Temperature Dependence of the ^{31}P Chemical Shifts of Nucleic Acids. A Probe of Phosphate Ester Torsional Conformations†

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ABSTRACT: The temperature dependence of the ^{31}P chemical shifts of the ribodinucleoside monophosphates, ApA, GpC, CpC, UpU, and ApU, of the deoxyribonucleic acids, d-ApT, TpT, d-ApA, and d-pApT, and of the homopolyribonucleic acids poly(G), poly(U), poly(C), and poly(A) is shown to provide information on the helix-coil transition in nucleic acids. The base stacked, helical structure with a gauche,gauche phosphate ester torsional conformation is 0.2–0.6 ppm upfield from the random coil conformation. In contrast, the ^{31}P chemical shifts of dimethyl and diethyl phosphate do not change significantly with temperature. These results support our earlier hypothesis that ^{31}P shifts are sensitive probes of torsional conformations with phosphate esters in gauche,gauche conformations having ^{31}P shifts upfield from nongauche conformations.

Nuclear magnetic resonance (NMR)[1] spectroscopy and x-ray crystallographic studies of simple monomer, dimer, and polymer models of nucleic acids have shown that the ribose ring and base form a rather rigid unit with the main conformational flexibility of the nucleic acid backbone being limited to the two P-O phosphate ester torsional angles (Sundaralingam, 1969; Kim et al., 1973; Ts'o et al., 1969). Unfortunately, of the six torsional angles that largely define this backbone structure (Sundaralingam, 1969), only the four involving the ribose ring have been shown to be amenable to analysis by NMR techniques (see, for example, Ts'o et al., 1969; Chan and Nelson, 1969; Lee et al., 1975; Tsuboi et al., 1969). We wish to propose that ^{31}P NMR spectroscopy is potentially capable of providing information on the most important remaining two torsional angles involving the phosphate ester bonds.

We have recently described an empirical (Gorenstein, 1975) and theoretical (Gorenstein and Kar, 1975) correlation of phosphate ester structure and ^{31}P chemical shifts. The salient feature of our proposal is that phosphate ester ^{31}P chemical shifts are primarily determined by the ester torsional conformations (which are also directly related to phosphate-oxygen bond angles; Gorenstein et al., 1976). We have proposed that the gauche, gauche (g,g) torsional conformation of phosphate diester monoanions (structure I) should have the further upfield chemical shift, while any eclipsed conformation (or simply less gauche conformation) should have a downfield ^{31}P chemical shift. Thus, our calculations (Gorenstein and Kar, 1975) indicate that a phosphate diester monoanion in a gauche, trans (g,t) conformation (structure II) should have a ^{31}P chemical shift approximately 10 ppm downfield from an ester in the g,g conformation. Although the magnitude of the shift is only poorly established and must await further x-ray structural data and ^{31}P chemical shifts, the direction of the shift appears to be qualitatively correct.

Our proposal that ^{31}P chemical shifts serve as a probe of torsional geometries is made ever more meaningful when one considers that other possible factors that could affect ^{31}P shifts in phosphate esters apparently are quite unimportant (Gorenstein, 1975; Gorenstein et al., 1976). Thus, we have recently noted (Gorenstein et al., 1976) that interaction of a mono- or dianionic phosphate monoester with strong hydrogen bonding donors results in small (if any) ^{31}P chemical shift perturbations.

As a test of our ^{31}P shift torsional angle hypothesis, we have studied the temperature dependence of the ^{31}P chemical shift of a number of dinucleoside monophosphates, a dinucleotide (hereafter, both referred to generally as dimers) and homopolyribonucleic acids. It has been established mainly through ORD, CD, uv/visible absorption, and ^1H NMR spectroscopy that both dimers and polymers undergo a "helix-coil" transition with a characteristic melting temperature (T_m) representing the temperature at which half the nucleic acid is in a base stacked, helix conformation and the other half is in a random coil, largely unstacked conformation (see for example, reviews such as Ts'o, 1975; Duchesne, 1973; Turchinskii and Shibaev, 1972; also Ts'o et al., 1969; Chan and Nelson, 1969; Brahms et al., 1967a,b; Warshaw and Tinoco, 1965; Warshaw and Cantor, 1970; Bobst et al., 1969). Since the phosphate ester conformation in the helical state is g,g (see Day et al., 1973 and references therein; Newton, 1973; Gorenstein et al., 1976; Perahia et al., 1974; Sasisekharan and Lakshminarayawan, 1969), it was hoped that upon raising the temperature of these phosphate diesters, a shift to lower field would occur, indicating an increase in the proportion of nongauche conformations.

Experimental Section

Nucleic acids were obtained from Sigma, PL Biochemicals, and Collaborative Research. The nucleic acids were freed of possible paramagnetic metal ion impurities by passing them through a Chelex-100 ion-exchange resin (Na^+ form). The solutions were lyophilized and the solid material was dissolved in a 10^{-3} M EDTA solution (20–30 mg/ml). The pH was

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; CD, circular dichroism, EDTA, (ethylenedinitrilo)tetraacetic acid; Act-D, actinomycin D; poly(A), poly(adenylic acid); poly(G) poly(guanylic acid).

TABLE I: ^{31}P Chemical Shifts of Phosphate Diesters.

