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# Phosphorus-31 Fourier Transform Nuclear Magnetic Resonance Study of Mononucleotides and Dinucleotides. 1. Chemical Shifts†

Patrick J. Cozzone‡ and Oleg Jardetzky*

ABSTRACT: A phosphorus-31 nuclear magnetic resonance (NMR) study of adenine, uracil, and thymine mononucleotides, their cyclic analogues, and the corresponding dinucleotides is reported. From the pH dependence of phosphate chemical shifts, p$K_a$ values of 6.25–6.30 are found for all 5′-mononucleotides secondary phosphate ionization, independently from the nature of the base and the presence of a hydroxyl group at the 2′ position. Conversely, substitution of a hydrogen atom for a 2′-OH lowers the p$K_a$ of 3′-monoribonucleotides from 6.25 down to 5.71-5.85. This indication of a strong influence of the 2′-hydroxyl group on the 3′-phosphate is confirmed by the existence of a 0.4 to 0.5 ppm downfield shift induced by the 2′-OH on the phosphate resonance of 3′-monoribonucleotides, and 3′,5′-cyclic nucleotides and dinucleotides with repect to the deoxyribosyl analogues. Phosphate chemical shifts and titration curves are affected by the ionization and the type of the base. Typically, deviations from the theoretical Henderson-Hasselbalch plots are observed upon base titration. In addition, purine displays a more deshielding influence than pyrimidine on the phosphate groups of most of the mononucleotides (0.10 to 0.25 ppm downfield shift) with a reverse situation for dinucleotides. These effects together with the importance of stereochemical arrangement (furanose ring pucker, furanose-phosphate backbone conformation, O-P-O bond angle) on the phosphate chemical shifts are discussed.

Many important biological molecules (nucleic acids, membrane phospholipids, some proteins and coenzymes, etc) are phosphorus-containing compounds. Their structures and interactions have been extensively documented using proton nuclear magnetic resonance over the past 15 years and, more recently, carbon-13 magnetic resonance. However, and quite surprisingly, only a limited attention has been given to the phosphorus magnetic resonance study of these molecules.

Recent instrumental improvements have rendered phosphorus NMR[1] more attractive despite the low sensitivity of this nucleus (about 7% of proton value at constant magnetic field) and the combined use of Fourier transform techniques, together with larger magnetic fields, larger sample tubes, proton noise decoupling and sensitivity enhancement devices (quadrature detection and single side band filters), allows one to carry out *routine* studies in the $10^{-4}$ M range of concentrations.

Phosphorus generally occurs as a phosphate group in biological molecules. The phosphate groups resonances appear in a wide range of chemical shifts (about 30 ppm) and their high sensitivity to chemical environment (pH, metal ions, etc) makes the study of biological phosphates a very informative

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; ATP, AMP, adenosine tri- and monophosphates; UMP, TMP, uridine and thymidine monophosphates.

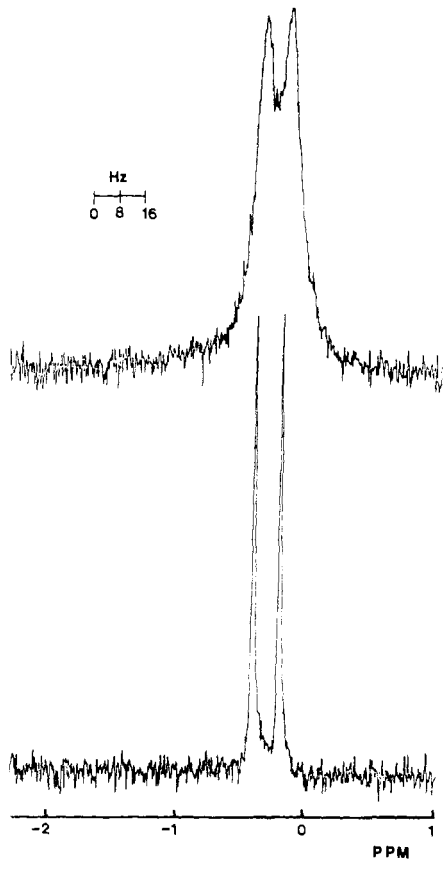


FIGURE 1: $^{31}P$ NMR spectra of thymidine 3′-phosphate. Upper spectrum: 5 mM 3′-TMP in $D_2O$; 1300 scans; 0.2 Hz digital resolution. Lower spectrum: same sample after purification (see Material and Methods); 190 scans.

one. In addition, spectra can be obtained easily from compounds in aqueous solutions, complex buffers, or biological fluids without any interference of solvent lines. $^{31}P$ NMR spectra of metabolites in intact tissues and cells have been recorded (Hoult et al., 1974; Salhany et al., 1975).

