Circular Dichroism and Ordered Structure of Bisnucleoside Oligophosphates and Their Zn^{2+} and Mg^{2+} Complexes†

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ABSTRACT: Circular dichroism, absorbance, hypochromicity, and the formation of Mg^{2+} and Zn^{2+} complexes have been measured for a series of bisnucleoside oligophosphates that contain adenosine, guanosine, and mixed guanosine/adenosine, guanosine/cytidine, and guanosine/uridine, as well as 7-methylguanosine and ribose-methylated purine nucleosides. All of the metal complex ions have stacking interactions at 2 °C, 10 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 8.0. There is a measurable degree of base stacking for all unsubstituted purine nucleotides that differs, however, from that of bases in nucleic acids. The degree of base stacking varies with the length of oligophosphate chains and the state of methylation. The effect of 7-methylation of guanosine is interpreted as causing a switch of nucleic acid base stacking from an atypical to a typical mode, which could be important for cap function in mRNA. The Mg^{2+} and Zn^{2+} complexes give rise to characteristic circular dichroism. In all instances excepting 7-methylated bisguanosine oligophosphates, the active secondary structures are disrupted, and in this regard, Zn^{2+} is more effective than Mg^{2+}. At least two sets of binding sites are involved. A single metal ion is bound tightly. Stability, in terms of equilibrium constants, increases by more than 1000-fold as a function of chain length varying from two to six phosphates. The consequences of methylation are only minor. Electrostatic attraction between metal ions and phosphates is the most likely mechanism of these phenomena as judged by the effect of high ionic strength.

Some years ago, in a study of the amino acid activation step in protein synthesis, we observed the development of unusual optical rotatory dispersion (ORD) and circular dichroic (CD) patterns on incubation of ATP, lysyl-tRNA synthetase, lysine, and Mg^{2+}, in Tris[1] buffer, pH 7.8 (Zamecnik et al., 1966; Randerath et al., 1966). A new biological compound was identified, adenosine(5′)tetraphospho(5′)adenosine (Ap_4A), formed in the back-reaction of the lysyl adenylation reaction, as follows:

enzyme·Lys–AMP + pppA ⇌ AppppA + Lys + enzyme

The enzymatically formed Ap_4A is identical with that synthesized chemically as a side reactant in the synthesis of ATP (Moffatt & Khorana, 1961; Reiss & Moffatt, 1965). Subsequently, Ap_4A was identified in living cells (Zamecnik & Stephenson, 1969) and has now been found in a wide variety of eukaryotic and prokaryotic organisms.

Its concentration rises 50–100-fold in synchronized eukaryotic cells between the G_0/G_1 and G_1/S phases of the cell cycle (Rapaport & Zamecnik, 1976). When cells are permeable, addition of 10^{-4} M Ap_4A to the resting cells promptly restores DNA synthesis and results in development of numerous replication eyes (Grummt, 1978). Hence, Ap_4A was characterized as a positive pleiotypic activator (Rapaport & Zamecnik, 1976), intimately related to DNA synthesis. It associates tightly but noncovalently with a subunit of DNA polymerase α (Grummt et al., 1979; Rapaport et al., 1981a). In an in vitro system consisting of highly purified DNA polymerase α, activated DNA, poly(dT), or double-stranded synthetic octadecamer from the origin-area sequence of SV40 DNA synthesis as a templete, Ap_4A serves as a good primer for DNA synthesis (Rapaport et al., 1981a,b; Zamecnik et al., 1982). Ap_4A, as the primer, attaches covalently to nascent DNA chains (Rapaport et al., 1981a; Zamecnik et al., 1982). Ap_4A can also serve as an initiator dinucleotide in the primase-catalyzed synthesis of a short ribonucleotide primer chain for DNA synthesis (E. F. Baril, unpublished observations).

Other functional observations related to Ap_4A include (1) stimulation of ADP ribosylation (Yoshihara & Tanaka, 1981), (2) inhibition of terminal deoxynucleotidyl transferase (Ono et al., 1980), (3) a high concentration in *Salmonella typhimurium* following exposure to toxic compounds (Bochner et al., 1982), and (4) that 2′–5′-oligoadenylate synthetase can add 5′-AMP in 2′–5′ linkage to Ap_4A (Cayley & Kerr, 1982).

In malignant cells grown in tissue culture, a shift from aerobiosis to anaerobiosis results in a 98% fall in the concentration of Ap_4A, while the ATP and ADP concentrations remain unchanged. Anaerobiosis also results in a cessation of initiation of DNA synthesis, and Probst et al. (1983) call attention to a possible relationship between the decline in Ap_4A concentration and the cessation of new DNA synthesis. The high level of Ap_4A present in the dense granules of platelets is of particular interest (Flodgaard & Klenow, 1982), since it is a finding of potential medical importance in relation to blood clotting.

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[1] Abbreviations: Ap_2A, adenosine(5′)diphospho(5′)adenosine; Ap_3A, adenosine(5′)triphospho(5′)adenosine; Ap_4A, adenosine(5′)tetraphospho(5′)adenosine; Ap_5A, adenosine(5′)pentaphospho(5′)adenosine; Ap_6A, adenosine(5′)hexaphospho(5′)adenosine; Gp_2G, guanosine(5′)diphospho(5′)guanosine; Gp_3G, guanosine(5′)triphospho(5′)guanosine; Gp_4G, guanosine(5′)tetraphospho(5′)guanosine; Gp_5G, guanosine(5′)-pentaphospho(5′)guanosine; Gp_6G, guanosine(5′)hexaphospho(5′)-guanosine; Gp_3A, guanosine(5′)triphospho(5′)adenosine; Gp_3C, guanosine(5′)triphospho(5′)cytidine; Gp_3U, guanosine(5′)triphospho(5′)uridine; Gp_3Gm, guanosine(5′)triphospho(5′)-2′-*O*-methylguanosine; Gp_3Am, guanosine(5′)triphospho(5′)-2′-*O*-methyladenosine; m^7Gp_3G, 7-methylguanosine(5′)triphospho(5′)guanosine; $m^7Gp_4m^7G$, 7-methylguanosine-(5′)tetraphospho(5′)-7-methylguanosine; m^7Gp_3Gm, 7-methylguanosine(5′)triphospho(5′)-2′-*O*-methylguanosine; $m^7Gp_3m^6Am$, 7-methylguanosine(5′)triphospho(5′)-N^6-methyl-2′-*O*-methyladenosine; ApcppcpA, adenosine(5′)phosphomethylenediphosphomethylene-phospho(5′)adenosine; Tris, tris(hydroxymethyl)aminomethane; AUFS, absorbance unit full scale; EDTA, ethylenediaminetetraacetic acid.

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The peculiar CD and ORD patterns characteristic of Ap_4A and Ap_3A were ascribed to unusual purine ring stacking in which the two rings facing one another in an α/α configuration (Scott & Zamecnik, 1969) is preferred over the usual α/β one of polynucleotides (Scott & Zamecnik, 1969; Tinoco et al., 1963; Tinoco, 1964).

It is now known that Zn^{2+} enhances the formation of both Ap_4A and Ap_3A when added to the amino acid activating enzymes for phenylalanine, lysine, and alanine (Plateau et al., 1981; Goerlich et al., 1982; Plateau & Blanquet, 1982; Brevet et al., 1982). This increased production of Ap_4A and Ap_3A is the result of Zn^{2+} inhibition in the formation of aminoacyl-tRNA and possibly of other as yet unknown Zn^{2+} effects on this catalytic system and its products. In this regard, zinc deficiency arrests development, growth, and differentiation and causes teratological abnormalities, manifesting variously in many different species, which have been particularly well studied in *Euglena gracilis* (Vallee & Falchuk, 1981). In this case, some observations can now be understood in terms of the known enzymology, but the explanation of others requires that zinc would have functions neither appreciated nor specified so far. Thus, zinc could affect overall growth, development, and differentiation by participating in gene expression and its control, as well as in cell division and its consequences. Such metabolic transformations require selective gene activation and/or repression, depending on molecules that bind to DNA or other materials that could modulate transcription (Vallee, 1983).

