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Assignment of Resonances in the Phosphorus-31 Nuclear Magnetic Resonance Spectrum of Poly[d(A-T)] from Phosphorothioate Substitution†

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ABSTRACT: Two phosphorothioate analogues of poly[d(A-T)] have been synthesized enzymatically. In one, poly[d(A_s-T)], dTMP is replaced by thymidine 5′-*O*-phosphorothioate; in the other, poly[d(T_s-A)], dAMP is replaced by 2′-deoxyadenosine 5′-*O*-phosphorothioate. The ^{31}P NMR spectrum of poly[d(A_s-T)] in solutions at low salt concentration shows two resonances at 51.80 and −4.25 ppm relative to trimethyl phosphate. The corresponding values for poly[d(T_s-A)] are 51.51 and −4.43 ppm. These data allow the assignment of the downfield resonance at −4.23 ppm in poly[d(A-T)] to the phosphate group of d(TpA) and the resonance at −4.41 ppm to that of d(ApT). Thus, strong evidence is provided for a repeating dinucleotide structure. A comparison of the ^{31}P NMR spectra of the various polymers in solutions of 2 M CsF reveals that both resonances are shifted upfield by approximately 0.9 ppm in the case of the phosphorothioates and by 0.2 or 0.4 ppm in the case of the phosphates. An upfield shift of about 0.18 ppm can also be observed for the two corresponding dinucleoside monophosphates. Thus, the upfield shift induced by high concentrations of CsF is not specific for the polymer backbone.

A variety of structural and spectroscopic studies suggest that the sugar phosphate backbone of the alternating copolymer of dAMP and dTMP (poly[d(A-T)][1] is not uniform but probably is best described by an alternating conformation [for a review, see Zimmermann (1982)]. Thus, ^{31}P NMR spectroscopy shows two signals for segments of helical poly[d(A-T)] less than 200 base pairs in length at 30 °C in solutions of low ionic strength (Shindo et al., 1979; Cohen et al., 1981). The difference in chemical shift disappears upon melting of the double helix but is increased in the presence of high salt concentrations, particularly CsF (Patel et al., 1981). For an interpretation of these spectra, an assignment of the two signals to the phosphorus of the two internucleotidic linkages d(ApT) and d(TpA) is necessary. We attribute the resonance at higher field to d(ApT) and that at lower field to d(TpA) on the basis of measurements performed with polymers carrying selective substitutions of the phosphate group by phosphorothioate. The upfield shifts observed upon an increase in ionic strength apply to all phosphates in the polymer as well as in the corresponding dinucleoside monophosphates.

Experimental Procedures

Materials

The S_P diastereomer of dATPαS was synthesized from the chemically synthesized mixture of diastereomers of dADPαS by reaction with phosphoenolpyruvate and pyruvate kinase (Burgers & Eckstein, 1979) as described for ATPαS (Eckstein & Goody, 1976). The mixture of diastereomers of dTTPαS was synthesized by reaction of acetyl phosphate and acetate kinase with the mixture of diastereomers of dTDPαS (Bartlett & Eckstein, 1982) which were synthesized from dTMPS as described for ADPαS (Eckstein & Goody, 1976). The diastereomers of d[Tp(S)A] were prepared as described (Romaniuk & Eckstein, 1982) and those of d[Ap(S)T] by an analogous procedure (S. Spitzer and F. Eckstein, unpublished results).

Poly[d(A-T)] and alkaline phosphatase from calf intestine were obtained from Boehringer Mannheim, Germany. *Micrococcus luteus* DNA polymerase I was from P-L Biochemicals, Milwaukee, WI. d(TpA) and d(ApT) were purchased from Sigma Chemical Co., St. Louis, MO. High-pressure liquid chromatography was performed on a Waters Associates