Protein–nucleic acid complexes are of critical importance in many biological processes. Since phosphate binding plays very often a key role in these associations, $^{31}P$ NMR is readily indicated as a potential highly informative technique of investigation of these associations. As a matter of fact, the clarity of the phosphorus spectra obtained from various interacting systems allows detailed structural interpretation, since the chemical shift, spin–spin coupling pattern, and relaxation parameters of the phosphate signals contain valuable information about the local environment of the phosphorus nuclei, the nature of the neighboring groups, and the dynamics of the interaction.

$^{31}P$ NMR has been already used successfully, so far, to study some complexes between proteins or enzymes and mononucleotides or short oligonucleotides, such as the association of various nucleotide inhibitors to ribonuclease A (Lee and Chan, 1971; Griffin et al., 1973; Gorenstein and Wyrwicz, 1973; Haar et al., 1973), the actinomycin D interaction with mono-, di-, and oligonucleotides (Patel, 1974a,b,c), the complex of ATP with G-actin (Cozzone et al., 1974; Nelson et al., 1974), and the binding of oligonucleotide substrate analogues to staphylococcal nuclease (Cozzone and Jardetzky, unpublished results).

Before investigating a nucleic acid–protein interaction by phosphorus NMR, a prerequisite is to know with great accuracy the behavior of the isolated nucleic acid structure. Several studies have been recently devoted to the $^{31}P$ NMR analysis of total nucleic acids (Guéron and Shulman, 1975) or to their basic components (e.g., Lee et al., 1975; Evans et al., 1975). In this laboratory, a detailed phosphorus NMR investigation of nucleic acid structure, focusing mainly on pH and temperature effects, has been undertaken in an attempt to contribute to the filling of the existing gaps in the literature.

This paper is the first of a series in which we report the analysis of the spectra obtained from adenine, uracil, and thymine mononucleotides, their cyclic analogues, and the corresponding dinucleotides. The effect of some important factors affecting chemical shifts and proton–phosphorus coupling constants have been documented yielding information on the conformational features of the nucleotide structures. This study is not intended to be exhaustive but is aiming at bringing pertinent information which will be relevant and useful when regarding the nucleic acid structure as one part of an interacting system.

## Materials and Methods

All mononucleotides and dinucleotides were obtained from Sigma Chemical Co. Deuterium oxide was from ICN Chemical and Radioisotopes Division. Paramagnetic contaminants were systematically removed from NMR samples by chromatography on Chelex-100 (Bio-Rad Laboratories) and treatment with dithizone (J. T. Baker Chemical Co.) in $CCl_4$ or 8-hydroxyquinoline in $CHCl_3$. This purification step has proved to be absolutely necessary, as illustrated in Figure 1. Phosphorus signal line widths of 0.3 Hz or less are then routinely obtained.

Phosphorus-31 nuclear magnetic resonance spectra were obtained in the Fourier transform mode on a Varian-Associates XL-100-15 NMR spectrometer operating at 40.5 MHz, equipped with a Nicolet Technology Corp. (Mountain View, U.S.A.) Fourier transform accessory and a Nicolet NIC-80 32K computer. The spectrometer was field-frequency locked on the deuterium resonance of deuterium oxide used as a solvent. Heteronuclear proton noise decoupling was achieved with a Varian Spin Decoupler Model V-4421. All spectra were recorded at 17 °C, unless otherwise stated, using 12-mm precision NMR tubes (Wilmad). A Varian temperature controller was used for temperature stabilization, with an accuracy of ±0.5 °C.

Chemical shifts were measured from 85% $H_3PO_4$ as an external standard with an accuracy of ±0.02 Hz. Spectral baseline stability was improved by automatically shifting the radio-frequency pulse phase from 0 to 180° after every two scans and, then, alternatively, adding and subtracting the signal into the analog-to-digital converter. Sensitivity was increased by using a single side-band crystal filter (Nicolet Technology Corp.).

Nucleotides solution were $0.5 \times 10^{-2}$ M or less to avoid possible self-associations (Jardetzky, 1964; Schweizer et al., 1968). Values of pH were measured on a Radiometer pH meter Model 26 employing a thin combination electrode. No correction was made for the deuterium isotope effect at the glass electrode.

## Results

The chemical shift of the phosphorus resonances of all mononucleotides studied is a very sensitive function of pH. When plotting values of chemical shifts against pH (Figure 2), titration curves reflecting the phosphate ionization are obtained. Total neutralization is accompanied by a 4 ppm upfield shift