In studies on other bisnucleoside oligophosphates, the pioneer observation of Finamore & Warner (1963) should be cited, in which guanosine(5′)tetraphospho(5′)guanosine (Gp_4G) was found in high concentrations in brine shrimp cysts. Recently, Furuichi and colleagues (Smith & Furuichi, 1982; Yamakawa et al., 1982) have found that compounds of the general structure Gp_4N (where N is any of the nucleosides) accumulate during transcription in vitro by purified insect cytoplasmic polyhedrosis virus and by vaccinia virus. Ap_4A and ApppGpp also are synthesized to high levels in *S. typhimurium* following exposure to a bacteriostatic quinone and to several other toxic compounds (Bochner et al., 1982). Further, Ap_5A is a potent inhibitor of adenylate kinase (Lienhard & Secemski, 1973).

The relationship of the ordered structure of bisnucleoside oligophosphates to their function has not been explored to any extent and forms the basis of the present study. It is known that methylation of the G residues in the cap structure of mRNAs is important to the initiation of message translation. Methylation of the cap structure and of adjacent nucleotides stolen from cellular mRNAs by a special nucleotidase present in influenza virus is also significant for the successful use of this constellation of nucleotides as a primer for synthesis of the plus strand of influenza virus by the viral RNA replicase (Krug, 1981). The structural basis for the above enhancements of catalytic functions is unknown however.

In the present studies the unusual base stacking initially proposed for Ap_4A has been found to occur also in a number of bisnucleoside oligophosphates. It is reasonable to suspect that this peculiar base stacking plays a role in enzyme recognition of this class of compounds, a consideration that underlies this investigation and contributes to its potential biological importance.

Materials and Methods

Sodium salts of bisnucleoside oligophosphates were purchased from P-L Biochemicals, Milwaukee, WI, except Ap_4A, Ap_5A, and Gp_5G, which were purchased as lithium salts from Boehringer, Mannheim, West Germany. ApcppcpA was a gift of Dr. Eliezer Rapaport, Boston, MA. Phosphodiesterase I (EC 3.1.4.1) from *Crotalus adamanteus* venom, 0.64 unit/mg of protein, was obtained from Sigma Chemical Co., St. Louis, MO. Magnesium sulfate and zinc sulfate were of spectral grade. Double-distilled water was used throughout. All other reagents were of highest available purity.

Bisnucleoside oligophosphates were assayed by high-performance liquid chromatography (HPLC). Waters HPLC equipment with A254 monitor, recorder, and integrator was employed, using a Whatman partisil PXS 10/25 SAX column and linear gradient elution of 0.007 M KH_2PO_4/0.007 M KCl, pH 4.0, to 0.25 M KH_2PO_4/0.50 M KCl, pH 5.0 (McKeag & Brown, 1978; Plesner et al., 1979), 1.5 mL/min in 30 min, at 0.2 AUFS sensitivity. The compounds were at least 96% pure, except Gp_2G (93%), m^7Gp_3G (92%), Ap_3A (92%), Gp_3Gm (91%), Gp_3G (88%), and $m^7Gp_4m^7G$ (70%).

Concentrations of all nucleotides were measured spectrophotometrically by employment of the molar absorptivities given in Table I. Absorption spectra were obtained with a Perkin-Elmer Lambda 5 UV–vis spectrophotometer.

Results for circular dichroism are given in units of deg cm^2 $dmol^{-1}$ calculated from

$$[\theta] = \frac{100\theta}{Ml}$$

where θ is the measured ellipticity, l is the optical path length in centimeters, and M is the molar concentration expressed as moles of mononucleoside oligophosphate or bisnucleoside oligophosphate per liter. Measurements were carried out in 10 mM Tris-HCl buffer with quartz cells of 1-, 3-, 5-, or 10-cm path and a Cary 61 spectropolarimeter. Spectra were the same at pH 7.5 and 9.7 (in the absence of metal ions, 22 °C). Spectra of Ap_4A at pH 5.5 (20 mM sodium acetate), 7.5, and 9.7 are indistinguishable at both 22 and 92 °C. The spectra in the presence and absence of metal ions (10 mM $MgSO_4$, 0.4 mM $ZnSO_4$) are pH independent in the range of 7.5–8.0.

Concentrations of nucleotides were 10^{-6}–10^{-3} M when the stoichiometry of metal binding was measured. Metal salts were added in microliter amounts of concentrated (millimolar to molar) solutions. Volume changes were less than 0.1%. Maximum metal ion concentrations were 0.4 mM at pH 8.0 or 3 mM at pH 7.5 for $ZnSO_4$ and 20 mM for $MgSO_4$. In this range, effects of changes in ionic strength are negligible.

In the ranges investigated, relations between ellipticities and nucleotide concentrations were linear save for guanosine nucleotides above 30 mM and in the presence of 2 mM $ZnSO_4$, when a slow change in the ellipticity in the trough between 280 and 300 nm could be observed, a process reversed on addition of EDTA. In general, the addition of excess (1–10 mM) EDTA demonstrated the reversibility of reactions of nucleotides with Zn^{2+} or Mg^{2+}.

Spectra of mixtures of mononucleotides of base composition identical with that of bisnucleoside oligophosphates were measured after digestion with 3×10^{-4} unit/mL phosphodiesterase in the presence of 10 mM $MgSO_4$. Reactions were followed until equilibrium had been achieved. Digestion was not observed in the presence of $ZnSO_4$, nor was ApcppcpA hydrolyzed by the enzyme.

Difference spectra refer to identical solutions (0.2–1.5 A_{260} units of nucleotide, 10 mM Tris-HCl, pH 8.0 at 25 ± 1 °C) in both reference and sample compartments, except that, at a maximum, 50 μL of metal salt solution was added to the sample cuvette (3 mL, 1-cm path length, quartz). Corrections for dilution were made by addition of identical amounts of distilled water to the reference cuvette.

Percent degree of hypochromicity was measured as

$$\frac{100[A_{260}(Mg^{2+} \text{ plus phosphodiesterase}) - A_{260}(\pm Mg^{2+})]}{A_{260}(Mg^{2+} \text{ plus phosphodiesterase})}$$

where $A_{260}(Mg^{2+}$ plus phosphodiesterase) refers to absorbance at 260 nm after digestion and $A_{260}(\pm Mg^{2+})$ to that in the presence or absence of 10 mM $MgSO_4$. The measurements were performed in sequence for each bisnucleoside oligophosphate, reading first A_{260} in the absence and then in the presence of Mg^{2+}, as well as after digestion.

Molar absorptivities in Table I were calculated from spectra in the absence of $MgSO_4$ and show the degree of hypochromicity. The calculation is based on a 1:1 (mole:mole) stoichiometry of bases in bisnucleoside oligophosphates and the following molar absorptivities for the nucleotide mixtures after digestion (in units of 10^3 M^{-1} cm^{-1} at 250 nm) (Handbook of Biochemistry and Molecular Biology, 1975): 15.4 (AMP), 13.4 (6MeAMP), 11.4 (GMP), 11.1 (7MeGMP), 7.5 (CMP), 10.0 (UMP). Ribose-methylated compounds were treated as if not methylated.

Stoichiometry and dissociation constants of nucleotide–metal complexes were determined as follows. First, a sample of relatively high concentration of nucleotide was titrated with $MgSO_4$ or $ZnSO_4$. A sharp break of the titration curve usually signified an equivalence. If subsequent measurements at lower concentration of nucleotide showed the value of the dissociation constant to be lower than that of the nucleotide concentration in the first experiment, this titration curve served to determine stoichiometry by the tangent method (Bartmann et al., 1975). Dissociation constants were determined routinely from Scatchard (1949) plots or, when nucleotide concentrations were much smaller than metal ion concentrations, from Eadie (1942) plots.

Nonlinearity of Scatchard and Eadie plots respectively were interpreted to indicate binding of metal ions to more than a single class of binding sites. To determine macroscopic titration constants in these instances, the stoichiometry and dissociation constant of the high affinity site were determined, neglecting the second site (2). The stoichiometry and dissociation constant of the low-affinity site(s) were then determined by introducing corrections due to binding of metal ions and changes in optical properties by saturation of the high-affinity site(s).

Reproducibility of CD spectra in terms of ellipticities was of the order of <20%. Errors due to base-line drifts were kept at a minimum by recording the reference values, usually air, intermittently during titration. Reproducibility of absorbance measurements was found to be better than 5%. Standard deviations for dissociation constants were in the range of 10% (circular dichroism) and 5% (absorbance).

