Determination of Microscopic Basic Ionization Constants of Guanylyl-(3′→5′)-guanosine. Structure and Optical Properties of Half-Protonated Guanylyl-(3′→5′)-guanosine and Their Models

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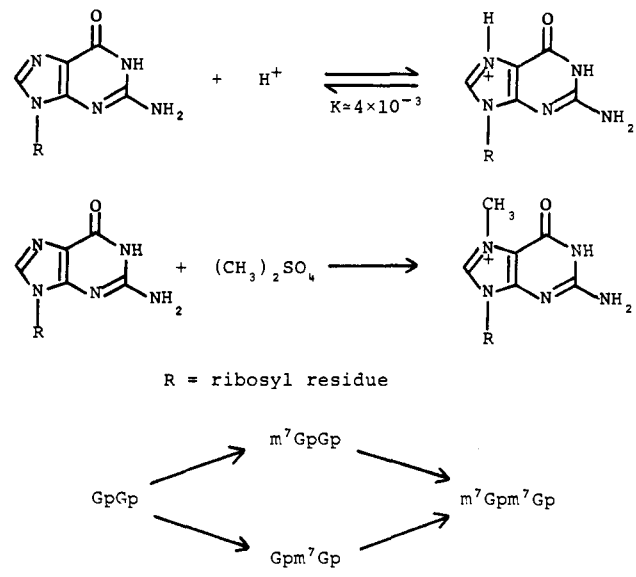
Abstract: Evidence is presented that GpG undergoes protonation at either and then both of two base residues within the pH range 0–5. Application of the least-squares computer method developed by Perrin to the CD-titration data gave pK values for two stages of protonation of GpG. Basic ionization constants are also reported for m^7G^+pG and Gpm^7G^+. From the measured values, the equilibrium between ^+GpG and GpG^+ in monoprotonated GpG is shown to favor the "more stacked" form, ^+GpG, and the quotient has been estimated. This constant has been found to agree with that determined by comparing the CD spectra of the monoprotonated species and their model compounds with fixed structures.

Investigations of the acid-base properties of simple oligonucleotides have been undertaken in several laboratories with the aim of uncovering details of changes in their conformation and conformational stability.[1-5] While these studies have contributed much to our knowledge of the ionization behavior of oligonucleotides, neither macro- nor microscopic ionization constants have been reported for oligonucleotides, which contain more than two ionizable groups of comparable acidity or basicity.

For guanine containing di- and trinucleotides, a particular feature of the change of CD[6] or optical rotatory dispersion (ORD) spectra upon acidification has been observed previously.[7-12] In this connection the present work was initiated in an attempt to establish microscopic protonation processes of GpG as a part of a systematic investigation of the acid-base chemistry of oligonucleotides. We have shown that m^7G^+pU serves as a model compound for the monoprotonated form of GpU, i.e., ^+GpU.[11] This preliminary study[11] has also suggested that N^7-methylation (quarternization) of the Guo residue in oligonucleotides can be used as a diagnostic tool in studying the protonation process and the conformational change on protonation of oligoguanylates. Evidence is presented that GpG undergoes protonation at preferentially one and then both of two basic N^7 atoms. The relation to other work is also discussed.

Scheme I



Experimental Section

Materials. Pure GpG and GpGp were prepared by the method as reported in previous papers.[13,14] m^7GpG and Gpm^7G were prepared by a partial methylation of GpGp according to a modification of the method of Jones and Robins,[15] followed by column chromatographic separation with a Dowex 1 X2 anion exchanger, and enzymatic dephosphorylation of m^7GpGp and Gpm^7Gp with alkaline phosphatase (Boehringer, Mannheim) at 35°, pH 7, for 24 hr. The diammonium salt of GpGp(3′) (0.014 mmol) was dissolved in 3 ml of distilled water and the pH of the solution was adjusted to 5 with 0.1 N HCl. Dimethyl sulfate (0.74 mmol, 70 μl.) was added to the dinucleotide solution, and the solution was left under stirring for 40 hr at room temperature. During the reaction the solution was pH-statted at pH 5 by the gradual addition of 0.5 N KOH solution. Since GpGp is believed to be protonated and methylated on the same nitrogen atom under the conditions used, GpGp is attacked simultaneously at N^7 of the 5′ and 3′ termini, the end product being m^7Gpm^7Gp (Scheme I). Chromatographic analysis is the reaction mixture revealed the presence of both intermediates, m^7GpGp and Gpm^7Gp, in a comparable yield. Methylation is irreversible and thus kinetically controlled. The base sequences of m^7GpGp and Gpm^7Gp were determined from the known specificity of ribonuclease T_1 and ascertained by *E. coli* alkaline phosphatase treatment followed by hydrolysis with ribonuclease T_2.

Methods. Changes in absorbance as a function of pH were measured as difference spectra, $\Delta A = A$ (pH) $- A$ (pH 6.0). The variation of the CD spectrum of GpG with pH was determined. All spectroscopic titrations were performed at 25° by the use of a thermostated cell jacket with a magnetic stirrer. For absorption and CD measurements a salt-free lyophilized sample of GpG (in ammonium form) was dissolved in glass-distilled water to give A_{252} values of approximately 5, the solution was heated in warm water (60–70°) for 30 min to dissociate the aggregates, and the resulting solutions were further diluted to give final A_{252} values of about 0.5–2.5 with appropriate buffer solutions to which sufficient NaCl had been added to give a final ionic strength of 0.5 M. No detectable degradation of dimers occurred during heating of the sample solutions. To evaluate apparent pK values for two stages of protonation which differ by only about 1 pK unit, the theoretical titration curves was fitted to absorption and CD data by use of a least-squares computer program written[16] in Fortran 4E for use on a HITAC 8800/8700 digital electronic computer at the Computer Centre of the University of Tokyo and kindly provided by Dr. D. D. Perrin, Australian National University.

