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Effect of the Base Stacking Association on the Phosphate Ionization of 2′-Deoxyguanosine 5′-Monophosphate

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The concentration dependence of ^{31}P chemical shifts at neutral pH has been measured for 2′-deoxyguanosine 5′-monophosphate and deoxyribose 5-monophosphate. The chemical shifts are sensitive to the variation of concentration, and are affected by the presence or absence of the base. The variations of ^{31}P chemical shifts correspond to those of $pK_{a'}$ values. The concentration dependence of ^{31}P chemical shift of deoxyribose 5-monophosphate can be explained by the ionic strength change accompanied by the concentration change, whereas that of the nucleotide can not be interpreted with the aid of it alone. The phosphate group of the nucleotide ionizes more readily at higher concentration. There is an additional effect other than ionic strength on the $pK_{a'}$ eand it is caused by the presence of the base. The appearance of the concentration effect owing to the base is attributable to the base stacking association. The measurements at different temperatures support this conclusion.

The association of naturally occurring nucleotides in aqueous solution has been studied by various experimental techniques such as microcalorimetry[1] and UV[2-4] and NMR[5-12] spectroscopy. It is now generally accepted that base stacking is a possible mode of self-association of nucleotides and it proceeds beyond dimer stages.[6,7,9] It is also well established that the stacking affinity of the nucleobases contributes greatly to the free energy sustaining the nucleic acid secondary structure.[13,14] The majority of studies have based on the concentration dependence of ^1H NMR chemical shift. A change of concentration should, however, affect the phosphate ionization of nucleotides through a change of ionic strength of solutions. The base stacking is also expected to affect the phosphate ionization through the formation of new molecular assemblies, since assembly of electrolytes in solution affects the pK_a value.[15] It seems that an understanding of the effect of the base stacking association on the phosphate ionization and on the conformation of nucleotides would promote the understanding of the secondary structure of DNA. In this work, we will focus on the phosphate ionization.

^{31}P NMR is a useful technique for studying an ionization state of phosphate group, since the chemical shift is a very sensitive function of pH.[16,17] The effect of pH on ^{31}P chemical shifts of mononucleotides has been studied by Cozzone and Jardetzky,[17] where the nucleotides were 0.5×10^{-2} M or less to avoid possible effects of self-associations.

In order to clarify the effect of the base stacking on the phosphate ionization of nucleotides, it is necessary to differentiate the effect of ionic strength from that of self-association both of which are included in the concentration dependence of ^{31}P chemical shifts. For the purpose of this subject, we will study, based on ^{31}P and ^1H NMR, the effect of concentration, ionic strength, and temperature on the phosphate ionization of 2′-deoxyguanosine 5′-monophosphate (5′-dGMP) and deoxyribose 5-monophosphate (5-dRP).

Experimental

Sample Preparation. Sodium salts of 5′-dGMP and 5-dRP were purchased from Sigma Chemical Co. Deuterium oxide (isotope purity >99.7%) was from Merck Sharp& Dohme, Canada Ltd.. Paramagnetic contaminants were removed from NMR samples by chromatography on Chelex-100 (Bio-Rad Laboratory), followed by lyophilization. All NMR sample tubes were soaked in 40% nitric acid prior to use. NMR samples were prepared in D_2O, containing 0.5×10^{-2} M† ethylenediaminetetraacetic acid (EDTA). The addition of EDTA did not affect the ^{31}P and ^1H chemical shifts. The pH of solution was adjusted to a desired value by an addition of very small amount of concentrated DCl and/or NaOD solutions in D_2O. The pH was measured with an accuracy of ±0.01 pH unit, both before and after the NMR experiments, using a Toko TP-101 pH meter with a combination electrode of Type CE-103 which can be directly used for NMR sample tube. No correction was made for the deuterium isotope effect on pH value. Concentrations were determined by dry weight and then adjusted to desired values, typically from 0.5×10^{-2} M to 0.2 M, by dilution using a volumetrically variable pipette 4710 (Eppendolf).

NMR Measurements. ^{31}P NMR spectra were obtained in the Fourier transform mode on a JEOL PS-100 NMR spectrometer operating at 40.5 MHz, equipped with a PFT-100 Fourier transform system and a JEOL EC-100 computer. Heteronuclear proton noise decoupling was achieved with a JEOL JNM HD-SC. The spectrometer was field-frequency locked on the deuterium resonance of deuterium oxide used as a solvent. ^{31}P chemical shifts were measured in Hz from 85% H_3PO_4 as an external reference, without any correction of bulk susceptibility. Digital resolution was 0.2 Hz.

