Phosphorus-31 Fourier Transform Nuclear Magnetic Resonance Study of Mononucleotides and Dinucleotides. 2. Coupling Constants†

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ABSTRACT: Stereosensitive $^3J_{PH}$ and $^4J_{PH}$ phosphorus–proton coupling constants have been measured directly from the phosphorus-31 nuclear magnetic resonance (NMR) spectra of a variety of adenine, thymine, and uracil 3′-mononucleotides, 5′-mononucleotides, their cyclic analogues, and the corresponding dinucleotides, under various conditions of pH and temperature. For all 5′-mononucleotides, the identical $^3J_{PH}$ coupling to phosphorus of the two $H_{5'}$ protons is found essentially independent of the nature of the base, the presence of a 2′-OH on the sugar ring, the temperature, and the pH; the "gauche–gauche" rotamers about $C_{5'}$–$O_{5'}$ and $C_{4'}$–$C_{5'}$ remain overwhelmingly (85%) preferred. The "gauche" arrangement during $C_{3'}$–$O_{3'}$ is favored in all cases for 3′-mononucleotides. However, while no sizeable pH effect is noted on 3′-monodeoxyribonucleotides, the pH dependence of $^3J_{PH_3}$ in 3′-monoribonucleotides strongly suggests an interaction between the 3′-phosphate and the 2′-OH. Molecular features affecting the magnitude of $^4J_{PH}$ coupling constants are discussed together with pH and temperature effects. The time-averaged preferential structural features of mononucleotides are found in dinucleotides with a higher probability; hence, dimerization induces an increase in the statistical conformational purity of the phosphodiester–sugar backbone, even at extreme pH. Temperature studies point out that the thermal unwinding of stacked dinucleotides occurs mainly via rotation about $P_{3'}$–$O_{3'}$ and $P_{5'}$–$O_{5'}$ bond axes.

NMR[1] spectroscopy has long since proved to be a method of choice to study interacting systems in solution. In the case of the ubiquitous protein–nucleic acid complexes, ^1H NMR is often hopeless or very limited due to the overlap of the protein and nucleic acid spectra. However, phosphorus NMR allows us to study directly and independently the behavior of the nucleic acid component, and the perturbation brought upon binding to the protein. It has thus become critical to provide detailed ^{31}P NMR analysis of nucleic acids and nucleotides in order to contribute to apprehending the potentials and limits of this spectroscopy when regarding a nucleic acid structure as one part of an interacting system.

In a preceding paper, we have discussed some of the major factors influencing the phosphorus chemical shift of phosphate groups in a vaariety of mono- and dinucleotides (Cozzone and Jardetzky, 1976). The present report deals with the analysis of the heteronuclear proton–phosphorus coupling constants in the same series of adenine, thymine, and uracil 3′- and 5′-mononucleotides, their cyclic analogues, and the corresponding dinucleotides.

Mononucleotides and dinucleotides contain both P–O–C–H and P–O–C–C–H linkages whose arrangement plays a key role in determining the phosphodiester backbone conformation of nucleic acids.

Evidence for the stereosensitivity of vicinal P–O–C–H coupling ($^3J_{PH}$) has been already presented in a variety of acyclic and cyclic phosphorus containing compounds (Tsuboi et al., 1967, 1968; Hall and Malcolm, 1968; Kainosho et al., 1969; White and Verkade, 1970). This vicinal coupling generally follows a Karplus-type variation in a manner similar to H–C–C–H (Karplus, 1959) or P–C–C–H (Benezra, 1973; Evelyn et al., 1973), although an accurate quantitative correlation of P–O–C–H coupling to the dihedral angle is not well established (Hall and Malcolm, 1972a) and seems to depend upon the class of the phosphorus compounds. Long-range four-bond proton–phosphorus couplings in P–O–C–C–H systems ($^4J_{PH}$) have also been reported in various derivatives (Takahashi et al., 1966; Hall et al., 1967; Hall and Manville, 1968; Albrand et al., 1969; Kainosho and Nakamura, 1969) and have been shown to be sensitive to geometrical parameters. The coupling constant through four α bonds in P–O–C–C–H systems, as well as in P–C–C–C–H (Benezra, 1969) and P–C–C–O–H (Bottin-Strzalko et al., 1974), displays a maximal value when the two interacting nuclei are oriented as a coplanar "W" (Sternhell, 1964). In addition, the sign of $^3J_{P\text{-}O\text{-}C\text{-}H}$ has always been found to be positive (Duval and Lucken, 1966; McFarlane, 1967), whereas $^4J_{P\text{-}O\text{-}C\text{-}C\text{-}H}$ can be positive or negative depending on the degree of coordination of phosphorus. For tetracoordinated phosphorus compounds, $^4J_{PH}$ is positive (Takahashi et al., 1966; McFarlane, 1967; McFarlane and Nash, 1969).

