(Trasylol) and Shell Development Co., Houston, Texas, for the use of their 90-MHz ^{13}C NMR facilities. We especially wish to thank Ms. Ruth Inners for obtaining initial spectra at 25 and 90 MHz and Dr. Dale Holecek of Shell Development for his assistance in obtaining the 90-MHz spectra.

References and Notes

(1) Abbreviations used: ^{13}C NMR, ^{13}C nuclear magnetic resonance; BPTI, basic pancreatic trypsin inhibitor (bovine); SM-BPTI, [*S*-[^{13}C]methyl methionine-52]-BPTI; ppm, parts per million; Z, benzyloxycarbonyl.
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(14) The methyl ester of [^{13}C]-SM-methionine at pH 10 exhibited a 0.9-Hz separation in the *S*-methyl signals at 90 MHz. Under these conditions the corresponding signal for [^{13}C]-SM-methionine amide showed an unresolved shoulder on the upfield side.
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^{1}H, ^{23}Na, and ^{31}P NMR Studies of the Self-Assembly of the 5′-Guanosine Monophosphate Dianion in Neutral Aqueous Solution in the Presence of Sodium Cations

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Contribution from the Institut de Chimie Organique et de Biochimie (B6), Université de Liège, Sart-Tilman par 4000 Liège, Belgium. *Received April 12, 1979*

Abstract: ^{1}H chemical shifts show at concentrations below 0.2 M a normal stacking process, with an apparent mean equilibrium constant of 3.8 M^{-1} at 299 K. Above a concentration of approximately 0.3 M at this temperature, an altogether different mode of self-ordering sets in. The multiplicity of H-8 resonances in the ^{1}H NMR spectra and that of phosphate resonances in the ^{31}P NMR spectra are consistent with the coexistence of two kinds of ordered structures, likely to be octamers and hexadecamers, respectively. ^{23}Na chemical shifts and line widths are also concentration dependent: below a critical concentration, they increase normally with the fraction of ion-paired sodium, just as in model systems (5′-AMP, 5′-ATP, 2′-GMP, D-ribose 5-phosphate), with binding constants of about 2 M^{-1}, at 300 K. Above a critical concentration, the ^{23}Na resonance undergoes a pronounced upfield shift, together with a considerable line broadening: these are diagnostic of a sharp disorder–order transition, which can be studied either by changing the concentration at constant temperature, or by varying the temperature at constant concentration. This disorder–order transition is highly cooperative, with a Hill coefficient of 6.1 ± 0.7. From the critical concentrations, determined either from the ^{23}Na chemical shifts or from line widths reduced to unit viscosity, a phenomenological phase-separation model yields $\Delta H^\circ = -17 \pm 2$ kcal·mol^{-1} and $\Delta S^\circ = -51 \pm 6$ cal·mol^{-1}·K^{-1}. The self-assembly is enthalpy driven, rather than determined predominantly by hydrophobic forces, like the normal stacking interactions. The binding of Na^+ contributes directly to the buildup of octamers and hexadecamers from hydrogen-bonded tetramers whose central cavity includes a sodium cation.

Introduction

Alone among the nucleotides, guanosine monophosphate (GMP) arranges itself into highly ordered aggregates, in aqueous solutions.[1] The 5′ isomer (5′-GMP) not only forms gels at acidic pHs,[1-5] but also self-orders at neutral and slightly basic pH. This phenomenon was discovered in 1972 by Miles and Frazier,[6] using infrared spectroscopy. Further X-ray[7] and IR studies provided nearly conclusive evidence for a planar tetrameric arrangement of GMP molecules held together by eight hydrogen bonds. ^{1}H NMR studies reported in 1975 the high rigidity of 5′-GMP aggregates at neutral pH: energy barriers of more than 15 kcal·mol^{-1} prevent fast exchange of monomer nucleotides between sites.[8] Two groups then independently discovered by NMR that the self-assembly process depends critically on the cation present: Pinnavaia et al.[9] using ^{1}H NMR and Laszlo et al.[10-13] using ^{23}Na and ^{39}K NMR showed a potassium-selective interaction of alkali metal cations with the central cavity delimited in the tetramers by the four O_6 oxygens.

5′-GMP aggregation is related to the exceptionally high rigidity displayed by poly(riboguanylic acid) [poly(G)] in neutral aqueous solution,[14-17] due to multistrand formation,[18] with a four-stranded form characterized by X-ray in the solid.[19-20]

The self-ordering of 5′-GMP could have had prebiotic significance: nucleotide formation has been documented under abiotic conditions,[21-23] and 5′-GMP self-assembles at the slightly basic pHs (7.8–8.4) characteristic of the present and plausibly also of the primeval ocean.[24-26] We have indeed already shown[27] that 5′-GMP aggregates discriminate between

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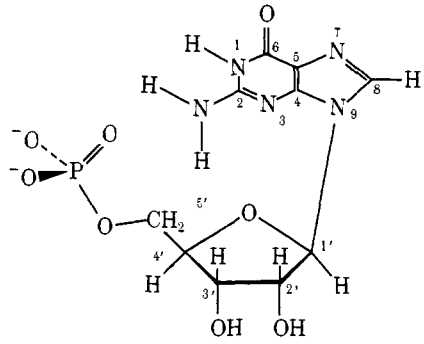
the two archaic[24,28-29] amino acids glycine and alanine. And such interactions with amino acids may have a bearing on the origin of the genetic code.[30-39]

Cation binding determines the self-assembly of the 5'-GMP dianion. Our investigation focuses on the binding modes of alkali metal cations, site binding or atmospheric condensation; on their binding sites, at the central cavity of the tetramers or on the doubly charged phosphate groups; and on the thermodynamics and microdynamics of binding and release of univalent cations by the self-ordered structures. These questions are answered using a combination of ^1H, ^{13}C, and ^{31}P NMR, and the corresponding heteronuclear Overhauser effects, together with direct examination of alkali metal nuclear magnetic resonance.[40,41]

The first article is devoted to an examination of the self-assembly of the sodium salt of guanylic acid, i.e., 5'-GMP. In the accompanying article,[42] we describe results obtained in the presence of other cations besides sodium, especially K^+ and NH_4^+.

The structure of 5'-GMP and numbering of the atoms are as shown.



Experimental Section

Preparation of Solutions. Sodium salts of the nucleotides 2'- and 5'-GMP, 5'-AMP, and 5'-ATP and of D-ribose 5-phosphate are of the best commercial grade (Sigma Chemicals). These are hydrated disodium salts, of known water content. Knowledge of the sodium content being fundamental for the ^{23}Na NMR measurements, we have checked the sodium composition using flame spectrometry, and we have determined the number of hydration molecules by the Karl Fischer amperometric method. We thus obtain a hydration number matching the label within ±15% and a sodium composition of 2 ± 0.03 sodium atoms per molecule of nucleotide. Solutions are made volumetrically in D_2O (Merck "Uvasol" 99.8%) or in twice-distilled water. Only at high concentrations of 5'-GMP, Na_2 (>0.5 M), homogenization of the solution requires time (~12 h) because of the high viscosity. The pH of solutions is measured, both before and after recording NMR spectra, with a Tacussel TS 4 N pH meter. The pH meter is calibrated with standard buffers (Merck Titrisol), and measurements are accurate to ±0.01 pH unit. The pD of the D_2O solutions is obtained from the following approximate relationship:[43] pD = pH + 0.4. Solutions used for the ^{23}Na measurements contain only the disodium salt of the nucleotide and the solvent. These solutions are not buffered to avoid undesirable competition between various anions and cations which would then coexist. As a consequence, the pH of these solutions varies slightly with concentrations: for example, for 5'-GMP, Na_2 it varies by 0.5 pH unit over the 5×10^{-3}–1 M concentration range. Solutions prepared for ^1H NMR chemical-shift measurements contain, in addition to 5'-GMP, Na_2 and D_2O 0.2 M NaCl, 1,4-dioxane (Merck "for analysis" 10^{-3} M) as an internal reference both for chemical shifts and line widths,[44,45] and EDTA (Merck "for analysis", 2×10^{-3} M) to complex paramagnetic impurities that might affect the line widths and proton chemical shifts.[46] The pH of these solutions is adjusted to the desired value using very small amounts of concentrated DCl or NaOD. For relative intensity measurements, ^1H NMR spectra are obtained for solutions of 5'-GMP, Na_2 twice lyophilized from >99.75% D_2O.

Viscosity Measurements. Viscosities of the aqueous solutions are measured at different temperatures, from 0 to 50 °C, and at various concentrations with a Desreux-Bischoff viscosimeter[47] for a reduced volume (2 mL) thermostated to within ±0.1 °C. Densities are determined with a pycnometer brought to the same temperature in the thermostated bath. Both the viscosimeter and the pycnometer are calibrated with pure water at different temperatures, using values for the densities and the viscosities from standard sources.[48] The standard deviation on the flow times (at least five measurements are averaged) is less than 0.25%. However, in order to decrease the time required for viscosity measurements, we have often used another procedure: the viscosity (η) of a solution is measured at different temperatures, and then at different concentrations. By interpolation on the resulting plot, we determine η as a function of nucleotide concentration at the different temperatures with an uncertainty of ±1%.

