in ethanol–dioxane (2:1) mixture (60 ml) over Pd/C (0.3 g) and the reaction mixture was worked up as described for **13c**. The syrupy product gave, after stirring with ether, a solid **13d** (0.24 g, 79%), homogeneous on tlc (S_2 and S_3), mp 140–142°. Crystallization of the product from ethanol raised the melting point to 150–151°, which was not depressed on admixture with a sample of the racemic compound (lit.[25] mp 165–168°): $[\alpha]^{25}D$ −33.4°, $[\alpha]^{25}_{436}$ −74.4°, $[\alpha]^{25}_{365}$ −136.6° (*c* 0.5, $CHCl_3$); uv max (95% ethanol) 260 nm, min 227; nmr ($CDCl_3$) δ 8.33 (s, 1, H_8), 7.92 (s, 1, H_2), 6.31 (t, 1, $H_{1'}$ partially overlapped with NH_2), 6.43 (broad s, 2, NH_2), 4.14 (q, 2, $H_{4'}$), *ca.* 2.31 (m, 4, $H_{2'} + H_{3'}$). Uv, ir, and nmr spectra correspond to those of the racemic product.

Anal. Calcd for $C_9H_{11}N_3O$: C, 52.68; H, 5.40; N, 34.13. Found: C, 52.47; H, 5.42; N, 33.92.

3-*N*-Methyl-5-fluoro-2′-deoxyuridine (9b).[32] A solution of 5-fluoro-2′-deoxyuridine (**9a**, 0.25 g, 1 mmol) and dimethylformamide dimethyl acetal (1 ml, *ca.* 10 mmol) in dimethylformamide (10 ml) was heated 5.5 hr at 90° (bath temperature) and the reaction mixture was evaporated to a syrup (0.1 mm, 50°). The addition of ether together with chilling to −20° produced a crystalline solid, 0.21 g (81% yield) of **9b**, mp 75–80°, which was homogeneous on tlc (S_2). The analytical sample was crystallized from ethyl acetate (0.15 g, 58%), mp 80–81° (sinters from 60°): $[\alpha]^{22}D$ +33.2°, $[\alpha]^{22}_{436}$ +89.6°, $[\alpha]^{22}_{365}$ +200.8° (*c* 0.5, dioxane); uv max (95% ethanol) 269 nm (ε 7400), min 236 (1100); nmr (CD_3COCD_3) δ 8.24 (d, 1, H_6), 6.32 (t, 1, $H_{1'}$), 4.50 and 3.97 (m, 2, $H_{4'} + H_{3'}$), 3.31 (s, 3, NCH_3), 2.35 (m, 2, $H_{2'}$), 2.89 (broad s, 2, OH), 3.84 (d, 2, $H_{5'}$).

Anal. Calcd for $C_{10}H_{13}N_2FO_5 \cdot H_2O$: C, 43.17; H, 5.41; N, 10.07. Found: C, 43.37; H, 5.50; N, 9.92.

(32) This compound was mentioned briefly by T. A. Khwaja and C. Heidelberger, *J. Med. Chem.*, 13, 64 (1970), without providing details of the method of preparation and without characterization.

Acknowledgments. The authors are indebted to Mr. N. Cvetkov for measuring uv, ir, and nmr spectra and optical rotations, and also to the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md., for providing us with a sample of racemic **13d**. The authors gratefully acknowledge the generous sample of 5-fluoro-2′-deoxyuridine provided by Hoffmann-La Roche Co., Nutley, N. J.