Results and Discussion

The essential conclusions drawn on the basis of the experimental results in the following sections are presented first. (1) Bisnucleoside oligophosphates have conformational structures involving stacking of bases. (2) Stacking, at least in the case of unsubstituted purine nucleotides, occurs through α/α face contacts of bases. (3) Stacking structures depend on the kind of base, the state of methylation (more particularly of the nucleobase than of the ribose), and the length of the oligophosphate chain that links nucleosides through their 5′-positions. (4) Mg^{2+} and Zn^{2+} form complexes with bisnucleoside oligophosphates involving at least two sets of binding sites, one of which has a high stability constant. (5) The stability of the metal ion complex varies with the kind of nucleobase, the state of methylation, and the length of the linking oligophosphate chain. (6) Binding of Mg^{2+} and Zn^{2+} ions is thought to cause redistribution between the stacked and unstacked forms and/or new conformers as a function of the kind of both bisnucleoside oligophosphate and metal ion involved.

Conformational Structure. The features of the circular dichroic spectra of the nucleotides studied are characteristic and grouped according to their overall appearance. The family of bisadenosine oligophosphates, except Ap_2A, has a trough at 276–280 nm (Figure 1A) and a peak at 254–262 nm. Crossover points occur at 264–267 and at 242–250 nm. The trough of the bisguanosine oligophosphate family is at 278–285 nm, its peak is at 255–263 nm, and a second trough is observed at 240–242 nm (Figure 2A). Crossover points occur at 267–276, 247–252, and 229–231 nm. The spectrum of Ap_2A is "inverted" with a peak replacing the trough and with crossover points at wavelengths similar to those of other bisadenosine oligophosphates (Figure 1A). The spectrum of the disphosphate Gp_2G does not exhibit this inverted spectrum (Figure 2A).

The ribose-methylated Gp_3Gm resembles Gp_3G except for decreased ellipticities and is similar to m^7Gp_3G/m^7Gp_3Gm (Figure 3A) and Gp_3A/Gp_3Am (Figure 4A). For the latter, however, ribose methylation does not decrease the ellipticity. Methylation of guanosine replaces the long-wavelength trough with a peak and the short-wavelength peak with a trough (m^7Gp_3G and $m^7Gp_3m^7G$ in Figure 3A; $m^7Gp_3m^6Am$ in Figure 4A). Spectra for the mixed nucleobase oligophosphates Gp_3A and Gp_3Am are reminiscent of parent spectra for Ap_3A and Gp_3G, whereas those for Gp_3U and Gp_3C, although related to one another, are not similar to that of Gp_3G (Figure 4A).

Comparison of spectra for nucleotide monomers (Figure 5), bisnucleoside oligophosphates at 22 °C (Figures 1A–4A), and bisnucleoside oligophosphates at 92 °C provides evidence for the existence of ordered structure (Figure 6). The difference between the monomers and the bisnucleoside oligophosphates relates to both chemical ligation by the phosphate chain and stacking structure, whereas that for bisnucleoside oligophosphates between low and high temperature relates to partial melting of ordered structure (Cantor & Schimmel, 1980). Figure 6 (inset) shows that the structure of Ap_4A is temperature dependent. Such "melting curves" serve to determine apparent thermodynamic parameters of conformational changes that give rise to the observed spectral differences at low and high temperature (Catlin & Guschlbauer, 1975). Changes for bisguanosine oligophosphates approach a "master" spectrum that, indeed, differs from the spectra for GMP and GTP (Figures 5 and 6).

The representative absorbance difference spectra in Figure 7 give additional evidence in favor of base stacking (Table I). Thus, Ap_3A, e.g., exhibits a positive and negative difference at 260 and 280 nm, respectively, at variance with the hypochromicity of DNA and homopolymers (Cantor & Schimmel, 1980). It could reflect the typical α/α contacts (see below) and environments for the two nucleobases with respect to ribose moieties and oligophosphate chain.

A tentative interpretation of the observed CD spectra in terms of structure rests on the assumption that bisnucleoside oligophosphates partition between one or more stacked and/or unstacked conformations, as assumed in the case of other oligonucleotides (Catlin & Guschlbauer, 1975). The spectra observed are a summation of the spectra of the various conformers owing to thermodynamic equilibrium. In the Ap_3A

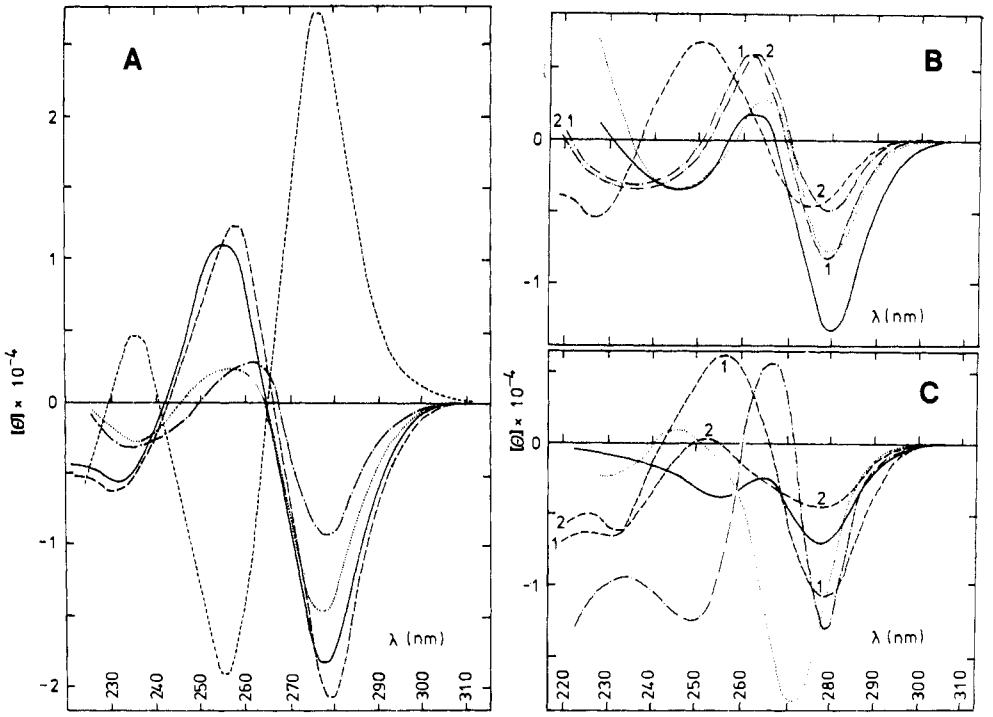

FIGURE 1: Circular dichroic spectra of bisadenosine oligophosphates in the absence (A) and in the presence of $MgSO_4$ (B) or $ZnSO_4$ (C). P/T is ratio of $[\theta]$ of main peak to $[\theta]$ of main trough. (---) Ap_2A, 10 mM $MgSO_4$ and 0.4 mM $ZnSO_4$ have no effect; (--) Ap_3A, P/T = 0.6 in (A), 8 mM $MgSO_4$ and P/T = 1.4 in (B); (curve 1) Ap_3A, 360 μM $ZnSO_4$ and P/T = 0.58 in (C); (curve 2) Ap_3A, 1.4 mM $ZnSO_4$ in (C); (—) Ap_4A, P/T = 0.6 in (A), 6 mM $MgSO_4$ in (B), 330 μM $ZnSO_4$ in (C); (···) Ap_5A, P/T = 0.18 in (A), 6.5 mM $MgSO_4$ and P/T = 0.35 in (B), 280 μM $ZnSO_4$ in (C); (-·-) Ap_6A, P/T = 0.30 in (A), 152 μM $ZnSO_4$ and P/T = 0.75 in (C); (curve 1) Ap_6A, 184 μM $MgSO_4$ and P/T = 0.70 in (B); (curve 2) Ap_6A, 5.4 $MgSO_4$ and P/T = 1.14 in (B).

