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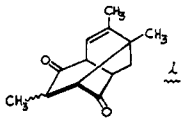

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# Titration Properties of Homodinucleoside Monophosphates. Determination of Overlapping Ionization Constants and Intramolecular Stacking Equilibrium Quotients of ApA, CpC, GpG, and UpU

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**Abstract:** In order to study the effect of ionization (protonation or deprotonation) upon the base-base stacking properties of homoribodinucleotides, the overlapping p$K$ values which differ by less than 2 were determined for four homodinucleoside monophosphates by means of a computer-assisted iterative least-squares treatment of spectrophotometric titration data. The p$K$ of component nucleic acid base residues of the four homodimers in the fully unstacked conformation was approximated by $\frac{1}{4}[pK_{3'\text{-mononucleotide}} + pK_{5'\text{-mononucleotide}} + 2pK_{\text{nucleoside}}]$. These were used to estimate the intramolecular stacking equilibrium quotients for the dimers at neutral pH and for the half-ionized form. The proportion of stacked species in the half-ionized molecules of a homodimer is found to be almost the same as that in the corresponding un-ionized dimer and is even enhanced in the case of GpG with the protonated guanine in the 3′-linked nucleoside as compared to that of the neutral GpG.

Although the acid-base chemistry of nucleic acid bases is well known at the monomer level, protonation-deprotonation reactions of oligonucleotides have been much less investigated. Known examples include ApA,[1,2] ApU,[1] UpA,[1] CpC,[3,4] GpG,[5] GpU,[6] GpUp,[6] UpC,[7] and UpU[1,7] as well as some of higher oligomers.[3,8-10] The possibility of creating and stabilizing a half-protonated form of dinucleoside monophosphates[4,6,11,12] by interaction of two side-chain bases, one protonated and the other un-ionized, has prompted further study of the conformational characteristics of half-protonated homodinucleoside monophosphates. First, in this paper spectrophotometric titration of the diacidic bases (ApA, CpC, and GpG) and dibasic acids (GpG and UpU) was selected as the tool to investigate the molecular behavior. No macroscopic ionization constants

$K_1$ and $K_2$ were known for these homodinucleotides until we published basic $pK_1$ and $pK_2$ for GpG.[5,21] The present paper describes similar determinations for a few other homodinucleotides. A knowledge of $pK_1$ and $pK_2$ can be used to give an estimate of the ratio of stacked to unstacked species of unionized and half-ionized dinucleotides if one makes some approximations. The clarification of these suppositions will be made in the text. Until now, the stacking equilibria of half-ionized homodinucleotides have not been studied except for some preliminary observations that half-protonated ApA has been erroneously concluded to exist as an unstacked form at 20 °C.[1] Secondly, in the following paper,[13] by combining the thermodynamic data, based on the analysis of the temperature-dependent optical properties, and the stepwise $pK$ values, determination of stacking equilibrium quotients for the unionized and half-ionized homodimers will be discussed separately. The agreement between the results on stacking equilibrium quotients of this work and those in the following paper is good, in view of the differences in experimental approach and experimental errors.

## Experimental Section

**Materials.** Pure ApA, CpC, GpG, and UpU were prepared by the method as reported in previous papers.[14,15] The purity of materials was examined by paper chromatography in two solvent systems before physical measurements. Nucleosides (Ado, Cyd, Guo, and Urd) and their 5′-phosphates were purchased from Boehringer Mannheim GmbH, Mannheim. Nucleoside 2′- and 3′-phosphates were separated from the mixtures in this laboratory. All the other chemicals used in this study were analytical grade.

**Methods.** Changes in absorbance due to ionization of nucleic acid bases as a function of pH were measured as difference spectra (ionization difference spectra), $\Delta A = A(\text{pH}) - A(\text{pH } 6.0)$ as previously described.[5]

**(a) $pK$ Determinations of Nucleosides and Mononucleotides.** All measurements were made by the spectrophotometric method. Solutions were made in a series of appropriate buffers in the presence of 0.1 M NaCl at 25 °C, standardized with a glass electrode. This series decreased in pH down to values where the change in the difference spectrum, corresponding to the step of protonation under study, ceased; and, similarly, it increased toward the alkaline direction. The $\Delta A$ values were read at a wavelength where the charged and uncharged species showed a maximum difference in extinction coefficient. Measurements were rejected and the experiment was repeated if a spread[16] in $pK$ values was not within 0.04 unit. Replicate experiments were carried out on a number of nucleosides and mononucleotides. The precision of the $pK$ values can be taken as of the order of ±0.03.