Compound[a]	$\delta_{5°}$[b] (ppm)	$\delta_{82°}$[b]	$-\Delta\delta$[c] (ppm)
Dinucleoside monophosphates			
CpC	0.995	0.460	0.535
UpU	1.005	0.705	0.300
ApA	1.170[d]	0.652	0.518
GpC (pH 9.72)	1.255[d]	0.612	0.643
ApU	1.215	0.726	0.489
Deoxydimers			
d-TpT[e]	1.225	0.995	0.230
d-ApA	1.213	0.890	0.323
d-ApT	1.380	1.075	0.305
d-pApT (diester)	1.455	1.225	0.230
Polynucleic acids			
Poly(C)[e]	1.522	1.045	0.477
Poly(A)	1.302[d]	0.890	0.412
Poly(U)[f]	0.768	0.482	0.286
Poly(U) (0.5 M NaCl)	1.188	0.788	0.400
Poly(G) (pH 10.00)	1.487[g]	0.950	>0.537
Poly(G) (pH 11.48)	0.546	0.415	0.131
Reference compounds			
Dimethyl phosphate	−2.780	−2.738	−0.042
Diethyl phosphate	−0.820	−0.855	0.035
Dibenzyl phosphate	−0.405	−0.560	0.155
2′,3′-Cyclic CMP	−20.05	−19.50	−0.500

[a] In 10^{-3} M EDTA, pH ~7.0, concentration 15–35 mg/ml unless otherwise specified. [b] ^{31}P chemical shifts at 5 and 82 °C. Positive numbers represent shifts to higher fields relative to room temperature 85% H_3PO_4. [c] Chemical shift difference, $\delta_{82°}-\delta_{5°}$. [d] Extrapolated from curve (Figures 3–5). [e] Contains 10^{-2} M cacodylate buffer. [f] Contains 2×10^{-3} M cacodylate buffer. [g] Value at 25 °C.

adjusted with 1 M NaOH or HCl to ca. 7.0 unless otherwise indicated. The dimethyl phosphate was prepared by reacting trimethyl phosphate (Aldrich Chemicals) with NaI in acetone. The NMR sample of dimethyl phosphate was purified and prepared in a similar manner as described for the nucleic acids. The diethyl and dibenzyl phosphates were obtained from Eastman and Aldrich Chemicals, respectively and were not specially purified.

The ^{31}P NMR spectra were recorded on a Bruker B-KR 3225 pulsed spectrometer/HFX-90 spectrometer at 36.4 MHz. Signal averaging in the pulsed Fourier transform mode of the spectrometer was accomplished with a Nicolet 1080 data system. ^{1}H broad band decoupling and a fluorine external lock using a coaxial lock with a coaxial inner cell of C_6F_6 were used. Several studies used an external ^{2}H lock with coaxial inner cell of D_2O. In these runs the ^{31}P chemical shifts were referenced back to the external ^{19}F lock frequency of C_6F_6. The temperature dependence of the ^{31}P signal of dimethyl phosphate in H_2O with external ^{19}F lock and with external ^{2}H lock provided a calibration to reformulate all ^{31}P shifts relative to room temperature 85% phosphoric acid (0 ppm; referenced in turn to external C_6F_6). Equation 1 may be used to convert chemical shifts referenced to a ^{19}F lock signal of C_6F_6, $\delta_{^{19}F}$, to chemical shifts referenced to a ^{2}H lock of D_2O, $\delta_{^2H}$:

$$\delta_{^{19}F} = \delta_{^2H} + 0.0091141(T - 274\ °C) \quad (1)$$

The chemical shifts at temperature, T, are in ppm from room temperature, 85% phosphoric acid. The sample temperature was controlled to better than ±1 °C with a Bruker B-St 100/700 temperature control unit.

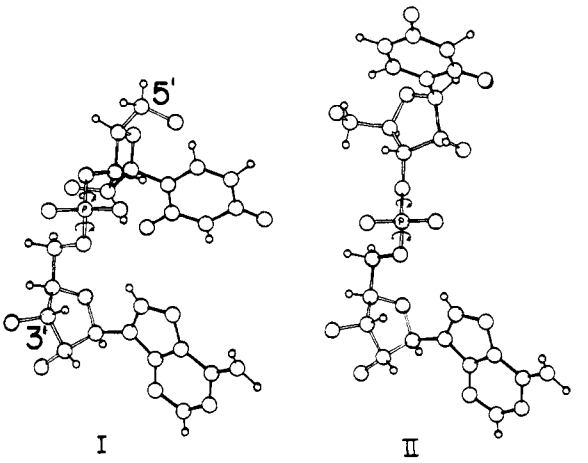


FIGURE 1: Structure of a dinucleoside monophosphate, UpA in the gauche, gauche, I, and gauche, trans, II, phosphate ester conformations. From Sundaralingam (1969).

Results and Discussion

Origin of Temperature Dependence to ^{31}P Shifts. As shown in Table I and Figures 2–5 the proposed shift in the ^{31}P signal of *all* dimers and polyribonucleic acids without exception is observed, with a 0.13–0.64 ppm downfield shift, $\Delta\delta$ (Table I), occurring with increasing temperature. Since these shifts are referenced to the ^{19}F lock signal of an external capillary of C_6F_6, it is important to establish that these ^{31}P signal changes are really the result of a "helix-coil" transition and not an artifact attributable to changes in the ^{19}F lock frequency or to changes in the bulk susceptibility in the solution, or to any number of other possible explanations. We will consider a number of facts that argue in favor of our proposed interpretation.

(I) First, shown in Figure 6 is the temperature dependence of the ^{31}P signal of dimethyl phosphate, diethyl phosphate, dibenzyl phosphate, and 2′,3′-cyclic cytidine monophosphate (cCMP) (pH ~7, 10^{-3} M EDTA). In contrast to the dimeric and polymeric nucleic acids, the dimethyl and diethyl phosphates do not show any significant ^{31}P chemical shift variation with temperature (in fact, there may be a slight ca. 0.04 ppm *upfield* shift with increasing temperature for the dimethyl phosphate). It is especially significant that the ^{31}P shift with temperature for cCMP is so dramatically different from that of the acyclic diesters, shifting 0.5 ppm *upfield* with increasing temperature. The cyclic mononucleotide, of course, contains a relatively rigid five-membered cyclic, monoanionic, phosphate diester (Lapper and Smith, 1973; Lavallee and Coulter, 1973). Since phosphate ester conformational effects probably only modestly influence the ^{31}P chemical shift temperature dependence of cCMP, this molecule is perhaps a better standard than the conformationally flexible, acyclic dialkyl phosphates. The upfield shift with temperature for cCMP is due simply to our choice of C_6F_6 as the lock standard, since the chemical shifts of the lock standards will themselves be temperature dependent. Using a D_2O lock, the cCMP signal actually shifts ca. 0.1 ppm downfield, but now, of course, relative to the D_2O lock the shift differences, $\Delta\delta$, for the nucleic acids would be ca. 0.6 ppm larger (i.e., the nucleic acids would shift 0.7–1.3 ppm downfield with increasing temperature under D_2O lock conditions). These model phosphate diester shifts indicate that the large downfield shifts for the ^{31}P signals of the nucleic acids are not an intrinsic property of phosphate diester monoanions or an artifact of the experimental design. The special