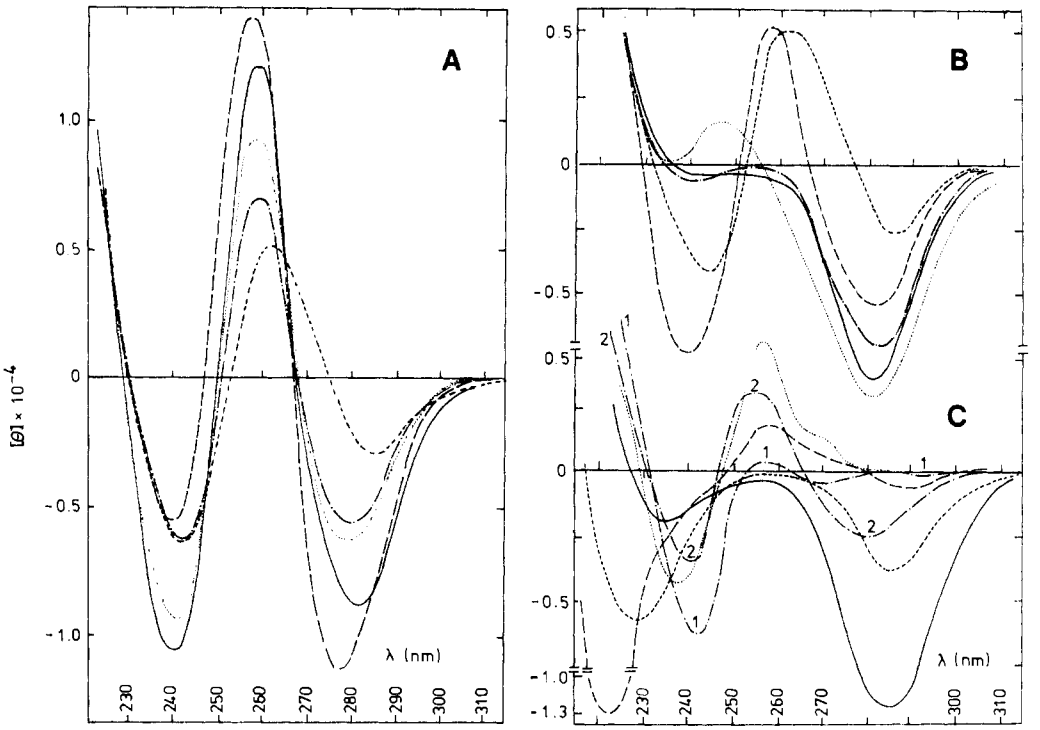

FIGURE 2: Circular dichroic spectra of bisguanosine oligophosphates in the absence of (A) and in the presence of $MgSO_4$ (B) or $ZnSO_4$ (C): (---) Gp_2G, P/T = 1.7 (near UV) and 0.81 (far UV) in (A), 8.4 mM $MgSO_4$ and P/T = 2.0 (near UV) and 1.2 (far UV) in (B), 380 μM $ZnSO_4$ in (C); (--) Gp_3G, P/T = 1.3 and 2.2 in (A), 10 mM $MgSO_4$ and P/T = 1.0 and 0.74 in (B), 215 μM $ZnSO_4$ in (C); (—) Gp_4G, P/T = 1.4 and 1.2 in (A), 9.4 mM $MgSO_4$ in (B), 220 μM $ZnSO_4$ in (C); (···) Gp_5G, P/T = 1.5 and 1.0 in (A), 3.2 $MgSO_4$ in (B), 28 μM $ZnSO_4$ in (C); (-·-) Gp_6G, P/T = 1.3 and 1.1 in (A), 7.7 mM $MgSO_4$ in (B); (curve 1) Gp_6G, 2.3 μM $ZnSO_4$ in (C); (curve 2) Gp_6G, 280 μM $ZnSO_4$ and P/T = 1.3 and 0.9 in (C).

through Ap_5A series a conformation has been proposed where the adenine rings contact one another through their α faces, i.e., the side of the adenine ring that faces the C_2-hydroxyl group in the anticonformation (Scott & Zamecnik, 1969; Kolodny et al., 1979). The α/α contact gives rise to the observed bathochromic trough whereas β/β contacts, as in Ap_2A, and α/β contacts, as in A-3′-p-5′-A, are thought to result in the positive ellipticities observed in the same region

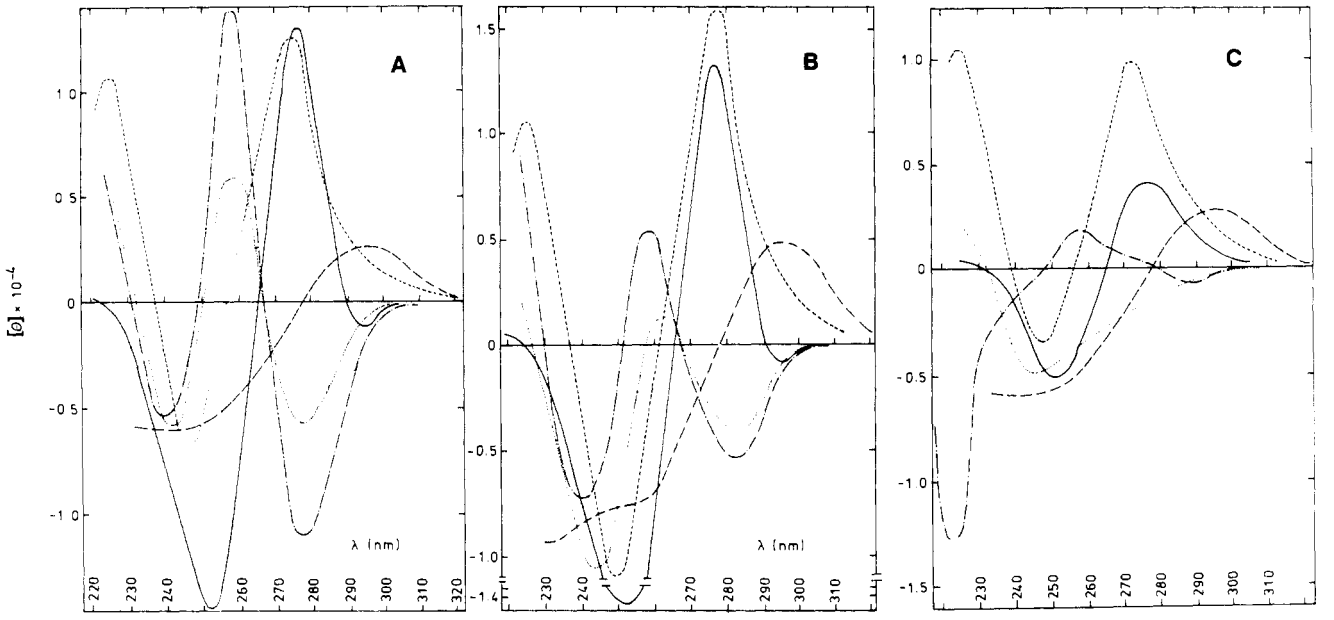

FIGURE 3: Circular dichroic spectra of methylated bisguanosine oligophosphates in the absence (A) and in the presence of $MgSO_4$ (B) or $ZnSO_4$ (C). The spectra for Gp_3G (-·-) are the same as in Figure 2 and are included for comparison. (···) Gp_3Gm, P/T = 1.0 (near UV) in (A), 9 mM $MgSO_4$ in (B), 270 μM $ZnSO_4$ in (C); (- - -) m^7Gp_3G, P/T = 1.9 in (A), 8 mM $MgSO_4$ and P/T = 1.2 in (B), 300 μM $ZnSO_4$ and P/T = 2.9 in (C); (—) m^7Gp_3Gm, P/T = 0.9 in (A), 12 mM $MgSO_4$ in (B), 250 μM $ZnSO_4$ and P/T = 0.8 in (C); (– –) $m^7Gp_4m^7G$, P/T = 0.6 in (A), 7 mM $MgSO_4$ and P/T = 0.6 in (B), 162 μM $ZnSO_4$ and P/T = 0.6 in (C).