**(b) $pK$ Determinations of Dinucleoside Monophosphates.** A desired amount of the lyophilized dinucleotide was dissolved in glass-distilled water to give an approximately $1\text{–}5 \times 10^{-4}$ M solution. Equal volumes of this solution and an appropriate buffer solution (0.02 M) containing 0.2 M NaCl were mixed to obtain $1\text{–}5 \times 10^{-4}$ M solutions of the sample in the buffer solutions (final ionic strength 0.10 M). The absorption spectrum of each of these solutions was measured against the sample solution at neutral pH. The pH of the sample solution was determined before and after the spectral measurement. The spectrophotometric titrations of GpG were done as previously described.[5] Not less than two independent titrations, with more than 15 data points each, were made in the pH range 0.5–7 for ApA, CpC, and GpG and 7–11.5 for GpG and UpU. Duplicate titrations of dinucleotides agree to ±1%, a precision comparable to those of nucleosides and mononucleotides.

**Instrumentation.** (a) Absorption measurements were made on a Hitachi Model 124 spectrophotometer. For spectrophotometric titrations the thermostatically controlled cell holder (Komatsu Electronics Inc., Tokyo) was used and the thermistor was inserted in the solution in the neck of the cell.

(b) The pH of the solution in the cuvette was measured by means of a Radiometer combination electrode No. GK2301C measuring the temperature range 0–60 °C that was used in conjunction with a Radiometer expanded scale pH meter Model PHM26 (Radiometer, Copenhagen). The pH meter was standardized at the required temperature (25 °C) before and after each set of measurements.

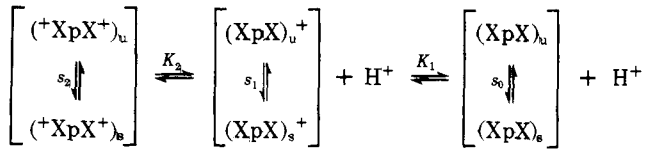
## Results and Discussion

**Determination of Overlapping $pK$ Values of Dinucleotides.** A knowledge of the ionization constants of a dinucleotide is of value both for purposes of characterization and for planning further work on the conformational equilibria. Values of the constants may be used to calculate the fraction of the stacked and unstacked molecular species and to extract the intensive molecular parameters for half-ionized species.

**(a) Basic $pK$ Values Representing Protons Gained by the "Un-ionized" Molecule, XpX.** The specific site of protonation on nucleic acid bases differs for various nucleosides. The most basic position in the simple adenine derivatives is N-1 and it may be the first to be protonated.[17] Similarly, based on methylation studies, N-3 in cytosine and N-7 in guanine are the most likely protonation sites in the simple nucleosides and nucleotides.[17] For oligonucleotides information of such nature is limited and that which is available only pertains to a few cases, two based on x-ray crystallographic studies[18–20] and one based on methylation studies.[12] Protonation may be thus expected to occur at the same sites in di- and other higher oligonucleotides; however, equilibria may exist involving nonprotonated, monoprotonated, and diprotonated forms in the case of a dinucleotide.

It is now suggested that XpX undergoes the stepwise protonation, although unequivocal experimental evidence for the existence of half-protonated species is lacking. XpX is subject to the equilibria illustrated in Scheme I, where "s" and "u"

Scheme I




denote "stacked" and "unstacked", respectively. Scheme I representing the protonation of XpX involves initial protonation at one of the two base residues. This step is followed by the second protonation to form $^+XpX^+$. It should be noted that XpX has two basic centers but is not of prime importance to know which base moiety first accepts the proton in aqueous solution at the moment. However, the value of the ionization constant will, of course, depend on whether 3′ base or 5′ base is protonated first. Thus, a comparison of the macroscopic ionization constants ($K_1$ and $K_2$) of XpX should be taken with some reserve.

No stepwise $pK$ values of any homooligonucleotides had been recorded up to late 1975.[5,21] No difficulty was found in titrating spectrophotometrically XpX because N-glycosidic hydrolysis or transesterification of the phosphodiester group, which would upset the equilibria shown in Scheme I, was inappreciable during the time in which the measurements were performed. Three homodinucleotides, ApA, CpC, and GpG, have been studied. The absorption spectra of ApA and protonated ApA were similar and resulted in small differences during the acid titration, suggesting use of difference spectrophotometry. The advantage was the increased difference between the spectra of the ApA at neutral pH and that at low pH by the use of a higher concentration of ApA up to $4.5 \times 10^{-4}$ M. A series of the difference spectra of ApA, adjusted to an appropriate pH, was recorded between 280 and 300 nm. The blank was $4.5 \times 10^{-4}$ M ApA at neutral pH. These difference spectra showed a maximum at 284 nm. When spectrophotometric titrations are carried out for CpC and GpG, their spectra undergo marked changes both in $\lambda_{max}$ and intensity.