Molecular extinction coefficients of GpG, m^7G^+pG, and Gpm^7G^+ were calculated from hyperchromicities at their absorption maxima upon hydrolysis with ribonuclease T_2 to component monomers. Hypochromicities were determined at 25° by ribonuclease T_2 hydrolysis of the samples with the use of the extinction

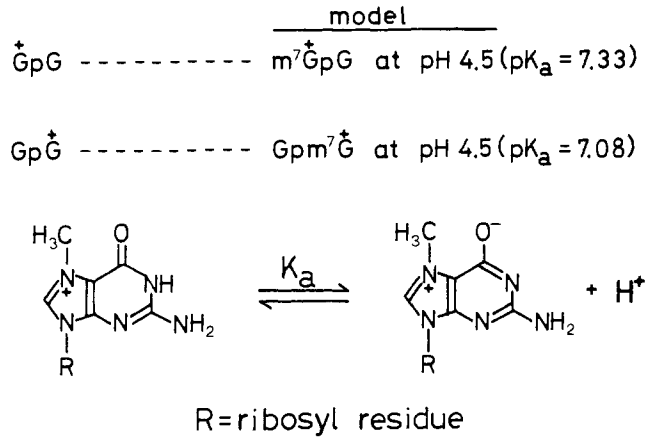


Figure 1. Model compounds for monoprotonated GpG and ionization of the m^7G residue.

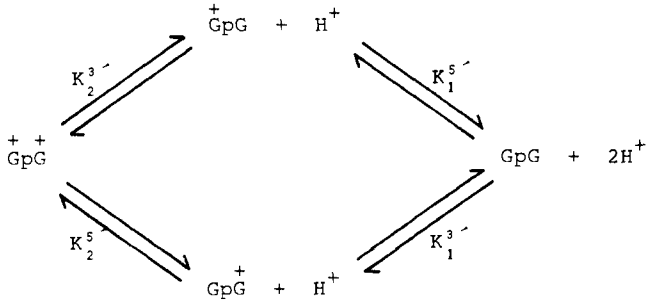
coefficients of monomers given below, and those necessary for CD data treatments are listed in Table I: m^7G^+p (3′), ϵ_{257} = 13.2 × 10^3 at pH 2;[17] m^7G^+, ϵ_{257} = 10.9 × 10^3 at pH 2;[17] Gp (3′), ϵ_{252} = 13.4 × 10^3 at pH 7; Guo, ϵ_{252} = 13.6 × 10^3 at pH 7.

Instrumentation. CD spectra were measured on a Jasco J-10 and/or J-20 circular dichrometer, with 3- and 10-mm quartz cells. The CD data are expressed in terms of $\epsilon_L - \epsilon_R$, which for dinucleoside monophosphates is calculated per mean residue. Light absorption spectra and differences in absorbance were obtained with a Hitachi Perkin-Elmer spectrophotometer, Model 124. pH measurements and pH-statting were performed using a pH meter Type PHM26 (Radiometer) equipped with an autotitrator and syringe buret. The pH meter was standardized with 0.05 *M* potassium hydrogen phthalate and 0.05 *M* sodium borate standard solutions.

Results and Discussion

Protonation of GpG and Model Compounds. Dinucleotides contain two bases, both of which are in principle capable of accepting a proton in acidic solutions. For the specific case of GpG the course of ionization of the two bases can be illustrated in Scheme II. Proton addition to guanosine,

Scheme II



which takes place on N^7, shifts the B_{2u} band slightly (by ~2 nm) and the B_{1u} band, by ~4 nm, to longer wavelengths. Replacement of the N^7 proton by a methyl group shifts both bands in the same direction, by almost the same amounts. Based on the structural similarities of m^7G^+ and m^7G^+pU and protonated Guo and GpU,[11] it was expected that m^7G^+pG and Gpm^7G^+ would be useful as model compounds for the specifically monoprotonated species of GpG involved in the equilibrium (Figure 1).

The anti → syn conversion was first proposed by Guschlbauer and Courtois[18] as an interpretation of the observed inversion of the Cotton effect of Guo and its nucleotides on their conjugate acid formation at pH 1. More recently, nuclear Overhauser enhancement measurements have been carried out to demonstrate that the guanosine nucleotide molecules are flexible and that lowering the pH tends to increase the contribution of the syn conformation.[19] It also

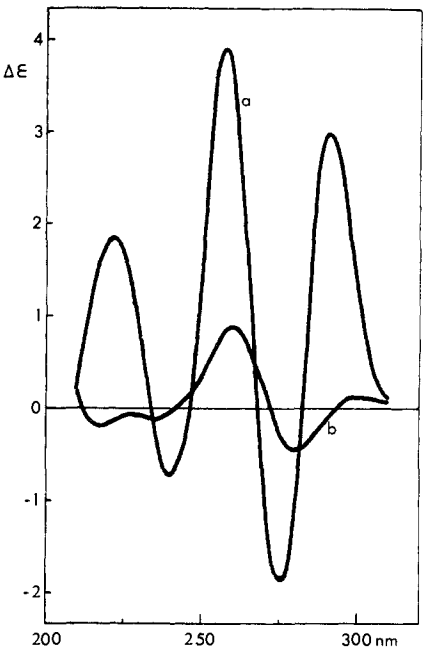


Figure 2. CD spectra of (a) m^7G^+pG and (b) Gpm^7G^+ at pH 4.5 and 25°.