In the case of nucleotides, several attempts have already been made at measuring the magnitude of the dihedral angle defined by the P, O, C and O, C, H planes in the P–O–C–H systems by determining the corresponding $^3J_{PH}$ coupling constant (Tsuboi et al., 1967; Kainosho et al., 1969; Lapper and Smith, 1973; Hruska et al., 1973; Sarma et al., 1973; Blackburn et al., 1973; Davies and Danyluk, 1974, 1975; Lee et al., 1975, Kondo and Danyluk, 1976). It is then possible to describe the favored conformations along the $C_{3'}$–$O_{3'}$, $C_{5'}$–$O_{5'}$, and $C_{4'}$–$C_{5'}$ bond axes of the sugar–phosphate backbone.

Most of these previous data have been obtained from ^1H NMR studies and required exhaustive interpretation of com-

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; AMP, TMP, UMP, adenosine, thymidine, and uridine monophosphates. $^3J_{PH}$ and $^4J_{PH}$ designate, respectively, proton–phosphorus coupling constants $^3J_{P\text{-}O\text{-}C\text{-}H}$ and $^4J_{P\text{-}O\text{-}C\text{-}C\text{-}H}$.

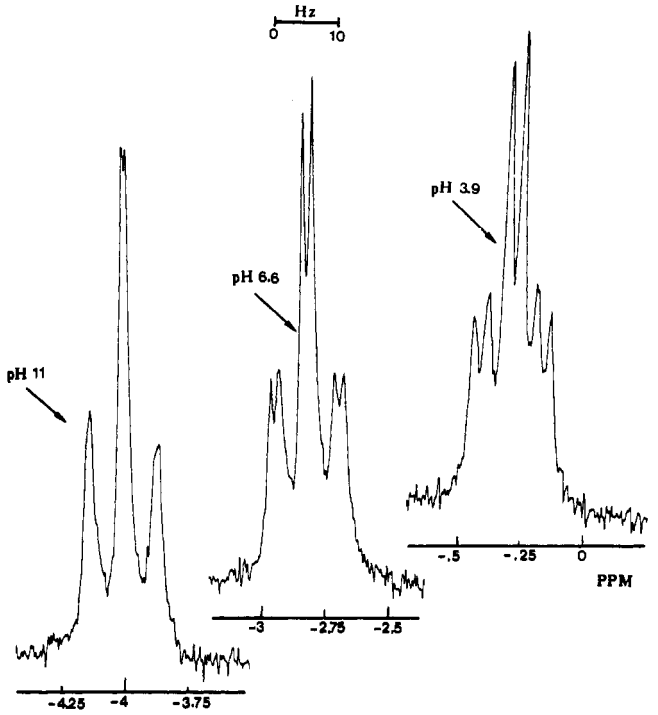

FIGURE 1: ^{31}P NMR spectra of 5 mM 5′-TMP at different pH. Three hundred transients were collected and Fourier transformed with a digital resolution of 0.1 Hz. Chemical shifts are from 85% H_3PO_4. Temperature: 20 °C.