Table I. Ionization of Homodinucleotides at 25 °C

| Dimer | Protons gained | | | | Protons lost | | | |
|---|---|---|---|---|---|---|---|---|
| | $pK_1$ | $pK_2$ | $pK_{app}$[a] | $\Delta pK$[b] | $pK_1$ | $pK_2$ | $pK_{app}$[a] | $\Delta pK$[b] |
| ApA | $3.89_6 \pm 0.07$ | $3.02_5 \pm 0.07$ | $3.46_1 \pm 0.07$ | $0.87_1 \pm 0.07$ | | | | |
| CpC | $4.51_9 \pm 0.05$ | $3.66_5 \pm 0.05$ | $4.09_2 \pm 0.05$ | $0.85_4 \pm 0.05$ | | | | |
| GpG | $2.50_5 \pm 0.03$ | $1.48_5 \pm 0.03$ | $1.99_5 \pm 0.03$ | $1.02_0 \pm 0.03$ | $9.75_6$ | $9.15_6$ | $9.45_6 \pm 0.04$ | 0.60 |
| UpU | | | | | $9.61_2$ | $9.01_2$ | $9.31_2 \pm 0.03$ | 0.60 |

[a] $pK_{app} = (pH)_{\alpha = 1/2} = \frac{1}{2}(pK_1 + pK_2)$. [b] $\Delta pK = pK_1 - pK_2$.

The iterative least-squares method has been used to extract the overlapping $pK_1$ and $pK_2$ values, as well as the intensive parameter, molar extinction coefficient, for half-protonated XpX.[22] Using synthetic data containing random errors, we found that whenever our program converged, it did so to give the same solution regardless of the initial guesses of $pK_1$ and $pK_2$. In cases where $\Delta pK = pK_1 - pK_2$ is close to 0.6, the iteration will generally not converge.[23] The iterative least-squares best values of $pK_1$ and $pK_2$ were determined for ApA, CpC, and GpG. The average values (two or three runs) of the overall ionization constants are shown in Table I. The uncertainties are the standard deviations. Some preliminary results on GpG obtained in this investigation were previously reported.[5] Additional data on GpG are given in ref 5; in order to facilitate comparison, some of the data have been included in the present tables.

Inspection of Table I reveals that homodinucleotides are distinctly weaker bases than the corresponding monomer, as measured by $pK$ difference, $pK_{monomer} - pK_{app}$. The base-weakening effect can be attributed to appreciable intramolecular stacking interactions in the unprotonated dimer conformation (see Appendix). The pH range over which ionization was observed was also broader than that of a monoacidic base and is indicative of a stacked structure. The $pK_1$ and $pK_2$ values are meaningless for purpose of direct comparison because they are a composite constant involving both stacked and unstacked species as shown in Scheme I. Detailed discussion will be seen in a later section.

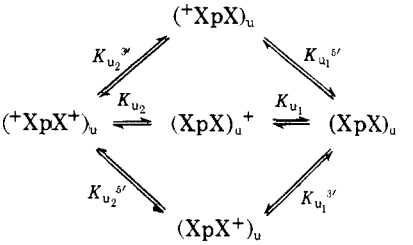
**(b) Acidic $pK$ Values Representing Protons Lost by the "Un-ionized" Molecule, XpX.** Guanine and uracil residues each have one titrable group over the range pH 7–12 so that GpG and UpU undergo the acid-base reactions on titration with alkali, in accordance with the equilibrium scheme similar to that shown in Scheme I. Convergencies to the correct solutions for $pK_1$ and $pK_2$ were not realized for GpG and UpU so that the $K_2/K_1$ values are expected to be quite close to the statistical factor of 4. Actually, the titrations of these dimers with alkali could be fitted to the simple Henderson-Hasselbach equation. Computer analysis of the data by the use of our least-squares program for a single $pK$ process gave $pK_{app} = 9.45_6$ for GpG and $pK_{app} = 9.31_2$ for UpU, with values for the standard error of fit of 0.04 and 0.03, respectively. The $pK_{app}$ value found for UpU is in good agreement with that found in earlier titrations[1] but somewhat less than that reported by Clauwaert and Stockx.[7] We thus obtained values of acidic $pK_1$ and $pK_2$ for GpG and UpU by making them respectively 0.3 $pK$ units higher and lower than the corresponding apparent $pK$ values. The results are included in Table I. The decrease in apparent acidity of the dimers as compared with their constituent monomers is also noted for GpG and UpU.

**Evaluation of the Stacking Equilibrium Quotients, $s_0$ and $s_1$. (a) Theoretical Consideration.** This investigation was initiated with the primary objective being to provide a quantitative measure for the relative stability of the half-ionized homodimers. It has been shown that the result of intramolecular interactions forms, at neutral pH, a folded or stacked structure with a conformation in which each nucleoside unit has an anti conformation and 3′-5′ right-handed screw sense.[26] However, experimental evidence for the stacking equilibrium conformation of half-ionized homodinucleotides has not been reported before.[21]

The influence of dinucleotide conformational equilibria on ionization constants was first studied for 3′-5′ dinucleotides containing adenine and uracil by Simpkins and Richards,[1] using the general principles set out by Cox.[8] Relationships between apparent ionization constants and stacked ⇄ unstacked conformational equilibria in the homo- and heterodinucleotide systems were derived on the basis of the two-state model. Let us first consider the equilibrium scheme for a diacidic base. In Scheme I the equilibrium quotient, $s_0$, for the stacking of $(XpX)_u$ to give $(XpX)_s$ is defined as $s_0 = [(XpX)_s]/[(XpX)_u]$. Similarly, the symbol $(s_1)$ is read, "the apparent equilibrium quotient for stacking when either the 3′- or 5′-linked nucleoside base is protonated". The other symbol $(s_2)$ has the corresponding meaning indicated by the subscript.