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[1] Abbreviations: dATPαS, deoxyadenosine 5′-*O*-(1-thiotriphosphate); dADPαS, deoxyadenosine 5′-*O*-(1-thiodiphosphate); dAMPS, 2′-deoxyadenosine 5′-*O*-phosphorothioate; dTTPαS, thymidine 5′-*O*-(1-thiotriphosphate); dTDPαS, thymidine 5′-*O*-(1-thiodiphosphate); dTMPS, thymidine 5′-*O*-phosphorothioate; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); poly[d(A-T)], alternating copolymer of dAMP and dTMP; poly[d(G-C)], alternating copolymer of dGMP and dCMP; poly[d(T_s-A)], alternating copolymer of dTMP and 2′-deoxyadenosine 5′-*O*-phosphorothioate; poly[d(A_s-T)], alternating copolymer of dAMP and thymidine 5′-*O*-phosphorothioate; d[Tp(S)A], 5′-*O*-(2′-deoxyadenosyl) 3′-*O*-thymidyl phosphorothioate; d[Ap(S)T], 5′-*O*-thymidyl 3′-*O*-(2′-deoxyadenosyl) phosphorothioate; d(TpA), 5′-*O*-(2′-deoxyadenosyl) 3′-*O*-thymidyl phosphate; d(ApT), 5′-*O*-thymidyl 3′-*O*-(2′-deoxyadenosyl) phosphate; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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chromatograph equipped with a Model 440 absorbance detector operating at 254 nm, using ODS-Hypersil (Shandon Products, Astmoore, Runcorn, U.K.) as the stationary phase with 0.1 M triethylammonium acetate, pH 7.0, containing 10% acetonitrile as the eluant for analysis of the dinucleoside phosphorothioates. ^{31}P NMR spectra were recorded at approximately 30 °C on a Bruker WP200SY spectrometer operating at 81.01 MHz with ^{1}H broad-band decoupling using aqueous trimethyl phosphate as the external standard. Chemical shifts are given in parts per million, positive values indicating downfield shifts from this standard. The polymers were sonicated under ice cooling with a Branson sonifier for 1 h before the spectra were recorded.

Methods

Synthesis of Phosphorothioate-Substituted Poly[d(A-T)]. Poly[d(A_sT)] and poly[d(T_sA)] were enzymatically synthesized in a reaction mixture containing 50 mM Tris-HCl, pH 8.3 (alternatively, 50 mM potassium phosphate, pH 7.4), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.02% sodium azide, 1 or 2 mM either of dATP or of dTTP (in some cases labeled with ^{3}H), 1 or 2 mM concentration of the S_P isomer of the phosphorothioate analogue of the complementary deoxynucleoside triphosphate dTTPαS (a 1:1 mixture of the S_P and R_P isomers) or dATPαS (pure S_P isomer), 0.04–0.2 mM poly[d(A-T)], and 8–12 units/mL *M. luteus* DNA polymerase I. Incubation was at 37 °C, and incorporation was monitored by the radioactivity or the enhancement of ethidium bromide fluorescence. In some cases, reactions were cascaded by successive dilution with fresh solution containing substrate but not template and starting with up to 150 units/mL enzyme. The yield was 25–50% after 40–50 h. The reactions were terminated by addition of EDTA and heating at 65 °C for 10 min. After centrifugation, the solutions were extracted twice with buffer-saturated phenol and then ether. The separation of the polymer product from the residual substrate was effected by chromatography on Sephadex G-50 in 5 mM Tris-HCl, pH 7.8, 10 mM NaCl, and 0.1 mM EDTA. The DNAs had chain lengths generally in excess of 400 base pairs by electrophoretic analysis. Thermal denaturation measurements were performed according to Pörschke & Jung (1982).

Digestion of Polymers. Approximately 3 A_{260} units of poly[d(A_sT)] and poly[d(T_sA)] were digested with *Escherichia coli* DNA polymerase I and alkaline phosphatase as described (Romaniuk & Eckstein, 1982). After incubation for 16 h at 37 °C an aliquot of each reaction was analyzed by HPLC, and the elution profiles were compared with those of the diastereomers of the chemically synthesized dinucleoside monophosphorothioates d[Ap(S)T] and d[Tp(S)A]. This analysis showed that each polymer had been degraded to the expected dinucleoside monophosphorothioate of the R_P configuration.