Table I. Hypochromicity and Molar Extinction Coefficients at λ_{max} (Values at 25°)

Dimer	pH	λ_{max}, nm	$\epsilon \times 10^{-3}$	*h*, %
GpG	7.2	252	25.0	6.2
m^7G^+pG	4.5	256	24.4	7.2
Gpm^7G^+	4.5	255	22.3	5.7

seemed safe to conjecture the conformation of m^7G^+pU from the structural analogy with $^+$GpU and the syn conformation with respect to the sugar-base torsion angle of the m^7G^+ residue in m^7G^+pU was shown to satisfy the geometrical disposition for greater stacking interaction, leading to enhanced intensity of the CD spectrum of m^7G^+pU compared with that of GpU.[20] Examination of space-filling CPK models of m^7GpU indicates that the strengthening of stacking interaction in m^7G^+pU seemed to be due to the adoption of right-handed (syn)m^7G^+p(anti)U conformation.

Were the enhancement of the contribution of the syn conformation for GpU on N^7 methylation the true origin of exhibiting the CD pattern similar to $^+$GpU, a similar effect would be anticipated for m^7G^+pG. This is what is observed, the corresponding effect being noticed as is demonstrated in Figure 2. The geometric parallel between m^7G^+pU and m^7G^+pG thus results in optically analogous consequences. It would be expected on comparing the vastly different CD spectra[21] of 8-bromoguanylyl-(3′→5′)-adenosine (brGpA) and adenylyl-(3′→5′)-8-bromoguanosine (ApbrG) that the CD spectra of a pair of sequence isomers, m^7G^+pG and Gpm^7G^+, would be quite different. Indeed, for m^7G^+pG large amplitudes of rotation are observed, and this fact may be taken as prima facie evidence for strong base-base stacking interaction; the sequence isomer, Gpm^7G^+, exhibits the CD spectrum of which intensity is as low as the sum of the CD of the component monomers.

Evidence for the Two-Stage Protonation of GpG. Determination of Overall Basic Ionization Constants. In a study of the protonation of GpG, a knowledge of the ionization constants of GpG is of value both for purposes of conformational characterization of monoprotonated GpG at the molecular level and for planning systematic study of the ther-

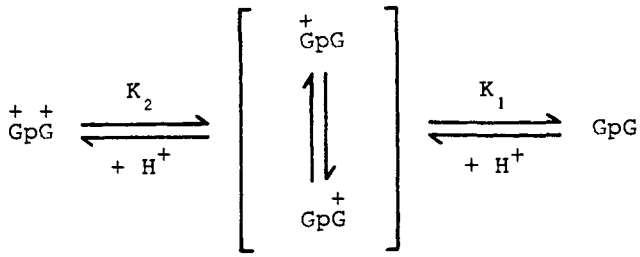
modynamic stability of monoprotonated species. In the case of guanylic acid, good isosbestic points (228, 257, 265.5, and 276 nm) were defined by the family of ultraviolet absorption spectral curves at pH 0–5 (proton gain). The basic pK_a values representing protonation of the base at N^7 were found as 2.37 ± 0.02 and 2.13 ± 0.01 at 25° and ionic strength of 0.5 for 5′-GMP and 3′-GMP, respectively.

Investigation of the protonation equilibria of GpG in acid solutions presented a bit of a problem. Ultraviolet spectral changes did not define clear isosbestic points between pH 0 and 5. This is illustrated in Figure 3a. This lack of isosbestic points indicates the presence of pH-dependent equilibria between more than two species. Plots of absorbance at a given wavelength against pH were approximately sigmoid. However, these curves showed some irregularities. The titration curve obtained at the wavelength where the absorptivities at pH 0 and 5 differ maximally does not fit a theoretical titration curve for a single pK (Figure 3b).

The structure of GpG has two basic moieties, either or both of which could receive a proton. These moieties are separated by a diribosylphosphate bridge which may not necessarily act as an insulator.

Scheme III shows the equilibria involved in the pH (or H_0) range −1 to 5, where the portion inside the square

Scheme III



brackets exists in dynamic equilibrium. Although GpG undergoes acid hydrolysis of the glycosidic bonds in moderately strong acidic media, the hydrolysis is relatively slow in the above pH range at 25°. Thus, these circumstances made it possible to study the prototropic equilibria by rapid spectroscopic titration of GpG. From the data based on the CD measurements of GpG at different pH values (0.4 ~ 4.2), we have noticed that, at particular wavelengths, the Δε rose with decreasing pH from 4.2 to a maximum at pH about 2.0, followed by a decrease with a further increase in hydrogen ion activity until eventually the Δε value of the fully protonated form of GpG is reached (Figure 4a). The desired wavelength is that at which the isotropic absorption is weak and the anisotropic absorption is rather strong (Figure 4b). Figure 5 shows a typical plot of Δε against pH at 292 nm. A change of this nature is convincing evidence for the existence of the intermediate monoprotonated species. At intermediate pH values the monoprotonated species, $^+$GpG plus GpG$^+$, appears to be important.

The macroscopic ionization constants defined in Scheme III are

$$K_1 = \frac{a_H[GpG]}{[^+GpG] + [GpG^+]} \quad (1)$$

$$K_2 = \frac{a_H([^+GpG] + [GpG^+])}{[^+GpG^+]} \quad (2)$$

If $\Delta\epsilon_1$, $\Delta\epsilon_2$, and $\Delta\epsilon_3$ are the circular dichroisms of the species $^+$GpG$^+$, $^+$GpG plus GpG$^+$, and GpG, respectively, the observed Δε of a solution containing a mixture of these species is given by

$$\Delta\epsilon = \frac{a_H^2\Delta\epsilon_1 + a_H K_2\Delta\epsilon_2 + K_1K_2\Delta\epsilon_3}{a_H^2 + a_H K_2 + K_1K_2} \quad (3)$$