Before considering further, it is necessary to deal with the effect of symmetry for the species participating in the equilibria. We first assume that there are no interactions whatsoever between the two bases in the fully unstacked homodimer, $(XpX)_u$; we consider that unstacked XpX is essentially symmetrical (see Scheme II). With this assumption, since $K_{u_1}{}^{3'}$

Scheme II




$= K_{u_1}{}^{5'} = K_{u_2}{}^{3'} = K_{u_2}{}^{5'} = K_0$ where $K_0$ is the intrinsic ionization constant for each site, we have $K_{u_1} = \frac{1}{2}K_0$ and $K_{u_2} = 2K_0$. The titration curve of the fully unstacked XpX will then be indistinguishable from that of a monomer with an ionization constant $K_0$. If the two heteroaromatic base rings are sufficiently close to one another by intramolecular stacking association, the two bases do not titrate independently. Thus, the stepwise overall ionization constants, $K_1$ and $K_2$, can be expressed as

$$pK_1 = (pK_0 + \log 2) - \log \frac{1 + s_0}{1 + s_1} \quad (1)$$

and

$$pK_2 = (pK_0 - \log 2) - \log \frac{1 + s_1}{1 + s_2} \quad (2)$$

For dibasic acids such as GpG and UpU, the corresponding relations can be written as

$$pK_1 = (pK_0 + \log 2) - \log \frac{1 + s_{-2}}{1 + s_{-1}} \quad (3)$$

and

$$pK_2 = (pK_0 - \log 2) - \log \frac{1 + s_{-1}}{1 + s_0} \quad (4)$$

It has been reported that the intensity of ORD and CD bands of ApA, CpC, and GpG changes markedly in going from pH 7 to 0. At lower pH where only the diprotonated species $^+ApA^+$, $^+CpC^+$, or $^+GpG^+$ is present, the intensity of the longer wavelength positive band decreases and approaches that of the corresponding nucleoside and nucleotide constituents at the same pH.[5,27,28] The ORD or CD patterns of each dimer at pH $\leq pK_{app} - 2$ are also close to those of its corresponding component monomers at the same pH. All of these experimental results strongly indicate that diprotonation elicits unstacking in these dinucleotides though the origin of the pH-induced unstacking of dimers in the presence of solvent-dimer interaction is not at present fully understood.[28] Using ultraviolet difference spectroscopy, Simpkins and Richards also concluded that ApA is unstacked at low pH. Under the actual experimental conditions (at 25 °C, $I = 0.1$) in the present study the percentage of the stacked diprotonated form $(^+XpX^+)_s$ is thus very small so that the above general expression (eq 2) of the overall secondary basic ionization constant (as $pK_2$) can be written as

$$pK_2 = pK_0 - \log 2 - \log (1 + s_1) \quad (5)$$

The basic $pK_1$ value for XpX depends on the relative stabilities of the stacked and the unstacked species ($s_0$ and $s_1$). Thus the $pK_1$ is raised if $s_1 > s_0$, and $pK_2$ is lowered if $s_1 > 0$ regardless of the magnitude of $s_0$. By combining eq 1 and 5, the effect of the stacking interaction in XpX reduces the $pK_{app}$ by $\frac{1}{2}$ log $(1 + s_0)$ as compared to $pK_0$. Hence, in addition to the ultraviolet hypochromicities, the ORD, or CD spectra, the apparent ionization constant of dimers can be diagnostic of stacking at neutral pH.

Two approaches can be taken to determine $s_0$ (and then $s_1$), one involving measurements of the temperature dependence of an intensive property and the other, measurements of the stepwise basic ionization constants of homodimers. The latter method is essentially the same as that first employed by Simpkins and Richards,[1] who used it to calculate $s_0$ and $s_1$ for ApA.