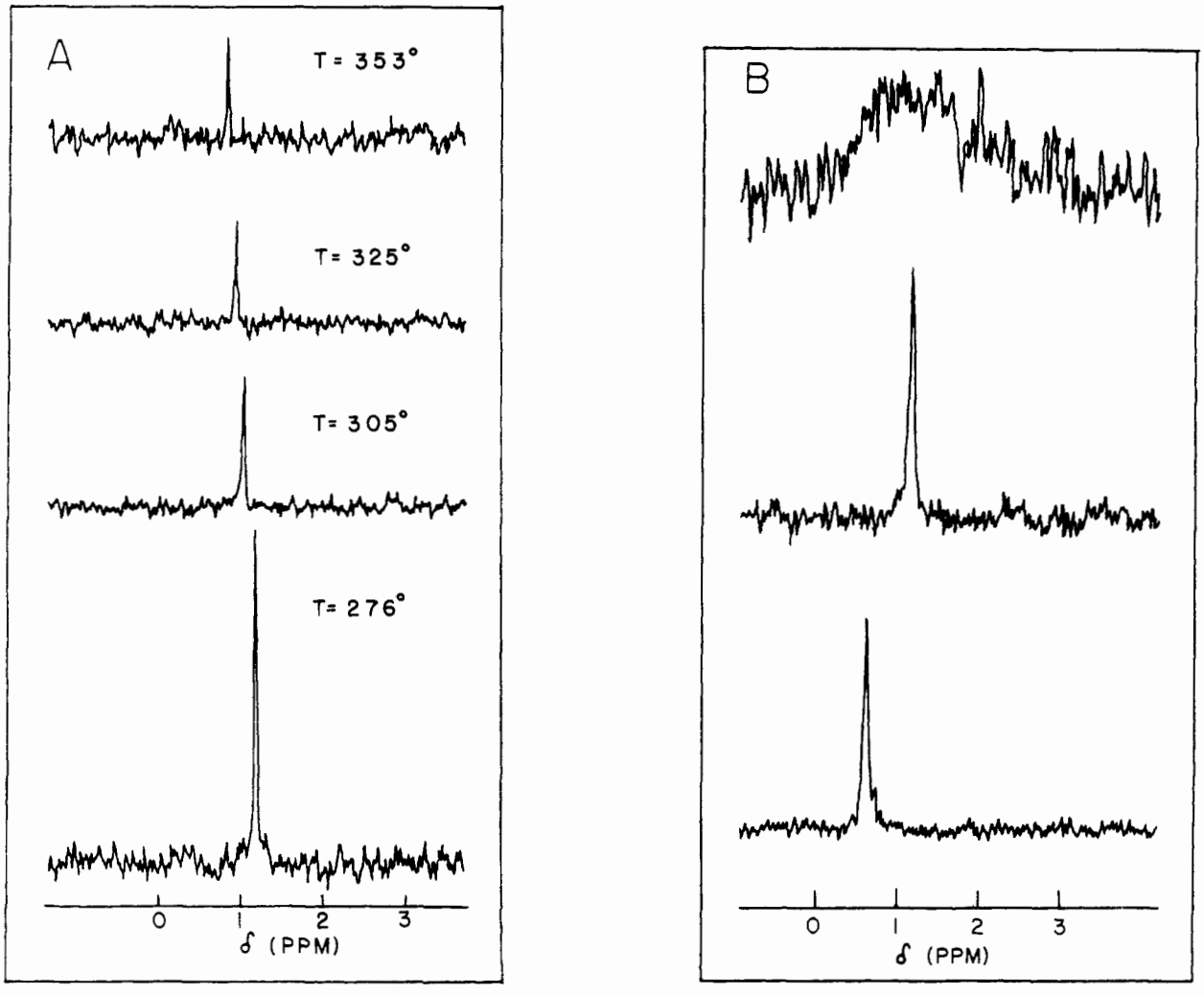


FIGURE 2: (A) Representative ^{31}P NMR spectra of d-ApA at different temperatures. (B) Representative ^{31}P NMR spectra of poly(G) at pH 10 (top), poly(A) at pH 7 (middle), and poly(U) at pH 7 (bottom).

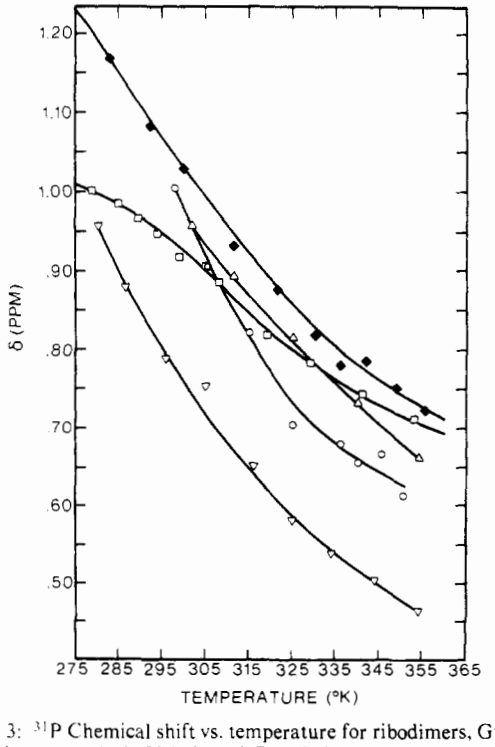


FIGURE 3: ^{31}P Chemical shift vs. temperature for ribodimers, GpC (○), UpU (□), ApA (△), ApU (♦), and CpC (▽).

case of dibenzyl phosphate will be treated separately later.