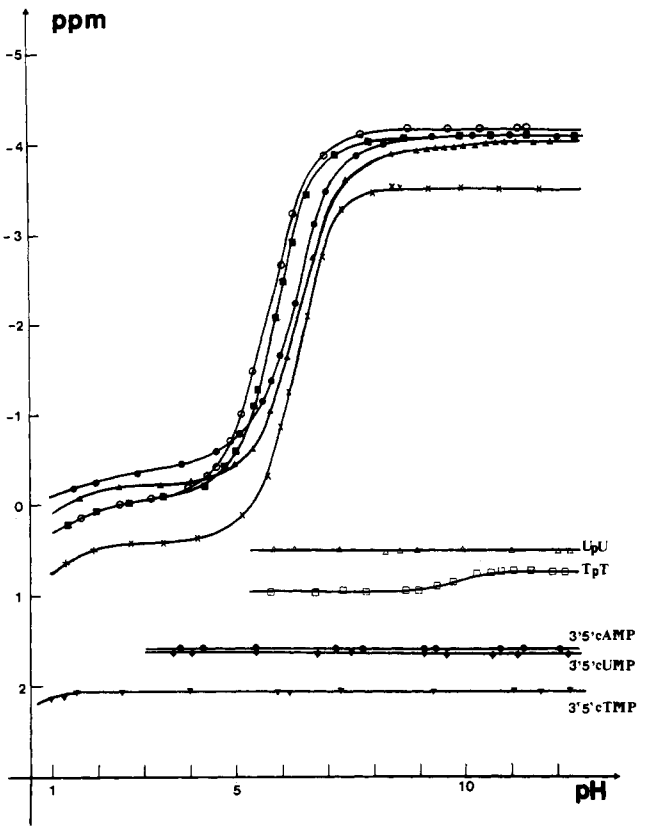


FIGURE 2: Phosphorus-31 chemical shift of various nucleotides as a function of pH. Signals position was recorded on spectra obtained from 5 mM nucleotide solutions (100 scans, 0.2 Hz digital resolution) under condition of full proton decoupling. 3′AMP (○—○); 5′AMP (●—●) 5′dAMP; 3′UMP (■—■); 5′UMP,5′TMP (▲—▲) 5′dUMP; 3′TMP (×—×) 3′dAMP.

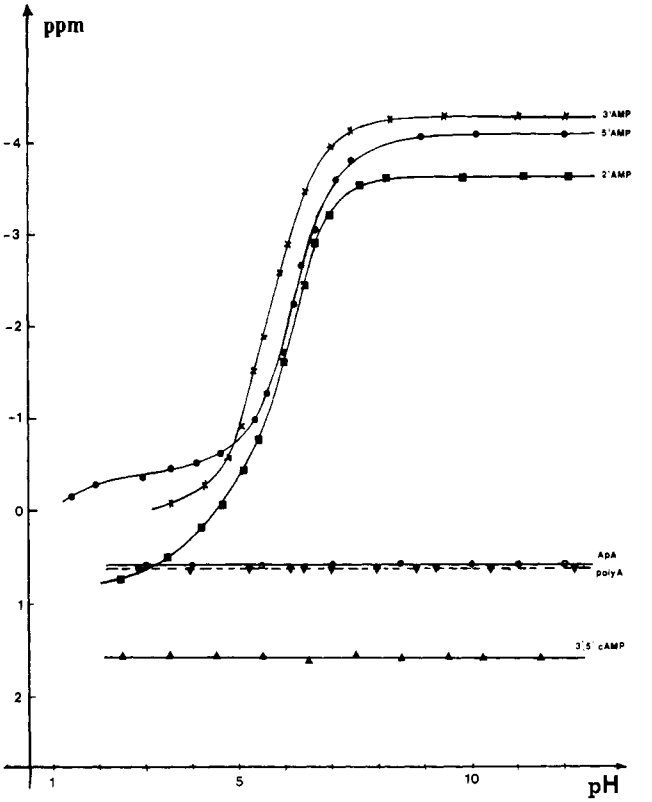


FIGURE 3: Phosphorus-31 chemical shifts of adenine-containing nucleotides as a function of pH. Conditions are the same as Figure 2.

and the magnitude of the shift is bigger for 3′-phosphate than 5′-phosphate by 0.4 to 0.5 ppm.

The pH dependence of the chemical shifts provides a very accurate method of measuring p$K_a$'s of the phosphate groups. Values in the range of 6.25–6.30 are found for the secondary phosphate ionization of all 5′-mononucleotides, in perfect agreement with thermodynamic determinations (Phillips et al., 1965). These values are not affected by the nature of the base and the presence or absence of an hydroxyl group at the 2′ position. In the case of 3′-mononucleotides, a significant difference is observed between ribonucleotides (p$K_a$ = 5.71–5.85) and deoxyribonucleotides (p$K_a$ = 6.25). An intermediate value of p$K_a$ = 5.95 is found for the 2′-mononucleotides. In all cases, the primary phosphate ionization appears in the 1.6–1.7 range of pH.

When comparing experimental titration curves with the theoretical behavior that one can calculate from the Henderson–Hasselbalch equation, significant deviations are found for 5′-mononucleotides. Deprotonation of adenine induces a downfield distortion of the curve in the vicinity of pH 4 (see 5′-AMP and 5′-dAMP in Figure 2). More pronounced is the effect on the 5′-UMP, 5′-TMP, and 5′-dUMP curves, since a new downfield inflection of 0.18 ppm is present, with a midpoint corresponding to a p$K$ of 10, thus reflecting the deprotonation of the pyrimidine bases. The effect of base ionization is present on the 2′-mononucleotide titration curves and, to a lesser extent, on the 3′-mononucleotides (Figure 3).