**(b) Determination of Basic and Acidic Ionization Constants of Nucleosides and Mononucleotides Having the 3′- or 5′-Phosphate Group.** Equations 1–4 may be used to afford the stacking equilibrium quotients, $s_0$ and $s_i$ (and/or $s_{-1}$), of homodinucleotides, provided that the doubly ionized form of XpX exists exclusively in an unstacked conformation; i.e., in the case of a diacidic base, $(XpX)_u^+$ and $(XpX)_s^+$ form a common diprotonated unstacked dimer $(^+XpX^+)_u$. As mentioned above, lowering the pH to $pK_{app} - 2$ unstacks ApA, and this applies to all the other basic homodinucleotides studied here. This information, together with stepwise basic $pK$ values and the intrinsic $pK_0$ value, enables the stacking equilibria of XpX and $(XpX)^+$ to be studied. In order to perform the calculations, we need estimates for the magnitude of $pK_0$ for XpX. Because of the assumption of independent ionization of each base in $(XpX)_u$, we may assume that the monomer, X, pX, or Xp, behaves as an unstacked residue in the acid-base reaction, so that estimates can be obtained by equating the $pK_0$ values to the weighted average of $pK$ values of the corresponding nucleoside and 3′- and 5′-mononucleotides.[29] Differences in charge should be considered to make the major contribution to the difference in the activity coefficient between nucleotides and their corresponding nucleosides. The $pK$ value of the secondary phosphate group is approximately 6.5 so that there seems to be little variation in the activity coefficient of monoesterified and the diesterified phosphate residues over the range of pH <4.5. We may, thus, as a first approximation, use the value of $\frac{1}{4}(pK_{3'\text{-mononucleotide}} + pK_{5'\text{-mononucleotide}} + 2pK_{\text{nucleoside}})$ as that for $pK_0$.[29] For dibasic acids, GpG and UpU, the values of acidic $pK_0$ are tentatively approximated by the same manner as for the diacidic bases, ApA, CpC, and GpG. There is disagreement between $pK$ values of various authors for some mononucleotides and nucleosides. Hence, we did spectrophotometric titrations under the same conditions as those used for the dimer titrations.

Table II. Ionization Constants of Nucleosides and Mononucleotides and Intrinsic Ionization Constant for Homodinucleoside Monophospliates (25 °C, $I = 0.1$)

| | $pK$ | | | $pK_0$ | |
|---|---|---|---|---|---|
| Compd | Protons gained | Protons lost | Compd | Basic | Acidic |
| Ado | 3.590 ± 0.015 | | | | |
| 3′-AMP | 3.663 ± 0.014 | | ApA | 3.67 ± 0.010 | |
| 5′-AMP | 3.839 ± 0.019 | | | | |
| Cyd | 4.133 ± 0.011 | | | | |
| 3′-CMP | 4.243 ± 0.006 | | CpC | 4.21 ± 0.007 | |
| 5′-CMP | 4.330 ± 0.009 | | | | |
| Guo | 2.143 ± 0.011 | 9.029 ± 0.033 | | | |
| 3′-GMP | 2.146 ± 0.019 | 9.321 ± 0.020 | GpG | 2.19 ± 0.009 | 9.22 ± 0.019 |
| 5′-GMP | 2.336 ± 0.021 | 9.461 ± 0.029 | | | |
| Urd | | 9.045 ± 0.044 | | | |
| 3′-UMP | | 9.233 ± 0.044 | UpU | | 9.19 ± 0.021 |
| 5′-UMP | | 9.425 ± 0.025 | | | |

A least-squares fit of data yielded $pK$ values listed in Table II. These values are consistent with earlier measurements by a different data treatment.[30] The $pK_0$ value of adenine is 3.67 at 25 °C in 0.1 M NaCl which is somewhat smaller than the corresponding value of 3.86[1] at 20 °C in 0.1 M NaCl. The latter value seems to be less certain because the value of $s_0$ obtained by these authors must be overestimated as judged from the thermal denaturation data of ApA at neutral pH. Results summarized in Table II show that 5′-nucleotides are in general a slightly better base than the 3′-nucleotide isomers, as evidenced by their $pK$ values.

**(c) Evaluation of Stacking Equilibrium Quotients $s_0$ and $s_1$ from Titration Data.** The fraction of molecules in stacked and unstacked conformations can be evaluated since $pK_1$, $pK_2$, and $pK_0$ are now known for ApA, CpC, GpG, and UpU. By applying eq 5 to ApA which has $pK_2 = 3.02_5 \pm 0.07$ and $pK_0 = 3.67_1 \pm 0.01$, we find $s_1 = 1.2_1 \pm 0.36$ corresponding to about 55% stacking in the half-protonated state. Equation 1 may then be used to afford $s_0 = 1.6_3 \pm 0.61$, so that approximately 62% of ApA molecules is present in the stacked conformation at neutral pH at 25 °C. Both of the above described data sets as summarized in Tables I and II were combined, and values of $s_0$, $s_1$, and $s_{-1}$ were computed for the four homodinucleotides. The results are listed in Table III.