(II) Secondly, the generally sigmoidal shape and effective T_m (midpoint of the sigmoidal curves) for the nucleic acids agree with melting curves and T_m's obtained by other physical techniques (see Ts'o, 1975, and references therein). Thus, for poly(A) the T_m of ~33 °C, the enthalpy of unstacking[2] ($\Delta H°$ ~ 10.1 ± 4.1 kcal/mol) and the entropy of unstacking ($\Delta S°$ ~ 30.8 ± 12.8 eu) calculated from the ^{31}P shift data agree within the experimental error with that obtained from circular dichroism spectra (T_m ~ 40 °C, $\Delta H°$ = 7.9 kcal/mol, $\Delta S°$ = 25 eu, pH 7.4, 0.1 M NaCl; Brahms et al., 1966). The T_m for the dimers are lower, generally less than 25 °C and thus only the intermediate and high-temperature limits can be studied. Note that the ^{31}P chemical shifts of all the dimers in Figures 3–5 begin to level off at higher temperatures as would be expected if we are monitoring a helix–coil transition. Even at the highest temperatures apparently dinucleoside monophosphates such as ApA are still extensively stacked (Ts'o et al., 1969). Disruption of the helical, stacked conformation can also be effected by protonation of the bases (Chan and Nelson, 1969). Thus, the ApA ^{31}P signal shifts 0.09 ppm downfield at room

[2] Obtained by a nonlinear least-squares computer fit of the van't Hoff equation, assuming a simple two-state (helix–coil) process. As discussed later, the assumption of a simple two-state model is probably a severe oversimplification.

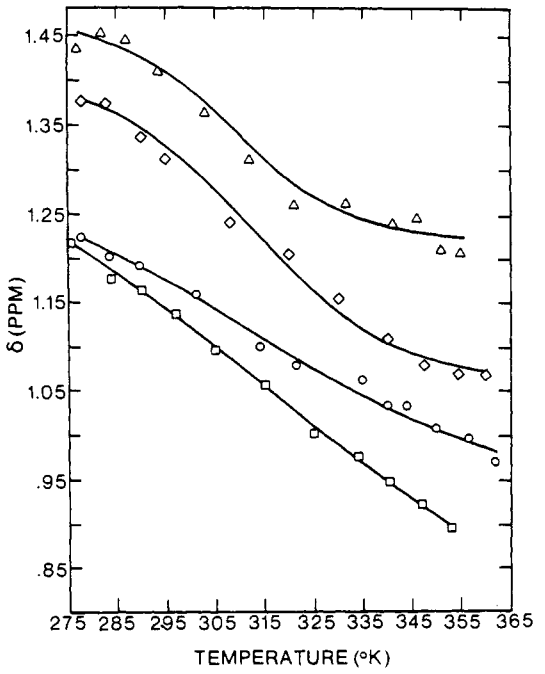


FIGURE 4: ^{31}P chemical shift vs. temperature for deoxyribodimers, d-pApT (diester signal only, △), d-ApT (◇), TpT (○), and d-ApA (□).

temperature upon lowering the pH to 3.0. Partial protonation of the bases at this pH unstacks the dimer and increases the proportion of nonhelical conformations.

Unlike ^{1}H NMR, ^{31}P NMR appears to more accurately monitor the melting of the nucleic acids. Temperature-dependent ^{1}H NMR spectra of nucleic acids apparently are influenced by changes in both base stacking and conformational populations (Ts'o, 1975; Chan and Nelson, 1969; Ts'o et al., 1969; Kondo et al., 1972) and thus it has not been possible to analyze the ^{1}H NMR derived melting curves on the basis of a simple two-state process. In fact, simple sigmoidal curves are rarely observed in these ^{1}H NMR studies.

(III) ^{31}P Chemical Shifts of Polyribonucleic Acids. Although the structure of the ordered, single-stranded polynucleic acid is not as well defined as the duplex polynucleic acid, the structures of the single-stranded polymers are at least better established than the dimers. Considerable structural information on the single-stranded homopolymers is available from NMR, optical, and Raman spectroscopy (see references in Ts'o, 1975; Duchesne, 1973) and theoretical calculations (Olson and Flory, 1972; Newton, 1973; Gorenstein and Kar, 1976; Perahia et al., 1974; Yathindra and Sundaralingam, 1974, and references therein). At low temperatures all data indicate that many of these single-stranded polynucleic acids (such as poly(A)) exhibit considerable organization with bases stacked and the polynucleic acid backbone arranged in a helical conformation. Specifically, the phosphate diester conformation is g,g. This fairly well-established structural information on the polymers significantly supports our ^{31}P shift hypothesis (comparing both the relative ordering of ^{31}P chemical shifts for the homopolymers and the shapes of the ^{31}P melting curves). Thus, at *all* temperatures the ^{31}P shift of poly(U) is 0.3–0.9 ppm downfield from the other homopolymers (the exception of poly(G) in strongly alkaline solution will be separately discussed below). This result is consistent with the present interpretation of the structures of these homopolymers, since poly(U) is apparently the least stacked, least helical homoribonucleic acid with the greatest amount of random coil