Results

As has been shown for *E. coli* DNA polymerase I as well as for T4 and T7 page DNA polymerases, the S_P isomer of dATPαS can replace dATP as a cosubstrate with dTTP in the polymerization reaction using poly[d(A-T)] as template-primer (Burgers & Eckstein, 1979; Brody & Frey, 1981; Romaniuk & Eckstein, 1982; Gupta et al., 1982; Brody et al., 1982). We show here that it is also a substrate for the enzyme from *M. luteus*, as is dTTPαS. Degradation of the resulting polymers, poly[d(T_sA)] and poly[d(A_sT)], respectively, with the 5′–3′-exonuclease activity of *E. coli* DNA polymerase I and alkaline phosphatase was carried out as first described for poly[d(A-T)] by Brody & Frey (1981) and later by Brody et al. (1982) as well as by Romaniuk & Eckstein (1982). The resulting dinucleoside phosphorothioates were analyzed by HPLC on a reverse-phase column (Table I). Degradation of poly[d(T_sA)] yielded d[Tp(S)A] which was shown by HPLC to have the R_P configuration by comparison with the chemically synthesized diastereomers (Romaniuk & Eckstein, 1982). Thus, the polymerization reaction proceeded with inversion of configuration at phosphorus as has been observed for all other DNA polymerization reactions so far. Similarly, the degradation of poly[d(A_sT)] yielded d[Ap(S)T] which was identical with the diastereomer of the chemically synthesized material which had been previously shown to be degraded by snake venom phosphodiesterase. Therefore, it also has the R_P configuration and indicates similarly that the S_P isomer of dTTPαS had been used as the substrate in this case.

Table I: HPLC Analysis of Dinucleoside Phosphorothioates

dinucleoside phosphorothioates		
chemically synthesized	isolated from digestion of	retention time (min)
d[Tp(S)A], R_P isomer		5.1
d[Tp(S)A], S_P isomer		6.2
	poly[d(T_sA)]	5.1
d[Ap(S)T], R_P isomer		6.6
d[Ap(S)T], S_P isomer		9.8
	poly[d(A_sT)]	6.6

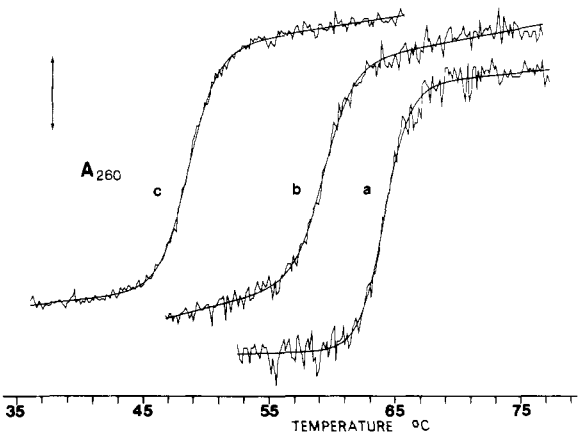

FIGURE 1: Thermal denaturation of poly[d(A-T)] (a), poly[d(T_sA)] (b), and poly[d(A_sT)] (c). The T_m's in 0.15 M NaCl + 3 mM buffer (Tris-HCl, pH 7.6) were 64.2, 59.3, and 48.8 °C for (a), (b), and (c), respectively. The corresponding ΔH's were 0.93, 0.77, and 0.72 MJ/mol. The transitions were accompanied by a 35% hyperchromicity and were fully reversible. T_m determinations of a second set of independently synthesized DNAs were within 1 °C of the above values. The arrow indicates a ΔA_{260} of 0.02, 0.03, and 0.04 for (a), (b), and (c), respectively.

To further characterize the phosphorothioate analogues of poly[d(A-T)], we determined the midpoint and cooperativity (ΔH) of their thermal denaturation. As shown in Figure 1, the T_m values for the modified polymers are dramatically lower than that for poly[d(A-T)], the effect being more prominent for substitution 5′ to the pyrimidine nucleoside (Δ = −15 °C) than 5′ to the purine nucleoside (Δ = −5 °C). A low T_m value has also been observed by others for poly[d(T_sA)] (S. Adler, R. S. Brody, P. A. Frey, and P. Modrich, unpublished results). Similar effects are seen with the phosphorothioate analogues of poly[d(G-C)] (Jovin et al., 1983; T. M. Jovin et al., unpublished results) but not of poly[r(A-U)], for which the T_m values are essentially the same as those for poly[r(A-U)] (Matzura & Eckstein, 1968; Eckstein & Gindl, 1970).