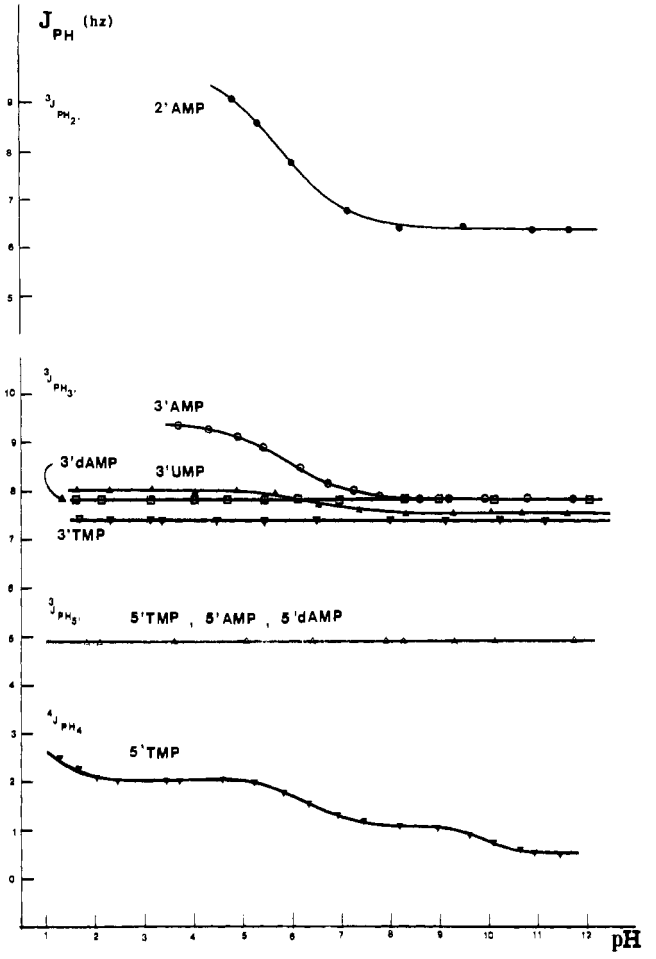

FIGURE 2: pH dependence of $^3J_{PH}$ and $^4J_{PH}$ coupling constants in various mononucleotides. All spectra were taken at 20 °C on 5 mM nucleotide solutions.

plex proton spectra with the help of lengthy and sophisticated computer simulations and iterative methods. In this report, it is clearly demonstrated that phosphorus NMR spectroscopy alone can be used to obtain *directly* unambiguous values for all $^3J_{PH}$ and $^4J_{PH}$ of mono- and dinucleotides. A straightforward interpretation of the simple first order ^{31}P NMR spectra allows us to analyze directly the rotations about three of the six bonds determining the sugar–phosphate arrangement of a nucleotide unit, namely the three torsional angles ϕ, ϕ', and ψ, according to Sundaralingham's nomenclature (1969). Temperature and pH effects on the magnitude of the phosphorus–proton coupling constants have been documented.

Material and Methods

All mononucleotides and dinucleotides (sodium salts) were from Sigma Chemical Co. and dissolved in deuterium oxide (ICN Chemical and Radioisotopes Division) at a concentration of 0.5×10^{-2} M or less. Paramagnetic contaminations were removed as already described (Cozzone and Jardetzky, 1976). ^{31}P NMR spectra were obtained on a Varian Associates XL-100-15 spectrometer interfaced with a Nicolet Technology Corp. Fourier transform accessory and a Nicolet NIC-80 32K computer. Experimental conditions are identical to those reported in the preceeding paper of this issue (Cozzone and Jardetzky, 1976). Temperature variation was achieved using a Varian temperature controller with an accuracy of ±0.5 °C. Coupling constants are measured with a precision of 0.1 Hz. Spectra simulation was performed on a NIC-80 computer equipped with a NIC-294 disk memory using the ITRCAL program from Nicolet. This program is the implementation on a minicomputer of the LAOCN3 algorithm.

Results

5′-Mononucleotides. Phosphorus-31 spectra of all 5′-mononucleotides display a very characteristic "split triplet" pattern shown in Figure 1. The two $H_{5'A}$ and $H_{5'B}$ protons born by the exocyclic carbon are equivalent and account for the triplet structure of the phosphate signal, which is further split in a pseudosextet by the four-bond long-range coupling to the sugar ring H_4 proton. The presence of an hydroxyl group at the 2′ position of the ribose does not affect the magnitude of $^3J_{PH_{5'}}$ couplings (see, for instance, 5′-AMP and 5′-dAMP, Table I); neither does the nature of the base. Also, in the whole range of pH, $^3J_{PH_{5'}}$ does not vary significantly (Table I, Figure 2), although a slight decrease of the coupling constant is noted for 5′-AMP upon ionization of the 5′-phosphate.

The four-bond coupling, $^4J_{PH_{4'}}$, is more sensitive to pH, since, for all 5′-nucleotides (Table I and Figure 1), a marked decrease of the coupling constant accompanies an increase in pH. Deprotonation of the various ionizable groups is reflected when plotting $^4J_{PH_{4'}}$ vs. pH (Table I and Figure 2) and the pH effect is more intense on the 5′-deoxyribonucleotides. As a typical example, the pH-dependent curve obtained for 5′-TMP (Figure 2, bottom) clearly shows the two ionizations of the phosphate group ($pK_{a_1} = 1.7$ and $pK_{a_2} = 6.30$) and the ionization of the thymine base ($pK_a = 10$); the magnitude of the coupling constant variation is 2.1 Hz between pH 1 and 2. For 5′-ribonucleotides (5′-AMP, 5′-UMP), the coupling constants vary to a lesser extent (0.5 Hz) over the same range of pH. No difference is found between purine and pyrimidine 5′-nucleotides.