The chemical shift of the 3′,5′-cyclic mononucleotides is not sensitive to pH variation (Figure 2) in the range of pH 3–12. In the case of 3′,5′-cyclic TMP, a shift is observed at very low pH and corresponds probably to the primary phosphate ionization. The base deprotonation is again reflected by a 0.17 to 0.19 ppm downfield inflection in the 2′,3′-cyclic mononucleotides (Table I).

Finally, ApA, dApdA, UpU, UpA, and ApU resonance positions are not affected by pH variation from 3 to 12. However, TpT titration displays, surprisingly, an inflection corresponding to the ionization of the thymine base (p$K_a$ = 9.9).

The phosphorus resonance of mononucleotides varies with the position of the phosphate on the sugar moiety (Figure 3) and, typically, the signals of the 2 phosphate groups present on thymidine 3′,5′-diphosphate are very well separated and distant by 0.5 ppm from each other (Figure 4).

The nature of the base has no effect on the 3′-ribo- and deoxyribonucleotides chemical shift when the phosphate is monoanionic and a slight effect on the 3′-ribonucleotides at pH above the second phosphate dissociation (3′-AMP resonance occurs 0.132 ppm lower field than 3′-UMP at pH 10). For the 5′-nucleotides a downfield shift (0.197 ppm at pH 1) is observed for purine nucleotides, with respect to pyrimidine nucleotides. The same trend is also found on the 3′,5′-cyclic nucleotides (Table I), with the purine base having a more deshielding effect on the phosphate resonance than the pyrimidine. However, the reverse effect is noted for dinucleotides and homopolynucleotides. It is thus reasonable to consider that other effects, like the conformational dynamics of the sugar ring, might also play a role in determining the magnitude of the phosphate chemical shift (see Discussion).

More pronounced is the influence of a hydroxyl group at the 2′ position of the ribose. The phosphorus signals of the 3′-ribonucleotides are always found at least 0.4 ppm downfield from the corresponding 3′-deoxyribonucleotides (Figure 2 and

TABLE I: Phosphate Chemical Shifts at Various pH.

| | pH 1 | pH 3 | pH 7 | pH 12 |
|---|---|---|---|---|
| 2′,3′-cAMP | −20.222 | −20.222 | −20.395 | −20.395 |
| 2′,3′-cUMP | −20.148 | −20.148 | −20.148 | −20.345 |
| 3′-AMP | 0.296 | −0.049 | −3.876 | −4.148 |
| 3′-UMP | 0.296 | −0.049 | −3.802 | −4.049 |
| 5′-AMP | 0.123 | −0.370 | −3.456 | −4.049 |
| 5′-dAMP | 0.123 | −0.370 | −3.456 | −4.049 |
| 5′-UMP | 0.074 | −0.222 | −3.382 | −3.975 |
| 5′-dUMP | 0.074 | −0.222 | −3.382 | −3.975 |
| 5′-TMP | 0.074 | −0.222 | −3.382 | −3.975 |
| 2′-AMP | nd[a] | 0.839 | −3.382 | −3.555 |
| 3′-dAMP | 0.740 | 0.395 | −3.037 | −3.481 |
| 3′-TMP | 0.740 | 0.395 | −3.037 | −3.481 |
| UpU | 0.691 | 0.493 | 0.493 | 0.493 |
| Poly(U) | nd[a] | 0.543 | 0.543 | nd |
| ApA | nd | 0.716 | 0.716 | 0.716 |
| Poly(A) | nd | 0.765 | 0.765 | 0.765 |
| TpT | nd | 0.938 | 0.938 | 0.716 |
| dApdA | nd | 1.111 | 1.111 | 1.111 |
| 3′,5′-cAMP | 1.580 | 1.580 | 1.580 | 1.580 |
| 3′,5′-cUMP | 1.629 | 1.629 | 1.629 | 1.629 |
| 3′,5′-cTMP | 2.123 | 2.049 | 2.049 | 2.049 |

[a] nd, not determined.

