears to affect the distribution of electrons in the base in poly(A) and poly(IPA), the observed hyperchromicity of Mg^{2+}-poly(A) and hypochromicity of Mg^{2+}-poly(IPA) might be caused, at least in part, by changed intrinsic extinction coefficients of the chromophores in addition to base stacking interactions determining conformation. However, the last transition in the melting profiles of both Mg^{2+} polynucleotides (Figures 3 and 4) shows large hyperchromicity above any redissolution of precipitates consistent with temperature-dependent conformational changes with unstacking of bases. Thus, both intrinsic ϵ_P and base stacking are changed by Mg^{2+} binding. However, no change in spectrum of either monomer is observed in the presence of Mg^{2+} (Figures 1 and 2) indicating that Mg^{2+}-dependent spectral changes require the polynucleotide structure.

The extent of base stacking appears much higher in the Mg^{2+} salts than the corresponding Na^+ salts particularly for poly(IPA) whose Na^+ salt shows only slight stacking interaction demonstrated by the slight hypochromicity relative to monomer and relatively small hyperchromicity on melting (Figures 2 and 4) but whose Mg^{2+} salt is highly hypochromic at 25° (240–284 nm) and whose absorbance at 267 nm triples on melting between 75 and 90° with no interference from precipitate.

The low degree of base stacking observed for the Na^+-poly(IPA) (Figures 2 and 4) suggests predominant perpendicular orientation of the isopentenyl group to the adenine for this salt as in poly(N^6-MeA), poly(N^6-HEA), and 2-MeS-IP-adenine (Griffin *et al.*, 1964; Van Holde *et al.*, 1965; McMullan and Sundaralingam, 1971; respectively). However Mg^{2+}-poly(IPA) shows a high degree of base stacking indicating that the isopentenyl group is predominantly coplanar with adenine. The contrast of the apparent orientations of the isopentenyl group in the two salts suggests that the necessary rotation around the C^6–N^6 bond is promoted by magnesium ion possibly as a consequence of its interaction with the base, as is also demonstrated by Mg^{2+}-poly(IPA)·poly(U) helix formation (see Results).

Another factor increasing the thermal stability of Mg^{2+}-poly(IPA) compared to that of Mg^{2+}-poly(A) (Figures 3 and 4) is the additional hydrophobic bonding afforded by the isopentenyl substituent. The Corey–Pauling–Koltun models of

segments of poly(IPA) and poly(IPA)·poly(U) with bases closely associated in a helical array (isopentenyl group coplanar with adenine) shows intimate contact of isopentenyl groups on adjacent adenines. Similar increased hydrophobic interaction of bases consequent to alkylation has been reported for bases, polynucleotide analogs, and polynucleotides (Shugar and Szer, 1962; Chan *et al.*, 1964; Szer and Shugar, 1966; Broom *et al.*, 1967; Helmkamp and Kondo, 1968; Browne *et al.*, 1968; Leonard *et al.*, 1969; Iwamura *et al.*, 1970).

The temperature-dependent hypochromic transitions of Mg^{2+}-poly(A) and -poly(IPA) (Figures 3 and 4) must be due in part to precipitation. However, hydrophobic interaction between stacked bases should increase with temperature in this range (Scheraga, 1963) and such bonding may contribute to the observed hypochromicity. Though quantitative estimates of the separate contributions of precipitation and solution phase base stacking to these hypochromic transitions is not possible, it appears that precipitation is predominant in the Mg^{2+}-poly(A) melting profile and base stacking in the Mg^{2+}-poly(IPA) profile. At 65°, where turbidity disappears in both cases, the Mg^{2+}-poly(A) shows a significant increase in absorbance consistent with dissolution of the substantial poly(A) precipitate but no such increase in absorbance of Mg^{2+}-poly(IPA) is observed until 80° suggesting that precipitate dissolution contributes little to absorbance and that the hypochromicity from 20 to 65° is mainly due to base stacking.

Poly(IPA)–Poly(U) Interaction. Two conditions must be met in order that base pairing occur to give a poly(IPA)·poly(U) helix: the paired bases in each strand must be close together to maximize stabilizing stacking interactions providing hypochromicity (Holcomb and Tinoco, 1965); and specificity of base pairing must be provided by hydrogen bonding between N^6 and N^1 of IPA with O^4 and N^3 of U, respectively (Watson and Crick, 1953), or N^6 and N^7 of IPA with the same atoms in U (Hoogsteen, 1963). The required close stacking of base pairs and the base pairing itself requires coplanarity of the isopentenyl group and the adenine ring in poly(IPA). A perpendicular orientation of these groups (McMullan and Sundaralingam, 1971) would make the N^6–H bond 90° out of the plane of the putative base pair thereby precluding either Watson–Crick or Hoogsteen base pairing. With isopentenyl group perpendicular to adenine, base pairing between Na^+-poly(IPA) and Na^+-poly(U) would be impossible without rotation around the C^6–N^6 bond in poly(IPA). The energy barrier to such rotation in Na^+-poly(N^6-alkyladenylates) appears to increase in the order poly(A) < poly(N^6-MeA) < poly(N^6-HEA) = poly(IPA) based on the T_m's of the sodium salts of these polynucleotides and duplex helix formation with poly(U) (Griffin *et al.*, 1964; Ikeda *et al.*, 1970; Van Holde *et al.*, 1965; this work; respectively).