Tautomerism of Nucleic Acid Bases. II. Guanine[1]

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Abstract: It has long been noted that the H_8 proton of guanosine and its related derivatives exhibits unusually broad resonances in the pmr spectra under certain conditions of temperature and pD. We have examined this phenomenon as a function of temperature, concentration, and solution pD, as well as the external magnetic field, and have shown that the line broadening arises from chemical exchange between the lactam and lactim tautomers of the guanine base. The observation of sharp H_8 resonances in guanine derivatives where the guanine base is frozen in only the lactam and lactim tautomeric structure supports this interpretation. This tautomeric exchange was found to be catalyzed by OD^- and the solvent D_2O. Analysis of the temperature and pD dependence of the H_8 line width for 2′-GMP led to the following rate law at 30°: $-(d[A]/dt)_f = 1.7 \times 10^9[OD^-][A] + 0.7[D_2O][A]$ M sec^{-1}, where A represents the major tautomeric species of 2′-GMP. Activation energies of 7 and 13 kcal/mol were deduced for the OD^-- and D_2O-catalyzed steps, respectively. The minor lactim tautomer was estimated to be present to the extent of 16 ± 3% at room temperature in neutral aqueous solution. In most guanosine derivatives, with the notable exception of 2′-GMP, the H_8 line width was also found to be strongly concentration dependent over the pD range 3–6. This concentration dependence has been interpreted in terms of the effect of intermolecular association on the lactam–lactim tautomeric equilibrium. Analysis of the data in terms of a rapid equilibrium involving the monomer, a hydrogen-bonded tetramer, and stacked aggregates of this tetramer yielded a tetramer formation constant of $2.5 \pm 0.5 \times 10^7$ M^{-3} and a tetramer stacking or association constant of 40 ± 10 M^{-1} in the case of 5′-GMP.

In a recent paper,[2,3] we showed that the unusual broadening of the cytosine H_5 resonance frequently observed in the pmr spectrum of cytosine and its nucleoside and nucleotide derivatives is due to tautomeric exchange between the amino and imino tautomers of the cytosine base. A detailed study of this line-broadening phenomenon has been made as a function of temperature, concentration, solution pH, and external magnetic field, and quantitative treatment of the linewidth data enabled us to ascertain both the equilibrium and the kinetics of this tautomerism. Contrary to what has generally been accepted,[4–6] the cytosine base was found to exist in a significantly high percentage in the abnormal tautomer (15 ± 3%) at room temperature. The kinetics data revealed both a solvent- and base-catalyzed step, but the rate of the tautomeric exchange was found to be quite slow. However, this slow kinetics is quite reasonable if the rate-determining step involves proton abstraction from the cytosine amino group by base or a solvent molecule.

This paper concerns a similar study with the guanine base. It has long been noted that the H_8 proton of guanosine and its derivatives also exhibits an unusually

(1) This work was supported in part by Grants GM 14523-04 and -05 from the National Institute of General Medical Sciences, U. S. Public Health Service, and Grant No. GP-8540 from the National Science Foundation.

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(5) A. R. Katritzky and A. J. Waring, *ibid.*, 1540 (1962).

(6) A. R. Katritzky and A. J. Waring, *ibid.*, 3046 (1963).

broad resonance in the pmr spectrum under certain conditions of temperature and solution pD.[7] We have examined this phenomenon as a function of temperature, concentration, and solution pD as well as external magnetic field, and have shown that the line broadening in this case arises from chemical exchange between the lactam and lactim tautomers of the guanine base. It has been postulated by Watson and Crick that the existence of minor tautomeric structures of the nucleic acid bases would be an important source of the imperfect base pairing which can lead to genetic mutation during DNA replication or RNA transcription.[8-10] In the case of guanine, the lactim form of the guanine (G) base has an electronic structure which is superimposable with an adenine (A) base to form an abnormal G–T base pair instead of the normal Watson–Crick G–C base pair (Figure 1). In view of the important biological implications of this proposal, it is desirable to obtain a detailed understanding of the thermodynamic as well as kinetic properties of the guanine lactam–lactim system.

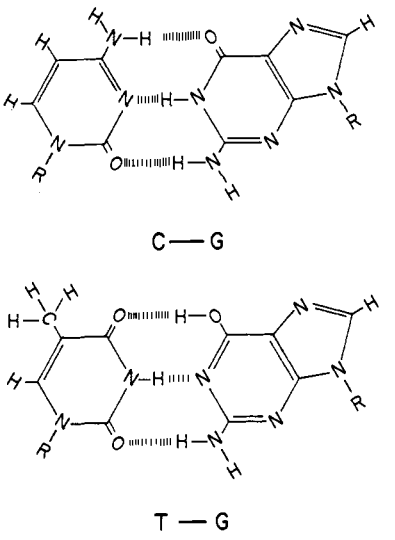

Figure 1. Abnormal G–T base pair and normal G–C base pair.