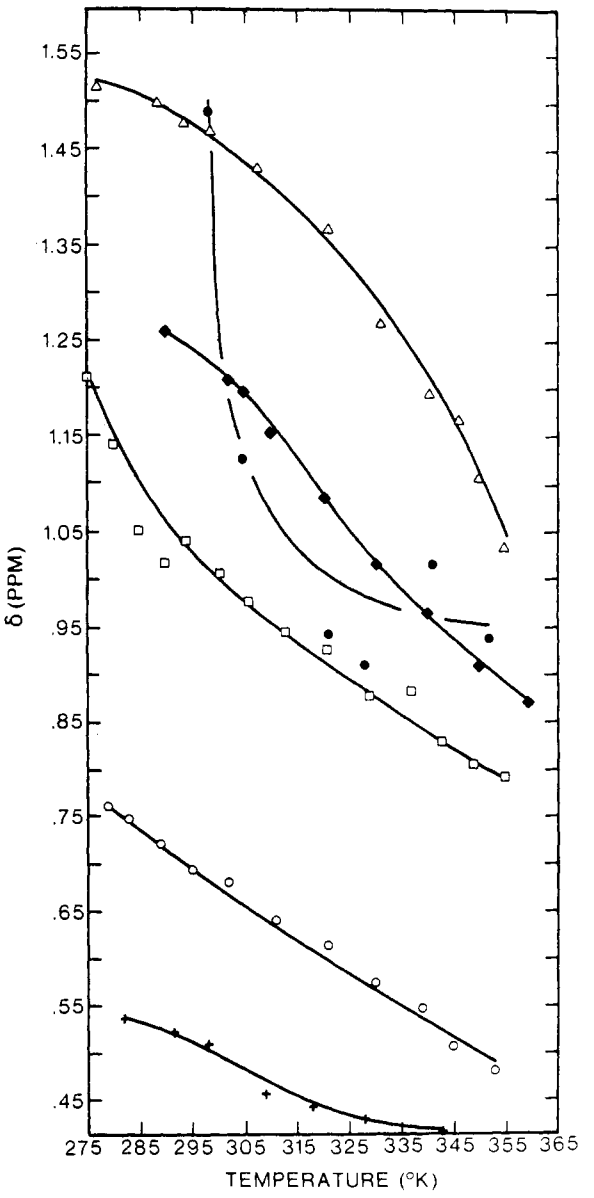


FIGURE 5: ^{31}P chemical shift vs. temperature for polynucleic acids, poly(C) (△), poly(A) (♦), poly(U) (0.5 M NaCl, □), poly(U) (○), poly(G) (pH 11.48, +), poly(G) (pH 10, ●).

(see Ts'o, 1975; Wagner, 1957; Lippset, 1960).

The ^{31}P chemical shift and line width for poly(G) is very pH dependent (see also Akasaka et al., 1975). The ^{31}P signal of poly(G) at pH 10.0 is quite broad (line width at half-height is >0.8 ppm, 25 Hz) and is shifted 1.0 ppm upfield from the much sharper poly(G) signal (2 Hz line width) observed at pH 11.48.[3] The signal broadness and upfield shift for the neutral poly(G) are indicative of the more rigid, helical conformation for this polymer. Aggregation of the poly(G) involving hydrogen-bonding interactions is also probably partially responsible for these differences. In the strongly alkaline solution the guanosine base is negatively charged, which will disrupt the base stacking and helical structure. The signal sharpness and the downfield shift (note even from poly(U)) reflect the

[3] This signal broadness leads to much poorer signal-to-noise ratios, resulting in less accurate chemical shift measurements. Fortunately only poly(G) in moderately alkaline solution has very broad ^{31}P signals. All other nucleic acids in dilute solution have line widths of 1–6 Hz. See Figure 2.

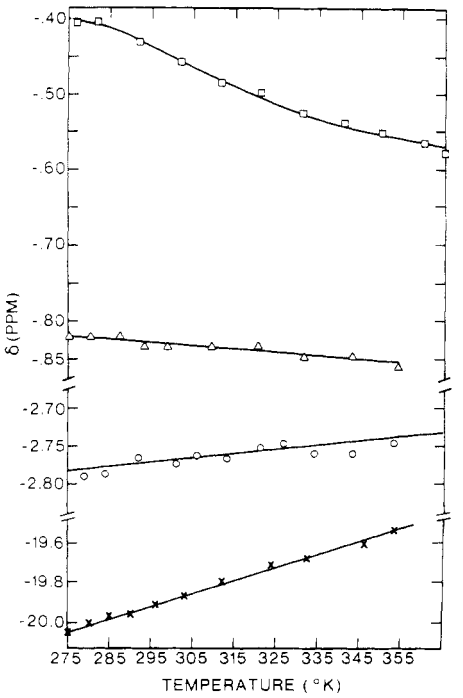


FIGURE 6: ^{31}P chemical shift vs. temperature for reference compounds, dibenzyl phosphate (□), diethyl phosphate (△), dimethyl phosphate (○), 2′,3′-cCMP (×). Note the ^{31}P chemical shift scale for cCMP has been reduced by one-fourth from other scales.

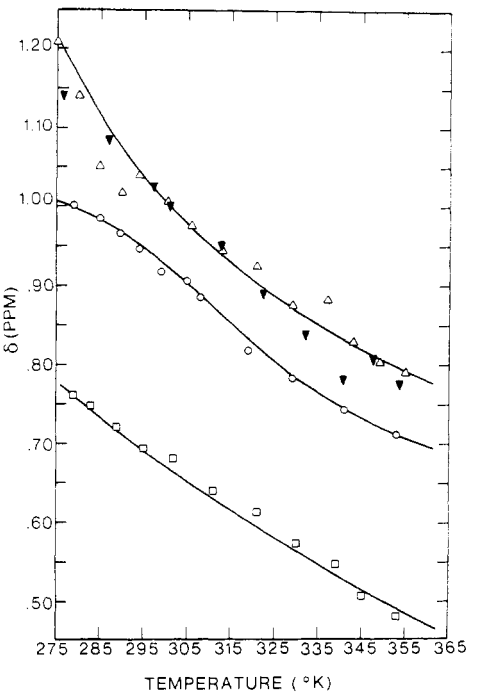


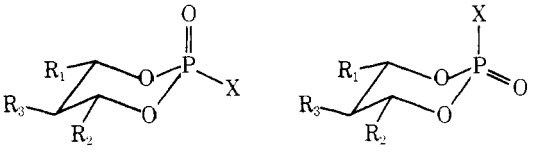
FIGURE 7: ^{31}P chemical shift vs. temperature for homouridine nucleic acids, poly(U) (1.0 M NaCl, ▼), poly(U) (0.5 M NaCl, △), UpU (○), poly(U) (□).