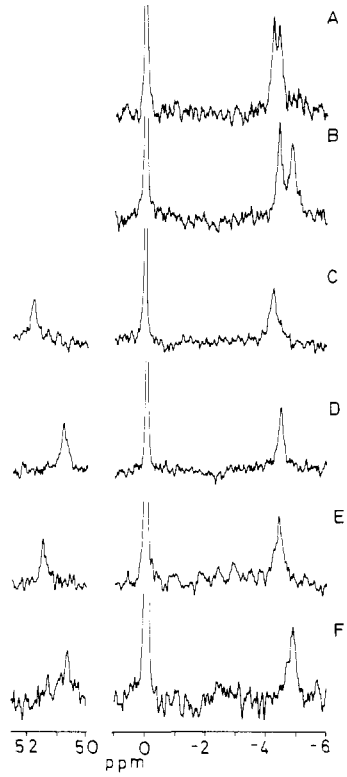

FIGURE 2: ^{31}P NMR spectra of poly[d(A-T)] (A, B), poly[d(A_sT)] (C, D), and poly[d(T_sA)] (E, F). Spectra A, C, and E at low ionic strength: 10 mM Tris-HCl, pH 8.0 (uncorrected for D_2O), 10 mM EDTA, and 2–4 mM nucleotide in approximately 90% D_2O. Spectra B, D, and F at high ionic strength: same as low ionic strength solutions except for the addition of 2 M CsF. The spectra were taken in 5-mm diameter tubes with approximately 13 000 transients. Conditions: 4.09-s acquisition time; 3-Hz line broadening; 2-kHz sweep width.

Table II: ^{31}P NMR Chemical Shifts of Poly[d(A-T)], Poly[d(A_sT)], and Poly[d(T_sA)]

polymer[a]	^{31}P shift (ppm)
poly[d(A-T)]	−4.23, −4.41
poly[d(A-T)] + 2 M CsF	−4.43, −4.84
poly[d(A_sT)]	−4.25, 51.80
poly[d(A_sT)] + 2 M CsF	−4.46, 50.84
poly[d(T_sA)]	51.51, −4.43
poly[d(T_sA)] + 2 M CsF	50.68, −4.80
assigned linkage	d(TpA), d(ApT)

[a] Data taken from Figure 2.

In the ^{31}P NMR spectrum of poly[d(A-T)] at low salt, two signals separated by 0.18 ppm can be seen (Figure 2, Table II). This value compares with 0.22 ppm reported for a poly[d(A-T)] 145 base pairs (Shindo et al., 1979) or 50–200 base pairs (Cohen et al., 1981) in length and with 0.13 ppm for a poly[d(A-T)] of unreported chain length (Patel et al., 1981). As expected, the resonances for the phosphorothioates in the two polymers with phosphorothioate substitutions are shifted downfield by about 55 ppm whereas the phosphate resonances at low salt concentration remain at −4.25 ppm for poly[d(A_sT)] and at −4.43 ppm for poly[d(T_sA)]. These values differ only by 0.02 ppm from those obtained for poly[d(A-T)]. Similarly, at 2 M CsF, the resonances for the phosphates in the two modified polymers (−4.46 and −4.80 ppm, respectively) are shifted upfield and deviate only very little from the corresponding values for poly[d(A-T)]. The upfield shift induced by addition of CsF is also reflected in the phosphorothioate resonances, the value being approximately 1 ppm.

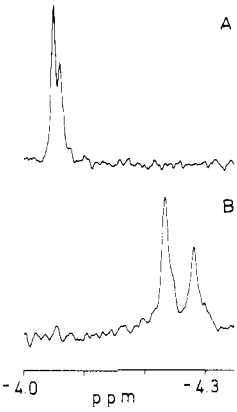

FIGURE 3: ^{31}P NMR spectra of a mixture of d(ApT) and d(TpA). Solutions at low ionic strength (A) were 10 mM Tris-HCl, pH 8.0 (uncorrected for D_2O), 10 mM EDTA, and about 5 mM nucleotide in approximately 90% D_2O. Solutions at high ionic strength (B) contained in addition 2 M CsF. Conditions: 10.24-s acquisition time; 0.2-Hz line broadening; 800-Hz sweep width.

Table III: ^{31}P NMR Chemical Shifts of d(ApT) and d(TpA)

dinucleoside phosphate[a]	^{31}P shift (ppm)
d(ApT)	−4.048
d(ApT) + 2 M CsF	−4.232
d(TpA)	−4.060
d(TpA) + 2 M CsF	−4.281

[a] Data taken from Figure 3. Each dinucleoside phosphate alone has the same chemical shift as in the mixture at low ionic strength.

The ^{31}P resonances in the spectra of the dinucleoside phosphates, d(TpA) and d(ApT), are also separated at low salt, albeit by a mere 0.012 ppm (Figure 3, Table III). The difference is increased to approximately 0.05 ppm concomitant with an upfield shift of approximately 0.2 ppm in solutions of 2 M CsF.