Experimental Section

Materials. The following guanine nucleotides were obtained either in the free acid or sodium salt form. Guanosine 2′-monophosphate (2′-GMP), 3′-GMP, and 5′-GMP were purchased either from Sigma Chemical Co., St. Louis, Mo., or from Calbiochem, Los Angeles, Calif., as grade A reagents. Guanosine and 2-*N*,*N*-dimethylaminomethyleneguanosine were obtained from Calbiochem. 1-Methylguanosine and 6-methoxypurine riboside were purchased from Cyclo Chemical Corp., Los Angeles, Calif.

Although the above compounds were purchased as highly pure grade A reagents, all materials were passed through a Dowex-50 cation exchanger to eliminate possible paramagnetic ion contaminants before use. Sample solutions were prepared at concentrations of 0.001–0.05 *M* in 99.7% D_2O supplied by Columbia Organic Chemicals, Columbia, S. C. The pD of each solution was measured with a Leeds and Northrup 7401 pH meter equipped with miniature electrodes and was calculated from the observed pH meter reading plus 0.4 (the standard correction).[11] pD adjustments were made with either 1 *M* DCl or 1 *M* NaOD.

Instrumentation. The pmr spectra of the various guanine nucleoside and nucleotide solutions were recorded on Varian HA-100 or HR-220 nmr spectrometers. A C-1024 time-averaged computer was used to enhance the signal-to-noise ratio. Chemical shifts were measured relative to an external TMS capillary, which also provided the field–frequency lock signal for HA-100 operation. The error in the measurement of the chemical shifts and line widths is ±1 Hz. The ambient probe temperature was 30 ± 1° in the case of the HA-100 spectrometer and 17 ± 1° for the HR-220. In the variable-temperature studies, the sample temperature was controlled to ±1° by means of a variable-temperature controller, and was measured using the methanol and ethylene glycol standards together with the calibration curves supplied by Varian.

Results

The solution properties of guanosine and its related derivatives have been studied by Gellert, *et al.*,[12] and Miles, *et al.*,[13] using uv, ir, and optical rotation as well as X-ray diffraction, and it has been found that some of these guanine derivatives aggregate in aqueous solution to form viscous gels at intermediate pH values around room temperature for sufficiently concentrated solutions. However, this tendency toward gel formation varies greatly with the compound. Guanosine and 5′-GMP have been shown to form viscous gels even at concentrations of the order of 10 m*M* whereas 2′-GMP and 2′-deoxyguanosine exhibit little tendency toward gel formation. This strong intermolecular association (pairing and stacking) can complicate the analysis of our nmr data, but if the data were obtained under conditions where gel formation is suppressed, these complications can be circumvented.

The pmr spectra of guanosine and several of its derivatives have been previously reported and discussed.[14] The guanine H_8 resonance appears approximately 8–9 ppm downfield from TMS. It is a singlet but it is often broadened, even at sufficiently low nucleoside or nucleotide concentrations where gel formation should no longer prevail and for those guanine derivatives which exhibit little tendency toward gel formation. The elucidation of this line broadening is the principal objective of this work.

pD and Concentration Studies. The variation of the chemical shift of the H_8 proton of guanosine and its derivatives with pH in aqueous solution has been previously reported.[14] A significant downfield shift of about 1 ppm occurs between pD 2.0 and 4.0 as the result of the protonation of the base (Figure 2). Above pD ~4.0 the chemical shift of the H_8 resonance of these compounds remains essentially constant, except in the case of 5′-GMP, where effects due to the ionization of the phosphate group are apparent, indicating that the base orientation is preferentially anti in this nucleotide.