Measurement of NMR Chemical Shifts and Relaxation Times. A. ^1H NMR spectra are obtained with a Bruker HFX-90 spectrometer equipped with Fourier transform, at 90 MHz, with a deuterium lock for field stabilization. Samples are maintained at 22 ± 1 °C. D_2O is used as the solvent, providing the internal lock; the residual HOD line, of weak intensity, overlaps little with the nucleotide resonances. Line widths are measured at half-height ($\nu_{1/2}$), and the line width of the inert 1,4-dioxane internal reference is subtracted in order to minimize the effect of inhomogeneous magnetic fields and the changes in viscosity of the solutions with the nucleotide concentration. The chemical shifts are also measured with respect to this same internal reference. Continuous wave ^1H NMR spectra were recorded on a 250-MHz Cameca spectrometer. ^1H chemical shifts are reported in parts per million with respect to a Me_4Si external reference, without bulk susceptibility corrections. Temperature control equipment was used in all experiments; temperatures were determined by measuring the chemical shift between the peaks in a methanol spectrum.

B. Sodium-23 NMR spectra are obtained at 21.16, 23.81, and 62.86 MHz with Bruker WP-80 (Liège), Bruker HFX-90 (Liège), and Cameca 250 (Orsay) spectrometers, all in the Fourier transform (FT) mode. Samples are introduced in the probe at least 20 min before recording the spectrum, in order to equilibrate to the probe temperature. Measurements are performed in the 0–50 °C temperature range. Temperature in the probe of the Bruker HFX-90 instrument is measured with a thermocouple and a micro-voltmeter; it is measured with a thermometer in the probes of the Cameca 250 and Bruker WP-80 instruments. Owing to the phenomenon studied, with a pronounced temperature dependence of the line width (up to 15 Hz per degree), it is indeed extremely important that spectra on different instruments be recorded not only on identical samples, but also at a closely similar temperatures. The line widths are reproducible and accurate within ±5%. For NaCl solutions 0.5 M in D_2O and H_2O, the ^{23}Na line width is directly proportional to the measured viscosity, at a given temperature: $\nu_{1/2} = 179.9\eta T^{-1} + 0.777$ ($\rho = 0.996$ for 16 experimental points, 8 in D_2O and 8 in H_2O). The transverse relaxation times T_2 are obtained from the line widths of Lorentzian lines compared to those of the NaCl reference: $\pi\Delta\nu_{1/2} = 1/T_2$. At constant temperature, the reference line width does not change by more than ±1 Hz with the field homogeneity. With non-Lorentzian lines, the fast and slow relaxation components T_2' and T_2'' are obtained by deconvolution of the composite absorption according to a procedure published elsewhere.[13] The longitudinal relaxation times T_1 are measured with the 180°-t-90° pulse sequence, at different t values.[49] The time variation of the line intensity follows the relationship: $\ln(I_\infty - I_t) = -t/T_1 + \ln(kI_\infty)$. A linear regression yields T_1 with a value for the constant $k = 1.8 \pm 0.2$, and an uncertainty of ±10%.

C. Durations of the exciting 90° pulses are 8.5 μs (Bruker WP-80) and 9 μs (Bruker HFX-90 and Cameca 250). The usual spectral windows are 6 or 12 kHz. Taking into account the line widths studied, always below 1 kHz in this study, the spectral distortion is negligible.[50] For a rather typical sample, with a line width at half-height $\Delta\nu_{1/2} \sim 100$ Hz at 23.81 MHz, the number of scans is 2000 (4K); the same sample, with a line width at half-height $\Delta\nu_{1/2} \sim 60$ Hz at 62.86 MHz requires 500 scans (8K). The S/N ratio for all of the absorptions is better than 40:1. Chemical shifts are measured with respect to a 3 M NaCl in water external reference, extrapolated to infinite dilution in D_2O or H_2O; since the solutions studied are also aqueous, no susceptibility correction is made; the attendant error is negligible with respect to the experimental uncertainty.[51-53]

D. ^{31}P spectra have been recorded in the FT mode: on the Bruker WP-80 spectrometer (Liège) at 32.37 MHz—12-μs 90° pulses; 1000-Hz spectral window; line widths of about 10–30 Hz include the

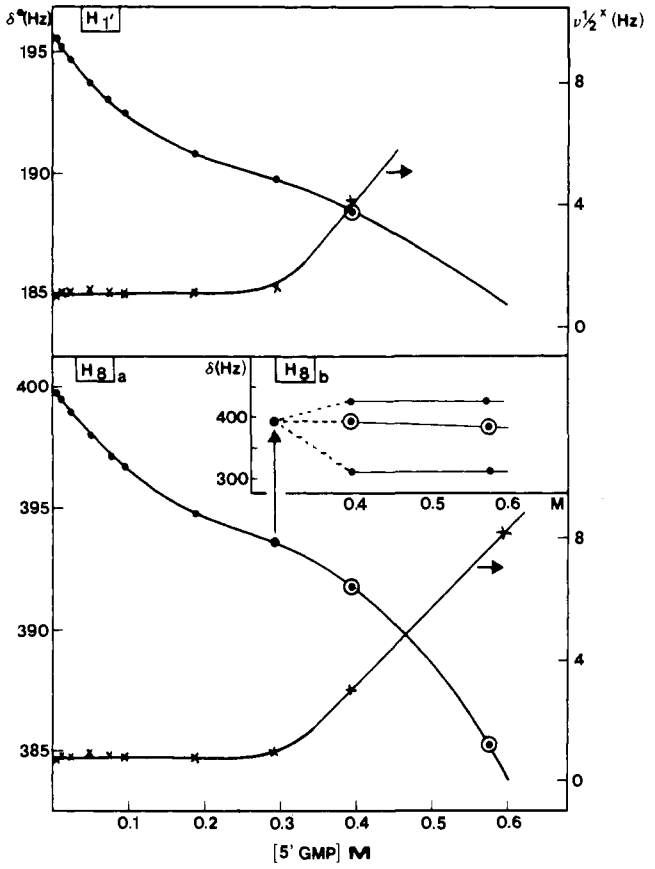


Figure 1. Variation of the chemical shift (●) and of the line width (×) for the H-1′ and H-8 protons as a function of 5′-GMP concentration at pH 7.5 and 22 °C. The inset displays the changes in the chemical shifts for the component resonances into which H-8 splits above a critical concentration of ca. 0.3 M (note the difference in scale).

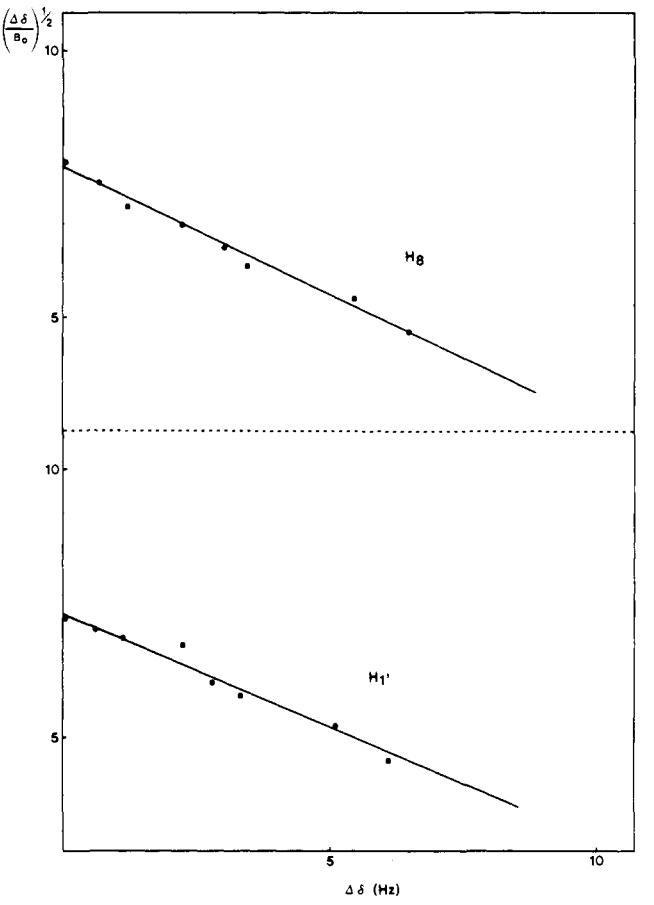


Figure 2. Plots used for the derivation of the mean self-association equilibrium constant $\bar{K}$, for 5′-GMP concentrations below 0.2 M at pH 7.5 and 22 °C.

coupling to adjoining protons, since these were undecoupled spectra; ~500 scans (8 K) are needed for a 40:1 S/N ratio; on the homemade spectrometer of Professor Guéron (Palaiseau) at 111.7 MHz—spectral width = 30 ppm; acquisition time = 1.22 s; rate of recurrence = 2.00 s; spectral resolution = 0.8 Hz; >40:1 S/N ratio with 100 scans; Bruker HFX-90 (Paris) at 36.43 MHz—9-μs 90° pulses; spectral width = 30 ppm; line widths of 10–20 Hz lead to 400 scans (4 K) needed for a 30:1 S/N ratio. Temperature control equipment was used in all experiments; temperature settings were recorded, but absolute temperatures were not determined. The chemical shifts are indicated in parts per million relative to H_3PO_4, 90% as external reference. Proton-decoupled ^{31}P NMR spectra were recorded at 111.7 MHz, at 281 and 288 K. These temperatures were measured by placing an alcohol thermometer in the probe.