much greater degree of motional freedom in the more flexible random coil. The very sharp transition around 15 °C for the poly(G) in moderately alkaline solution is likely attributable to the partial disruption of aggregation at higher temperatures (Ts'o, 1975). The small change in ^{31}P shift with temperature for poly(G) in strongly alkaline solution is consistent with our interpretation that poly(G) has little helical structure under these conditions.

The poly(A) and poly(C) ^{31}P signals are 0.3–1.0 ppm upfield from their respective homodimers. This likely reflects a slightly more ordered, helical (g,g) composition for the polymers. Poly(U) appears to be an exception, coming ca. 0.3 ppm downfield from UpU. These results are in agreement with CD, ORD, and ^{1}H NMR spectra of poly(A), poly(C), poly(U), and their corresponding homodimers (Cantor et al., 1966; Brahms et al., 1966; 1967a,b; Simpkins and Richards, 1967; Gray et al., 1972; Ts'o and Schweizer, 1968). Thus, poly(A) and poly(C) ORD spectra are greater in magnitude than expected by comparison to the dimer and calculated spectra. The poly(U) ORD spectrum is comparable to the *monomer* Up spectrum (Cantor et al., 1966; Simpkins and Richards, 1967). Addition of 0.5–1.0 M NaCl to the poly(U) sample shifts the signal 0.3 ppm upfield. Most interesting is the sudden increase in slope for the poly(U)–salt samples at low temperatures. As shown in Figure 7, the slope of the melting curves of the four uridine nucleic acids at higher temperatures are quite similar except for the low-temperature region of the poly(U)–salt samples. Other evidence has been presented that suggests that poly(U) takes on a more ordered structure (hairpin loop–double helix) at low temperatures in the presence of added salt (Simpkins and Richards, 1967).

The pH dependence of the ^{31}P chemical shift for the polyribonucleic acids has recently been reported by Akasaka et al. (1975). Their results are consistent with our interpretation of the conformational dependence of these shifts.

(IV) ^{31}P Shifts of Model Compounds. Our ^{31}P chemical shift–torsional angle hypothesis evolved from molecular orbital calculations on a small number of phosphate diester monoanions possessing well-defined structures. Unfortunately, insufficient data present exists to better define this relationship. However, a similar dependence of ^{31}P shifts on torsional geometry is expected (Gorenstein and Kar, unpublished results) for other phosphates as well. It is therefore significant that the same general trend of ^{31}P shifts is observed in cyclic trisubstituted phosphates and phosphoramidates of general structure III and IV:



III IV

(a) X = -OCH_3
(b) X = -$N(CH_3)_2$

Mosbo and Verkade (1972) and Bentrude and Tan (1973) have recently determined the ^{31}P chemical shifts of the isomeric sets of cyclic phosphates and phosphoramidates III and IV, and the results are shown in Table II. Note that molecules with the X group equatorial have ^{31}P chemical shifts 1.6–3 ppm downfield from the isomeric phosphates with the X group axial. In III the equatorial X group is locked into a trans torsional conformation relative to the endocyclic P–O ester bonds. In IV the arrangement is gauche. Once again, a trans conformation shows a ^{31}P chemical shift downfield from a gauche conformation.

Finally, the 2 ppm downfield shift observed by Griffin et al. (1973) in the binding of 3′-methyleneuridylyl-3′,5′-adenosine phosphonate to ribonuclease A likely reflects the binding of this dinucleotide analogue in a more open, g,t conformation as found in the x-ray crystal structure of the complex (Richards and Wyckoff, 1971).

TABLE II: ^{31}P Chemical Shifts of Isomeric Cyclic Phosphates and Phosphoramidates.

Compound	R_1	R_2	R_3	$\delta_{^{31}P}$ (ppm)
IIIa	CH_3	CH_3	H	+4.98[a]
IVa	CH_3	CH_3	H	+7.06[a]
IIIb	CH_3	CH_3	H	−6.58[a]
IVb	CH_3	CH_3	H	−3.49[a]
IIIa	H	H	$(CH_3)_3C$-	5.34[b]
IVa	H	H	$(CH_3)_3C$-	6.96[b]

[a] Mosbo and Verkade, 1972. [b] Bentrude and Tan, 1973.

TABLE III: ^{31}P Chemical Shifts of Nucleotide and Nucleotide–Drug Complexes from Patel (1974a,b).

	δ[c] (ppm)
d-pGpC[a]	+0.48
Act-D-dpGpC (1:2)	−1.20
d-ApTpGpCpApT[b]	0 to +1
Act-D-dApTpGpCpApT (1:2)	(−2.2, −1.2, −0.55)[d]
	(+0.4, +0.7)[e]

[a] Patel (1974b), pH 7.88, D_2O, 8 °C. [b] Patel (1974a), pH 7.0, H_2O, 10 °C. [c] Referenced to 16% phosphoric acid in D_2O. Internucleotide phosphates only. [d] Integrates for 1 phosphate/signal. [e] Broad doublet integrating for 7 phosphates.