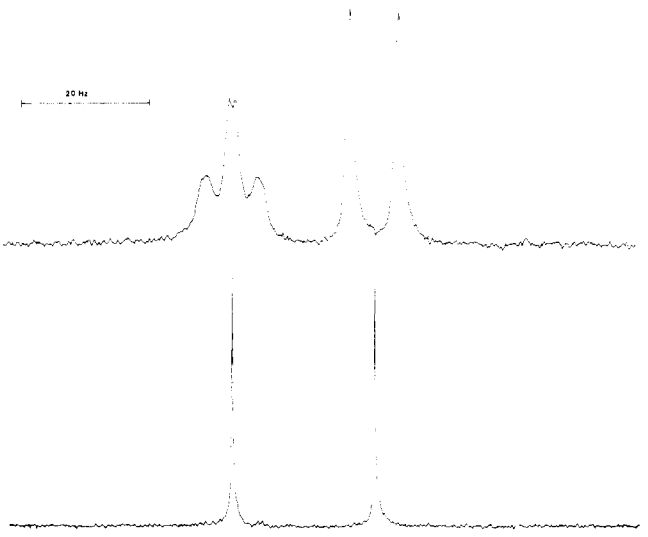

FIGURE 4: $^{31}P$ NMR spectrum of 3′,5′-thymidine diphosphate. Upper spectrum: "natural" phosphorus spectrum of 4 mM, 3′,5′-pTp, pH 9.60, digital resolution, 0.2 Hz, 600 scans. Left signal (lower field) corresponds to the 5′-phosphate, while the upfield doublet corresponds to the 3′-phosphate. Lower spectrum: proton-decoupled phosphorus spectrum of the same sample; 200 scans. The two signals are separated by 23 Hz (0.56 ppm). Chemical shifts of 3′- and 5′-phosphates are, respectively, −3.45 and −4.01 ppm with respect to 85% phosphoric acid.

Table I). This effect is independent of the nature of the base and exists for both purine and pyrimidine nucleotides. A similar effect of same magnitude is observed for 3′,5′-cyclic nucleotides, with 3′,5′-cUMP resonating 0.420 ppm downfield from 3′,5′-cTMP, which is similar to the deviation observed (0.444 ppm) between two homologous 3′-mononucleotides bearing a monoanionic phosphate (e.g., 3′-dAMP and 3′-AMP at pH 3). The deshielding effect of the 2′-OH is also marked on the dinucleotides, since the phosphate signals of UpU and ApA occur at 0.445 and 0.395 ppm downfield from TpT and dApdA, respectively (Table I). However, no such effect is apparent in the 5′-mononucleotides. Of interest is the comparison of the chemical shifts of the different compounds at pH 3 (Table I), that is to say when all nucleotides bear only one negative charge on the phosphate group and the bases are protonated. Thus, the ionization states of all nucleotides are comparable. Going downfield, one finds successively the 3′,5′-cyclic nucleotides (2.1–1.5 ppm), the dinucleotides (1.1–0.5 ppm), the 3′-mononucleotides (0.4–0.0 ppm), the 5′-mononucleotides (−0.2–−0.4 ppm), and the 2′,3′-cyclic nucleotides (−20 ppm). In this latter case, the dominant effect on chemical shift is conformational and pertains to different geometrical parameters around the phosphorus atom. For instance, the main difference, at the phosphate level, between 2′,3′-cUMP and 3′,5′-cUMP concerns the O–P–O bond angle whose deformation is reflected by a 22 ppm shift in the phosphorus signal position.

## Discussion

*pH Effect.* The screening of a nucleus, which is the origin of the chemical shift, is generally considered to be mainly due to the diamagnetic shielding of the neighboring electrons, i.e., a direct function of the electron density. Hence, one would expect deprotonation of the nucleotide phosphate groups to induce an increased electronic shielding of the phosphorus nucleus leading to a shift of the signal to higher field, as is often found for proton resonances. However, experimentally, the reverse effect is observed for phosphates whose chemical shift decreases to downfield when pH increases. This effect is general to all phosphates and has been found for orthophosphate solutions (Jones and Katritzky, 1960), condensed phosphates (Crutchfield et al., 1962), as well as for a variety of phosphate-containing biological molecules (Cohn and Hughes, 1960; Blumenstein and Raftery, 1972; Sarma and Mynott, 1972; Moon and Richards, 1973; Haar et al., 1973; Cozzone et al., 1974). The downfield shift observed upon deprotonation suggests that the charge density variations on the phosphorus atom are not accounted for on the basis of the chemical in-

ductive effect. Similar observations have been made for carbon resonances in amino acids, amines, and carboxylic acids by Horsley and Sternlicht (1968) who have proposed that deprotonation would cause a polarization effect on vicinal bonds "occurring both through the bonds as an inductive effect and through space as an electric field effect". According to this hypothesis, protonation of the amino group of an amino acid would increase the charge density on the $\alpha$ carbon. A similar mechanism could be operative in the case of the phosphates. Other authors (Jones and Katritzky, 1960) have proposed that loss of a proton on the phosphate group would expand the electronic cloud on the phosphorus as a predominant effect thus deshielding the nucleus. A more general and consistent explanation has been given by Moedritzer (1967) on a quantum mechanical basis. The phosphorus chemical shift would be affected by pH variation in terms of the sum of three contributions: variation in the electronegativity of the oxygen atoms adjacent to phosphorus, variation in the O-P-O bond angles, and occupation of phosphorus d$\pi$ orbitals. Depending on the modulation of the pH effect on these three factors, the deprotonation-induced downfield shift of the phosphorus signal of the phosphates is readily explained, as well as the deprotonation-induced high-field shift of the phosphonate signals. This latter reverse effect is characteristic of salts of phosphonic acids and has also been found in various nucleotide phosphonate inhibitors (Cozzone and Jardetzky, unpublished results).