Discussion

The structures of several oligodeoxynucleotides revealed by single-crystal X-ray analysis have provided a detailed insight into the multitude of conformations into which nucleotides can be arranged in such molecules (Dickerson et al., 1982; Zimmermann, 1982). Of particular interest most recently has been the realization that oligo- and poly[d(G-C)] can, under appropriate conditions, adopt a left-handed Z rather than a right-handed A or B helix conformation (Pohl & Jovin, 1972; Wang et al., 1981). On the basis of the X-ray structural analysis of d(pApTpApT), a B-DNA-type polymer conformation has been suggested for poly[d(A-T)] (Viswamitra et al., 1978, 1982). A striking feature of this structure is the alternation of the sugar conformation between a C3′-endo and a C2′-endo pucker depending on whether the sugar is attached to a purine or a pyrimidine base. Evidence for the C3′-endo conformation, i.e., a salt-dependent varying ring pucker, in the solution structure of poly[d(A-T)] has been obtained recently by Raman spectroscopy (Thomas & Peticolas, 1983). Thus, for poly[d(A-T)], it has been proposed that the repeating unit is not a mononucleotide but rather a dinucleotide (Klug et al., 1979; Viswamitra et al., 1982). In the classical B-DNA structure, the sugar adopts a uniformly C2′-endo conformation. However, a detailed X-ray analysis of the dodecamer d-[CGCGAATTCGCG] (Fratini et al., 1982) shows that the sugar conformation is not unique but that for pyrimidines the conformation tends toward C2′-endo and for purines toward C3′-endo but without reaching the extreme positions. An alternating B-like structure for poly[d(A-T)] is consistent with

a number of biochemical properties of this DNA. An early finding was the highly preferential cleavage of the d(ApT) internucleotidic bond by pancreatic DNase I (Scheffler et al., 1968). Shindo et al. (1979) and Cohen et al. (1981) observed two resonances in the ^{31}P NMR spectrum of short segments of poly[d(A-T)] in solution at low salt concentration and at temperatures below the T_m. Two resonances have also been seen by ^{31}P NMR spectroscopy of poly[d(A-T)] fibers arranged parallel relative to the magnetic field (Shindo & Zimmermann, 1980). CD measurements at high CsF concentrations give additional evidence that poly[d(A-T)] adopts a conformation with different geometries at the purine and pyrimidine nucleotides (Vorlickova et al., 1980), as has been substantiated by ^{31}P, ^{1}H, and ^{13}C NMR studies (Patel et al., 1981; Kypr et al., 1981; Cohen et al., 1981; Shindo, 1981). Thus, ^{31}P NMR spectroscopy is a valuable tool for monitoring conformational states of poly[d(A-T)] as it is for the B → Z transition in poly[d(G-C)] (Patel et al., 1979). Such ^{31}P NMR studies would be even more valuable if one could unambiguously determine whether the two resonances are due to the two phosphate groups in d(ApT) and d(TpA) and, if so, assign them.

It seemed to us that the use of phosphorothioate analogues of poly[d(A-T)] could furnish an answer to these questions since it is known that phosphorothioates have chemical shifts in the ^{31}P NMR spectrum located approximately 50 ppm downfield from the corresponding phosphates (Eckstein & Goody, 1976; Eckstein et al., 1977). Studies with phosphorothioate analogues of poly[d(G-C)] have indeed provided the assignment of the two resonances observed when this polymer exists in the Z conformation (Jovin et al., 1983; T. M. Jovin et al., unpublished results).

The ^{31}P NMR spectra for the two phosphorothioate polymers poly[d(T_sA)] and poly[d(A_sT)] as well as for poly[d(A-T)] were recorded in solutions of low ionic strength and at 2 M CsF (Figure 2). The data are summarized in Table II. The phosphate resonance for poly[d(A_sT)] at −4.25 ppm appears at the same position as the low-field resonance of poly[d(A-T)] at −4.23 ppm at low ionic strength. Similarly, the resonance of poly[d(T_sA)] at −4.43 ppm is at the position of the high-field resonance of poly[d(A-T)] at −4.41 ppm. These results lead to the assignment of the downfield resonance of poly[d(A-T)] to the phosphate group of d(TpA) and that of higher field to that of d(ApT), in agreement with the tentative assignment made by Shindo & Zimmermann (1980) for poly[d(A-T)] fibers. Thus, our data provide strong support for a dinucleoside repeat structure of poly[d(A-T)] possibly corresponding to the alternating B model of Klug et al. (1979). The fact that the two ^{31}P NMR resonances exhibit equal areas under a variety of conditions argues against the proposal advanced by Sobell et al. (1983) invoking a dynamic equilibrium between two different structures of poly[d(A-T)]. The anomalous internucleotidic linkage is evidently d(TpA) as judged by the downfield shift of the ^{31}P NMR resonance, the non-B phosphate diester conformation (torsional angle $\omega' \simeq -125°$; Viswamitra et al., 1982), and the reduced susceptiblity to pancreatic DNase I. The underlying structure is not obviously related to that arising in the B → Z transition of poly[d(G-C)], in which the downfield shift is associated with the purine–pyrimidine linkage d(GpC). A common denominator, however, could be the correlation between a gauche, trans conformation and a downfield shift as opposed to the more general gauche, gauche conformation and a more upfield resonance [for a review, see Gorenstein (1981)].