By contrast, the line width of the guanine H_8 resonance in many guanine derivatives is broad over the pD range 2–8. In some of these derivatives, it is also strongly concentration dependent. The extent of broadening as well as the concentration and pD range over which the broadening is important are strongly dependent on the compound. We therefore discuss each case separately.

(a) 2′-GMP, 2-*N*,*N*-Dimethylaminomethyleneguanosine and Adenylyl-(3′–5′)-guanosine (ApG). It is known

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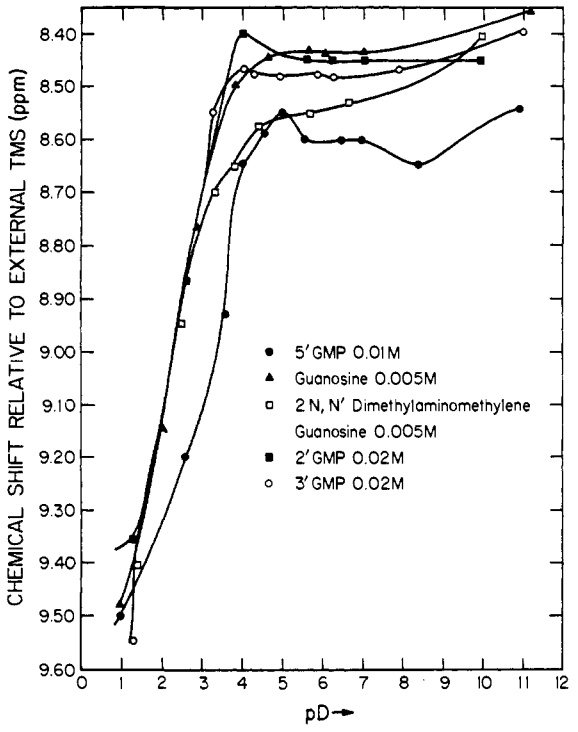


Figure 2. pD dependence of the chemical shifts of the H_8 resonance of guanosine and its related derivatives.

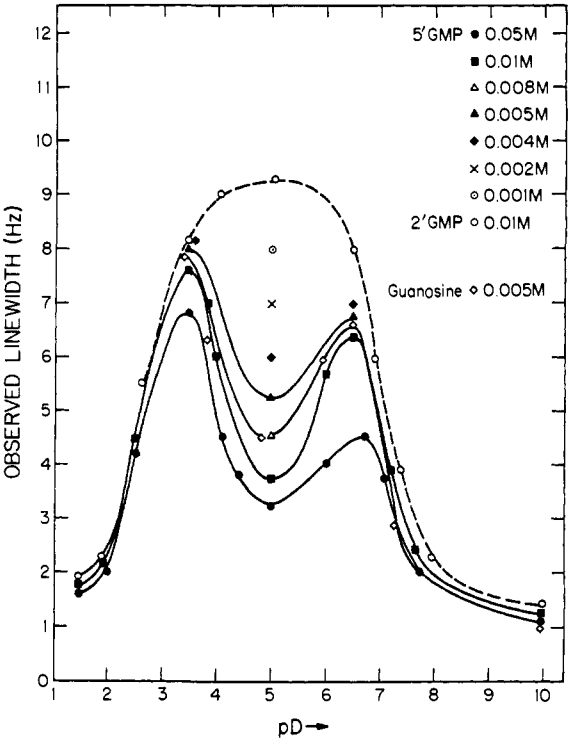


Figure 3. pD and concentration dependence of the line widths of the H_8 resonance of guanosine and its related derivatives at 30° (100 MHz).

that these compounds do not undergo gel formation. Accordingly, the line widths of the guanine H_8 resonances in these compounds were found to be concentration independent.