*Influence of 2′-Hydroxyl Group.* No significant difference is detected when comparing the $^{31}$P NMR data on the ribose and deoxyribose 5′-nucleotides. This is not surprising, since potential interaction is unlikely, due to the distance separating these two groups either through space or along the $\sigma$ backbone.

Conversely, a strong effect of the 2′-OH has been found on 3′-mononucleotides and 3′,5′-cyclic nucleotides and dinucleotides. The presence of the hydroxyl group is primarily reflected by a 0.4 to 0.5 ppm downfield shift of the phosphate signal at any pH. In addition to the deshielding effect, the p$K_a$ of the ribose 3′-nucleotides is decreased by 0.4–0.5 unit with respect to deoxyribose 3′-nucleotides. These findings, together with changes in the proton–phosphorus coupling patterns (Cozzone and Jardetzky, 1976), demonstrate a strong influence of the 2′-hydroxyl on the 3′-phosphate group. This effect could be accounted for on the basis of the substitution of a hydrogen atom by a more electronegative 2′-OH (interaction through $\sigma$ bonds) and/or, more likely, a direct hydrogen bond type spatial interaction between the 2′-OH and the 3′-phosphate. Actually, such an association has been found in the crystalline state, since hydrogen bonding from the hydroxyl to an oxygen atom of the adjacent phosphate group has been described for transfer RNA (Spencer et al., 1962). It is assumed that this intramolecular extra bond helps to stabilize the structure which, in the case of RNA, is largely unchanged over a broad range of humidity. In addition, this binding could be considered as a first step facilitating the reaction leading to the formation of cyclic phosphates. Our findings, thus, do not seem to favor the hypothesis of the possible existence of a strong intramolecular hydrogen bond between the 2′-OH and the base in the 3′-ribomononucleotides. Such an interaction between the 2′-OH and the N-3 of purine or the 2-keto of pyrimidine has, however, been detected by $^1$H NMR for 5′-ribomononucleotides (Schweizer et al., 1968; Ts'o, 1970). In this latter case, the phosphate group occupies an unfavorable position to allow any competing interaction with the 2′-hydroxyl.

*Influence of the Base.* The presence and the nature of the base are reflected in the chemical shifts and by anomalies in the phosphate titration curves. When going from the deoxyribose to the ribose 3′-mononucleotides, sensitivity to the nature of the base appears. While 3′-TMP and 3′-dAMP display superimposable normal titration curves and identical chemical shifts, adenine has a more deshielding effect at high pH on the phosphate of 3′-AMP than uracil on 3′-UMP (Figure 2). The base affects the half-neutralization of the 3′-phosphate, since 3′-AMP has a lower p$K_a$ (5.71) than 3′-UMP (p$K_a$ = 5.85). Also, 3′-AMP and 2′-AMP curves present a deviation from the theoretical Henderson–Hasselbalch plots, suggesting again an influence of the base ionization on phosphate titration. It is unlikely that a field effect would account for this interaction considering the large distance between the 2′- or 3′-phosphate and the base, especially when the base is in "anti" conformation. Rather, the effect of the base would propagate along the $\sigma$ backbone and be absent in the 3′-deoxyribonucleotides due to the lack of the "hydroxyl relay" at the 2′ position of the ribose.

The influence of the base through the covalent linkage could also explain a larger effect on 2′-AMP than on 3′-AMP, and, also, the sensitivity of the 2′,3′-cUMP and 2′,3′-cAMP phosphate titration to protonation of the base.

The case of 5′-nucleotides appears somewhat different. It is known (Schweizer et al., 1968) that the 5′-phosphate group interacts by an electrostatic field effect with the bases, the results being a specific deshielding of the protons H-8 of purines and H-6 of pyrimidines. One can thus postulate that ionization of the base could, in turn, affect the 5′-phosphate by means of a "through-space" interaction, explaining the deviations of the pH-dependence curves from the theoretical Henderson–Hasselbalch plots. A similar deviation has also been reported for 5′-CMP (Haar et al., 1973); it is particularly apparent for the pyrimidine 5′-nucleotides (Figure 2). However, the base influence is not large enough to affect significantly the ionization properties of the phosphates, since the same p$K_a$ value can be calculated from the titration curves for all 5′-nucleotides, in agreement with independent potentiometric titrations (Philips et al., 1965).

The behavior of dinucleotides is more complex, since a reverse situation is observed with UpU and TpT resonating at a lower field than ApA and dApdA, respectively (Table I). It is likely that, in this case, the expected deshielding effect of purine is largely cancelled by other factors, mainly stereochemical, affecting to a larger extent the phosphate resonance (vide infra).