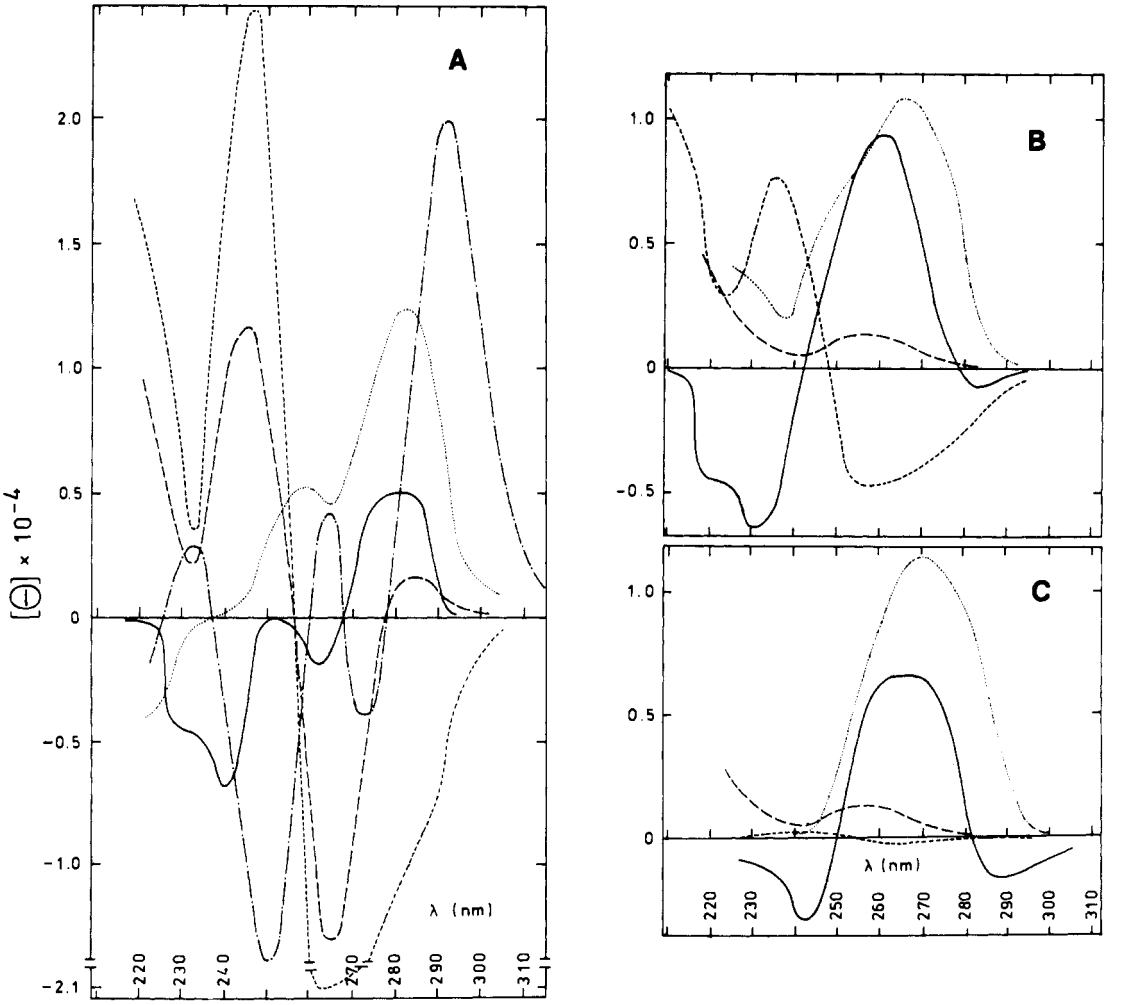

FIGURE 4: Circular dichroic spectra of mixed bisnucleoside oligophosphates in the absence (A) and in the presence of $MgSO_4$ (B) or $ZnSO_4$ (C). (- - -) Gp_3A, P/T = 2.1 in (A), 21 mM $MgSO_4$ in (B), 474 μM $ZnSO_4$ in (C); (– –) Gp_3Am, P/T = 0.88 in (A), 3.7 mM $MgSO_4$ in (B), 360 μM $ZnSO_4$ in (C); (-·-) $m^7Gp_3m^6Am$, 10 mM $MgSO_4$ and 200 μM $ZnSO_4$ had no effect; (···) Gp_3C, 10 mM $MgSO_4$ in (B), 1.7 mM $ZnSO_4$ (pH 7.5) in (C); (—) Gp_3U, 10 mM $MgSO_4$ in (B), 1.6 mM $ZnSO_4$ (pH 7.5) in (C).

Table I: Properties of Bisnucleoside Oligophosphates and of Their Complexes with Mg^{2+} and Zn^{2+}

compd	ϵ_{260} (10^3 M^{-1} cm^{-1})[a]	hypochromicity (%)[b] +10 mM $MgSO_4$	hypochromicity (%)[b] −10 mM $MgSO_4$	dissociation constants (μM) Mg^{2+}	dissociation constants (μM) Zn^{2+}
Ap_2A	25.1	18.1	18.4	9300 (A_{250})[c]	560 (A_{280})
Ap_3A	25.1	16.4	18.5	370 (θ_{278})[d]	170 (θ_{278}) (1)[e]
Ap_4A	27.1	15	13	28 (θ_{278}) (1) 6200 (θ_{278})[f]	15 (θ_{278}) (1) 900 (θ_{278})[f]
Ap_5A	27.8	11.8	9.5	30 (θ_{275}) 1000 (θ_{275})	0.5 (θ_{285}) (1)
Ap_6A	27.6	11.4	10.5	0.8 (θ_{270}) (1) 180 (θ_{270}) (A_{271}) (6-7)	0.17 (θ_{270}) (1) 150 (θ_{278})
Gp_2G	21.6	8	9.5	1300 (θ_{262})	400 (θ_{262})
Gp_3G	21.6	8.3	9.6	240 (θ_{258}) (1) 250 (A_{270})	27 (θ_{258}) (1)
Gp_4G	22	6.8	4.6	9 (θ_{258}) (1) 2000 (θ_{258})	1 (θ_{260}) (2)
Gp_5G	22.3	6.8	5.6	23 (θ_{282}) (1) 900 (θ_{278})	0.88 (A_{280}) (1)
Gp_6G	23.1	4.8	4.8	<<3 (θ_{259}) (1) 100 (θ_{259}) 60 (A_{267})	<<3 (θ_{258}) (1) 20 (θ_{258})
Gp_3Gm	21.6	7.2	8.0	280 (θ_{258})	150 (θ_{258})
m^7Gp_3G	21.8	7.7	2.7	1200 (A_{255})	300 (A_{275})
m^7Gp_3Gm	21.8	7.9	1.9	310 (A_{275})	130 (A_{278})
$m^7Gp_4m^7G$	21.5	4.3	−9.9	230 (θ_{255})	50 (θ_{260})
Gp_3A	24	9.6	11.5	390 (A_{265})	81 (A_{265})
Gp_3C	18.4	5.4	4.9	360 (A_{267})	160 (A_{267})
Gp_3U	22	3.5	4.6	450 (θ_{265}) 360 (A_{287})	120 (θ_{265})
Gp_3Am	23.6	10.9	12.6	100 (θ_{265}) 600 (θ_{265})	70 (θ_{264}) 100 (A_{265})
$m^7Gp_3m^6Am$	20.4	18	12	750 (A_{255})	210 (A_{255})
ApcppcpA	27.1	nd[g]	nd	2.4 (A_{260}) (1)	0.26 (A_{260})

[a] Molar absorptivity. [b] Measured at 260 nm. [c] Measured at 250 nm, absorbance; A = absorbance. [d] Measured at 278 nm, circular dichroism; θ = ellipticity. [e] Stoichiometry, number of moles of metal ion bound per mole of bisnucleoside oligophosphate. [f] High values refer to binding at second sites of bisnucleoside oligophosphates. [g] nd, not determined.

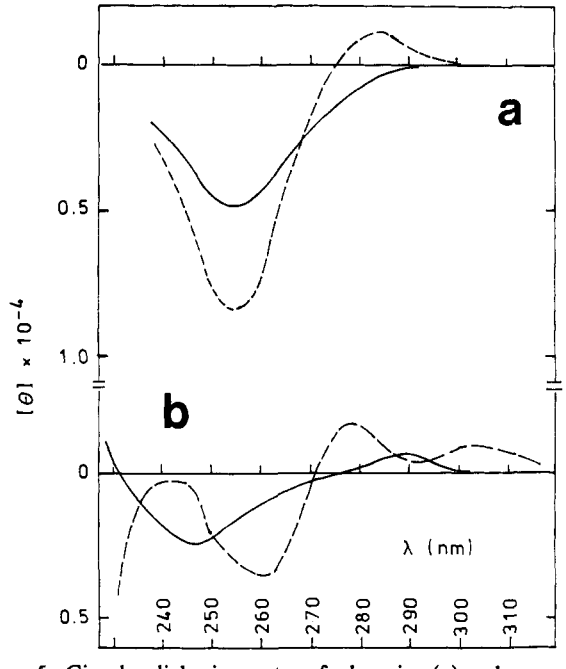

FIGURE 5: Circular dichroic spectra of adenosine (a) and guanosine (b) mononucleotides at pH 7.5: (—) AMP, AMP plus 10 mM $MgSO_4$, and AMP plus 2 mM $ZnSO_4$ in (a) and GTP, GTP plus 10 mM $MgSO_4$, GMP, and GMP plus 10 mM $MgSO_4$ in (b); (--) ATP plus 2 mM $ZnSO_4$ in (a) and GTP plus 2 mM $ZnSO_4$ in (b).