(*V*) *Other ^{31}P Data on Nucleic Acids.* Recent ^{31}P NMR studies by Patel (1974a,b) further support the proposal that these ^{31}P shifts are sensitive probes of phosphate ester torsional conformations. Patel's ^{31}P data are presented in Table III and demonstrate that significant downfield shifts of the internucleotide phosphates in both the dideoxyribonucleotide duplex, 2(d-pGpC), and in the hexadeoxyribonucleotide duplex, 2(d-ApTpGpCpApT), occur upon binding the intercalating drug, actinomycin D (Act-D). Jain and Sobell (1972) have proposed models for these intercalated complexes that involve partial unwinding of a specific section of the double helix. This requires significant disruption of the normal, g,g phosphate diester conformation, which must be responsible for the 2–3 ppm downfield shift.

Solution Structures of Nucleic Acids from ^{31}P Shifts. Assuming that the above evidence does support our claim that these temperature-dependent ^{31}P shifts do provide information on the phosphate ester torsional conformation, several conclusions may be reached regarding the solution structures of nucleic acids.

Duplex Dimers. First, at low temperature the ^{31}P signals of self-complementary dimers (ApU, GpC, d-ApT, and d-pApT) are approximately 0.1–0.2 ppm upfield from their respective homodimers (UpU, CpC, ApA, dApA, and TpT). Considerable evidence is now accumulating (Young and Krugh, 1975; Patel, 1975b) that indicates that these small self-complementary dimers do form miniature double helices at low temperature. The structure of these duplexes is more clearly defined than the single-stranded helix and there is little doubt that the phosphate ester torsional conformation is g,g (Sundaralingam, 1969). Raising the temperature disrupts the duplex and the dinucleotide reverts to some mix of single-stranded helix and random coil. It is therefore quite satisfying (and further supportive of our hypothesis) that these duplexes that must be more constrained to the g,g conformation than either the single-stranded helix or random coil show ^{31}P signals upfield from nucleic acids which can only form single-stranded helices.

Deoxyribose vs. Ribose Dimers. The deoxyribose dimers also show ^{31}P signals ca. 0.1–0.3 ppm upfield from the ribose dimers. As we have noted in the case of the polynucleic acids, this suggests that the deoxyribose dimers have a more helical secondary structure than the ribose dimers. Fang et al. (1971) and Kondo et al. (1972) have concluded on the basis of ^{1}H NMR studies that d-ApA is more stacked than r-ApA. Presumably, this originates from a difference in the conformations of the furanose ring in the stacked and unstacked states (Fang et al., 1971; Hruska and Danyluk, 1968). The differences in furanose conformation must in some way affect the populations of the phosphate ester conformations.

Polymer vs. Dimer Structure. The upfield shift and hence the increased helicity of the polynucleic acids likely reflects the effect the additional 5′-phosphate has on the 3′–5′ internucleotide phosphate conformation. Supporting this interpretation, note that the melting curve of d-pApT is also shifted ca. 0.1 ppm upfield from d-ApT. In addition, recent energy calculations on phosphate ester torsional potentials for dinucleoside monophosphates and dinucleotides indicate that fewer nongauche conformations are accessible to the dinucleotides (Yathindra and Sundaralingam, 1974).

Origin of Secondary Structure Stabilization. At first reflection, it is rather surprising that the range of ^{31}P chemical shifts of the nucleic acids is so small (Table I and Figures 3–5). Theory and model studies rather crudely suggest a 2–10 ppm downfield shift for a phosphate ester in the g,t conformation. Yet the full range of chemical shifts observed for the nucleic acids over all temperatures is only 1.1 ppm. The individual melting profiles cover an even smaller range—0.1–0.64 ppm. If ^{31}P shifts really are more sensitive to torsional changes than actually observed, then one conclusion that could be reached is that all the nucleic acids whether double helices, single-stranded helices, or random coils have remarkably similar phosphate ester torsional conformations. Considerable evidence does exist to suggest that the phosphate conformation in the single-stranded helix is similar to that in the double helix, but it is rather surprising that the phosphate conformation in the "random" coil appears to be not so random. The various conformations accessible to the unstacked nucleic acids in the random coil must on average be quite similar to those in the double or single helix, i.e., predominantly gauche,gauche. This conclusion is consistent with recent torsional energy calculations. Thus, we have recently calculated, using quantum mechanical procedures, a torsional angle–energy contour map for dimethyl phosphate (Gorenstein and Kar, 1975), as have several other groups (Newton, 1973; Perahia et al., 1974; Sasisekharan and Lakshminarayanan, 1969), and have shown that the g,g conformation is ca. 3 kcal/mol more stable than the g,t conformation. This must explain why the high temperature, random coil ^{31}P shift in the ribonucleic acids is shifted only 0.3–0.64 ppm downfield from the helical, ^{31}P shift, since even in the "random" coil the torsional conformation of the diester will still be predominantly g,g with only a small contribution from the g,t conformation.

Olson (1975) has recently compiled a statistical population analysis for phosphate torsional conformations in the nucleic acid random coil based upon various energy calculation methods and crystallographic data. Thus, ab initio calculations predict ca. 12% g,t (including both + and − gauche conformations, g^+t, tg^+, g^-t, tg^- which should give equivalent ^{31}P shifts and 88% g,g (including g^+g^+, g^+g^-, g^-g^+, g^-g^-).

Classical and some semiempirical calculations predict a much larger population for the g,t conformations (≥50%), and much smaller g,g populations (3–35%). Even some t,t conformations (5–39%) are predicted in the classical calculations. X-ray structures show populations closer to the ab initio results (19% g,t, 81% g,g, 0% t,t).