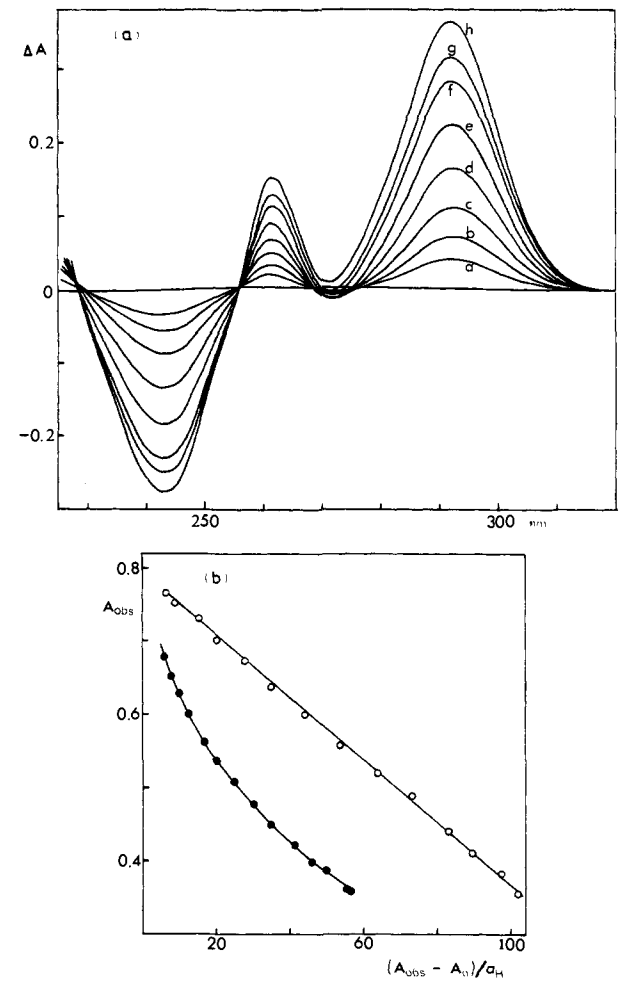


Figure 3. (a) Difference ultraviolet absorption spectra resulting from change of the pH of solutions of GpG from 6.0 to (a) 3.15_1, (b) 2.86_2, (c) 2.57_6, (d) 2.26_1, (e) 1.96_2, (f) 1.66_8, (g) 1.50_4, and (h) 1.22_0: temperature 25°; ionic strength 0.5; [GpG] = 1.49×10^{-4} M. (b) A_{obsd} vs. $(A_{obsd} - A_0)/a_H$ plots at 292 nm, showing a single-stage protonation for 5′-GMP (-O-O-O-) and a nonsingle-stage protonation for GpG (-●-●-●-). For a monoacidic base, and when the absorbance of the conjugate acid, A_+, is not directly obtainable, the following straight-line relationship leads to the desired pK_a, $A_{obsd} = A_+ - K_a(A_{obsd} - A_0)/a_H$, where A_0 is the absorbance of the molecular species (conjugate base) at an analytical wavelength.

Equation 3 is an exact description of the relation between Δε and the hydrogen ion activity of solutions. Conversely, the experimental determination of a sufficient number of corresponding values of Δε and a_H will suffice to delineate the graph of eq 3 (Figure 5), whence, in principle, the values of the five unknowns, $\Delta\epsilon_1$, $\Delta\epsilon_2$, $\Delta\epsilon_3$, K_1, and K_2, can be calculated. By applying the least-squares computer method developed by Perrin and his associates[16] to the presently observed CD data, we are now able to estimate the apparent macroscopic constants, K_1 and K_2, and $\Delta\epsilon_2$. $\Delta\epsilon_1$ and $\Delta\epsilon_3$ are obtained directly from the CD measurements in 0.5 M HCl and at pH, say, 5, respectively. Computer analysis of the data gave pK_1 = 2.51, pK_2 = 1.49, $\Delta\epsilon_2 = 1.50_9$ l./(cm mol), and a value of 0.04_1 for the standard error of fit.[22] The bell-shaped CD-titration curve at 292 nm for GpG fits a theoretical curve for these values (Figure 5). It should be noted that the $\Delta\epsilon_1$ value of $^+$GpG$^+$ obtained from the direct measurement in 0.5 M HCl has been found to agree, within the limits of experimental error, with that estimated from the method of Ang.[23,24] (The Ang's method[23] is particularly useful when $\Delta\epsilon_1$ is unknown or cannot be determined directly.)