3′-Nucleotides. Three bond coupling constants, $^3J_{PH_{3'}}$

TABLE I: Vicinal ($^3J_{P-H}$) and Four-Bond ($^4J_{PH}$) Phosphorus–Proton Coupling Constants in Hz for Various 5′-Nucleotides and 3′-Mononucleotides at Different pH and 20 °C.

Coupling Constant		pH 3	pH 5.5	pH 8.5	pH 11
$^3J_{P\ H5'A,B}$[a]					
	5′-AMP	4.9	4.9	4.7	4.7
	5′-dAMP	4.8	4.8	4.8	4.8
	5′-UMP	4.9	4.9	4.8	4.8
	5′-dUMP	4.9	4.9	4.8	4.8
	5′-TMP	4.9	4.9	4.9	4.9
$^4J_{P\ H4'}$					
	5′-AMP	1.9	1.7	1.5	1.5
	5′-dAMP	2.0	1.8	1.1	1.1
	5′-UMP	2.0	2.0	1.5	1.5
	5′-dUMP[b]	1.9	1.8	1.0	0.8
	5′-TMP[b]	2.1	2.1	1.2	0.5
$^3J_{P\ H3'}$					
	3′-AMP	9.4	8.8	7.8	7.8
	3′-dAMP	7.8	7.8	7.8	7.8
	3′-UMP	8.0	7.9	7.4	7.3
	3′-TMP	7.4	7.5	7.5	7.5

[a] Phosphorus coupling constants to $H_{5'A}$ and $H_{5'B}$ are identical. [b] At pH 1, $^4J_{P-H4'}$ values for 5′-dUMP and 5′-TMP are, respectively, 2.2 and 2.6 Hz.

TABLE II: Vicinal Phosphorus–Proton Coupling Constants in Cyclic Mononucleotides.

Coupling Constant	3′,5′-cAMP	3′,5′-cUMP	3′,5′-cAMP	2′,3′-cUMP pH 7	2′,3′-cUMP pH 11
$^3J_{P\ H5'}$	20.1	20.2	18.5		
$^3J_{P-H5''}$	1.2	2.0	3.4		
$^3J_{P-H3'}$	0.6	1.0	0.7	11.6	11.9
$^3J_{P-H2'}$				6.8	8.3

[a] Coupling constants in Hz were measured on spectra taken at pH 7 and 27 °C.

TABLE III: Vicinal and Four-Bond Phosphorus–Proton Coupling Constants in Dinucleotides as a Function of Temperature.[a]

Coupling Constant	Temp (°C)	ApA	dApdA	UpU	TpT
$^3J_{PH5'}$	20	4.5	4.0	6.0	4.1
	80	5.1	4.9	6.0	4.9
$^4J_{PH4'}$	20	2.0	2.4	2.0	2.5
	80	1.8	1.9	1.9	1.9
$^3J_{PH3'}$	20	8.4	6.4	6.1	7.0
	80	9.0	7.2	6.2	7.9

[a] Coupling constants in Hz were measured at pH 7.

(Table I), display higher sensitivity towards the nature of the mononucleotide. For 3′-ribonucleotides, decreasing values of $^3J_{PH3'}$ are obtained when pH is increased and the secondary phosphate ionization is again reflected on the pH-dependent curves (Figure 2). However, phosphorus–proton coupling in 3′-dAMP is pH insensitive, while in 3′-TMP the coupling constant increases slightly with pH, thus indicating that the absence of 2′-OH either cancels or reverses the effect noted for 3′-ribonucleotides.

For all 3′-mononucleotides, long-range $^4J_{PH}$ coupling constants between phosphorus and $H_{2'}$ and $H_{4'}$ protons are also detected. Due to their small magnitude (0.3–0.9 Hz) they usually do not perturb the spin–spin coupling pattern of the spectra but appear as a "line-broadening" contribution which is particularly apparent at low pH.

For the sake of comparison, $^3J_{PH2'}$ has been studied in 2′-AMP (Figure 2). The magnitude of the coupling constant and its pH dependence are similar to what is observed for 3′-AMP, although the value at high pH is lower for 2′-AMP (6.5 vs. 7.8 Hz). Four-bond coupling constants between phosphorus and $H_{1'}$ and $H_{3'}$ protons are found in the range of 0.9–1.1 Hz.