Olson (1975) concludes on the basis of calculated and experimental values for the characteristic ratio of polynucleic acids that an appreciable population (>20%) of the nucleic acids exists in the trans conformation (the torsional energy difference between gauche and trans conformations must then be less than 1 kcal/mol). She has pointed out that there exists no other physical-chemical technique to test these predictions. We suggest that the ^{31}P data could be used to calculate the populations of the various conformations. The low-temperature ^{31}P chemical shift of the double-helical dimers is likely quite close to the best chemical shift for a "locked" g,g diester. Since the g,t conformation is likely to have a ^{31}P chemical shift of at least 2 and possibly as large as 10 ppm downfield from this value, the percentage of g,t conformers in the random coil will therefore be $\Delta\delta/2$–10 ppm. With $\Delta\delta$ varying between 0.3 and 0.62 ppm, the percentage of g,t conformations can only be ca. 20 ± 10%, the remainder being g,g. It is unlikely that there exists any significant population of t,t conformations, since we would predict a very far downfield shift (~25 ppm) for this conformation (Gorenstein and Kar, 1975). In addition, most semiempirical and ab initio molecular orbital calculations predict very small populations for the t,t conformations. The ^{31}P results are therefore consistent with these energy calculations and the x-ray results but are not consistent with the classical energy calculations.

The "unstacking" or "melting" of the dimers and polymers as monitored by ^{31}P shifts likely results largely from an increase in the population of the g,t conformations. In these open conformations the nucleic acid bases must swing away from each other and thus cannot base stack. This explains why even nucleic acids with bases that should not base stack (i.e., uridine) still show significant melting behavior. Of course, if strong base stacking exists (such as in A- or G-containing nucleic acids), this will favor the g,g state over the g,t state. Conversely, if base stacking is disfavored (such as found in poly(G) at high pH) then the open g,t state will be "disfavored less." It is also significant that the properties of the "random coil" or the high temperature "unstacked" nucleic acids reflect the properties of only 10–30% of the open, g,t, conformation. The g,g conformation still predominates.

These conclusions are not inconsistent with those reached by Fang et al. (1971) from ^{1}H NMR melting data. Ts'o and co-workers conclude that two processes are responsible for changes in the ^{1}H NMR shifts with temperature. At low temperature, the g,g conformation is favored and the bases are stacked. In addition, the in-plane oscillation amplitude of the base planes relative to the fully-stacked state is small. At higher temperatures, the population of the open form increases and the oscillation amplitude of the bases in the stacked population increases as well. This is, in effect, the oscillatory model of Davis and Tinoco (1968). ^{1}H NMR will monitor both effects, while ^{31}P NMR should only be sensitive to the relative population of closed (g,g) or open (g,t) forms. There is no one "destacked" state but a multitude of partially and completely destacked states.

The separation of the secondary structure stabilization into backbone and stacking effects explains many inconsistencies in the literature regarding the structure of the nucleic acids. At nearly all temperatures poly(U) behaves as a random coil (as monitored by spectroscopic techniques sensitive to base stacking interactions). However, it apparently still retains quite large coil dimensions. Thus, Inners and Felsenfeld (1970) conclude that "even in the absence of the secondary structure conferred by base stacking, it remains a highly extended structure, and must therefore be relatively limited in the range of configurations available to it." This highly significant conclusion suggests why we observe a "helix–coil" type transition in the ^{31}P NMR of poly(U), while other spectroscopic techniques fail to observe any alteration of structure with temperature. Other techniques are sensitive to base stacking interactions—which we would generally agree must be minimal in poly(U). ^{31}P NMR is sensitive to the phosphate ester *backbone* conformation that is still largely g,g and hence helical. Our picture of poly(U) is one in which the secondary structure is largely helical and held together by the preference of phosphate diesters for this g,g conformation. The bases still "stack" but are able to oscillate widely relative to each other because of the weakness of this uridine–uridine base stacking interaction.

We conclude therefore that the major driving force for formation of a single-stranded helix is the preference for a phosphate diester to exist in the lowest energy g,g conformation. As noted earlier, this interpretation does not rule out base stacking interactions contributing to stabilization or even destabilization of the single helix. The considerable structure in poly(U) and other random coils (Inners and Felsenfeld, 1970) must result from highly restricted rotation about the six bonds of the nucleic acid backbone. Tsuboi et al. (1969), Sundaralingam (1969), Arnott (1970), Mantsch and Smith (1972), Olson and Flory (1972), and others have concluded that the backbone torsional angles about the $C_{2'}$–$C_{3'}$, $C_{3'}$–$O_{3'}$, $O_{5'}$–$C_{5'}$, and $C_{5'}$–$C_{4'}$ bonds are likely quite similar for different nucleic acids. The extent of base stacking and secondary structure differences are considered to reflect the differences in rotation about the $O_{3'}$–P and P–$O_{5'}$ bonds (see, for example, Sundaralingam, 1969). Whether the relatively *small* change with temperature in phosphate ester conformation is sufficient to account for the melting phenomenon remains to be determined. Alternatively, melting might reflect higher rotational amplitudes for the P–O bond torsional rotations. In our interpretation of this model, the oscillations of the bases is then largely due to rotational oscillations about the phosphate ester bonds.

A further indication that base stacking contributes to stabilization of the helix is demonstrated by the ^{31}P melting curve for dibenzyl phosphate (Figure 6). Unlike dimethyl and diethyl phosphates, the dibenzyl ester shows a sigmoidal melting curve similar to that of the nucleic acids. Examination of CPK molecular models reveals that significant stacking of the aromatic rings is possible in the g,g conformation and the "melting" behavior of dibenzyl phosphate likely reflects the disruption of this structure at higher temperatures. The actual shift of the signal over 80 °C is only 0.16 ppm and indicates that a smaller change in structure is occurring than is true for the nucleic acids.

Additional ^{31}P studies of nucleic acids are clearly in order to assess the extent of the conformational information available from ^{31}P chemical shifts (in progress). No other physical-chemical technique with the possible exception of ir and Raman spectroscopy (see for example, Hartman et al., 1973) has provided *direct information in solution about the phosphate ester geometry in nucleic acids.* Since it is now thought that the ribose ring and nucleic acid base form a fairly rigid unit, the conformational flexibility in nucleic acids appears to

be limited to the phosphate ester moiety. ^{31}P NMR therefore holds great promise in being able to provide detailed solution structures of the nucleic acids.

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