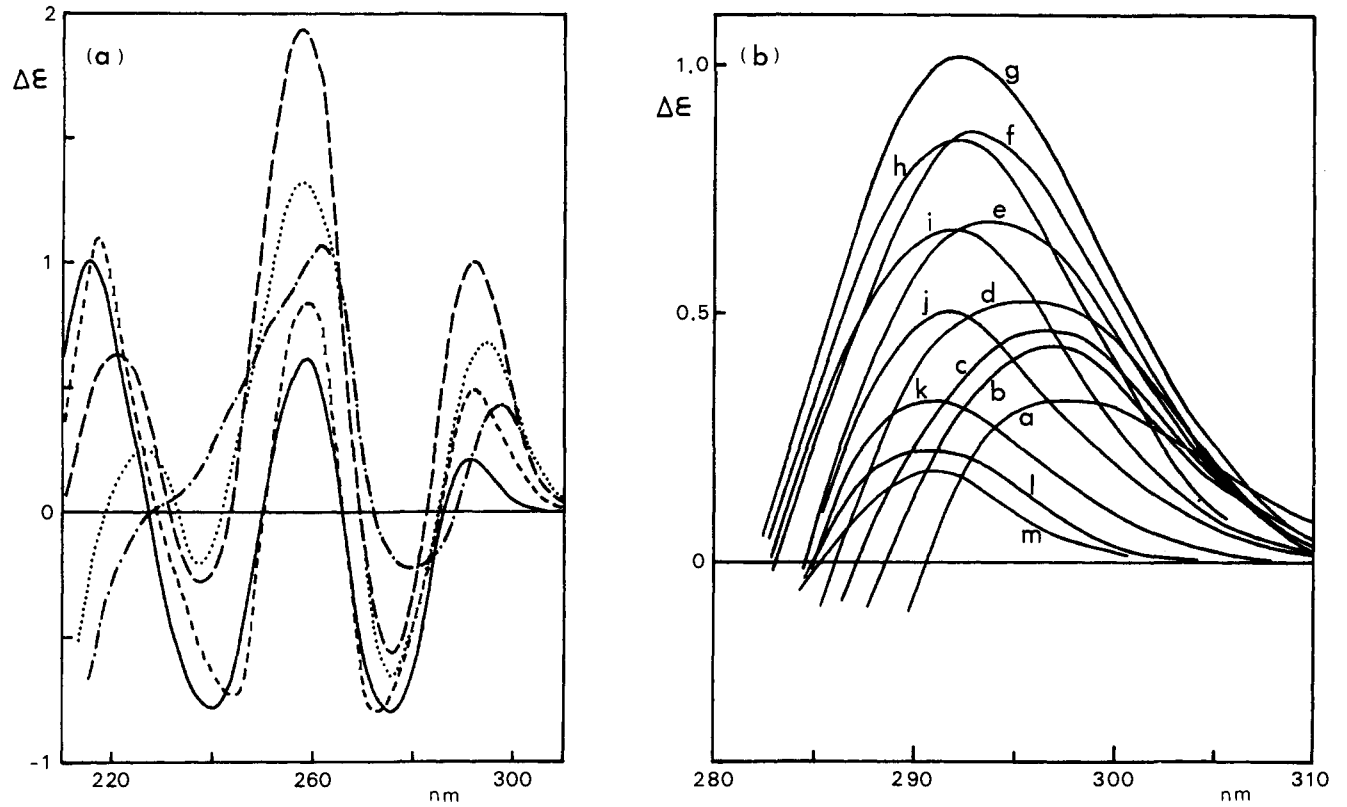


Figure 4. (a) Variation of the CD spectrum of GpG with pH at 25°: (——) pH 4.37; (- - - -) pH 3.04; (— — —) pH 2.01; (· · · · · · · ·) pH 1.32; (- · - · -) pH 0.20. (b) Effect of pH on the longer wavelength positive CD band. The symbols indicate the pH at which the spectra were recorded: (a) pH −0.69; (b) pH 0.20; (c) pH 0.55; (d) pH 0.98; (e) pH 1.32; (f) pH 1.65; (g) pH 2.05; (h) pH 2.41; (i) pH 2.70; (j) pH 3.04; (k) pH 3.44; (l) pH 4.37; (m) pH 7.02.

Determination of Microscopic Ionization Constants and Equilibrium Quotient (K_t = [$^+$GpG]/[GpG$^+$]) by Basicity Measurements. Apparent ionization constants, K_1 and K_2, thus obtained are composite. The individual, or microscopic, ionization constants, such as $K_1^{3'}$ and $K_2^{5'}$ (see Scheme II), are defined by the relations

$$K_1^{3'} = \frac{a_H[GpG]}{[GpG^+]}; \qquad K_1^{5'} = \frac{a_H[GpG]}{[^+GpG]};$$

$$K_2^{3'} = \frac{a_H[^+GpG]}{[^+GpG^+]}; \qquad K_2^{5'} = \frac{a_H[GpG^+]}{[^+GpG^+]}$$

The overall ionization constants, K_1 and K_2, of GpG can then be related to the above-defined true constants of $^+$GpG ($K_1^{5'}$ and $K_2^{3'}$) and GpG$^+$ ($K_1^{3'}$ and $K_2^{5'}$) by the expression

$$\frac{1}{K_1} = \frac{1}{K_1^{3'}} + \frac{1}{K_1^{5'}} \tag{4}$$

$$K_2 = K_2^{3'} + K_2^{5'} \tag{5}$$

The equilibrium quotient, K_t = [$^+$GpG]/[GpG$^+$], being constant regardless of the pH is also given by

$$K_t = \frac{K_1^{3'}}{K_1^{5'}} = \frac{K_2^{3'}}{K_2^{5'}} \tag{6}$$

To analyze the system in practice, values had to be assumed for either p$K_2^{3'}$ or p$K_2^{5'}$; they could not be obtained directly by experiment. From the structural similarities of m^{7}G$^+$pG and Gpm^{7}G$^+$ to $^+$GpG and GpG$^+$, respectively, it was expected that the basic ionization constants of $^+$GpG and GpG$^+$ ($K_2^{3'}$ and $K_2^{5'}$) would be approximated by the second constants of m^{7}G$^+$pG and Gpm^{7}G$^+$, respectively.[11,26]

Using a spectrophotometric method, the apparent pK values for m^{7}G$^+$pG and Gpm^{7}G$^+$ were determined:[27] for m^{7}G$^+$pG, pK = 1.72, and for Gpm^{7}G$^+$, pK = 1.95. With these values, the equilibrium quotient, K_t, and the microscopic constants could then be calculated by means of the above equations. The results are given in Table II. From these results, it is suggested that the monoprotonated form with a proton on the base at a 5′ terminus preponderates over that with a proton on the guanine base at a 3′ terminus; at 25° the equilibrium ratio of [$^+$GpG]/[GpG$^+$] is 1.7 in favor of $^+$GpG. The basic pK of the first stage of protonation of GpG on a 5′-terminal guanine base is slightly above