Fourier transformed ^1H NMR spectra were taken at 100 MHz on the same spectrometer. All ^1H chemical shifts were measured from 2-methyl-2-propanol as an internal reference. Although this reference is known to be shifted upfield in the presence of high concentration of aromatic solute,[18] the shift is negligibly small in the concentration range of our experiments (up to 0.2 M).

All spectra were recorded at 27 °C, unless specified other-

† 1 M=1 mol dm^{-3}.

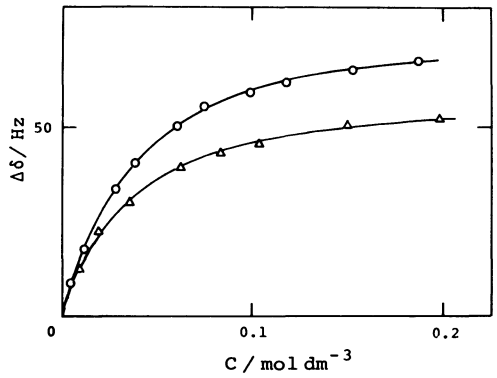

Fig. 1. Variation of the ^{31}P chemical shift differences ($\Delta\delta$) for 5'-dGMP(○) and 5-dRP(△) as a function of concentration(C) at pH 6.60, where $\Delta\delta$ is the difference between the ^{31}P chemical shift at each concentration and that at an infinite dilution.

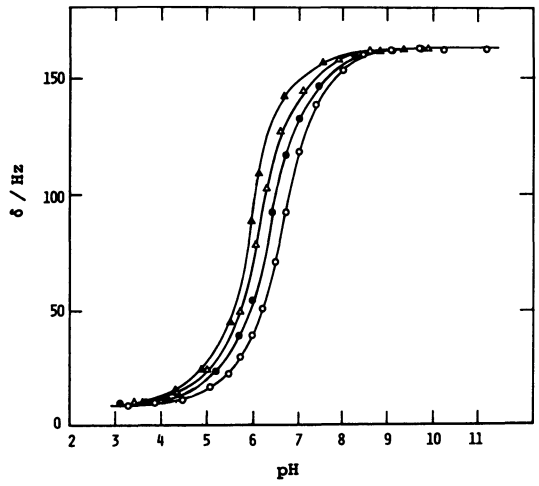

Fig. 2. ^{31}P chemical shift(δ) of 5'-dGMP as a function of pH at various 5'-dGMP concentrations, 0.5×10^{-2} M(○), 4×10^{-2} M(●), 0.1 M(△), and 0.2 M (▲).

wise. A JES VT-3 temperature controller was used for the stabilization, with an accuracy of ±2 °C.

Results and Discussion

Concentration dependence of ^{31}P chemical shifts of 5'-dGMP and 5-dRP was measured at a constant pH (6.60) near the pK_a for secondary ionization of phosphate groups (Fig.1). The ^{31}P resonances of these compounds show remarkable downfield shifts with increasing concentration. ^{31}P chemical shifts of these compounds are identical with each other at lower concentration (<0.02 M), but they are differentiated at higher concentration and the difference increases with increasing concentration. The magnitude of shift is larger for 5'-dGMP than for 5-dRP.

In order to clarify whether the concentration dependence of the chemical shift of each compound is owing to a change of pK_a value or not, titration curves were measured for 5'-dGMP at various concentrations (Fig. 2). The pK_a value decreased with increasing concentration. The ^{31}P resonance at pH 6.60 undergoes a remarkable downfield shift with a small decrease of the pK_a value. At an alkaline pH (>9), the ^{31}P chemical shift is independent of the concentration. It was found that 5'-dGMP and 5-dRP have the same thermodynamic pK_a (6.87±0.01) and chemical shift at an infinite dilution (65±0.5 Hz). The variation of chemical shifts of these compounds with concentration corresponds to the change of pK_a value. The concentration dependence of ^{31}P chemical shifts in Fig. 1, therefore, shows that the phosphate group of 5'-dGMP ionizes more readily than that of 5-dRP. The presence of base moiety may influence on the ^{31}P chemical shift.