Molecular orbital (CNDO/2 and MINDO SCF) treatments of the effects of nucleic acid-base protonation on intermolecular stacking interactions have appeared.[11] Jordan-Sostman's treatment, in which the stacking energies were calculated including monopole–monopole, monopole-induced dipole, and dispersion terms between two bases as a function of the state of protonation, yields that all half-protonated homogeneous pairs have increased stabilities over their neutral

Table III. Estimated Stacking Equilibrium Quotients for "Unstacked ⇄ Stacked" from the Data Based on Titration Studies (25 °C, $I = 0.1$)[a]

| Compd | From basic p$K$'s | | | From acidic p$K$s | | |
|---|---|---|---|---|---|---|
| | $s_0$ | $s_1$ | $s_2$ | $s_0$ | $s_{-1}$ | $s_{-2}$ |
| ApA | 1.63 ± 0.61 | 1.21 ± 0,36 | ~0 | | | |
| CpC | 0.72 ± 0,29 | 0,75 ± 0,20 | ~0 | | | |
| GpG | 1,48 ± 0.25 | 1.55 ± 0.19 | ~0 | 1.96 ± 0.50 | 0.71 ± 0.17 | ~0 |
| UpU | | | | 0.78 ± 0.23 | 0,32 ± 0.11 | ~0 |

[a] The probable errors given for $s_0$ and $s_1$ (or $s_{-1}$) values are based on estimated maximal errors in $pK_0$, $pK_1$ and $pK_2$ in $\sigma_{s_1} = 2.3\,(1 + s_1)\cdot[(\sigma_{pK_0})^2 + (\sigma_{pK_2})^2]^{1/2}$ and $\sigma_{s_0} = 2.3(1 + s_0)[(\sigma_{s_1})^2/5.29(1 + s_1)^2 + (\sigma_{pK_0})^2 + (\sigma_{pK_1})^2]^{1/2}$.

Table IV. Expected Relationships between Stacking Equilibrium Quotients and Titration Properties of Homodinucleotides[a]

| | $\Delta pK = pK_1 - pK_2$ | $\lvert d\alpha/dpH)_{\alpha=1/2}\rvert$ | Dev from Henderson curve | $pK_{app} = \frac{1}{2}(pK_1 + pK_2)$ | Known example |
|---|---|---|---|---|---|
| $\sqrt{1+s_0} < 1 + s_1$<br>$\sqrt{1+s_0} < 1 + s_{-1}$ | >2 log 2 or $K_2/K_1 > 4$ | <0.576 | Broader | ≤ basic $pK_0$<br>≥ acidic $pK_0$ | $(^+GpG^+)_u \rightleftharpoons \begin{bmatrix}(GpG)_s^+\\ \Updownarrow \\ (GpG)_u^+\end{bmatrix} \rightleftharpoons \begin{bmatrix}(GpG)_s\\ \Updownarrow \\ (GpG)_u\end{bmatrix}$ |
| $\sqrt{1+s_0} = 1 + s_1$<br>$\sqrt{1+s_0} = 1 + s_{-1}$ | =2 log 2 or $K_2/K_1 = 4$ | =0,576 | No dev | ≤ basic $pK_0$<br>≥ acidic $pK_0$ | $\begin{bmatrix}(UpU)_s\\ \Updownarrow \\ (UpU)_u\end{bmatrix} \rightleftharpoons \begin{bmatrix}^-(UpU)_s^-\\ \Updownarrow \\ (UpU)_u^-\end{bmatrix} \rightleftharpoons (^-UpU^-)_u$ |
| $\sqrt{1+s_0} > 1 + s_1$<br>$\sqrt{1+s_0} > 1 + s_{-1}$ | <2 log 2 or $K_2/K_1 < 4$ | <0.576 | Sharper | < basic $pK_0$<br>> acidic $pK_0$ | |

[a] $s_2$ and $s_{-2}$ are assumed to be negligibly small.[26]

counterparts. It is thus anticipated that the singly protonated dinucleoside monophosphates $(XpX)^+$ are further stabilized by a salt bridge possibly formed between a protonated base and an internucleoside phosphate anionic site. In this connection the comparably strong stacking interactions found for the half-protonated and unprotonated XpX are noteworthy, $s_1$ being almost equal to $s_0$.

The only quantitative discussion to date on the stacking behavior of half-protonated homodinucleotides is that of Simpkins and Richards[1] who examined the titration data for ApA and concluded that the half-protonated ApA is unstacked, i.e., $s_1 \simeq 0$. The present investigation suggests a pattern considerably different from that of Simpkins and Richards, and this may arise partly from the choice of analytical methods in the two treatments. Contrary to their opinion, half-protonated ApA at 25 °C has an appreciable fraction of the bases in a stacked conformation. This seems to apply to other homodinucleotides studied here.[33]