Cyclic Nucleotides. Spectra of 3′,5′-cAMP, 3′,5′-cUMP, and 3′,5′-cTMP have been analyzed, and the proton–phosphorus coupling constants were determined (Table II). The values reported are in excellent agreement with the values obtained after computer analysis of the 220 MHz proton spectra (Blackburn et al., 1973) of the compounds. However, in the case of 3′,5′-cUMP, analysis of the proton spectra had proved to be unsuccessful in providing the coupling constants reported here, because of the complexity of the overlapping proton–proton and proton–phosphorus spin–spin coupling patterns. One of the multiple advantages of phosphorus NMR is to allow a straightforward determination of phosphorus–proton coupling constants, since the phosphorus spectra are generally directly interpretable.

^{31}P spectra of 3′,5′-cyclic nucleotides are characterized by a particular spin–spin coupling pattern due mainly to the nonequivalence of the two protons at 5′ position whose couplings to phosphorus are different. In the case of 3′,5′-cTMP, phosphorus–proton coupling constants for the two $H_{5'}$ protons are, respectively, 3.4 and 18.5 Hz, and accounting for the two doublets observed. The rather large line width observed includes actually the small coupling of phosphorus to vicinal proton $H_{3'}$ (0.5 Hz).

Two 2′,3′-cyclic nucleotides have also been analyzed and the set of coupling constants is shown in Table II. The values reported here are obtained directly and are in good agreement with the results of a computer analysis of the corresponding 220 MHz proton spectra (Lapper and Smith, 1973) using the LAOCNPLT program (Castellano and Bothner-By, 1964). While the spin–spin coupling pattern is not significantly affected by pH for 3′,5′-cyclic nucleotides, a higher sensitivity is found in 2′,3′-cyclic analogues. For instance, $^3J_{PH2'}$ in 2′,3′-cUMP increases by 1.5 Hz upon deprotonation of the uracil base (Table II). This pH-dependent variation parallels the effect of base ionization on chemical shift already reported for 2′,3′-cyclic nucleotides and absent for 3′,5′-cyclic nucleotides (Cozzone and Jardetzky, 1976). Protons H_1 and H_4 exhibit similar 0.8 Hz long-range coupling to phosphorus, accounting for the pseudotriplet structure observed for each of the four signals corresponding to the phosphate.

Dinucleotides. The phosphorus NMR spectra are more complex and appear as the superimposition of mainly the three couplings of phosphorus to the $H_{5'}$, $H_{3'}$, and $H_{4'}$ protons. However, $^3J_{PH}$ and $^4J_{PH}$ are easily obtained and their magnitude was checked by computer simulation of experimental spectra. Coupling constant values at pH 7 are reported on Table III. When comparing values for ApA and dApdA, significant differences are found in the various $^3J_{PH}$ and $^4J_{PH}$, hence emphasizing the structural influence of the 2′-OH. However, within the class of dideoxyribonucleotides, the nature of the base does not play an important role, since similar values are found for dApdA and TpT. Conversely, purine and pyrimidine homodiribonucleotides display different behavior (ApA and UpU). In all cases, the two $H_{5'}$ protons are equivalent and their coupling to phosphorus identical. Also, the

four-bond coupling constants are closed to the maximal values, as found for dianionic 5′-nucleotides.

Only slight variations in the coupling constants are found for all dinucleotides over the whole range of pH. Temperature influence has been documented from 20 to 80 °C. No change is observed between 20 and 40 °C; at higher temperatures, only slight changes in the coupling pattern are apparent (Table III). The spectrum of UpU is not altered, while an increase of $^3J_{PH_{5'A}}$ and $^3J_{PH_{5'B}}$ to 5.2 Hz at 80 °C is found for ApA, TpT, and dApdA. Three bond couplings $^3J_{PH_{3'}}$ are also increased by approximately 1 Hz between 4 and 80 °C, while the long range $^4J_{PH_{4'}}$ shows a slight tendency to decreasing down to 1.8 Hz at 80 °C.