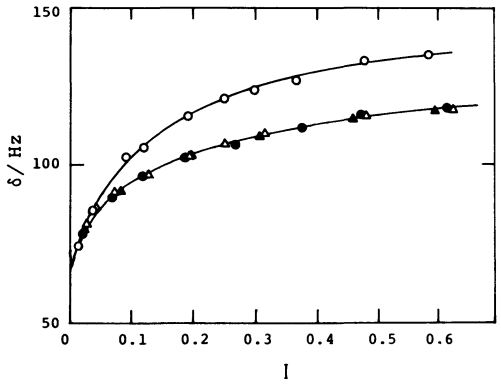

Fig. 3. Dependence of ^{31}P chemical shifts(δ) of 5'-dGMP (circle) and 5-dRP(triangle) on ionic strength (I) at pH 6.60. Open symbols, the ionic strengths calculated from the concentration of each compound in Fig. 1; filled symbols, those adjusted by adding NaCl to 0.5×10^{-2} M solution of each compound.

The conditional pK_a(p$K_{a'}$) changes with concentration by the change in the activity coefficient induced by that of the ionic strength.[15,19] To estimate the extent of this effect, the dependence of ^{31}P chemical shift on the ionic strength is observed at pH 6.60 by adding neutral salt (NaCl) to the 0.5×10^{-2} M solution of each compound (Fig. 3, all of filled symbols). At this concentration, the degree of self-association of nucleotides is negligibly small. The ionic strength dependence of ^{31}P chemical shift in this case is little affected by the presence of the base. In Fig. 3 is also shown the dependence of ^{31}P chemical shifts of both compounds on the ionic strength in which the ionic strength is changed with its own concentration (open symbols). As can be seen in Fig. 3, ^{31}P chemical shift change of 5-dRP lacking the base moiety depend only on the ionic strength, while those of 5'-dGMP can not be interpreted by the ionic strength alone. This also indicates that the concentration dependence of ^{31}P chemical shift of 5-dRP represents an concentration effect on the phosphate ionization of the nucleotide in the case where any self-association does not occur. There is an additional effect other than ionic strength in the concentration effect on the phosphate ionization of the nucleotide, which should be owing to the existence of base moiety of the nucleotide.

Above results suggest that the additional effect is

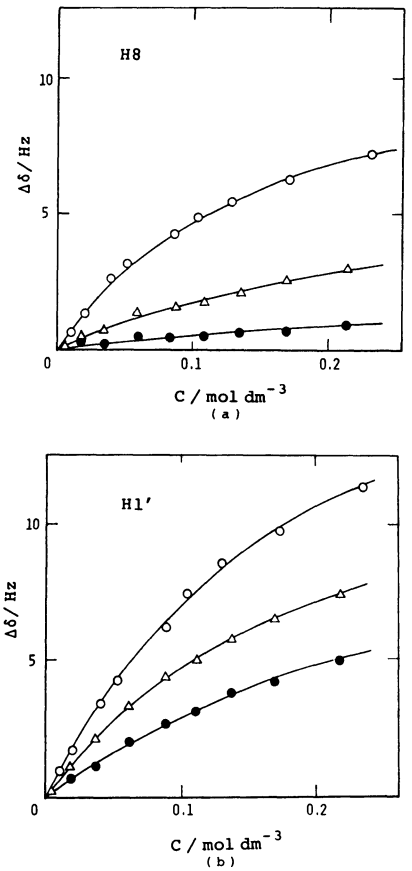

Fig. 4. Effect of temperature on the concentration(C) dependence of H-8(a) and H-1′(b) ^{1}H chemical shift differences($\Delta\delta$) of 5′-dGMP at pH 6.60. 27 °C(○); 45 °C(△); 60 °C(●). $\Delta\delta$ is the difference between the chemical shift of a particular proton at each concentration and that at an infinite dilution.

owing to the formation of the base stacking aggregates. Effects of temperature on the phosphate ionization of the nucleotide were studied to confirm this view. It is known that base stacking aggregates break down with increasing temperature.[11] First the concentration dependence was measured for H-8 and H-1′ chemical shifts of 5′-dGMP at three temperature, 27, 45, and 60 °C (Fig. 4). These resonances shift upfield with increasing concentration. The chemical shift changes are consistent with the isodesmic model of indefinite non-cooperative stacking.[20] The magnitude of shift decreases with increasing temperature. From the concentration dependence of ^{1}H chemical shifts at each temperature, the association constants (K) for the base stacking were estimated with the aid of the isodesmic model (Table 1). The K values derived from both ^{1}H chemical shifts decrease with increasing temperature, indicating the decrease of the stacking affinity.