All the results are reported with combined uncertainties of $\pm 3\sigma$, i.e., at the >95% confidence level.

Results

Self-Association at Low Concentrations. Base stacking is the normal mode of self-association in aqueous solution for nucleic acid bases, for nucleosides, and for nucleotides: numerous studies have vouched for this proposition.[54,55] Thermodynamic parameters are accessible through osmometry[56-59] and calorimetry,[60] often[57-59] under the isodesmic approximation of equal equilibrium constants in the successive equilibria $B_{n-1} + B \rightleftarrows B_n$. Values of $\Delta H°$ range between −2 and −8 kcal·mol^{-1}, while $\Delta S°$ values vary between −7 and −20 cal·mol^{-1}·K^{-1}.[57,59] Hydrophobic effects have been implicated;[61] however, they are not the sole determinants for the interaction.[53,62] Because of the relatively high concentrations at which self-association occurs, between 10^{-3} and 1 M, nuclear magnetic resonance methods could be applied. They have been focused mainly on the geometric disposition of monomer units within the clusters. Proton and carbon chemical shifts map the nearest neighbor relationships; because of the ring currents present in purine and in pyrimidine bases, whose effects have been computed,[63-65] local structures could be drawn in a number of cases. Self-association of pyrimidine derivatives[61] is weaker than for purine derivatives,[66] and ring currents in the former[63,64,67] do not induce chemical shifts of substantial magnitude. Hence, more is known about adenine derivatives.[59,66,68-73] The upfield chemical shifts of H-1′, H-2, and H-8 upon stacking of adenosine 5′-monophosphate, together with proton relaxation times[70] and paramagnetic line broadening in the presence of Mn(II),[69] were used to specify well-defined geometries in the dimers. However, 5′-AMP and other adenine derivatives self-associate past the dimer stage and form indefinite stacks.[62,68-76]

We have followed the proton chemical shifts of the H-1′ and H-8 protons of 5′-GMP as a function of concentration;[77] very similar upfield shifts occur at pH 5, 6.5, and 7.5. At the latter pH, where gels do not form, the H-1′ and H-8 resonances shift upfield in a normal manner up to a critical concentration of ca. 0.3 M (Figure 1). Then, the line widths increase markedly and the resonances split into several components (Figure 1); a different process sets in. It was first reported at a pH close to neutrality by Pinnavaia, Miles, and Becker.[8]

We analyze the variations in the H-1′ and H-8 chemical shifts at concentrations below 0.2 M using a standard treatment;[76] in addition to the isodesmic hypothesis, it makes the reasonable assumption that the chemical shift is determined predominantly by nearest neighbor molecules in the stacked array. If B_0 is the total concentration and B the free monomer concentration, δ_B is the chemical shift for a given proton in the monomer and δ_{B_2} in the dimer, while δ is the observed chemical

shift; with $\Delta\delta = \delta - \delta_B$, then:

$$(\Delta\delta/B_0)^{1/2} = (K/2\Delta\delta_{B_2})^{1/2}(2\Delta\delta_{B_2} - \Delta\delta) \quad (1)$$

Hence, a plot of $(\Delta\delta/B_0)^{1/2}$ against $\Delta\delta$ (Figure 2) yields from the slope and the ordinate the values of the mean equilibrium constant for self-association $\overline{K}$, together with the value of the dimerization chemical shift $\Delta\delta_{B_2}$. The H-8 resonance yields $\overline{K} = 3.8 \pm 0.5$ M^{-1} and $\Delta\delta_{B_2} = 0.09 \pm 0.02$ ppm. The corresponding values from H-1′ are 3.3 ± 0.5 M^{-1} and 0.09 ± 0.02 ppm, in good agreement.

The value of the equilibrium constant $\overline{K} = 3.5 \pm 0.5$ M^{-1} is commensurate with those reported for self-association in a number of cases: purine = 2.1 M^{-1};[54,76] adenosine = 4.5-15 M^{-1};[54,66] 5′-AMP = 1.7 M^{-1};[78] ATP = 1.3-5.1 M^{-1}.[66,73] Using the above value of $\overline{K}$, we calculate the distribution of 5′-GMP between monomers, dimers, trimers, etc., for various total concentrations in 5′-GMP.[77] At 10^{-2} M, the monomer predominates to the extent of 94%; only 6% of dimer is present. At 0.2 M, 5′-GMP still exists predominantly as the monomer (46%), coexisting with the dimer (30%). On the basis of this simplified model, in which 5′-GMP molecules interact only by the stacking of monomeric units, it is only above a concentration of ~1 M that the percentage of dimers plus trimers overtakes that of monomers. However, it is clear that above a critical concentration, ca. 0.3 M at 22 °C, an altogether different mode of self-ordering occurs, leading to a different type of aggregation than normal base stacking.

Ion Pairing of Na^+ with Nucleotides. We shall examine first the concentration dependence of the ^{23}Na chemical shifts and line widths of several nucleotides capable of "normal" self-association. For all these compounds, the extreme narrowing condition is fulfilled so that determination of the line width at half-height $\nu_{1/2}$ is equivalent to measuring the transverse relaxation rate: $R_2 = T_2^{-1} = \pi\nu_{1/2}$.[40] We find an increase of $\nu_{1/2}$ with the concentration in D_2O, between 0 and 0.6 M, of the disodium salts of D-ribose 5-phosphate, adenosine 5′-monophosphate, adenosine 5′-triphosphate, and guanosine 2′-monophosphate. Even when the observed line width $\nu_{1/2}$ is divided by the measured bulk viscosity η, the resulting line widths reduced to 1 mP $\nu_{1/2}$* are still found to increase with concentration.[77] In all these cases, the line width $\nu_{1/2}$ decreases when the temperature increases; there is fast exchange of the Na^+ cation between the different sites it can occupy.[79] Then by describing the system as a two-state equilibrium, the observed reduced line widths $\nu_{1/2}$* and chemical shifts δ are given by:

$$\nu_{1/2}{}^* = p_F\nu_{1/2}{}^F + p_B\nu_{1/2}{}^B \quad (2)$$

$$\delta = p_F\delta_A + p_B\delta_B \quad (3)$$

where the letters F and B refer to the free and bound states, respectively. As the concentration of each disodium salt increases, so does the fraction p_B of ion-paired sodium in the equilibrium:

$$RP^{2-} + Na^+ \overset{K_s}{\rightleftharpoons} RP^{2-} \cdot Na^+ \quad (4)$$

Denoting x as the equilibrium concentration of ion pairs and C_0 as the analytical concentration in disodium salt:

$$p_B = x/2C_0 \text{ and } K_s = x/(C_0 - x)(2C_0 - x) \quad (5)$$

it follows that:

$$(\nu_{1/2}{}^* - \nu_{1/2}{}^F)/(\nu_{1/2}{}^B - \nu_{1/2}{}^F) = x/2C_0 \quad (6)$$

By combining eq 5 and 6, the experimental results for D-ribose 5-phosphate can be analyzed.[77]

The cases of the nucleotides are more difficult to analyze, because the self-association of the nucleotide is superimposed on the ion pairing described by eq 4. A qualitative comparison of the results will serve as a background for description and analysis of the results for 5′-GMP, which are in an altogether different class. At the higher temperatures (18 and 25 °C), the curves for 5′-AMP and for D-ribose 5-phosphate are very similar: obviously, at these temperatures self-association of the nucleotide is small (corresponding to an equilibrium constant $\overline{K} = 2$ M^{-1} at ambient temperatures[72]). By contrast, the greater tendency of ATP toward self-association ($\overline{K}$ in the range 5-50 M^{-1} according to the conditions and methods of study[73,80,81]) shows as a much more abrupt increase of $\nu_{1/2}$* with concentration at 5 °C. At the same temperature of 5 °C, the dependence of the reduced ^{23}Na line width for 2′-GMP shows another striking difference: the initially convex curve becomes concave above a concentration of ~0.45 M. Interestingly, there is sufficient resemblance between the concentration dependence of the ^{23}Na chemical shift, for the same compound and under the same conditions, that the $\Delta\nu_{1/2}$* and $\Delta\delta$ variations are linearly related to one another (Figure 3).