The pD dependence of the guanine H_8 resonance width of 2′-GMP at the concentration of 0.01 *M* is shown in Figure 3. The H_8 line width can be seen to be strongly pD dependent. In the low pD region, the line width increases with increasing pD, until it reaches a maximum of 9 Hz at pD ~5.0, beyond which it narrows with increasing solution pD until it reaches the limiting line width of ~1 Hz at pD >8.0. Insofar as we were able to ascertain, these observations are independent of the concentration over a 100-fold concentration from 0.001 to 0.1 *M*. Similar observations were noted for 2-*N*,*N*-dimethylaminomethyleneguanosine and the dinucleotide ApG. In the latter case, both the H_2 and H_8 resonances of the adenine residue remain sharp (2–3 Hz) throughout the whole pD range over which the line broadening of the H_8 resonance of the guanine residue was observed.

(b) Guanosine, 5′-GMP, and 3′-GMP. Guanosine and 5′-GMP are known to form viscous gel even at low concentrations. These solutions are often viscous even at concentrations as low as 5 m*M*, the concentration level which was normally used in our nmr measurements.

The pD dependence of the H_8 resonance of guanosine at a concentration of 0.005 *M* is shown in Figure 3. This concentration corresponds to the solubility limit of this compound. As in the case of 2′-GMP, the spectral width of the H_8 resonance is strongly pD dependent. In fact, except in the pD range 4–6, the line-width behavior is quite similar. In the low pD region, the line width increases with increasing pD until it reaches a maximum width of 8 Hz at pD 3.2. Beyond this pD, it decreases abruptly to a minimum width of 3.8 Hz at pD 5.0. As the pD of the solution is further increased, the H_8 line width increases again to a maximum of 6.5 Hz at pD 6.5, and, at still higher pD values, it narrows again with increasing solution pD until it approaches the limiting line width of ~1 Hz at pD >8.0.

The line-width behavior for the H_8 resonance of 5′-GMP at various pD values is similar to that for guanosine. For a 0.01 *M* solution of 5′-GMP, the two linewidth maxima were found to be 7.8 and 5.8 Hz at pD 3.5 and 6.5, respectively, while the line-width minimum at pD ~5.0 is 3.5 Hz. However, in contrast to the conspicuous lack of dependence of the line width on the concentration in the case of 2′-GMP, the line width of the H_8 resonance here is strongly concentration dependent, particularly at low nucleotide concentrations and in the intermediate pD region 3–6. This behavior is also true in the case of guanosine although we did not study the concentration dependence here in detail. As shown in Figure 3, at pD 5.0 the line width of the H_8 resonance of 5′-GMP increases gradually from 3.0 Hz at the concentration of 0.05 *M* to the limit of about 9.0 Hz for concentrations lower than 0.001 *M*. At pD 3.5, the H_8 line width increases from 6.0 to 8.0 Hz as the concentration is decreased from 0.05 to 0.008 *M*, but then remains unchanged as the solution is further diluted. At pD 6.5, the observed line width changes from 4.5 to 7 Hz as the concentration is decreased from 0.05 to 0.005 *M*. Below this latter concentration level, the line width remains essentially constant at ~7 Hz. Despite these dramatic changes in the H_8 line width, there was no noticeable change in the spectral position of this resonance over the tenfold concentration range of 0.005–0.05 *M*.

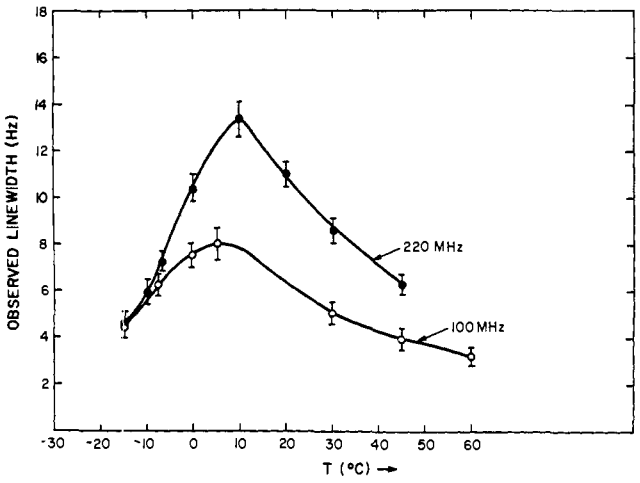


Figure 4. Temperature and frequency dependence of the H_8 line width of 2′-GMP at pD 7.0 ± 0.2.