(Scott & Zamecnik, 1969). By the same token, we conclude that bisguanosine oligophosphates also make α/α contacts and that methylation in position 7 again favors α/β contacts (m^7Gp_3G or $m^7Gp_3m^6Am$ would behave like DNA).

The effects of oligophosphate chain length and of ribose 2′-*O*-methylation can be discerned by inspection of Figures 1A–4A. For variable chain length, the crossover points remain the same within a narrow range of wavelength. Where ratios of ellipticities between peak and trough, P/T, are identical or similar, the orientation between bases is conserved (Catlin & Guschlbauer, 1975). Such preconditions are fulfilled on the long-wavelength sides of spectra for members of the bisguanosine family, for Ap_3A and Ap_4A, for Gp_3G and Gp_3Gm, for Gp_3Gm, and for Gp_3A and Gp_3Am. Decreased ellipticity is then tentatively explained as a shift in the equilibrium population away from the stacked conformer (Catlin & Guschlbauer, 1975) as a function of either oligophosphate chain length or methylation of the ribose. A destabilization of stacking could be expected, considering the increase in internal motion of the two bases as a result of an increased chain length. Another possibility is that removal of strain by the extended chain length allows new conformations. Such could be the case especially with Ap_5A and Ap_6A, which have P/T values that differ from those for Ap_3A and Ap_4A (Figure 1A). In the case of Gp_3Gm, we found by model building that rotation of the guanine residues is hindered in comparison to Gp_3G. This may lead to preference of other than stacked conformations.

Metal Ion Complexes. The presence of metal ions, Mg^{2+} and Zn^{2+}, evokes dramatic spectral changes (Table I) in both the CD (Figures 1–4) and the absorption spectra (Figure 8). The changes in the CD spectra are interpreted to indicate destacking of bases or new conformers on the basis of com-

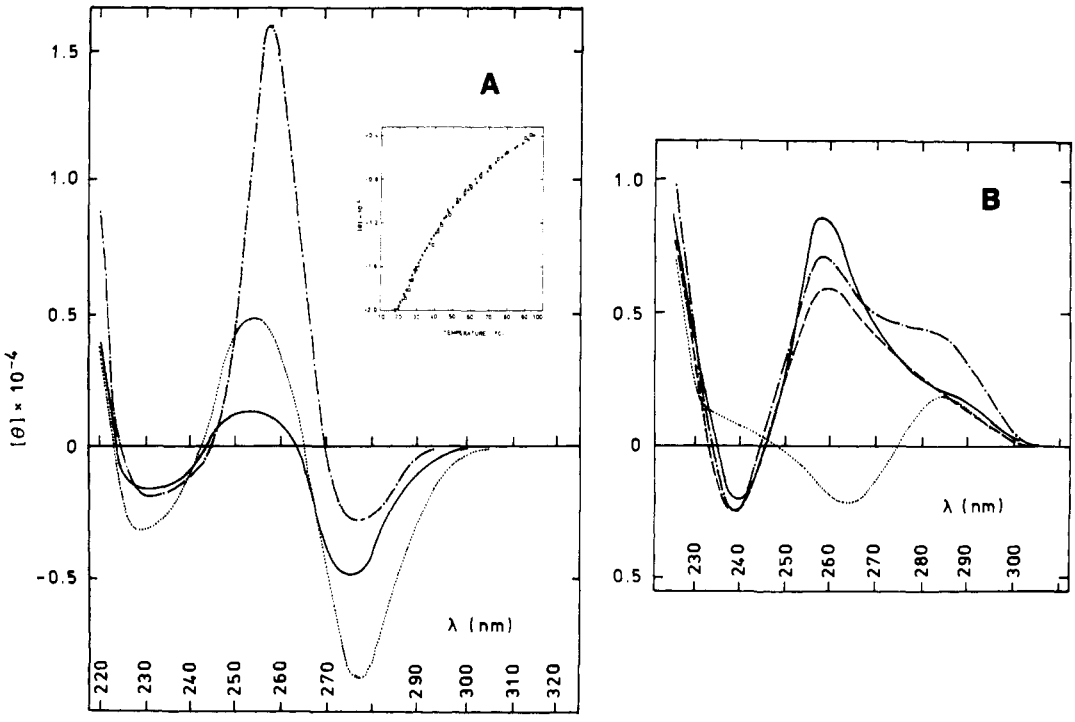

FIGURE 6: Circular dichroic spectra at 92 °C. (A) (···) Ap_3A, (—) Ap_4A, (-·-) Ap_6A; (B) (--) Gp_3Gm, (—) Gp_4G, (-·-) Gp_6G, (···) Gp_3A; (inset) temperature dependence for Ap_4A at 278 nm, 10 mM Tris-HCl, pH 7.5 (25 °C). Symbols refer to different heating/cooling cycles.

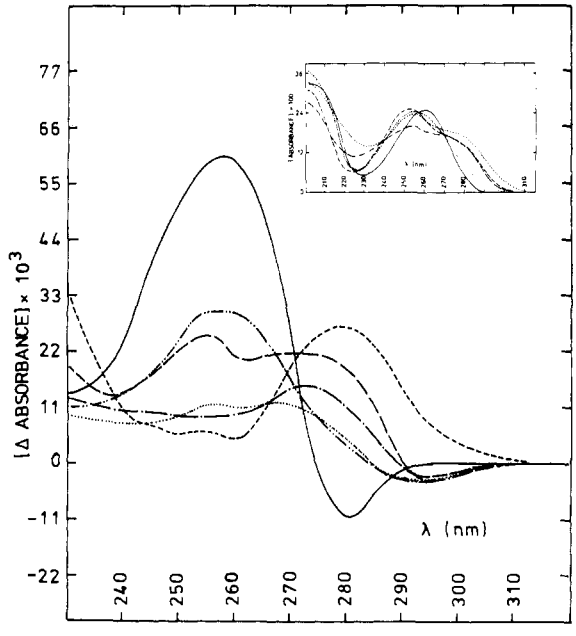

FIGURE 7: Difference absorption spectra of digested (sample) vs. undigested (reference) bisnucleoside oligophosphates. Only the sample contains 10 mM $MgSO_4$, which does not give rise to an absorbance difference. Spectra are corrected for absorbance by phosphodiesterase (almost negligible). Concentrations of bisnucleoside oligophosphates are 10 μM: (—) Ap_3A, (--) Gp_3G, (-··-) Gp_3A, (-·-) Gp_3C, (···) Gp_3U, (---) m^7Gp_3G; (inset) spectra before digestion, symbols are the same except (···), Gp_3Am instead of Gp_3U.

parison of the P/T values. Partial destacking of bases is assumed for Ap_3A in the presence of 360 μM $ZnSO_4$ (P/T ratios in Figure 1A,C), for Gp_6G with 280 μM $ZnSO_4$ (Figure 2A,C), and for m^7Gp_3Gm with 250 μM $ZnSO_4$ (Figure 3A,C). In the presence of Mg^{2+} or Zn^{2+}, the ellipticity of the mixed-base nucleotide Gp_3Am and that of Gp_3A (in the presence of Zn^{2+}) (Figure 4) suggests complete destacking.

The CD spectra of destacked bisnucleoside oligophosphates had to be defined to decide whether or not ordered structure was destroyed. Zn^{2+} but not Mg^{2+} induced spectral changes of mononucleotides probably by interaction with N(7) of the purine rings (Figure 5; Martin & Mariam, 1979). The spectra of bisnucleoside oligophosphate metal complexes are grossly different and probably do not only reflect metal–nucleobase interactions. Further, at a given metal ion concentration, a bisnucleoside oligophosphate is not saturated necessarily, owing to the large value of the dissociation constant. In the absence of suitable criteria, we equate a residual ellipticity of less than 40% of that in the absence of metal ions with destruction of ordered structure. This was the case for Ap_3A (1.4 mM $ZnSO_4$, Figure 1C), Ap_4A (330 μM $ZnSO_4$, Figure 1C), Gp_3G (215 μM $ZnSO_4$, Figure 2C), Gp_6G (2.3 μM $ZnSO_4$, Figure 2C), and Gp_3Gm (270 μM $ZnSO_4$, Figure 3C). The examples show that Zn^{2+}, in contrast to Mg^{2+}, tends to dissolve ordered structures of the nucleotides.