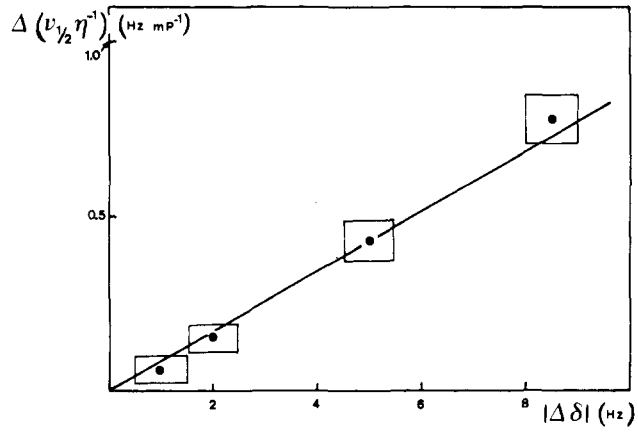


Figure 3. Variations of the ^{23}Na reduced line width $\nu_{1/2}\eta^{-1}$ against those of the ^{23}Na chemical shift in solutions of 2′-guanosine monophosphate, disodium salt, at 20 °C, and for various concentrations.

Under fast exchange conditions, such a proportionality indicates, on one hand, the complete absence of line broadening from chemical exchange, and also a common origin for the changes in the line widths and in the chemical shifts. The most reasonable assumption is for both types of parameters to increase with the fraction p_B of ion-paired sodium: at this temperature of 20 °C (Figure 3) self-association of 2′-GMP has a negligible influence on the sodium line width. Analyzing the chemical-shift variation in terms of equilibrium 4, and using eq 7, which is the counterpart of eq 6:

$$(\delta - \delta_A)/(\delta_B - \delta_A) = x/2C_0 \quad (7)$$

we find a K_s value for 2′-GMP at 20 °C of 1.5 ± 0.4 M^{-1}, commensurate with the stability constant of 2.2 ± 0.2 M^{-1} determined at 25 °C using pH measurements and at an ionic strength of 0.2 for the Na^+-5′-AMP^{2-} ion pair.[82] As for the variation $\Delta\delta$ of the ^{23}Na chemical shift of bound sodium in the ion pair, as compared to free sodium at infinite dilution in D_2O, it is equal to 1.3 ± 0.3 ppm toward lower field.

Self-Assembly at Higher Concentrations. Proton NMR and phosphorus-31 NMR can be used to follow 5′-GMP self-assembly. ^1H NMR spectra (250 MHz) of a 0.7 M solution of the sodium salt of 5′-GMP in D_2O were obtained in the temperature range 273-307 K. They agree with those published previously.[8] The spectra (H-8 region) obtained at 273 and 307 K are shown (Figure 4) with the labeling of H-8 peaks to be used. Deconvolution of the H-8 region of the proton spectra obtained in the range 273-307 K yielded the relative populations of Table I and the chemical shifts shown in Figure 5.

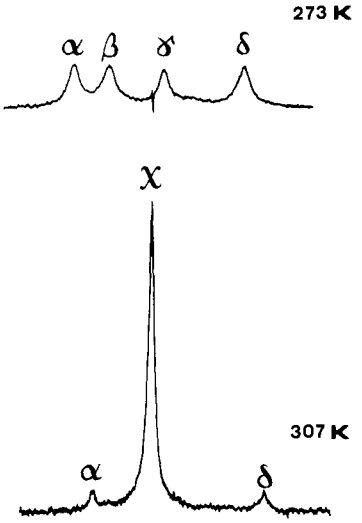


Figure 4. H-8 region of 250-MHz ^{1}H NMR spectra of a 0.7 M solution of 5′-GMP, Na_2 in D_2O at 273 and 307 K. These spectra show the labeling of peaks in the H-8 region used in the text.

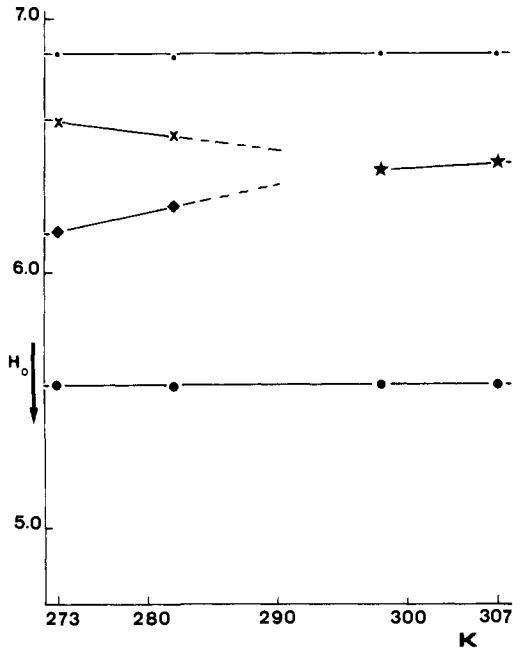


Figure 5. Temperature dependence of the chemical shifts, in parts per million from a Me_4Si-containing capillary, for H-8 region resonances in 250-MHz ^{1}H NMR spectra; other conditions are as in Figure 3: (•) α peak; (×) β peak; (♦) γ peak; (●) δ peak; (★) χ peak.

^{31}P NMR spectra were also recorded at 36.44 MHz for the same samples under identical conditions. A single phosphorus resonance is seen at the highest temperature examined, ca. 307 K. As the temperature is lowered, however, a second peak appears about 2.2 ppm upfield from the original resonance. The relative positions of these two peaks did not change with temperature, down to the lowest temperature examined (273 K). The relative weights of the two phosphorus resonances are plotted as a function of temperature settings in Figure 6. Comparison of Figure 6 with the data of Table I shows that the temperature behavior of the high-field ^{31}P resonance mirrors that of the α and δ ^{1}H resonances. Proton decoupling did not affect the relative populations of the two phosphorus resonances observed at 36.44 MHz.

Of these two resonances observed at 36.44 MHz, the low-field component is resolved into two peaks at higher field. Three phosphorus resonances are in fact observed at 111.7 MHz for

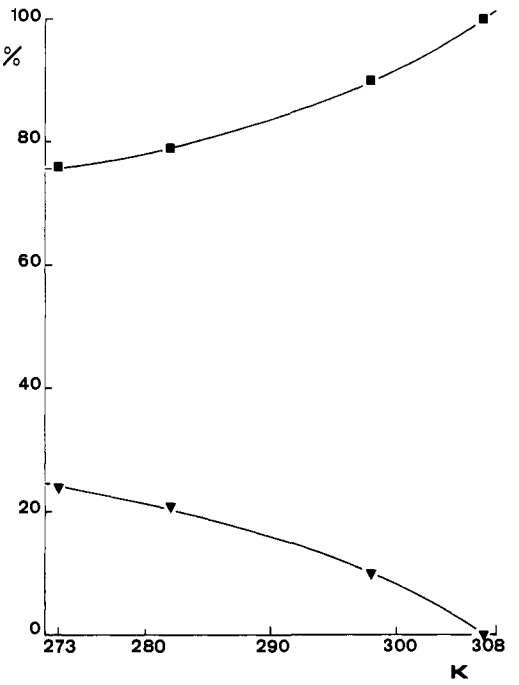


Figure 6. Temperature dependence of the relative populations of the peaks in the 36.44-MHz ^{31}P NMR spectra. Other conditions are as in Figures 3 and 4: (■) low-field peak; (▼) high-field peak.