Discussion

The $^3J_{PH}$ coupling constants measured for 5′-mononucleotides and 3′-mononucleotides reflect the weighted average of the three 60° staggered rotamers constrained, respectively, to the $C_{5'}$–$O_{5'}$ and $C_{3'}$–$O_{3'}$ bond axes (Figure 3a,b). These rotamers undergo rapid interconversion on the NMR time scale. If one can measure with the maximum of accuracy the $^3J_{PH}$ coupling constants when the phosphorus atom and the proton are in pure "gauche" and "trans" arrangements, it is then possible to estimate from the magnitude of the observed coupling constant the percentage of the statistically preferred rotamers. On the basis of our direct determination of $^3J_{PH}$ in cyclic nucleotides and after examination of other values reported in the literature for various acyclic and cyclic phosphates (Hall and Malcolm, 1968; Kainosho et al., 1969; White and Verkade, 1970; Blackburn et al., 1973), we have estimated the $^3J_{PH}$ constants in the gauche and trans conformations to be, respectively, 3.5 and 21.5 Hz. The molar fraction of each rotamer (α_i is the molar fraction of rotamer i) can then be readily computed using classical equations (Hruska et al., 1973; Davies and Danyluk, 1974, 1975).

For all 5′-mononucleotides (Table I), the two $H_{5'}$ protons display identical coupling to phosphorus, with an average constant of 4.8 Hz. This constant is practically independent of the nature of the base, the presence of a hydroxyl group at the ribose 2′-position, the temperature, and the pH. Accordingly, the value of $\alpha_I = 0.85$ obtained for the gauche–gauche rotamer (I), in good agreement with other studies (e.g., Davies and Danyluk, 1974), remains essentially constant, whatever the perturbation.

In the case of dinucleotides, equivalent coupling of the two $H_{5'}$ protons to phosphorus is also found (Table III), in disagreement with previous reports (Tsuboi et al., 1969). With the exception of UpU, the magnitude of the $^3J_{PH_{5'}}$ coupling constant is smaller for dinucleotides than for 5′-mononucleotides, indicating that, after dimerization, the gauche–gauche conformation (I) (Figure 3a), constrained to $C_{5'}$–$O_{5'}$, is not only dominant but even more favored than for the monomers, since α_I values of 0.88, 0.94, and 0.93 are determined for ApA, dApdA, and TpT, respectively. In the case of UpU, the same conformation is also preferred with $\alpha_I = 0.72$. Other studies of some dinucleotides in solution (Hruska et al., 1975; Lee et al., 1975; Kondo and Danyluk, 1976) or in the crystalline state (Sundaralingam, 1975) have led to similar conclusions.

Two factors have a limited influence on the extent of this conformational purity. The absence of hydroxyl at the ribose 2′ position confers more "rigidity" along $C_{5'}$–$O_{5'}$ and the probability of the gauche–gauche conformation is higher for dideoxyribonucleotides than for diribonucleotides. The role of the base stacking interaction is less clear, since gauche–gauche conformation is 16% less favored in unstacked UpU than in stacked ApA, while no significant difference is found between dApdA and TpT. In this latter case, the destabilizing effect of base unstacking might be totally canceled by the stabilizing influence of the deoxyribose ring.

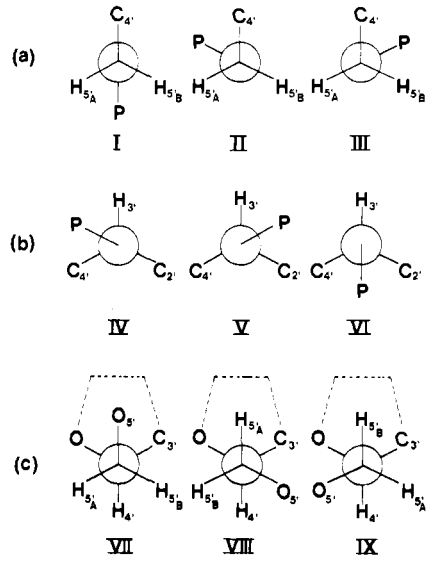

FIGURE 3: Classical 60° staggered rotamers for (a) the $C_{5'}$–$O_{5'}$ bond: (I) gauche–gauche, (II) gauche–trans, (III) trans–gauche; (b) the $C_{3'}$–$O_{3'}$ bond: (IV) gauche, 1, (V) gauche 2, (VI) trans; (c) the $C_{4'}$–$C_{5'}$ bond: (VII) gauche–gauche, (VIII) trans–gauche, (IX) gauche–trans.

The $^3J_{PH}$ coupling constants observed in 3′-mononucleotides are of greater magnitude than in 5′-mononucleotides (Table II). Also, their sensitivity to pH variation provides information on the intramolecular interactions between the 3′-phosphate and the 2′-OH of ribonucleotides.