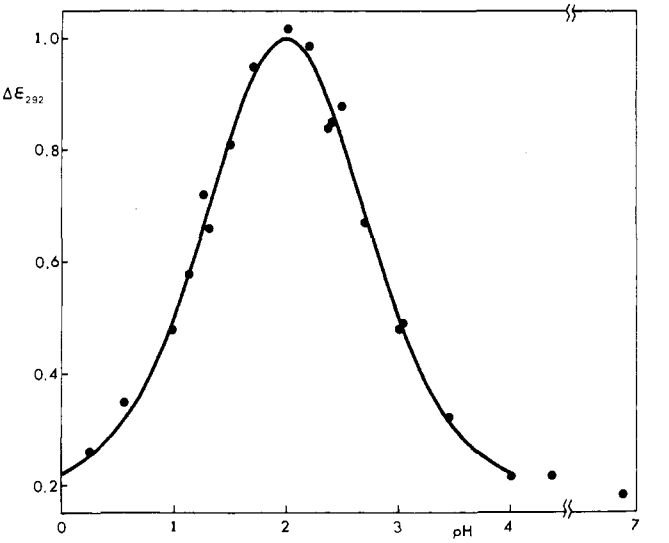


Figure 5. CD–pH titration curve of GpG at 292 nm. The points on the plot are experimental and the curve is theoretical, being derived from

$$\Delta\epsilon = \frac{(0.18a_H^2 + 4.89 \times 10^{-2}a_H + 1.84 \times 10^{-5})}{(a_H^2 + 3.24a_H + 1.02 \times 10^{-4})}$$