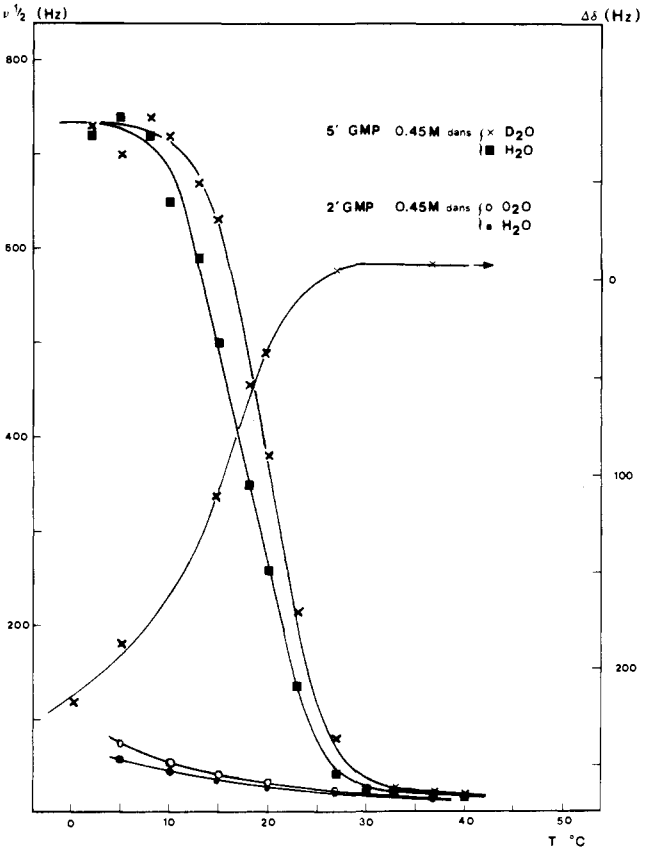


Figure 7. ^{23}Na line width $\nu_{1/2}$ and chemical shift $\Delta\delta$ as functions of temperature for solutions of disodium salts of 5′-guanosine monophosphate (0.45 M) and of 2′-guanosine monophosphate (0.45 M) in D_2O and H_2O solution.

an 0.8 M solution of 5′-GMP, Na_2 in D_2O at 281 and 288 K.[12] Deconvolution of these two 111.7-MHz ^{31}P NMR spectra shows equality of the populations of the highest and lowest field ^{31}P peaks at both temperatures investigated. These two peaks

Table I. Temperature Dependence of the Relative Populations of Peaks in the H-8 Region of 250-MHz Spectra of a 0.7 M Solution of 5′-GMP, Na_2, in D_2O

peaks	T, K			
	273	282	298	307
α	27	23	13	5
β	31	26	73[a]	90[a]
γ	15	27		
δ	27	24	14	5

[a] χ peak (see Figure 3).

Table II. Critical Concentrations (CC1, See Text) Determined from Line Widths Reduced to Unit Viscosity ([a]) and from Chemical Shifts ([b])

T, K	solvent	CC1, M
273	D_2O	0.12 ± 0.02[a]
273	D_2O	0.134 ± 0.015[b]
278	D_2O	0.15 ± 0.02[a]
278	D_2O	0.16 ± 0.02[b]
278	H_2O	0.17 ± 0.02[a]
288	D_2O	0.27 ± 0.04[a]
288	D_2O	0.29 ± 0.03[b]
288	H_2O	0.30 ± 0.05[a]
293	D_2O	0.34 ± 0.04[a]
293	D_2O	0.38 ± 0.04[b]
300	D_2O	0.43 ± 0.06[a]
300	D_2O	0.53 ± 0.08[b]
300	H_2O	0.45 ± 0.06[a]
310	D_2O	0.70 ± 0.15[a]
310	H_2O	0.70 ± 0.10[a]

are separated by 2.3 ppm at 281 and 288 K. The central ^{31}P resonance appears ~0.15 upfield from the lowest field ^{31}P peak at both these temperatures.

Self-Assembly: Phenomenological Approach. Sodium-23 nuclear magnetic resonance[40] is a choice method for studying binding of the sodium cation with biomolecules in aqueous solution. Binding of even a few percent of the Na^+ ions to a slowly reorienting macromolecule or molecular assembly will show up through an enhancement of the quadrupolar relaxation rate; attachment of Na^+ ions to antibiotic ionophores,[83-85] cation binding proteins,[86,87] synthetic[88] or natural[41,89-91] polyelectrolytes, and nucleic acids[92-94] has been studied in this manner.

^{23}Na NMR chemical shifts and line widths are measured as a function of 5′-GMP concentration and temperature, in H_2O or D_2O solution.[77] By plotting these parameters as a function of temperature, at constant nucleotide concentration one obtains sigmoidal curves (Figure 7), highly reminiscent of the melting curves reported in the literature for the aggregates formed in aqueous solution by guanylic acid at pH 8, either from proton NMR or from infrared data.[6,8] By contrast, no transition is observed with another nucleotide, 2′-GMP (Figure 7). Comparison between these sodium-23 results and the proton NMR results displayed earlier (Figure 1) shows that both spectroscopies are sensitive to the same phenomenon, an order-disorder transition as the temperature is raised. If, conversely, the temperature is kept constant, increasing the nucleotide concentration leads to: (a) splitting of the H-8 proton resonance into several lines, whose widths are augmented; (b) a pronounced upfield shift of the ^{23}Na resonance for the Na^+ counterions; (c) a considerable increase in the line widths for these sodium ions.

Before exploiting the latter parameter, we normalize line widths to unit viscosity using the measured viscosities for the solution.[77] A convenient representation of the changes of the ^{23}Na normalized line widths $\Delta\nu_{1/2}^{-1}$ and chemical shifts $\Delta\delta$

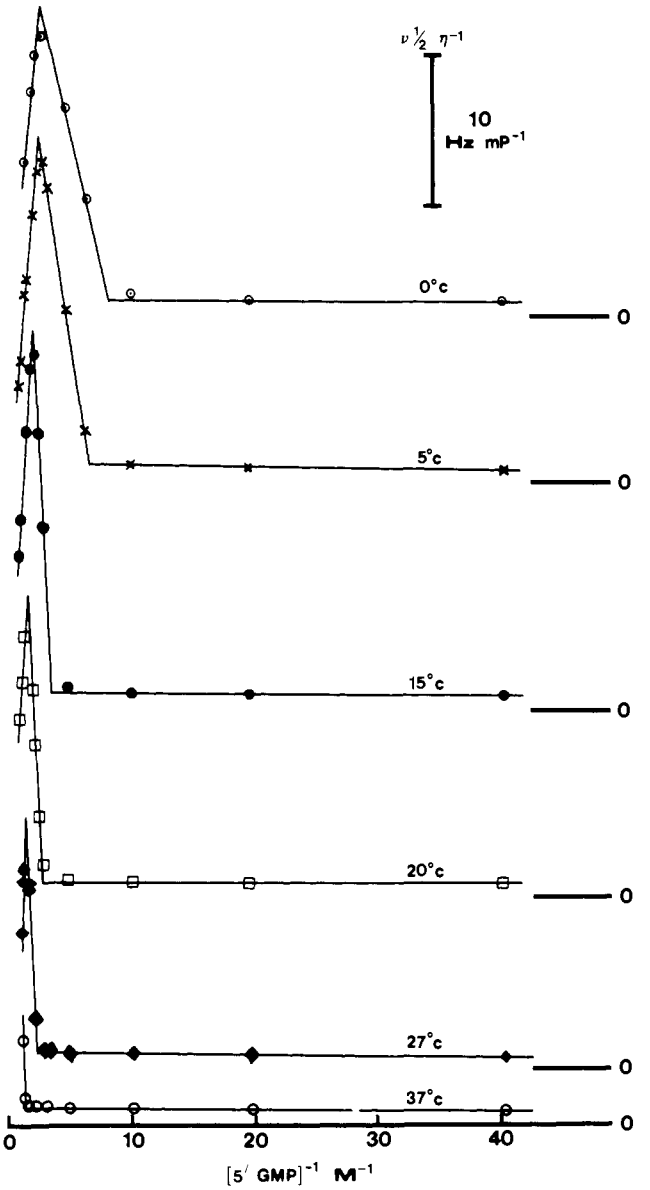


Figure 8. ^{23}Na-reduced line widths $\nu_{1/2}\eta^{-1}$ as a function of reciprocal 5′-GMP concentration in D_2O solution, at various temperatures.

upon self-assembly is to plot them as a function of reciprocal nucleotide concentration[10,95] (Figures 8 and 9). No significant solvent isotope effect (H_2O-D_2O) is seen on either of these observables.[77]

At each temperature, the chemical-shift variation is seen to consist of two straight lines with a sharp intersection: the chemical shift remains approximately constant up to a critical concentration, where it drops to strongly negative values (Figure 9). Correspondingly, the line widths are also invariant *up to the same critical concentration as the chemical shifts*, where they increase abruptly until a maximum is reached at a second critical concentration (Figure 8). We shall denote CC1 and CC2 these two critical concentrations. We shall be examining the significance of CC1 and CC2 in a later section. The critical concentrations CC1 are listed in Table II. There is very good agreement, within the combined uncertainties, between the critical concentrations determined from the line widths and from the chemical shifts.