With 3′-GMP, gel formation occurs only at concentrations in excess of 0.1 *M*. At the concentration levels normally used in our nmr experiment (<0.005 *M*), the line-width behavior of the H_8 resonance of 3′-GMP is similar to that observed for 2′-GMP.

(c) 2′-Deoxyguanosine, 5′-dGMP, 5′-GTP. Although these compounds do not form viscous gels in aqueous solution, the line-width behavior of the H_8 resonance in these compounds is similar to those observed for guanosine or 5′-GMP over the same pD range. The H_8 line-width minimum around pD 5.0 is, however, somewhat greater in these guanine derivatives than that for 5′-GMP at the same concentration.

(d) 1-Methylguanosine and 6-Methoxypurine Riboside. For these two compounds, the H_8 resonances remain sharp throughout the whole pD range of interest. In these methylated derivatives, the guanine base is frozen in the lactam form in the case of 1-methylguanosine and the lactim form in the case of 6-methoxypurine riboside.

Effect of Temperature and Magnetic Field. We have investigated the effect of temperature and magnetic field (or frequency) on the H_8 line width in several of the guanine derivatives.

The results for a 0.05 *M* 2′-GMP solution containing saturated sodium chloride at pD 7.0 ± 0.2 are summarized in Figure 4. For convenience of discussion, we have divided the whole temperature range into three regions. In the high-temperature region (>30°), the H_8 resonance of 2′-GMP was found to be strongly temperature and field dependent. The H_8 line width is much broader at 220 MHz than at 100 MHz and decreases gradually with increasing temperature. In the low-temperature region (<0°), the H_8 line width becomes essentially independent of field and decreases with decreasing temperature. The greatest line broadening was observed in the intermediate temperature region. At 220 MHz, the maximum H_8 line width is 13.0 ± 1 Hz at 10° and at 100 MHz the H_8 resonance reaches a maximum width of 8.0 Hz at 0°. Temperature studies of the H_8 line width at various solution pD values reveal a similar behavior, except that the temperature at which the observed H_8 line width is a maximum varies with the pD of the solution. At a given magnetic field, the temperature corresponding to the maximum line width was found to be greatest at pD ~5.0.

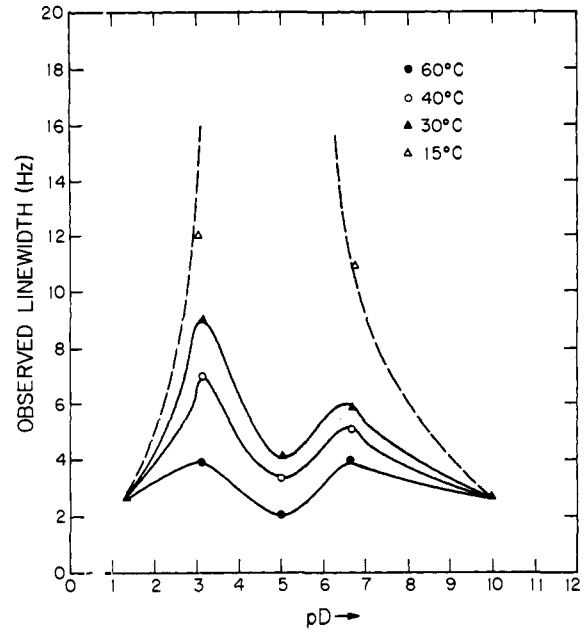


Figure 5. Temperature and pD dependence of the H_8 line width of 5′-GMP at 0.05 *M* and 100 MHz.

The pD dependence of the H_8 line width of a 0.05 *M* 5′-GMP solution is given at several temperatures in Figure 5. Perhaps the most striking feature of these data is the abrupt discontinuous increase in H_8 line width near 20° over the pD range 3–7. These observations together with the concomitant increase in the viscosity of the sample point to a phase transition from a disordered to a highly ordered system. Outside this pD range and above the "phase transition" temperature, the variation of the H_8 line width with temperature is similar to that observed for 2′-GMP.