*Influence of the Ribose Ring Pucker and Ribose–Phosphate Backbone Conformation.* The furanose ring of nucleotides can be discussed as a dynamic two-state equilibrium between two puckered forms designated as $^3$E ($C_3$′-endo, $C_2$′-exo) and $^2$E ($C_2$′-endo, $C_3$′-exo) (Altona and Sundaralingam, 1973; Sarma and Mynott, 1972). The relative population of these two forms, which can be regarded as equivalent to the population of N and S conformers, respectively, has been determined for a variety of mononucleotides (Davies and Danyluk, 1974; Lee et al., 1975a; Davies and Danyluk, 1975) and more recently for some dinucleotides (Lee et al., 1975b; Davies and Danyluk, 1975). Considering the close correlation between the conformation of the furanose ring and the orientation of the phosphate group attached either to the 3′- or 5′ position, one can reasonably expect that differences in sugar pucker among nucleotides, as well as the nature of the base and the presence of a 2′-OH group, will influence the phosphate chemical shift.

In the case of 3′-ribomononucleotides, one could propose

that the higher percentage of N conformer for 3′-UMP (56%) with respect to 3′-AMP (34%) could contribute to the higher field resonance found for the 3′-UMP phosphate at neutral pH. However, this finding cannot be generalized, since a reverse correlation is noted when comparing the series of 3′-ribomononucleotides and 3′-deoxyribomononucleotides (Table I). For instance, 3′-AMP resonates more than 0.8 ppm downfield from 3′-dAMP, although only 23% of 3′-dAMP molecules are N conformers (Lee et al., 1975a). The direct effect of 2′-OH seems predominant in this case.

Within the series of 5′-mononucleotides, a clear-cut correlation between the percentage of sugar pucker and the phosphate chemical shift is even harder to work out. Although 5′-AMP and 5′-UMP display similar amounts of $^3$E sugar pucker (40%) at neutral pH (Davies and Danyluk, 1974), their phosphate resonance are 0.074 ppm apart. Conversely, 5′-TMP and 5′-dUMP, with only 28% of $^3$E pucker, have the same chemical shift as 5′-UMP. Also, the 10% excess of S conformer found in 5′-deoxyribonucleotides with respect to the 5′-ribonucleotides (Davies and Danyluk, 1974) is not reflected in the phosphate $^{31}$P chemical shifts, which remain essentially ordered as a function of the nature of the base (Table I). One can thus assume that from the phosphorus chemical shift standpoint, differences in sugar ring conformation do not affect significantly the behavior of the exocyclic phosphate of 5′-mononucleotides, whose chemical shift remains essentially determined by other factors.

It is reasonable to consider that a different distribution of the rotamer population along the $C_{3'}$–$O_{3'}$ and $C_{5'}$–$O_{5'}$ bond axes, determining the statistically favored orientation of the 3′- or 5′-phosphate, would affect the chemical shift of the corresponding phosphorus signal. However, an identical percentage (85%) of the preferential "gauche–gauche" rotamer along $C_{5'}$–$O_{5'}$ (see following paper in this issue) is found for all 5′-mononucleotides. Similarly, the small differences noted in the predominant "gauche" rotamer population along $C_{3'}$–$O_{3'}$, for all 3′-mononucleotides, do not seem significant enough to account for the observed distribution of chemical shifts (Table I).

The case of dinucleotides is more complex. Considering the increased rigidity, the more restricted dynamics, and the interdependence of conformational features brought by dimerization, one can imagine a larger role of the spatial arrangement on the phosphate chemical shift. The nature of the predominant sugar ring pucker, as well as the relative conformer population along $C_{4'}$–$C_{5'}$, $C_{3'}$–$O_{3'}$, $C_{5'}$–$O_{5'}$, P–$O_{3'}$, and P–$O_{5'}$ bond axes (corresponding, respectively, to $\psi$, $\phi'$, $\phi$, $\omega'$, and $\omega$ angles according to Sundaralingam's nomenclature), are then expected to influence directly the environment of the phosphorus atom and hence its chemical shift.