Deeper insight into the self-assembly process, and sodium binding, is afforded by analysis of the ^{23}Na NMR line shapes, at concentrations above CC1, in the 273-300 K temperature range. The ^{23}Na absorption for Na^+ exchanging between the

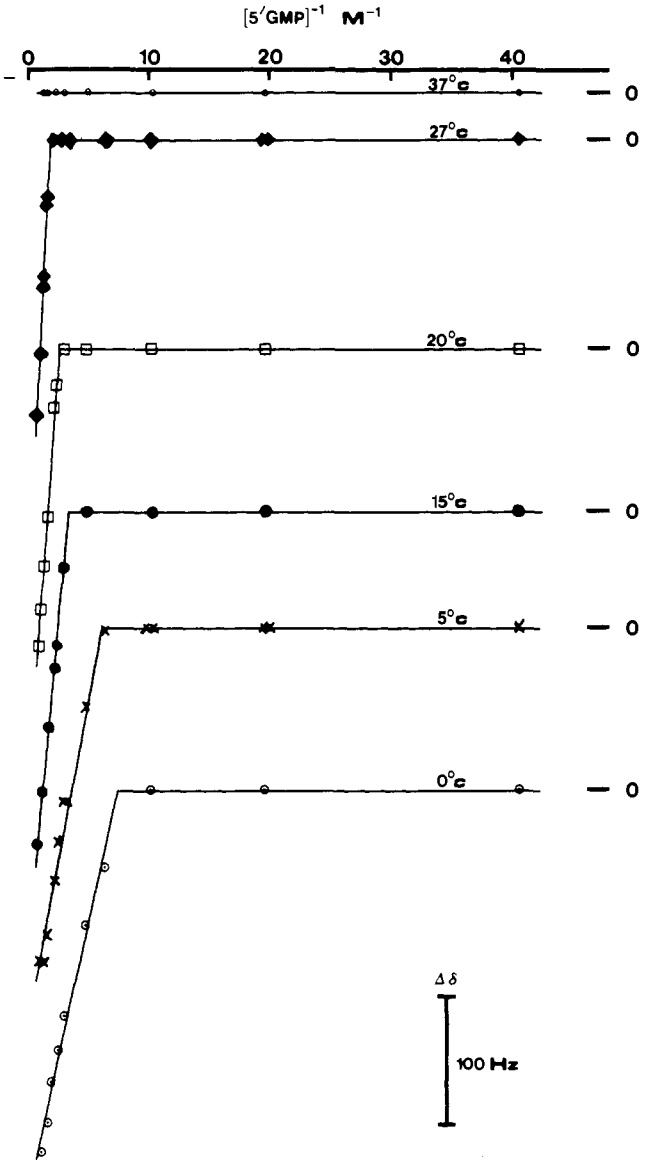


Figure 9. Variation of the ^{23}Na chemical shift as a function of reciprocal 5′-GMP concentration in D_2O solution, at various temperatures.

free state in aqueous solution and the bound state on a slowly reorienting macromolecule is the superposition of two Lorentzians, corresponding to a fast relaxation (60% of the total intensity) and a slow relaxation (40% of the total intensity). Separation of these two components is achieved by full digital deconvolution of the experimental line shape.[13] One thus obtains the values for the product $p_B\chi^2$, where p_B is the mole fraction of sodium ions in the bound state and χ is their quadrupolar coupling constant, together with τ_c, the correlation time for these bound sodium ions (Table III). In order to check the accuracy of this analysis, we have run a series of spectra for the same samples at 62.86 MHz. Given the orders of magnitude for the $p_B\chi^2$ and the τ_c terms in Table III, it is predicted from the appropriate equations[13] that only the slow relaxation rate component should be visible at this higher frequency. This is indeed the case. Whereas at 23.81 MHz the observed sodium line is distinctly non-Lorentzian, consisting of the superposition of two Lorentzian lines with differing widths, at 62.86 MHz only the narrow component (40% of the total intensity) can be seen. Furthermore, the relaxation rates $1/T_{2B}''$ measured at the higher frequency agree well with those calculated from the $p_B\chi^2$ and τ_c values obtained at the lower frequency (Table III).

Scheme I. Self-Assembly of the 5′-Guanosine Monophosphate Dianion in Neutral Aqueous Solution in the Presence of Sodium Cations

regular, ordered structure

α peak, δ peak

a relatively stable form containing two different but equally populated types of ^{1}H-8 and ^{31}P environments.

⇄

regular, ordered structure

β peak

a form containing a single type of ^{1}H-8 and ^{31}P environment.

γ peak

time-averaging of two or more classes of ^{1}H-8 protons and ^{31}P nuclei in rapid chemical exchange.

Comparing the third and the penultimate column, τ_c does not follow the bulk viscosity η, even though η changes by more than 300% between the less and the more viscous solutions. This already suggests that the measured correlation time τ_c cannot be identified with the reorientational correlation time τ_R for the aggregates. If one assumes that the species formed in the sole presence of sodium ions and the potassium-containing aggregates[42] have comparable aggregation numbers and similar structures, then the τ_R values of 9.4 ± 1 ns obtained for the (G_8, K^+, nNa^+) species[42] translate into τ_R values of 16 ns at 25 mP, 25 ns at 40 mP, 38 ns at 60 mP, and 50 ns at 80 mP. The correlation times τ_c listed in Table III *do not follow viscosity,* and they are significantly smaller than the values expected for the reorientation of a (G_8, Na^+, nNa^+) species. The inference is that the correlation times τ_c are predominantly determined by the residence time τ_B of the sodium ions on the aggregates. With $\tau_c^{-1} = \tau_R^{-1} + \tau_B^{-1}$,[96] the resulting τ_B values are also indicated in Table III. Because of accumulated errors, only an order-of-magnitude estimate is obtained; Na^+ cations stay on the aggregate for approximately 30 ns.

Discussion

A conspicuous feature of the aggregates formed by 5′-GMP, Na_2 is the coexistence in the ^{1}H NMR or in the ^{31}P NMR spectra of three singlet resonances: two minor outer resonances and a major inner resonance.[12,13] We have measured their relative intensity ratio R at different temperatures in the ^{31}P spectra (Table IV). It is readily apparent that the $p_B\chi^2$ values, obtained by the deconvolution of the experimental ^{23}Na line shapes, follow a similar trend (Table IV). The correlation between the two sets of parameters is very good, with a correlation coefficient of 0.984 for five points. The temperature dependence of R corresponds to $\Delta H = -17 \pm 2$ kcal·mol^{-1} and to $\Delta S = -63 \pm 6$ cal·mol^{-1}·K^{-1}.

Likewise, ^{1}H and ^{31}P results are interrelated: joint consideration of the two kinds of spectra suggests that the lowest field and the highest field ^{31}P resonances correspond to phosphorus-31 nuclei belonging to the same chemical species as the lowest field (α) and the highest field (δ) H-8 protons, respectively. This chemical species has a regular, ordered structure. The central ^{31}P peak in like manner corresponds to the β and γ ^{1}H-8 resonances. In Scheme I, we thus extend to the phosphorus-31 resonances the same labeling used for the H-8 proton resonances: lowest field ^{31}P peak = α; central ^{31}P resonance = β,γ; highest field ^{31}P peak = δ. This scheme outlines one of the simplest possible interpretations of the ^{31}P and ^{1}H-8 NMR data (this description has been hinted at in the litera-

Table III. Values of $p_b\chi^2$ (±15%), of τ_c (±15%), and of τ_B for a Series of Samples at Various Temperatures[a]

[5′-GMP^{2-}, Na_2^+], M	T, K	η, mP	$1/T_{2B}'$, Hz	$1/T_{2B}''$, Hz	$p_B\chi^2$, 10^{10} Hz^2	τ_c, ns	τ_B, ns
0.152	273	30.0	2 810	573	7.87	15.1	75 ± 40
0.203	273	32.5	2 080	1060	10.5	(6.3)	
0.324	273	40.0	6 800	1700	22.5	12.3	25 ± 15
0.370	273	43.5	7 460	1750	23.9	12.9	25 ± 15
0.450	273	51.0	10 500	1870	29.3	15.7	30 ± 15
0.600	273	75.0	9 920	1830	28.7	15.3	25 ± 15
0.896	273	*b*	9 200	1620	25.4	15.8	
0.152	278	25.0	468	201	1.66	9.2	20 ± 10
0.203	278	27.0	2 780	704	9.01	12.5	50 ± 25
0.324	278	34.0	9 940	1730	27.5	15.8	60 ± 30
0.370	278	36.5	7 990	1750	24.8	13.6	35 ± 20
0.450	278	42.0	7 910	1750	24.7	13.4	30 ± 15
0.600	278	58.0	7 280	1690	23.3	13.0	20 ± 10
0.744	278	80.0	8 460	1660	24.9	14.6	20 ± 10
0.896	278	*b*	6 670	1450	20.6	13.6	
1.00	278	*b*	6 510	1390	19.9	13.8	
0.324	288	24.0	1 870	641	7.26	9.6	25 ± 15
0.450	288	30.0	6 780	1570	21.8	12.9	40 ± 20
0.600	288	41.0	11 900	1800	30.9	17.2	50 ± 25
0.744	288	56.0	12 800	1820	32.2	17.9	40 ± 20
0.896	288	*b*	9 720	1620	26.9	15.9	
1.00	288	*b*	9 560	1490	25.1	16.9	
0.450	293	26.0	1 930	820	8.72	7.7	15 ± 7
0.600	293	35.0	6 270	1410	19.9	13.2	35 ± 20
0.744	293	48.0	6 940	1590	22.3	13.0	25 ± 15
0.896	293	*b*	9 200	1810	25.8	15.6	
1.00	293	*b*	6 360	1440	20.3	13.1	
0.600	300	30.0	3 080	825	10.6	11.6	30 ± 15
0.744	300	40.0	3 340	1180	13.6	9.1	15 ± 7
0.753	300	41.0	4 840	1180	16.0	12.4	25 ± 15
0.800	300	45.0	8 120	1430	22.8	15.6	35 ± 20
0.896	300	*b*	8 940	1500	24.5	16.0	
1.00	300	*b*	(15 000)	(1700)	33.8	20.4	

[a] The fast and slow relaxation rates (±10%) are also indicated. The Larmor frequency is 23.81 MHz. [b] Not determined.