$MgSO_4$ seems to reinforce the α/β-like contacts of $m^7Gp_4m^7G$ and of m^7Gp_3G (P/T ratios in Figure 3A,B), similar to the stabilization of DNA stacking by Mg^{2+} (Helene, 1975). Zn^{2+} more than Mg^{2+} induces conformation changes in other bisnucleoside oligophosphates as apparent from wavelength shifts and P/T values. Though conformations are changed, stacking is maintained, as judged from a persistence of the long-wavelength trough. Examples include (in the presence of $MgSO_4$) Ap_4A, Ap_5A, Ap_6A, Gp_4G, Gp_5G, Gp_6G, Gp_3Gm, and, in the presence of $ZnSO_4$, Ap_5A, Ap_6A, Gp_2G, and Gp_4G. The mixed bisnucleoside triphosphates Gp_3U and Gp_3C respond by an increase in positive ellipticity and by an elimination of a small trough at 264 nm. It is possible that the metal ions support the secondary structure of mixed purine/pyrimidine triphosphates but dissolve that of mixed purine triphosphates (Figure 4).

Metal-dependent changes are not observed for Ap_2A, m^7Gp_3Gm ($MgSO_4$), mGp_4m^7G ($ZnSO_4$), and $m^7Gp_3m^6Am$. While this might signify weak metal binding to Ap_2A (Table I), in a few cases binding metal neither affects stacking nor conformation. In general, Mg^{2+} and Zn^{2+} affect nucleotide structure, and among compounds of biological interest, one

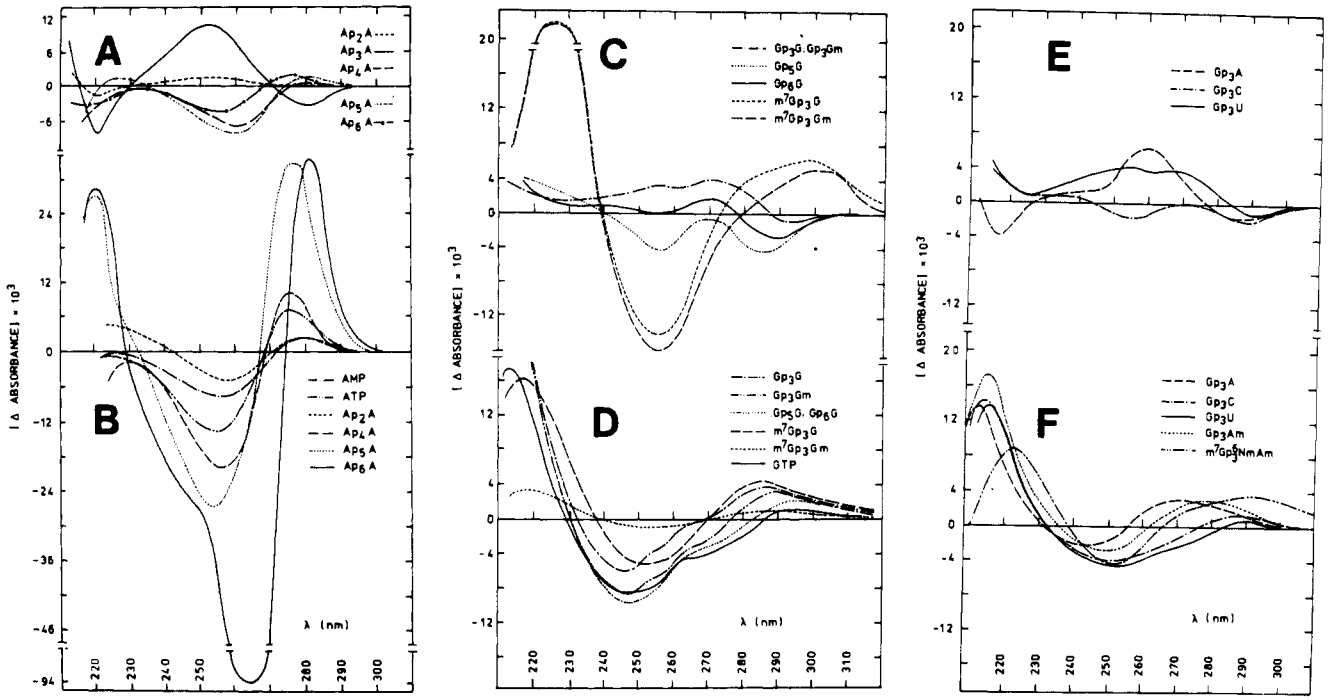

FIGURE 8: Difference absorption spectra of complexes of bisnucleoside oligophosphates with Mg^{2+} (10 mM) in (A), (C), and (E) or with Zn^{2+} (0.4 mM) in (B), (D), and (F). Concentrations of bisnucleoside oligophosphates were 10 μM in (A), 25 μM in (B), 10 μM in (C), 5 μM in (D), 10 μM in (E), and 5 μM in (F). Spectra are measured in 10 mM Tris-HCl, pH 7.5, 25 ± 1 °C.

group, Ap_3A, Ap_4A, Gp_3G, Gp_4G, and Gp_3A, is particularly sensitive while another, distinguished by its guanine 7-methyl group, tends to be insensitive.

Hypochromicity of nucleic acids and oligonucleotides has served as an index of base stacking (Cantor & Schimmel, 1980). With Ap_3A (Mg^{2+}, Figure 8A), Gp_3A (Mg^{2+}, Figure 8E), Gp_3G (Mg^{2+}, Figure 8C), and Gp_3Gm (Mg^{2+}, Figure 8C), hyperchromicity (260 nm) is equivalent to the degree of destacking as derived from CD data. In other cases, particularly in the presence of Zn^{2+}, the results diverge. This relates to the observation that Zn^{2+} perturbs the absorbance of purine by binding to N(7) (Martin & Mariam, 1979) as revealed for mononucleotides in Figure 8B,D while Mg^{2+} does not. Indeed, comparison of the absorption difference spectra (Figure 8) reveals a dominant common spectrum in the presence of Zn^{2+} but not of Mg^{2+}. Hence, their characteristics probably indicate both nucleotide–metal ion complexes and effects due to changes in stacking structure.

Stoichiometry and Dissociation Constants. The formation of metal bisnucleotide oligophosphate complexes is the source of the spectral effects of the metal ions. The changes of ellipticity and absorbance follow saturation dependencies with respect to metal ion concentration. In many instances, Eadie plots are biphasic (not shown). The first (low concentration) phase involves a single Mg^{2+} or Zn^{2+} ion in almost all cases (Table I). The weak affinity for the second sites requires high concentrations of nucleotides for measurements of stoichiometry, and this was determined only for Ap_6A due to lack of material. But this result suggests that second sites could be numerous. Dissociation constants of the various complexes are listed in Table I.

Several relations between stability constants of the Mg^{2+} and Zn^{2+} complexes and structural variations of bisnucleoside oligophosphates can be enumerated. (a) Stabilities increase as a function of the oligophosphate chain length, more than 10^4-fold from Ap_2A to Ap_6A in the case of $ZnSO_4$ and 10^4-fold for the same series with $MgSO_4$; the results for bisguanosine oligophosphates are analogous. Equivalent complexes containing guanine moieties are tighter than those containing adenine. (b) Methylation tends to decrease stabilities of the complexes involving the 7-methylguanine moiety, producing larger effects than the *O*-methylribose moiety. As seen for $m^7Gp_4m^7G$, 7-methylation does not abolish complexation with Zn^{2+}. (c) The stability constants of Zn^{2+} are higher than those of Mg^{2+} complexes. Dissociation constants often differ by factors of 3–4 and, in the case of Ap_5A, by a factor of 60.