For instance, the preferential sugar rings pucker in dApdA and TpT remains predominantly $^2$E–$^2$E as in the case of independent mononucleotides, while a more significant percentage of $^3$E–$^3$E is found in ApA (Lee et al., 1975a,b; Hruska et al., 1975; Kondo and Danyluk, 1976). Moreover, it has been shown using a semiempirical conformational energy calculation (Yathindra and Sundaralingam, 1975) that a direct correlation exists between the sugar pucker and the internucleotide P–O bond conformations. Consequently, some of the low energy domains in the conformational energy surface computed for the rotations $\omega'$ and $\omega$ around P–$O_{3'}$ and P–$O_{5'}$ are not found to be accessible to the same extent by all dinucleotides, depending on the pucker form of the sugar rings. The low energy domain present for $\omega'/\omega$ around 300°/300° is one of the favored domains for $^3$E–$^3$E diribonucleotides (significantly represented in ApA, Kondo and Danyluk, 1976) but becomes hardly accessible to $^2$E–$^2$E analogues. The environment of the corresponding phosphate will thus be different on a time-averaged basis and the phosphorus chemical shift will be affected accordingly. From the analysis of our $^{31}$P NMR data, it could be tentatively proposed that a higher field chemical shift might be correlated to the predominance of $^2$E ($C_{2'}$-endo) pucker form for both sugar rings of dinucleotides (TpT, dApdA). The lower-field resonance found for UpU and ApA would be correlated with a higher content of $^3$E ($C_{3'}$-endo) forms together with the deshielding effect of 2′-OH group. However, many other stereochemical factors are also influencing directly or indirectly the phosphate chemical shifts. If one examines the series of dinucleotides' chemical shifts, the base stacking effect seems to be correlated with a high field trend within each class of structural analogues (Table I). Also, the increase in "gauche–gauche" conformer percentage around $C_{5'}$–$O_{5'}$ bond seems to parallel an upfield shift of phosphate resonance among the diribonucleotides (UpU: 0.493 ppm, 72% of "gauche–gauche" conformer. ApA: 0.716 ppm, 88% of "gauche–gauche" conformer; Cozzone and Jardetzky, 1976).

The extent of the significance of these multiple stereochemical effects on phosphate chemical shifts is somehow hard to appreciate, if not questionable in some cases. Although reflecting real phenomena, these contributions are often difficult to separate, since, for instance, sugar pucker, $\omega'$, and $\omega$ angles, base stacking are interdependent and coordinated. Interpretation of data in this respect is, thus, not straightforward and the overall trend observed on the chemical shifts is only the resultant of several effects whose relative weights cannot be readily determined. Comparisons and conclusions have to be limited to families of close structural analogues, and the finding of reliable consistent patterns throughout the whole series of nucleotides will require more experimental and theoretical data.

*Influence of O–P–O Bond Angle.* In the series of monoanionic nucleotides shown on Table I, the phosphorus chemical shifts increase in the order 5-membered monomer < acyclic monomer < acyclic dimer < 6-membered monomer. This arrangement and especially the ring size effect upon phosphorus chemical shifts is similar to what is found with other classes of phosphoryl compounds like phosphites, phosphonates, phosphinates, etc. (Blackburn et al., 1971). Although the theoretical calculations (Lechter and Van Wazer, 1966) show that P-31 chemical shifts are complex functions involving several parameters, it seems justified to consider that, within a family of compounds like the mononucleotides, all parameters ($\pi$-electron overlap of phosphorus, electronegativity of adjacent nuclei, etc.), except for the stereochemical, remain essentially constant. In the case of nucleoside phosphates, and after taking into consideration any pH and base effects, it appears that the changes in chemical shifts can be correlated primarily to changes in $\sigma$-bond angles and consequent hybridization differences. The most striking effect is in the comparison of 2′,3′- and 3′,5′-cyclic mononucleotides. The 103° O–P–O bond angle in 3′,5′-cUMP (Coulter, 1969) is decreased to 96° in 2′,3′-cUMP (Coulter and Greaves, 1970) and this 7° variation is accompanied by a 22 ppm downfield shift of the phosphate resonances (Table I).

However, a straightforward relationship between the $^{31}$P chemical shift and the O–P–O bond angle cannot be proposed at the moment. Other stereochemical effects affecting the phosphorus nucleus of the phosphate group (e.g., rotation around O–P bond axis) are likely to play an additional role. For

instance, the resonance of 3′,5′-cUMP (+1.6 ppm) is found significantly higher than the resonance of 2′,5′-ApU or GpC (+0.5 ppm), although both compounds display an identical O-P-O bond angle of 103° (Sussman et al., 1972; Day et al., 1973).

This dependence of chemical shift upon conformation is of considerable interest for the study of interacting systems, since any distortion of the nucleotidic part of a complex will be readily reflected in the chemical shifts. On this basis, it has been proposed, for instance, that the binding of a dinucleotide phosphonate to ribonuclease A occurs without deformation of the substrate (Griffin et al., 1973), while the association of ATP to G-actin could involve a modification of the O-P-O bond angle of ATP (Nelson et al., 1974; Cozzone et al., 1974). In a similar way, upfield or downfield shifts of phosphorus resonances have been observed upon complexation of several dinucleotides with actinomycin D (Patel 1974a,b,c, and personal communication).

The high sensitivity of the phosphorus nucleus to variation of charge density due to different geometrical arrangements is also likely to account for the difference in chemical shift between inside and outside resonances of the phospholipid bilayers (Cozzone and Jardetzky, unpublished data; Berden et al., 1974).

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