Table IV. Intensity Ratio R (Sum of Outer Peaks vs. Central Peak Intensity) and $p_B\chi^2$ for 5′-GMP^{2-}, $2Na^+$ (0.7 M) as a Function of Temperature

T, K	R	$p_B\chi^2$, 10^{10} Hz^2	T, K	R	$p_B\chi^2$, 10^{10} Hz^2
275	0.37	16.2	290	0.22	12.8
280	0.36	15.7	295	0.16	10.9
285	0.30	13.7			

ture[8]) for the 5′-GMP, Na_2 system. It appears to fit well the ^{31}P and ^1H-8 chemical shifts, line widths, and relative populations.

Scheme I is characterized by the coexistence of species with small values of their reorientational correlation time τ_c (γ peak), and of ordered structures with long τ_c values (β peak and α,δ peaks). The sodium-23 NMR results indeed can be analyzed quite accurately in terms of the coexistence of a fast reorienting and of slow reorienting bound states, which agrees with the characteristic appearance of the plots in Figures 8 and 9 and with the finding of a critical concentration CC1.

Using a simplifying two-state, phase separation model, the standard free energy $\Delta G°$ of self-assembly is given by:[97,98]

$$\Delta G^\circ = RT[1 + (m/n)] \ln (CC1) \qquad (8)$$

where m is the number of counterions Na^+ and n is the number of nucleotide units within the aggregate, so that m/n is the fraction of charge neutralized for each monomer unit within the n-meric aggregate. The term $[1 + (m/n)]$ varies between 1 and 2 according to the degree of ionization of the monomers in the aggregates. We have chosen a value of 5/16 for the m/n ratio, for reasons that will be apparent from the later sections. Also, the system is far from being ideal at the critical concentrations of Table II; therefore, we determine thermodynamic parameters from eq 9, in which activities replace concentrations:

$$\Delta G° = 1.31RT \ln a \qquad (9)$$

We obtain these activities a by multiplying the CC1 values from Table I by the activity coefficients f calculated from the ionic strength I with the Davies formula[99] (eq 10):

$$\log f = -0.5z^2\left(\frac{I^{1/2}}{1 + I^{1/2}} - 0.3I\right) \qquad (10)$$

The ionic strength, taken as $I = (1/2)\Sigma c_i z_i^2$, is probably a slight underestimate because of the high charge of the aggregates, which, on the other hand, have very low concentrations at the critical concentration.

This phenomenological treatment works nicely; the ln a values are indeed linear with reciprocal temperature as implied by eq 9 (correlation coefficient = 0.993 for five points), yielding the following thermodynamic parameters: $\Delta H° = -17 \pm 2$ kcal·mol^{-1} and $\Delta S° = -51 \pm 6$ cal·mol^{-1}·K^{-1}.

These values are very slightly at variance from those reported in a preliminary communication[10] and which were obtained from the use of eq 8 instead of the more correct eq 9. There is excellent apparent agreement between these values, obtained from the chemical shifts and relaxation rates for the Na^+ counterions, and those determined by the completely different route of intensity ratios from ^{31}P spectra for the 5′-GMP^{2-} dianion. However, this could also be an accidental coincidence, with the different methods looking at distinct phenomena, a point which we now examine.

By analogy with the potassium-containing system,[42] and because of the similarity of the ^{23}Na correlation times, *octamers G_8 appear reasonable as the predominant aggregates.* A first possibility is that, at a rather high 5′-GMP concentration of 0.7 M (that of Figures 3 and 5 and Table IV), the $\alpha + \delta$ peaks correspond to octamers, while the central β peak corresponds to tetramers. The correlation obtained with $p_B\chi^2$ (Table IV) implies greater binding (or condensation) of the sodium cations upon the octamers. While such conclusions are qualitatively plausible, we were unable to effect a quantitative analysis using this sole model.

The second interpretation, which we tend to favor, is that the two types of measurements "see" different processes, albeit characterized by very similar parameters; the phenomenological approach of critical concentrations (^{23}Na results) describes predominantly the $2G_4 \rightleftarrows G_8$ equilibrium, while the intensity ratio measured at 0.7 M (^{31}P results) refers to *another* equilibrium, the dimerization of two G_8 units into a hexadecamer: $2G_8 \rightleftarrows G_{16}$. The G_8 species can exist in conformations with C_{4h} or S_8 symmetry, leading to observation of a single resonance for either the H-8 proton or the ^{31}P nucleus in the phosphate group (β peak). Dimerization of G_8 into a hexadecamer may lead to a species with effective twofold symmetry: simple stacking of tetramers will result in two "inner" and two "outer" tetramers, with 1:1 ratio ($\alpha + \delta$ peak). Alternatively, if a sodium cation sandwiched in between two phosphate groups effects the dimerization of two G_8 units into a G_{16} species, the observed spectra would require fast interconversion between the proximal and the distal positions. Whatever the structures present, it is reasonable to assume that, to a good approximation, all the 5′-GMP molecules are aggregated at this rather high 0.7 M concentration. Furthermore, in a similar way as G_8 binds Na^+ cations more than G_4, because of the bringing together of negatively charged phosphate groups, the G_{16} species would display increased sodium binding for a similar reason, thus accounting for the $p_B\chi^2$ variations listed in Table IV.

A third interpretation should be mentioned: the ($\alpha + \delta$) and the β peaks *all* correspond to octameric G_8 structures; indeed, it is possible to group the various G_8 conformers in two distinct manifolds: the stacking of planar tetramers occurring either face-to-face, leading to a single (β) resonance, or face-to-back, in which case diastereotopic α and δ resonances can be expected, at least for certain conformations.

The first two interpretations we have just presented agree with one another in ruling out the possibility that the two coexisting species have identical aggregation numbers, differing only in structure. However, it is impossible to check this point directly, since, as noted above, the correlation time τ_c is not determined by the reorientational time τ_R, but by the residence time τ_B (taking into account the coexistence of G_8, for which $\tau_R \simeq 10$ ns, together with 10-50% G_{16}, for which $\tau_R \simeq 20$ ns, does not affect significantly the values of τ_B given in Table III). A simple test will show that the above description is reasonable: by analogy with the (G_8, K^+, nNa^+) species,[42] one may assume that the octamer binds 5 out of the 16 total Na^+ cations, and that the octamer is indeed, above the critical concentration CC1, the major species. This implies a p_B value of 5/16, above CC1 at each temperature. In other words, we attribute the small variations with temperature of $p_B\chi^2$ at the concentration of 0.7 M (Table IV) to the presence of a small amount of hexadecamers, while the large variations of $p_B\chi^2$ with concentration at a given temperature (for instance, it jumps tenfold from 1.66 to ca. 25 at 278 K when the concentration goes from 0.15 to above 0.3 M), in Table III, are due to the $G_4 \rightleftarrows G_8$ equilibrium.