The nature of the interaction between bisnucleoside oligophosphates and metal ions is best considered for the high-affinity site of the bisnucleoside triphosphate series. With a few exceptions, values of dissociation constants are quite independent of the kind of nucleobases (100–390 μM for Mg^{2+} and 81–210 μM for Zn^{2+}). It suggests that metal ions interact with the phosphate chain. Indeed, pyrophosphate is known to bind Mg^{2+} (Lambert & Watters, 1957). In a further experiment, we found that high ionic strength disfavored binding of Mg^{2+} to Ap_4A (dissociation constants were 0.028, 0.3, 0.8, and 5 mM at 0, 120, 240, and 830 mM added NaCl, respectively, as measured by difference absorbance at 260 nm). However, somewhat higher values of dissociation constants are observed with m^7Gp_3G and $m^7Gp_3m^6Am$ in the bisnucleoside triphosphate series. The positive charge of the purine ring and/or steric hindrance might account for this. It follows that, at least in the case of Mg^{2+}, metal ions affect secondary structure through electrostatic interaction and possibly by stiffening of the oligophosphate chain.

The binding of Zn^{2+} involves interaction with N(7) of one of the purine rings (McCormick & Levedahl, 1959; Cohn & Hughes, 1962; Martin & Mariam, 1979) and gives rise to destacking in DNA and oligonucleotides (Shin, 1973; Zimmer et al., 1974; Helene, 1975). It could be an explanation for the tighter binding and pronounced effects on secondary structure of Zn^{2+} relative to Mg^{2+}. It also explains why complexes of Zn^{2+} and Ap_4A withstand high ionic strength (15 μM, 20 μM, and 0.4 mM in the presence of 0, 240, and 830 mM NaCl, respectively; experimental conditions as for binding of Mg^{2+}). This could be an important biological difference.

Zinc and Cell Biology. The present and past data point to the important role played by zinc in many enzymatic systems. The essentiality of zinc to the growth and development of all living matter was just recognized within the last decade in spite of abundant, long-standing evidence pointing in this direction. A series of major, closely interrelated biochemical advances in nucleic acid chemistry changed the perspective rapidly.

Histochemical observation indicated early that zinc has a role in nucleic acid metabolism and is present in the nucleus, nucleolus, and chromosomes (Fujii, 1954). Substantial quantities of firmly bound zinc stabilize the structures of RNA (Wacker & Vallee, 1959; Fuwa et al., 1960), DNA (Shin & Eichhorn, 1968), and ribosomes (Prask & Plocke, 1971). EDTA inhibits DNA polymerase activity in cell cultures (Lieberman & Ove, 1962), and zinc deficiency lowers the activities of both DNA and RNA polymerases in various tissues and organisms, observations that intimated an extensive involvement of zinc in the metabolism of nucleic acids (Swenerton et al., 1969; Sandstead & Rinaldi, 1969; Falchuk et al., 1975).

Chelating agents also inhibit the highly purified terminal deoxynucleotidyl transferase from calf thymus. On that basis Chang & Bollum (1970) suggested that it is a zinc metalloenzyme. Metal analyses soon confirmed the presence of zinc in nucleotidyl polymerases isolated from viruses, prokaryotes, and eukaryotes. Thus, within a year of Bollum's observations, the RNA (Scrutton et al., 1971) and DNA polymerases (Slater et al., 1971) from *Escherichia coli* were shown to contain 2 and 1 mol of zinc/mol, respectively, though the latter has recently been contested (Walton et al., 1982). At present, approximately 20 nucleotidyl polymerases are known to contain zinc. The role of zinc in these enzymes has been studied by standard approaches that include the concomitant reversible abolition and restoration of activity by chelating agents and of zinc.

Zinc is thus indispensable to the function of enzymes critical to DNA, RNA, protein synthesis, and—as was long known—protein degradation. However, but a short time ago, little or nothing was known about any role of zinc in cellular metabolism.

Conclusions

Bisnucleoside oligophosphates have well-defined ordered structures, which depend on subtle chemical changes (such as methylation), as well as formation of Mg^{2+} or Zn^{2+} complexes (and in all probability with other cations). This qualifies them to serve as effector molecules of biological reactions such as presumed for DNA replication and other examples outlined in the introduction. Promotion of certain conformers by Mg^{2+} and Zn^{2+} is important if the proteins involved interact favorably only with a single form. The binding of lactate dehydrogenase with the destacked form of nicotinamide adenosine nucleotide is an example (Holbrook et al., 1975). Similarly, as mentioned in the introduction, G residues in the cap structure are methylated in position N(7) before optimal translation of mRNA. It can be speculated that without methylation the Gp_3N terminus has α/α contacts between the terminal bases and interferes with the α/β contacts of the polynucleotide chain of mRNA. After methylation, the terminal bases switch to α/β contacts and now join the conformation of the mRNA. Enzyme recognition of the cap structure of tRNA or of primer structure in the case of influenza and other viruses (Krug, 1981; Yamakawa et al., 1982) may also depend on the conformation of the bisoligonucleoside phosphate, as influenced by methylation or metal interaction. Although data have been obtained only for Mg^{2+} and Zn^{2+}, it may be expected that other cations like Co^{2+}, Mn^{2+}, spermidine, and spermine exert similar effects, all of which could be decisive for such in vivo reactions.

Registry No. Ap_2A, 2596-55-6; Ap_3A, 5959-90-0; Ap_4A, 5542-28-9; Ap_5A, 41708-91-2; Ap_6A, 56983-23-4; Gp_2G, 34692-44-9; Gp_3G, 6674-45-9; Gp_4G, 4130-19-2; Gp_5G, 78101-73-2; Gp_6G, 83269-95-8; Gp_3A, 10527-47-6; Gp_3C, 79192-37-3; Gp_3U, 79192-36-2; Gp_3Gm, 66642-51-1; Gp_3Am, 62319-68-0; m^7Gp_3G, 79192-44-2; $m^7Gp_4m^7G$, 86862-70-6; m^7Gp_3Gm, 86862-71-7; $m^7Gp_3m^6Am$, 79192-39-5; ApcppcpA, 86854-97-9.

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Reaction of Human α_2-Macroglobulin Half-Molecules with Plasmin as a Probe of Protease Binding Site Structure†

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ABSTRACT: Human α_2-macroglobulin (α_2M) half-molecules were prepared by limited reduction and alkylation of the native protein. Reaction with plasmin resulted in nearly quantitative cleavage of the half-molecule M_r ~180 000 subunits into M_r ~90 000 fragments. Subunit cleavage was significantly less complete when plasmin was reacted with α_2M whole molecules. The plasmin and trypsin binding capacities of the two forms of α_2M were compared by using radioiodinated proteases. α_2M half-molecules bound an equivalent number of moles of plasmin or trypsin. Native unreduced α_2M bound only half as much plasmin as trypsin. These data are consistent with the hypothesis that the two protease binding sites are adjacent in native α_2M. α_2M half-molecule–plasmin complexes reassociated less readily than half-molecule–trypsin complexes, supporting this interpretation. The frequency of covalent bond formation between plasmin and α_2M was considerably higher than that previously observed with other proteases. Approximately 80–90% of the plasmin that reacted with α_2M whole molecules or half-molecules became covalently bound. The reactivities of purified α_2M–plasmin complexes were compared with small and large substrates. Equivalent k_{cat}/K_m values were determined at 22 °C for the hydrolysis of H-D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride by whole molecule–plasmin complex and half-molecule–plasmin complex (40 mM^{-1} s^{-1} and 39 mM^{-1} s^{-1}, respectively, compared with 66 mM^{-1} s^{-1} determined for free plasmin). Complexes of plasmin and the two different forms of α_2M digested fibrinogen at comparable rates (slowly compared with free plasmin); however, the half-molecule–plasmin complex demonstrated increased reactivity with soybean trypsin inhibitor. α_2M half-molecule–plasmin complex cleared rapidly from the circulation of mice, reflecting receptor binding and endocytosis.

The plasma protease inhibitor human α_2-macroglobulin (α_2M) is composed of four equivalent M_r ~180 000 polypeptide chains that are associated into pairs by disulfide bonds and whole molecules by strong noncovalent interactions (Swenson & Howard, 1979a; Harpel, 1973; Hall & Roberts, 1978). Each mole of tetrameric α_2M can bind up to 2 mol of α-chymotrypsin or trypsin (Barrett et al., 1979; Swenson & Howard, 1979a; Pochon et al., 1978), but only 1 mol of plasmin (Ganrot, 1967a; Gonias et al., 1982a; Pochon et al., 1978) or 1 mol of a synthetic α-chymotrypsin dimer (Pochon et al., 1981). This variation in molar binding ratios may be explained by a model of α_2M structure that includes two

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