The $s_0$ value reported by Simpkins and Richards ($s_0 = 4.25 \pm 0.7$ at 20 °C) is much higher than our own ($s_0 = 1,6_3 \pm 0.61$ at 25 °C) for a following reason; they assumed the $pK_0$ value as 3.86, which is larger by 0.19 unit than that presently estimated, and their value of $s_1$ is negligibly small, while our $pK_{app}$ value (3.46 ± 0.07 at 25 °C) agrees quite well with that (3.56 ± 0.02 at 20 °C) of Simpkins and Richards. The uncertainty of our procedure arises from the estimate of the values of $pK_0$. The values of $s_0$ listed in Table III are somewhat higher but almost of similar magnitude when compared with the data obtained from thermal denaturation experiments (see the accompanying paper[13]). Further support for the stacked half-protonated conformation can be found in the helical $ApA^+$ fragment demonstrated by the x-ray crystallographic analysis of the $Ap^+ApA^+$ crystals.[20,34]

The change of absorptivity on increasing pH from 7 to 12, observed for GpG and UpU, is characteristic of the single-stage deprotonation. It is likely therefore that the difference in overlapping $pK$ must be very close to a statistical factor of 0.6. This is merely corresponding to the existence of the relationship, $(1 + s_0)^{1/2} = 1 + s_{-1}$, and this is not necessarily the case with all dinucleotides as dibasic acids (see Table IV). [We have found the $\Delta pK$ value of $1.0_0$ for d(TpT) (M. Sakurai, I. Tazawa, and Y. Inoue, unpublished results).] It should be noted that the $s_0$ and $s_{-1}$ values estimated from the data based on alkali titrations have less quantitative significance because of the uncertainty in $pK_0$; the secondary phosphate ionization of 3′- and 5′-mononucleotides would be expected to affect the ionization of both guanine and uracil residues. The inadequacy of the acidic $pK_0$ approximation seems to be the origin of an overestimation of $s_0$ and $s_{-1}$ values. A better approximation is needed for the problem considered. Furthermore, as with the assumption of $s_{-2} \simeq 0$, speculation on the stacking nature of dideprotonated XpX, i.e., $^-GpG^-$ and $^-UpU^-$, is not warranted. For the above reasons, the results on $s_0$ and $s_{-1}$ based on the alkali titrations of GpG, UpU, and their component monomers must be considered with caution.

It is known that stacking tends to suppress the ionization of the base residues and thus may modify the shape of the degree of ionization ($\alpha$) vs. pH curve.[1,8] A rough estimate of the relative extent of stacked to unstacked form of XpX, $(XpX)^+$, and $(XpX)^-$ can be made if either $pK_1$ and $pK_2$ or $(d\alpha/dpH)_{\alpha=1/2}$ is known (Table IV). As is seen from the above discussion and Table IV, the $\Delta pK$ is very sensitive to the intramolecular stacking equilibrium conformation of homodinucleotides. In this view, it is rather strange that no investigations have been carried out on the effect of stacking interactions on $K_2/K_1$ ratio of XpX.[21]

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## Appendix

The equilibria in homodinucleotides can be summarized by the diagram shown in Scheme I (see text). At any given pH the ratios $[(XpX)_s]/[(XpX)_u]$, $[(XpX)_s^+]/[(XpX)_u^+]$, and $[(^+XpX^+)_s]/[(^+XpX^+)_u]$ are constant. The overall degree of protonation, $\alpha$, of XpX may be expressed by

$$\alpha = \frac{[(XpX)_u^+] + [(XpX)_s^+] + 2[(^+XpX^+)_u] + 2[(^+XpX^+)_s]}{2[[(XpX)_u] + [(XpX)_s] + [(XpX)_u^+] + [(XpX)_s^+] + [(^+XpX^+)_u] + [(^+XpX^+)_s]]} \quad (6)$$

Thus, we obtain

$$pH = pK_0 + \log (1 - \alpha)/\alpha - \log \frac{K_0(1 + s_0) + a_H(1 + s_1)}{K_0(1 + s_1) + a_H(1 + s_2)} \quad (7)$$

or

$$pH = pK_2 + \log (1 - \alpha)/\alpha - \log \frac{2K_1 + a_H}{K_2 + 2a_H} \quad (8)$$

The pH at the half-ionized point can be then written as

$$(pH)_{\alpha=1/2} = pK_{app} = pK_0 - \tfrac{1}{2} \log \frac{1 + s_0}{1 + s_2} \quad (9)$$

or

$$(pH)_{\alpha=1/2} = pK_{app} = \tfrac{1}{2}(pK_1 + pK_2) \quad (10)$$

Differentiating eq 7 and 8 with respect to pH gives, respectively

$$\frac{d\alpha}{dpH} = -\frac{2.303a_HK_0[K_0^2(1 + s_0)(1 + s_1) + 2a_HK_0(1 + s_0)(1 + s_2) + a_H^2(1 + s_1)(1 + s_2)]}{[K_0^2(1 + s_0) + 2a_HK_0(1 + s_1) + a_H^2(1 + s_2)]^2} \quad (11)$$

and

$$\frac{d\alpha}{dpH} = -\frac{2.303a_HK_2(K_1K_2 + 4a_HK_1 + a_H^2)}{2(K_1K_2 + a_HK_2 + a_H^2)^2} \quad (12)$$