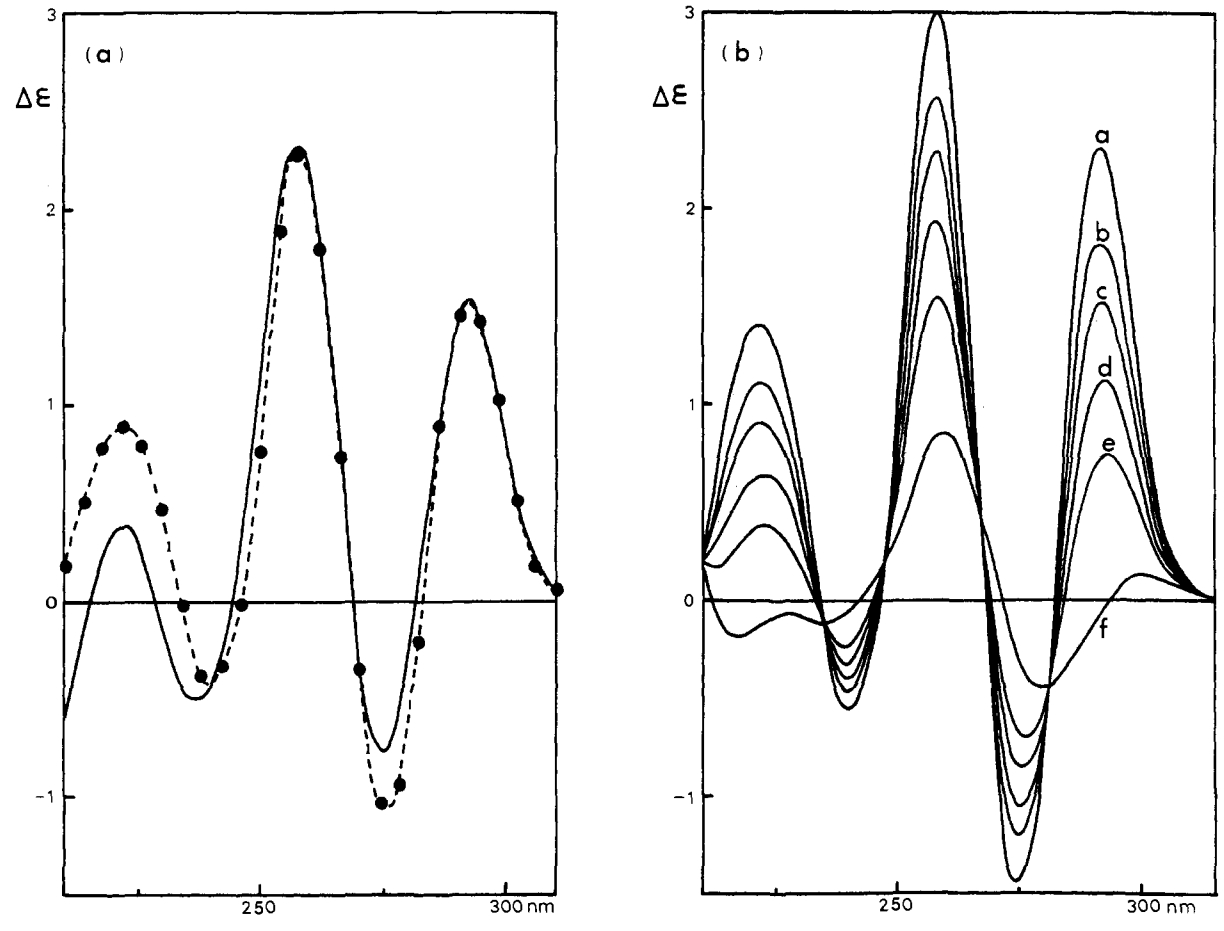


Figure 6. (a) CD spectra of monoprotonated GpG (——) and a model system comprised of m^7G^+pG and Gpm^7G^+ (- - -). The solid line is a plot of $\Delta\epsilon_2$, computed by a least-squares method, against wavelength. The dashed line is the result of the sum of the two CD curves for m^7G^+pG and Gpm^7G^+ with the ratio of $[m^7G^+pG]/[Gpm^7G^+] = 2$ after correction for a methyl substitution effect as described in the text and caption for Figure 6b. (b) Variation in the CD curves of $m^7G^+pG + Gpm^7G^+$ as a function of the ratio of $[m^7G^+pG]/[Gpm^7G^+]$. The family of curves is generated from the equation $\Delta\epsilon_{[^+GpG+GpG^+]} = [K_t/(1 + K_t)]\Delta\epsilon_{[^+GpG]} + [K_t/(1 + K_t)]\Delta\epsilon_{[GpG^+]} = A(\Delta\epsilon_{[m^7G^+pG]}/1.3) + B\Delta\epsilon_{[Gpm^7G^+]}$, where $K_t = [^+GpG]/[GpG^+]$ and $A + B = 1$, for the following six pairs of the values of A and B: for A (a) 1, (b) 0.8, (c) $\frac{2}{3}$, (d) 0.5, (e) $\frac{1}{3}$, (f) 0; for B (a) 0, (b) 0.2, (c) $\frac{1}{3}$, (d) 0.5, (e) $\frac{2}{3}$, (f) 1; for K_t (a) ∞, (b) 4, (c) 2, (d) 1, (e) 0.5, (f) 0 (after correction for the CD enhancement effect due to substitution of a methyl group for a proton in m^7G^+pG by a factor of 1/1.3; see text).

Table II. Overall Ionization (K_1 and K_2), Microscopic Ionization ($K_1^{3'}$, $K_1^{5'}$, $K_2^{3'}$, and $K_2^{5'}$), and Tautomeric ($K_t = [^+GpG]/[GpG^+]$) Constants of GpG at 25°

pK_1	pK_2	K_t	$pK_1^{3'}$	$pK_1^{5'}$	$pK_2^{3'}$	$pK_2^{5'}$
2.51	1.49	1.7	2.08	2.31	1.69	1.92

that on the 3′-terminus base. The lesser acidity of ^+GpG is as might have been expected from the observed changes in CD spectra during titration of GpG, as will be described in the next section.

Determination of Equilibrium Quotient, K_t, by Comparison of CD Spectral Data. An additional experimental approach was employed in checking the K_t value calculated from ionization constants. Because the value of the constant lies not far from unity, the equilibrium constant may be approximated by comparing the CD spectra of ^+GpG plus GpG^+ and a pair of sequence isomers m^7G^+pG and Gpm^7G^+.

The observed CD at a given pH can be considered to be made up of a number of terms each of which is the product of a concentration and an intrinsic CD for one of the species in the solution. Depending on the relative magnitude of $\Delta\epsilon_1$, $\Delta\epsilon_2$, and $\Delta\epsilon_3$, the observed $\Delta\epsilon$ value at a given wavelength varies in different ways with the pH of the solution, so that the CD of the monoprotonated species (^+GpG plus GpG^+) has been calculated by solving the set of n simultaneous equations by a least-squares minimization of their fit to the computed graph of circular dichroism vs. pH as a function of wavelength. The results are plotted in Figure 6a (the solid line).

As mentioned in the preceding section, m^7G^+pG and Gpm^7G^+ can be distinguished unambiguously by means of CD (Figure 2), though the absorption spectrum of the former resembles that of the latter. Gpm^7G^+ lacks the large positive CD band at 292 nm, which is characteristic of m^7G^+pG. The monoprotonated GpG exhibits the positive CD band at 292 nm associated with the intermediate ^+GpG species to an appreciable extent. Methyl groups, for the most part, exert only second-order effects on the position and intensity of allowed transitions, and accordingly the CD spectra of N-methyl derivatives, m^7G^+pG and Gpm^7G^+, can be taken as good models for the CD characteristics of ^+GpG and GpG^+. On this assumption the relative amounts of the ^+GpG and GpG^+ forms of the monoprotonated GpG may be estimated from the intensities of the selected CD bands in the spectra of a mixture of ^+GpG and GpG^+, and a pair of isomers, m^7G^+pG and Gpm^7G^+. However, the spectra of N^7-methyl derivatives allow a lower limit to be calculated for K_t, as the intensity of the main bands above 240 nm in the CD spectrum of m^7G^+pG is believed to increase by about 30% relative to ^+GpG as judged from the case with m^7G^+pU and ^+GpU.[11] The corresponding enhancement in the intensity was not observed for unstacked

Upm^7G^+ relative to UpG^+. When this correction is introduced, we are able to show how the CD spectrum of the monoprotonated GpG would vary as a function of K_t as illustrated in Figure 6b. In a regular manner, as the K_t values is increased, the bands near 258 and 292 nm get more positive and the band near 275 nm gets more negative.

The high degree of coincidence between the CD spectrum of the monoprotonated form, ^+GpG plus GpG^+, and the combined CD curve "c" ($K_t = 2$) in Figure 6b is shown in Figure 6a.[28] The K_t values obtained by the two methods, i.e., the pK method and the CD method, agree satisfactorily. The balance of evidence accordingly suggests that ^+GpG is more stable than the isomer, GpG^+, in aqueous solution. The greater stability is presumably due to stacking interaction; the stacking energy of ^+GpG is probably greater than that of GpG^+, since in the former the anti → syn conversion of the Guo residue at the 5′ end occurs on protonation, allowing the two base residues to overlap more effectively.

Evidence that ApA is less stacked in the mono- and diprotonated forms is presented and is thought to show that the protonation of ApA leads directly to the formation of the diprotonated species.[3] By contrast, we have shown that GpG gives clearly distinguishable CD spectra due to monoprotonation in solutions for which the acidity was insufficient to cause diprotonation. The observed relatively high K_2/K_1 ratio (=10.5) for GpG seems to be in contrast to the case of ApA or UpU, and this should be ascribed to stabilization of the species ^+GpG through a preferential protonation on one of two guanine bases in a molecule which accompanies the simultaneous augmentation of the syn conformation in a 5′-terminal nucleoside. Thus, an intramolecular enhanced stacking interaction in the monoprotonated form, right-handed (syn)Gp(anti)G, would facilitate the first ionization step and make the second one more difficult, thereby increasing the K_2/K_1 ratio.