In contrast to Mg^{2+}-poly(N^6-MeA)·poly(U) which has a T_m increased only 2° above the sodium salt (Griffin *et al.*, 1964) and the lack of interaction between Mg^{2+}-poly(N^6-HEA) and Mg^{2+}-poly(U) (Van Holde *et al.*, 1965), a duplex Mg^{2+}-poly(IPA)·poly(U) helix is formed which is stable to high temperature (T_m near 90°, Figure 7). Clearly, formation of this helix requires that the isopentenyl group be coplanar with the adenine ring just as in neutral Mg^{2+}-poly(IPA). The lack of helix formation with the sodium salts contrasted with ready formation of Mg^{2+}-poly(IPA)·poly(U) suggests that the site binding of Mg^{2+} to the poly(IPA) component allows base pairing and close base pair stacking; in other words, Mg^{2+} site binding to the hypermodified base lowers the energy barrier to rotation around the C^6–N^6 bond. A further inference from these considerations is that Mg^{2+} does not cause rotation about C^6–N^6 in poly(N^6-MeA) and poly(N^6-HEA) possibly because Mg^{2+} site binding to the purine base requires or is at least facilitated by the N^6-isopentenyl group.

The three different optically observable transitions of Mg^{2+}-poly(IPA)·poly(U) (Figure 7) indicate the existence of at least four different structures between 0 and 95°. Assuming that a minimum of two hydrogen bonds are required for forming a base pair, the N^6-isopentenyl group would preclude formation of triple-stranded helices like poly(A)·poly(U)$_2$ at any temperature or in any solvent. Thus, the three structures existing below 80° (Figure 7) must be duplex helices. The high temperature form above 95° is most likely a mixture of the two random coils. Further experimentation is required to define the helical structure at each stage of melting; however, some interpretation can be made.

The low-temperature transition, T_m = 8°, is at about the same temperature as the low-temperature transition of Mg^{2+}-poly(IPA) (Figure 4). Both transitions show a change in the spectrum of helix (the A_{280}/A_{267} ratio is an example) but the relatively small hyperchromicity suggests that unstacking of bases is not extensive. The fact that the changes in A_{280}/A_{267} in the two cases are in opposite directions indicates that paired uracil residues are affecting the spectrum of Mg^{2+}-poly(IPA)·poly(U) as would be expected. The hypochromic transition between 25 and 65° most likely is from closer stacking of IPA–U base pairs due to hydrophobic interactions of the N^6-isopentenyl residues as already indicated for Mg^{2+}-poly(IPA) (Figure 4). The helix → coil transition, incomplete at 95°, is less cooperative than expected on the basis of melting Mg^{2+}-poly(A)·poly(U)$_2$ (Stevens and Felsenfeld, 1964) (10^{-3} M Mg^{2+}) or the melting of Mg^{2+}-poly(N^6-MeA)·poly(U) (Griffin *et al.*, 1964) (0.016 M Mg^{2+}). At present, we have no hypothesis explaining this reduced cooperativity.

Implications for tRNA Structure. Magnesium is the most abundant divalent cation in cells being present in concentrations of about 2–15 mM (*i.e.*, 5–30 mequiv per kg wet weight) (Williams and Wacker, 1967; Wacker and Parisi, 1968). The binding of Mg^{2+} to the base in poly(IPA) occurs throughout this range as indicated by typical Mg^{2+}-poly(IPA) spectra at $MgCl_2$ concentrations as low as 2 mM, by typical absorbance–temperature profiles in this concentration range, and by incomplete reversion of the poly(IPA) spectrum from Mg^{2+} form to Na^+ form when mixed with equimolar Na_2EDTA. The binding of Mg^{2+} to adenine in poly(A) seems to occur at $MgCl_2$ concentration >10 mM but is not apparent at lower concentration. The results presented here demonstrate a Mg^{2+} dependence for duplex poly(IPA)·poly(U) helix formation and for an ordered structure of poly(IPA) in the physiological range of Mg^{2+} concentration and they suggest the possibility that site binding of Mg^{2+} to the hypermodified base ligand may allow interactions contributing to the maintenance of higher order structure of certain tRNAs.

Several recent studies using widely different approaches indicate multiple classes of divalent ion binding sites in tRNAs (Sander and Ts'o, 1971; Danchin, 1972; Rialdi *et al.*, 1972). Though these studies were limited to free Mg^{2+} (or Mn^{2+}) concentrations mostly lower than those used in this work and only approaching the physiological range, it seems possible that one such class of Mg^{2+} binding site above 2 mM free Mg^{2+} may be the hypermodified base in certain tRNAs. Present evidence is inconclusive in this regard and it would certainly be difficult to obtain since there is only one hypermodified base per tRNA molecule where they occur. This singularity of the hypermodified base in tRNA compared to the multiplicity

in poly(IPA) makes the homopolynucleotide a poor model for base stacking or base pairing interactions of the hypermodified base in tRNA. However, the results demonstrate that such interactions of the hypermodified base can occur in the presence of physiological Mg^{2+} concentrations.

It has been concluded from calorimetric studies of Mg^{2+} binding to $tRNA^{Phe}$ (Rialdi *et al.*, 1972; Levy and Biltonen, 1972) and spectrophotometric studies of the thermal transitions of Na^+ and Mg^{2+} salts of several pure tRNAs (Cole *et al.*, 1972) that Mg^{2+} merely stabilizes an existing tertiary structure of tRNA required for biological activity (Adams *et al.*, 1967). Structural alterations of the tRNAs caused by interaction with Mg^{2+} and observed spectrophotometrically are definite but small (Cole *et al.*, 1972) as would be expected of formation of a single base pair or addition of the single IPA to a stack of bases. The possibility that interaction of the single hypermodified base in some tRNAs with Mg^{2+} causes a small structural change essential for biological competence of the tRNA should be explored further with other models and with tRNAs.

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