In solutions containing 2 M CsF, the comparison of the ^{31}P NMR data indicates that the assignments remain the same: a low-field resonance for d(TpA) and a high-field resonance for d(ApT). Thus, it can be concluded that both phosphate groups are shifted upfield in 2 M CsF, a finding not available from observation of the poly[d(A-T)] spectrum which shows a resonance at −4.4 ppm at both low and high ionic strength. This component is high field in one case and low field in the other, thus leaving the question unresolved as to whether both resonances are shifted upfield by 0.2 and 0.4 ppm, or only one resonance by 0.6 ppm (Patel et al., 1981). From the present study, it is clear that the phosphate groups in both dinucleoside phosphates experience a change in their environment although the magnitude is greater for d(ApT) (0.4 ppm) than for d(TpA) (0.2 ppm).

The question also arises as to whether the conformational change, induced by CsF and also by tetraalkylammonium and 3α,5β,17β-dipyrrolidinium steroid dimethiodide cations (Patel et al., 1981) as manifested by the upfield shift of the ^{31}P resonances, is a reflection of a conformational change in the helical structure of poly[d(A-T)] or whether it can also be seen with the basic units constituting the polymer, i.e., the two dinucleoside phosphates. To answer this question we recorded the ^{31}P NMR spectra of d(ApT) and d(TpA) as well as of a mixture of the two dinucleotides at low and high ionic strength (Figure 3, Table III).

It can be seen that both resonances appear at lower field than when the dinucleoside phosphates are incorporated into poly[d(A-T)], in agreement with the observation of others (Gorenstein et al., 1976) that in the helical conformation the ^{31}P NMR resonances of nucleotides experience an upfield chemical shift. It is also worthy to note that d(TpA) resonates at higher field than d(ApT), which is in contrast to what is observed in the polymer. However, the difference is small, being only 0.012 ppm as opposed to 0.2 ppm in the polymer.

^{31}P NMR chemical shifts of nucleotides are influenced by a variety of factors such as pH, O–P–O bond angles, and ester torsional angles (Gorenstein, 1981; Cozzone & Jardetzky, 1976; Gorenstein & Luxon, 1979) so that a simple interpretation of the shift observed in 2 M CsF solution is not possible. However, it is clear from this study that the phosphorus of d(ApT) experiences a greater change in its environment than that of d(TpA). Patel et al. (1981) as well as Kypr et al. (1981) have found in their ^{1}H NMR studies of poly[d(A-T)] at low and high salt concentrations that the largest shifts are seen for H8 of deoxyadenosine and one of the sugar C1′ protons [which has been attributed by Kypr et al. (1981) to deoxyadenosine]. Since both of these protons are sensitive to changes in the glycosidic torsion angle which influences the sugar pucker and consequently the torsional angle of the phosphate group, the ^{1}H NMR and the ^{31}P NMR data are consistent with the idea that the most dramatic change involves the deoxyadenosine.

While small in magnitude, the ^{31}P NMR splitting in the spectrum of poly[d(A-T)] reported here and previously is also observed in the case of a number of other alternating purine–pyrimidine sequences (Cohen et al., 1981; Patel et al., 1981; McIntosh et al., 1983) for which the proposed dinucleotide structure may therefore be quite general.

Acknowledgments

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Registry No. Poly[d(A-T)], 26966-61-0; poly[d(T_sA)], 86594-48-1; poly[d(A_sT)], 86550-16-5; d(TpA), 19192-40-6; d(ApT), 23339-47-1;

dATP, 1927-31-7; dTTP, 365-08-2; S_P-dATPαS, 80875-87-2; S_P-dTTPαS, 83199-32-0; R_P-dTTPαS, 83199-35-3.

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