Incidentally, the present investigation of the acid-base chemistry of GpG has been encouraged by the recent theoretical findings[29] that the stacking interactions are invariably more favorable for half-protonated pairs than for neutral pairs in decreasing order of stability: $^+G\text{-}G > {}^+T\text{-}T > {}^+C\text{-}C > {}^+A\text{-}A$. In this connection the strong enhancement effects of the CD band intensity in the monoprotonated ^+GpG are noteworthy.

Some additional results in the area of the acid-base chemistry of homodinucleotides will be reported in a forthcoming paper.

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References and Notes

(1) R. A. Cox, *Biochem. J.*, **100**, 146 (1966).
(2) H. Simpkins and E. G Richards, *J. Mol. Biol.*, **29**, 349 (1967).
(3) H. Simpkins and E. G. Richards, *Biochemistry*, **6**, 2513 (1967).
(4) J. Clauwaert and J. Stockx, *Z. Naturforsch. B.*, **23**, 25 (1968).
(5) K. Javaherian, *Biopolymers*, 11, 2053 (1972).
(6) Abbreviations used are CD = circular dichroism; GpG = guanylyl-(3′→5′)-guanosine; ^+GpG and GpG^+ = half-protonated GpG at either a 5′-terminal base or a 3′-terminal base; m^7GpG = N^7-methylguanylyl-(3′→5′)-guanosine; m^7GpU = N^7-methylguanylyl-(3′→5′)-uridine; brGpA = 8-bromoguanylyl-(3′→5′)-adenosine; Guo = guanosine.
(7) M. M. Warshaw and I. Tinoco, Jr., *J. Mol. Biol.*, **20**, 29 (1966).
(8) Y. Inoue, S. Aoyagi, and K. Nakanishi, *J. Am. Chem. Soc.*, **89**, 570 (1967).
(9) W. Guschlbauer, I. Fric, and A. Holý, *Eur. J. Biochem.*, **31**, 1 (1972).
(10) S. K. Podder, *Biochemistry*, **10**, 2415 (1971).
(11) Y. Watanabe and Y. Inoue, *FEBS Lett.*, **35**, 344 (1973).
(12) J-F. Chantot, T. Haertle, and W. Guschlbauer, *Biochimie*, **56**, 501 (1974).
(13) K. Satoh and Y. Inoue, *Biochem. J.*, **114**, 271 (1969).
(14) H. Ikenaga and Y. Inoue, *Biochemistry*, **13**, 577 (1974).
(15) J. W. Jones and R. K. Robins, *J. Am. Chem. Soc.*, **85**, 193 (1963).
(16) G. Heys, H. Kinns, and D. D. Perrin, *Analyst*, **97**, 52 (1972).
(17) S. Hendler, E. Fürer, and P. R. Srinivasan, *Biochemistry*, **9**, 4141 (1970).
(18) W. Guschlbauer and Y. Courtois, *FEBS Lett.*, 1, 183 (1968).
(19) T-D. Son, W. Guschlbauer, and M. Guéron, *J. Am. Chem. Soc.*, **94**, 7903 (1972).
(20) Cf., 1,7-dimethylguanosine iodide has been examined by single-crystal X-ray diffraction methods and shown to exist as the syn conformer: E. Shefter, S. Singh, and P. Sackman, *Chem. Commun.*, 261 (1974).
(21) A. M. Michelson, C. Monny, and A. M. Kapuler, *Biochim. Biophys. Acta*, **217**, 7 (1970).
(22) The ionization constants are identical, within the limits of experimental error, with those obtained from the absorbance–pH titration at 292 nm by a least-squares computer method (see Figure 4a): $pK_1 = 2.54$ and $pK_2 = 1.51$.
(23) K. P. Ang, *J. Phys. Chem.*, **62**, 1109 (1958).
(24) GpG is found to follow eq 8 and 9 in the Ang's paper,[23] and a least-squares computer analysis of the readings taken from the middle portions (above pH 0.98) of the steep sections of the $\Delta\epsilon_{292}$ vs. pH gave $pK_1 = 2.52$ and $pK_2 = 1.46$ from the gradients of the relations and the values of 1.49 and 0.17 for $\Delta\epsilon_2$ and $\Delta\epsilon_1$ from the intercepts.[25] Thus, the close similarity in the $\Delta\epsilon_1$ value of $^+GpG^+$ estimated from the method of Ang and that obtained from the direct measurement in 0.5 *M* HCl indicates that ionization effects of the primary phosphate group should be small (pK value of the primary phosphate group is near 0).
(25) O. Ogasawara and Y. Inoue, *Chem. Lett.*, 547 (1975).
(26) P. O. P. Ts'o, "Fine Structure of Proteins and Nucleic Acids", G. D. Fasman and S. N. Timasheff, Ed., Marcel Dekker, New York, N.Y., 1970, p 94.
(27) Determination of the basic ionization constants for m^7G^+pG and Gpm^7G^+ in acidic solution was difficult because at pH below 1 acid hydrolysis of the glycosidic bond of the m^7G residue, which upsets the equilibrium, was appreciable during the time in which the spectrophotometric measurements were performed. This difficulty was overcome by computer analysis of the absorbance data taken at the analytical wavelength at pH above 0.9. (The absorbance of the dication, $m^7G^+pG^+$ or $^+Gpm^7G^+$, was not obtained from direct readings.)
(28) When the newly obtained value of 2.0 for K_t is combined with the values of pK_1 and pK_2, another set of the microscopic ionization constants can be determined as $pK_1^{3'} = 2.03$; $pK_1^{5'} = 2.33$; $pK_2^{3'} = 1.67$; $pK_2^{5'} = 1.97$.
(29) F. Jordan and H. D. Sostman, *J. Am. Chem. Soc.*, **95**, 6546 (1973).