If the additional reduction of p$K_{a'}$ value for the phosphate group of 5′-dGMP with increasing concentration is owing to the base stacking association, the magnitude of ^{31}P shift at pH 6.60 should decrease with increasing temperature. To verify this idea, the concentration dependence of ^{31}P chemical shift of 5′-dGMP was observed at different temperatures, 27, 45, 60 °C (Fig. 5). With increasing temperature, a reduction of induced shift is observed and the variation of shift with concentration at 60 °C agrees well with that of 5-dRP at 27 °C, indicating a validity of the interpretation based on the base stacking association. An essential effect of temperature on the p$K_{a'}$ value of 5′dGMP must be considered, but it was very small (d pK_a/ d T=0.5×10^{-3}).[21] Previous ^{1}H NMR studies have demonstrated that temperature variation has little effect on the nucleotide conformation.[22-24] There is, therefore, little contribution of conformational changes to the temperature variation of ^{31}P chemical shift.

Table 1. Effect of temperature on the association constant K[a] for the base stacking of 5′-dGMP at pH 6.60

T/°C	K/M^{-1}		K_{av}/M^{-1} [b]
	H-8	H-1′	
27	2.6	2.8	2.7
45	2.0	2.2	2.1
60	1.2	1.4	1.3

a) Estimated errors for these association constants are 10—15%. b) K_{av} is the mean of the individual results. (1 M = 1 mol dm^{-3})

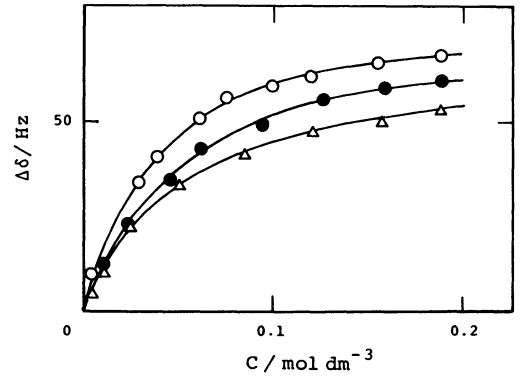

Fig. 5. Effect of temperature on the concentration(C) dependence of the ^{31}P chemical shift differences($\Delta\delta$) of 5′-dGMP at pH 6.60. 27 °C(○); 45 °C(●); 60 °C(△). $\Delta\delta$ is defined as in Fig. 2.

Base stacking should cause a change of nucleotide conformation: the ribose-phosphate backbone,[24-26] the ribose ring[25,26] and the ribose-base torsion.[26-29] One can postulate that ^{31}P chemical shift could be affected by these changes. However, the influence of these conformational changes is not large enough to affect significantly ionization of the phosphate groups, since the same pK_a value can be obtained from the titration curves for various nucleoside 5′-monophosphates with different conformation.[17,24] Identical percentages of the preferred "gauche-gauche" rotamers around C$_{5'}$–O$_{5'}$ and C$_{4'}$–C$_{5'}$ bonds are found for various nucleoside-5′-monophosphates.[24] Although a change of the rotamer populations around the C$_{5'}$–O$_{5'}$ and C$_{4'}$–C$_{5'}$ bonds, determining

the statistically preferred orientation of the 5′-phosphate group, should affect the chemical shift of the corresponding ^{31}P signal, constancy of conformer populations was found from the measurements of coupling constants $^{3}J_{PH}$ (*ca.* 4.8 Hz) and $^{4}J_{PH}$ (*ca.* 1.5 Hz) for 5′-dGMP over the range of experimental concentration at pH 6.60.

The effects on the ^{31}P shift of the ring current fields, as well as the diamagnetic and paramagnetic components of the atomic magnetic anisotropy, seem also insignificant, since ^{31}P chemical shifts are independent of concentration at alkaline pH (>9) (Fig. 2). Even in this pH region, base stacking can also occur.[7]

The effect of concentration on the 5′-phosphate ionization of 5′-dGMP should be attributed to the molecular condensation effect owing to the base stacking association.

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