Using the plateau values for $p_B\chi^2$ of 25-30 $\times 10^{+10}$ from Table III, together with $p_B = 5/16$, yields a quadrupolar coupling constant $\chi = 0.9 \pm 0.1$ MHz. Such values are slightly greater than those corresponding to simple phosphate binding of 0.68 ± 0.08 MHz, in the (G_8, K^+, nNa^+) species;[42] this is consistent with occupation of the core position by one sodium cation in the corresponding (G_8, Na^+, nNa^+) species; with $n = 4$, the core position is characterized by a quadrupolar coupling constant of 1.8 MHz (assuming that the observed χ is the weighted average between the inner and the outer sites). Such a high value, still in the 0.2-2 MHz range of sodium-23 quadrupolar coupling constants,[40] is in good qualitative agreement with the pronounced high-field shift of the ^{23}Na resonance upon aggregation: Kintzinger and Lehn[100] have reported such a χ-δ correlation in sodium cryptates, in line with the theory by Deverell.[101]

Going to the interpretation of the τ_B data in Table III, simple ion pair dissociation-recombination, with a binding constant equal to that in the monomers about 2 M^{-1}, would lead from $\Delta G^{\ddagger} > \Delta G$ and $k_- = KT/h \exp[-(\Delta G^{\ddagger}/RT)]$ to residence times τ_B in the nanosecond to picosecond time range, too small by at least one or two orders of magnitude with respect to the experimental. Another possibility would be for exchange to occur whenever a 5′-GMP unit with the Na^+ cation rigidly ion-paired to it broke away from the oligomer, such as the monomer $\rightleftarrows$ n-mer exchange. Then the applicable ΔG^a energy barrier would have to be at least 15 kcal·mol^{-1} from the chemical-shift differences observed in the ^1H spectrum.[8] This would correspond to $\tau_B > 1.7 \times 10^{-2}$ s, i.e., to a completely different process than the relatively fast sodium exchange monitored by ^{23}Na NMR. A better explanation is provided by other features in our data: let us consider as the source for the ΔG^a barrier the ΔG terms we determine with the phenomenological treatment for sodium-binding cum-aggregation. With $\Delta H \simeq -17$ kcal·mol^{-1} and $\Delta S \simeq -50$ cal·mol^{-1}·K^{-1}, $\Delta G^{\circ} \simeq -3$ kcal·mol^{-1} at 273 K. Taking ΔG^a as the amount at least necessary to overcome this stabilizing energy, say 3 kcal·mol^{-1}, leads to $\tau_B = 16$ ns at 300 K, i.e., to the correct order of magnitude. In short, observation of $\tau_B \simeq$ 30 ns rules out conclusively sodium binding to a single phosphate, and is consistent only with sodium binding by several groups—perhaps a coalition of oxygen and nitrogen atoms from phosphate groups, the ribose sugar, and the guanine base are all involved in the formation of these sodium binding sites. This conclusion should be strongly emphasized: *sodium cation binding contributes directly to the buildup of octamers and hexadecamers.* However, it does not follow from this conclusion that cation release from the aggregates destroys them; whereas sodium cations exchange with rates of 10^7-10^8 s^{-1}, the nucleotide counterions exchange with much slower rate constants, of less than 10^2 s^{-1}.

A point worthy of remark is the magnitude of the *limiting* chemical shift of ^{23}Na in the aggregates. The observed chemical shift of ~ -15 ppm is the weighted average between the limiting values for occupancy of the inner sites, of the outer sites, and for free sodium ions in the solution (or atmospherically condensed sodium ions around the aggregates). With various assumptions made about the limiting chemical shift for the peripheral Na^+ (−20 or −10 ppm), and for atmospherically condensed sodium ions (shifted upfield by 3-5 ppm by the ring currents of the guanines), one comes up with a limiting chemical shift for Na^+ in the central position of the tetramers in the range of −100 to −160 ppm. There are precedents in the literature for such pronounced changes of the sodium-23 chemical shift with the environment of the sodium ion.[102] Such a value is uniquely consistent with Na^+ inclusion in the central cavity of the tetramers, and indicates that it coordinates there with atoms of strongly differing types: beside the four O-6 oxygens, OH oxygens or guanine nitrogen appear to be also involved. Correspondingly,[100,101] the quadrupolar coupling constant for this central sodium has also a very high value of 1.8 MHz.

True, the use of sodium-23 NMR suffers from the disadvantage that the Na^+ cations exchange fast between several binding sites, as well as with the unbound or atmospherically condensed ions. Despite this limitation, it is a very useful adjunct to the NMR of covalently bound nuclei such as ^1H and ^{31}P: it provides direct information about the local environment of the Na^+ ions in the bound sites. From the large magnitudes of the limiting chemical shift *and* quadrupolar coupling constant, we have inferred occupancy of the central cavity of the tetramers by sodium cations. In addition, ^{23}Na NMR provides values of the correlation times τ_c for bound sodium ions. By using an attribution for the ordered structures that is strongly suggested by the ^1H and ^{31}P evidence (Scheme I), and by analogy with the (G_8, K^+, nNa^+) structures formed in the presence of potassium ions,[42] we could derive values τ_B for the residence times of Na^+ ions in the bound state(s). They show that Na^+ ions are indeed directly involved in the stabilization of G_8 and G_{16} ordered structures.

Our phenomenological analysis of the increase in ^{23}Na NMR relaxation rates above a critical concentration CC1 is made with a two-state model. It lumps together the two kinds of ordered structures, corresponding to the β peak and to the ($\alpha + \delta$) (G_8 and G_{16}); from the viewpoint of ^{23}Na NMR, these are seen as a single entity characterized by a single value of the limiting relaxation rate, because the corresponding correlation times are very similar, differing (at most) by a factor 2. Despite the simplification inherent in the use of such a phase-separation model, it is firmly based in the precise experimental determination of a critical concentration CC1 at each of several temperatures (Figures 8 and 9).

Another and altogether different model can be applied successfully to the data, and it leads to very similar conclusions. The order-disorder transition undergone by 5'-GMP has cooperative character, as shown by sigmoidal curves such as in Figure 7. Using a plateau value of $30 \times 10^{+10}$ for the term $p_B\chi^2$ (Table III), percentages of saturation ($y/(1-y)$) can be determined at each temperature, in the rising portion of the $p_B\chi^2$ vs. [Na^+] curve. They fit the Hill equation:[103]

$$\ln (y/(1-y)) = \ln K + n \ln [Na^+] \qquad (11)$$

The corresponding parameters are listed in Table V.

The resulting Hill coefficient $n = 6.1 \pm 0.7$ indicates positive cooperativity for sodium binding to the 5'-GMP aggregates, and its value is nicely consistent with the above model, in which octamers with a total of five binding sites (one inner plus four outer sites) as the major species coexist with hexadecamers with ten binding sites (two inner plus eight outer sites), as the minor species. The equilibrium constant K corresponds to $\Delta H = -53 \pm 5$ kcal·mol^{-1} and $\Delta S = -180 \pm 20$ cal·mol^{-1}·K^{-1} ($\rho = 0.992$ for $\Delta G = \Delta H - T\Delta S$ at the four temperatures of Table IV). While the signs of these thermodynamic parameters are of course adequate for a self-ordering process, their values should be compared neither to the thermodynamic parameters obtained from critical concentrations, which monitor the $2G_4 \rightleftarrows G_8$ equilibrium, nor to those obtained from ^{31}P intensity measurements, which monitor the $2G_8 \rightleftarrows G_{16}$ equilibrium. We are looking with the Hill formalism at the overall process of sodium binding to a $G_8 + G_{16}$ mixture. One may note here that analysis of the melting curves[77] exemplified by Figure 7 with the crude equation:[104]

$$\left(\frac{dx}{dT}\right)_{T_m} = \frac{\Delta H}{4RT_m^2} \qquad (12)$$

yields $\Delta H \simeq -45$ kcal·mol^{-1}, in semiquantitative accord with the result from the Hill plot.

A recurring feature in our results is the finding that self-assembly is *enthalpy driven*, since the entropy change upon self-association is strongly negative. This is reminiscent of the self-assembly of polynucleotides. Furthermore, this differentiates the 5'-GMP self-assembly from aggregates of micellar type: we are not dealing here with micelles (despite recourse in an earlier report to a formalism used to describe micelles[95]); the formation of micelles is characteristically entropy driven; their monomer concentration remains equal to the critical micelle concentration (cmc) above the cmc; the usual cmc's are smaller by two or three orders of magnitude than the critical concentrations we see. Likewise, the aggregation numbers for typical micelles are of the order of 100,[105-107] i.e., markedly greater than those applicable to 5'-GMP self-assembly. The value of the enthalpy change found here for the $2G_4 \rightarrow G_8$ process, viz. -22 to -26 kcal·mol^{-1}, is strikingly similar to values characteristic of interactions between tetranucleotides such as -19 kcal·mol^{-1} for the UUCC-UG_mAA interaction.[108,109] There is a balance between the loss of translational entropy for the nucleotides in the $2G_4 \rightleftarrows G_8$ and $2G_8 \rightleftarrows G_{16}$ equilibria, and the gain of translational entropy for some of the water molecules released by the alkali metal cations as they bind to the aggregates; that sodium cations are only partly dehydrated upon site binding to the aggregates is apparent from the magnitude of the observed quadrupolar coupling constant. However, the observed entropies are strongly negative. This would appear to imply a structuring of solvent molecules about the aggregates. Self-assembly of the 5'-GMP nucleotide does not occur because of the attendant release of numerous water solvent molecules. The D_2O-H_2O solvent isotope effect is below the limit of experimental uncertainty. Self-assembly of 5'-GMP, unlike a normal stacking interaction,[61] is not determined predominantly by hydrophobic forces. Cation binding plays the important role, together with and reinforcing the hydrogen bonding of the guanines into planar tetramers.

Table V. Hill Coefficient n and ln K as a Function of Temperature for Self-Assembly of 5'-GMP, Na_2

T, K	n	ln K	T, K	n	ln K
278	6.86	5.33	293	5.38	−0.30
288	6.45	1.70	300	5.57	−1.61

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