At $\alpha = \tfrac{1}{2}$, $(a_H)_{\alpha=1/2} = K_0[(1 + s_0)/(1 + s_2)]^{1/2}$ and $(a_H)_{\alpha=1/2} = [K_1K_2]^{1/2}$, so that inserting these relations into eq 11 and 12 gives the slope of the $\alpha$ vs. pH curve at half-ionization;

$$\left(\frac{d\alpha}{dpH}\right)_{\alpha=1/2} = -\frac{2.303}{2 + \frac{2(1 + s_1)}{\sqrt{(1 + s_0)(1 + s_2)}}} \quad (13)$$

and

$$\left(\frac{d\alpha}{dpH}\right)_{\alpha=1/2} = -\frac{2.303}{2 + \sqrt{K_2/K_1}} \quad (14)$$

If $pK_1 - pK_2 > 2 \log 2$, the slope $|(d\alpha/dpH)_{\alpha=1/2}|$ of the $\alpha$ vs. pH curve will be less than that of a monomeric base or fully unstacked XpX.

## References and Notes


(1) H. A. Simpkins and E. G. Richards, *Biochemistry*, **6**, 2513 (1967).

(2) Abbreviations used: in oligonucleotides A, C, G, and U represent adenosine, cytidine, guanosine, and uridine, respectively, and p to the left of a nucleoside symbol indicates a 5′-phosphate and to the right it indicates a 3′-phosphate. The protonation to the 3′- and 5′-linked nucleoside base in a homodinucleoside monophosphate, XpX, is denoted by $^+$XpX and XpX$^+$, respectively, and to both nucleoside bases by $^+$XpX$^+$. Throughout this paper, such terms as "un-ionized", "half-protonated" (or "singly ionized"), and "fully protonated" (or "double ionized") XpX are used in a convenient sense to represent XpX, $(XpX)^+$ (=$^+$XpX plus XpX$^+$), and $^+$XpX$^+$, without implying the ionization state of the primary phosphate group. For economy of space, nucleosides and mononucleotides will be occasionally referred to as monomers and dinucleoside monophosphates, e.g., ApA, as dinucleotides or dimers. Ado = adenosine; Cyd = cytidine; Guo = guanosine; Urd = uridine; CD = circular dichroism; ORD = optical rotatory dispersion.

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(21) A referee has pointed out that similar work has been carried out by M. Topal for dinucleoside monophosphates containing adenine and cytosine [M. Topal, Ph.D. Thesis, New York University, 1974; see also *Chem. Abstr.*, **81**, 136433y (1974)]. We learned that stepwise p$K$ values for homodimers ApA and CpC have been independently determined by Topal. He obtained $pK_1 = 4.62$ and $pK_2 = 3.72$ for CpC from the uv titrations at 25 °C and $I = 0.001$, and from analysis of the CD titrations data of ApA he also obtained $pK_1 = 4.03$ and $pK_2 = 2.35$. On the basis of previous considerations concerning the effect of increasing the ionic strength on the basic ionization constants of mononucleotides,[7] his results on the basic p$K$ values of CpC are in excellent accord with ours. However, the significant discrepancy between our results (see Table I) and those of Topal is noted for the p$K$ values of ApA. He derived those values from the CD titrations and the discrepancy may be attributed to differences in certainty in the uv and CD titration data. We believe that our values for ApA seem to be more reliable because our uv titration data as absorbance differences (see the text) showed smaller scatter and computer analysis of the duplicate titration data gave acceptable p$K$ values as judged from the small root mean square values ($\simeq 2 \times 10^{-3}$).

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(29) It can be seen from Table II that the presence of a phosphate group, either 3′ or 5′, can cause small but detectable differences in the p$K$ of monomers at $I = 0.1$. Furthermore, the ratio of base to phosphate (B/P) is 2 in dinucleoside monophosphate XpX; thus, we have chosen the average of the p$K$'s of the nucleoside, X, and the mononucleotides, pX and Xp, as the intrinsic p$K$ value for $(XpX)_u$, i.e., $pK_0 = \tfrac{1}{4}(pK_{3'\text{-mononucleotide}} + pK_{5'\text{-mononucleotide}} + 2pK_{\text{nucleoside}})$, rather than $pK_0 = \tfrac{1}{2}(pK_{3'\text{-mononucleotide}} + pK_{5'\text{-mononucleotide}})$. That the inclusion of the p$K$ of the nucleoside in the average is not bad as an approximation is evident when one compares the values of $s_0$ obtained from melting experiments with those predicted for ApA, CpC, and GpG. In any case, the relative $s_0$ and $s_1$ values should probably be more accurate than their absolute values.

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