For all 3′-mononucleotides, the gauche arrangement is favored with values of α_{IV+V} ranging from 0.67 ($^3J_{PH(av)} = 9.4$ Hz) up to 0.79 ($^3J_{PH(av)} = 7.3$ Hz). In the case of 3′-UMP, it has been proposed (Schleich et al., 1972) that conformation V (Figure 3b) was favored over conformation IV. For this arrangement, $O_{3'}$-P is gauche to $C_{3'}$–$C_{2'}$ and trans to $C_{3'}$–$C_{4'}$. A careful analysis of carbon–phosphorus vicinal coupling constant has definitely shown that the gauche conformation (V) (Figure 3b) is favored over the other analogue (Mantsch and Smith, 1972). The pH dependence of the $^3J_{PH_{3'}}$ coupling constant shows that ribo- and deoxyribonucleotides display a different behavior. No pH effect is found on 3′-dAMP, and 3′-UMP shows evident influence of pH on $^3J_{PH_{3'}}$ with a pronounced inflection corresponding to the secondary ionization of the phosphate group. The coupling constants decrease when pH increases. This shows that the conformational purity increases when 3′-AMP and 3′-UMP are dianionic. Statistically, the gauche conformation (V) (Figure 3b) then becomes more probable at high pH with a gain of 9% for 3′-AMP (67% at pH 3 vs. 76% at pH 11) and about 4% for 3′-UMP. This typical pH effect on the 3′-ribonucleotides can be correlated to a specific interaction between the 3′-phosphate and the 2′-OH. This interaction, which affects also the chemical shift of the phosphorus signal (Cozzone and Jardetzky, 1976), favors, as expected, the gauche conformation (V) when the phosphate group bears two formal negative charges.

The $^3J_{PH_{3'}}$ coupling constants have also been measured in the dinucleotides (Table III). The comparison of their values with those obtained at pH 3 for the corresponding 3′-mononucleotides, when the phosphate group is in the same ionization state (Table II) as in the dinucleotidic phosphodiester linkage,

shows that in the dimers, the gauche conformation is not only preferred but even more favored than in the monomeric units. Values of 0.71, 0.82, 0.86, and 0.81 are computed for α_{IV+V} in ApA, dApdA, UpU, and TpT, respectively, vs. values of 0.67, 0.76, 0.75, and 0.77 for the corresponding 3′-mononucleotides.

The magnitude of the four-bond coupling constants $^4J_{PH}$ has been directly measured for all 21 compounds. A few recent direct determinations (Lee et al., 1975) and prior computer analysis of ^{1}H NMR spectra (Sarma et al., 1973) of some compounds of this study have led to similar results at neutral pH and room temperature. Maximum value of $^4J_{PH}$ (2.7 Hz; Hall and Malcolm, 1972b,c; Donaldson and Hall, 1972) corresponds to a coplanar "W" arrangement for the P-O-C-C-H linkage. In the case of 5′-mononucleotides, it is clear that the maximum value is reached when the exocyclic group is constrained to the gauche-gauche conformation (I) (Figure 3a) along $C_{5'}$–$O_{5'}$ and the gauche-gauche conformation (VII) (Figure 3c) along $C_{4'}$–$C_{5'}$. The measurement of $^4J_{PH_{4'}}$ is then a reliable way to monitor the occurrence of two interdependent classes of rotamers (Sarma et al., 1973).

The variation of the magnitude of $^4J_{PH_{4'}}$ as a function of pH (Table I and Figure 2) reveals the role played by the state of ionization of the phosphate group on the distortion of the "W" arrangement. The maximum value is observed when the two acidic functions of the phosphate are protonated (2.6 Hz at pH 1 for 5′-TMP); then $^4J_{PH_{4'}}$ decreases with higher pH. The two phosphate deprotonations are clearly reflected on the pH-dependent curves (Figure 2), hence showing the destabilizing effect of the negative charge on the phosphorus. Minimum values are obtained at pH 11 and the ionization of the pyrimidine base induces an additional reduction of the coupling constant for the two 5′-deoxyribonucleotides, 5′-dUMP, and 5′-TMP. No significant difference is found among the nucleotides when the phosphate group is monoanionic. However, higher values of 1.5 Hz are noted for 5′-ribonucleotides after the second phosphate ionization, indicating a better preference for the gauche-gauche conformations I and VII (Figure 3a,c), as compared to the 5′-deoxyribonucleotides ($^4J_{PH_{4'}}$ = 1.0 Hz in 5′-dUMP).

In ApA and UpU (Table III), the values observed (2 Hz) over the whole range of pH are constant and correspond to the values reported for a monoanionic 5′-mononucleotide. However, for dApdA and TpT, $^4J_{PH_{4'}}$ is greater than for the component monomers, indicating both a loss in conformation flexibility and a gain in gauche-gauche rotamers population along $C_{5'}$–$O_{5'}$ and $C_{4'}$–$C_{5'}$ with an almost perfect "all trans" arrangement for the P-$O_{5'}$-$C_{5'}$-$C_{4'}$-$H_{4'}$ bond system.

Four-bond coupling constants are also measured for the 3′-mononucleotides and the 3′ moiety of dinucleotides. Values for $^4J_{PH_{2'}}$ and $^4J_{PH_{4'}}$ are smaller than for $^4J_{PH_{5'}}$ and they are found in the 0.5–1 Hz range. The preference of the 3′-phosphate for a gauche orientation (IV and/or V) has been shown above and it has been further suggested than conformation V (Figure 3b) was more populated on a time-average basis. The magnitude of the $^4J_{PH}$ coupling constant strongly supports this hypothesis, since should conformation IV (Figure 3b) be predominant, an almost perfect "W" relationship would be observed for the P-$O_{3'}$-$C_{3'}$-$C_{2'}$-$H_{2'}$ linkage. A coupling constant $^4J_{P\cdot H_{2'}}$ of the order of 2.5–2.7 Hz would then be expected, whereas a value smaller than 1.0 Hz is experimentally observed for 3′-mononucleotides and close to 1.0 Hz for dinucleotides. Consequently, it is clear that in both series of compounds, the phosphate group occupies preferentially the gauche conformation V (Figure 3b) along the $O_{3'}$–$C_{3'}$ bond axis. In this arrangement, $O_{3'}$–P bisects the angle formed by $H_{3'}$–$C_{3'}$ and $C_{3'}$–$C_{2'}$ bonds.

The analysis of the temperature influence on the various phosphorus–proton coupling constants in dinucleotides is particularly informative (Table III). Between 20 and 80 °C the vicinal couplings display a slight tendency to increasing up to the values reported for the corresponding "free" 3′- and 5′-mononucleotides. Similarly, the four-bond couplings $^4J_{PH_4}$ decrease down to 1.8–1.9 Hz; it is then clear that the gain in conformational purity noted upon dimerization of mononucleotides is lost upon heating. This trend is what one would expect from a temperature elevation effect, since less favored conformations, such as II or VI (Figure 3), can be reached more often. However, it is noteworthy that considering the very limited variations in the coupling constants magnitude, the same rotamers along the $C_{4'}$–$C_{5'}$, $C_{5'}$–$O_{5'}$, and $C_{3'}$–$O_{3'}$ bonds are overwhelmingly favored at 20 and 80 °C, meaning that the corresponding torsional angles ψ, ϕ, and ϕ' are almost insensitive to temperature. For instance, the gauche-gauche rotamer (I) (Figure 3a) in ApA is only 6% less favored at 80 °C (82%) than at 20 °C (88%).

Previous ^{1}H NMR studies (Ts'o et al., 1969; Schleich et al., 1972) have demonstrated that temperature variation has little effect on the ribose or deoxyribose ring conformation, as well as on the sugar–base torsional angle. Considering our complementary finding that ψ, ϕ, and ϕ' angles display low temperature sensitivity, it appears that the only possible site of flexibility in the phosphodiester–sugar backbone involves the P–O ester bonds. Similar sets of phosphorus–proton coupling constants have been found for stacked or unstacked dinucleotides (Table III), showing that base–base interaction has little effect in defining most of the dimer backbone conformation. The P–$O_{5'}$ and P–$O_{3'}$ bonds present, obviously, the major degree of freedom in the dinucleotides, and rotation along the P–O axes (angles ω and ω') is the one possibility to allow complete unwinding of a stacked arrangement when temperature is increased. This conclusion that we proposed to be general on the basis of our ^{31}P NMR experiments has been reached in the case of ApA by Kondo and Danyluk (1976) in a parallel work.

The present study shows again that in solution the main time-averaged conformational features of a dinucleotide phosphodiester–sugar backbone are those of the constituting independent mononucleotides. The monomeric units gain in conformational purity upon condensation mainly through a flexibility decrease involving the P–O torsional angles whose rotation is restricted by extra forces (e.g., electrostatic interaction, hydrogn bonding, and base–base stacking) brought by dimerization. These effects will be further discussed in a forthcoming paper dealing with the phosphorus NMR analysis of single-stranded polynucleotides.

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