Intensity Measurements. Experimentally we have observed that the intensity of the guanine H_8 resonance of 2′-GMP changes with temperature. We have studied this intensity variation in some detail and have summarized the results in Table I. These intensity measurements were made on a sample containing approximately 0.008 *M* 2′-GMP and 0.01 *M* 3′-AMP in a D_2O solution saturated with NaCl at pD 6.3. It was necessary to use 3′-AMP as a secondary intensity standard, as the guanine H_8 resonance of 2′-GMP appears significantly downfield from the ribose H_1' resonance, which could otherwise have been used as the primary intensity standard. In our intensity measurements we have therefore compared the intensity of the guanine H_8 resonance of 2′-GMP with the sum of the adenine H_2 and H_8 resonances of 3′-AMP, and this intensity ratio, $I_{H_8(G)}/[I_{H_2(A)} + I_{H_8(A)}]$, was in turn compared with the intensity ratio of the $H_{1'}$ resonances between these two nucleotides, that is, $I_{H_1'(G)}/I_{H_1'(A)}$.

At 20° and higher temperatures, the intensity ratio between the H_8 resonances of 2′-GMP and the average of the H_2 and H_8 resonances of 3′-AMP, namely, $I_{H_8(G)}/{}^1/_2[I_{H_8(A)} + I_{H_2(A)}]$, was found to be 0.77 ± 0.01. This value can be compared with the measured intensity ratio of 0.78 ± 0.01 for $I_{H_1'(G)}/I_{H_1'(A)}$ at the same temperature. At −10°, the intensity ratio $I_{H_8(G)}/{}^1/_2[I_{H_2(A)}$

Table I. Summary of Intensity Measurements[a]

Temp, °C	Intensity (arbitrary units) $H_8(G)$	Intensity (arbitrary units) $H_2(A) + H_8(A)$	$I_{H_8(G)}/\frac{1}{2}[I_{H_2(A)} + I_{H_8(A)}]$, %	Intensity (arbitrary units) $H_{1'}(G)$	Intensity (arbitrary units) $H_{1'}(A)$	$I_{H_{1'}(G)}/I_{H_{1'}(A)}$, %
20	56	146	77	110	140	79
20	90	234	77	150	190	79
20	108	276	78	115	150	77
20	70	184	76			Av 78 ± 1
20	44	110	80			
20	55	148	76			
20	115	304	76			
30	60	154	78			
50	55	145	78			
			Av 77 ± 1			
−10	40	120	67	120	160	75
−10	43	130	66	110	142	77
−10	46	130	71	95	125	76
−10	38	114	67			Av 76 ± 1
−10	20	56	71			
−10	29	84	69			
−10	34	102	67			
−10	42	126	67			
−10	69	200	69			
−10	86	268	64			
			Av 68 ± 2			

[a] Sample: 0.008 *M* 2′-GMP, 0.01 *M* 3′-AMP, pD 6.3, saturated NaCl.

$+ I_{H_8(A)}$] was found to be diminished to 0.68 ± 0.02, whereas the corresponding ratio for the $H_{1'}$ resonances, $I_{H_{1'}(G)}/I_{H_{1'}(A)}$, remained essentially unchanged at 0.76 ± 0.01. From these measurements it was concluded that the H_8 resonance of 2′-GMP at −10° accounted for only 88 ± 3% of the intensity at 20° or higher temperatures.

Discussion

Mechanism of Line Broadening. A number of mechanisms could cause broadening of the H_8 resonance of guanosine and its derivatives. These include contamination by paramagnetic impurities, spin coupling of the H_8 proton with neighboring ^{14}N nuclei, extensive molecular aggregation, and chemical exchange processes with rates observable by nmr.[2,3]

In our present work, we can rule out paramagnetic ion complexation as a source of line broadening in view of our extensive efforts to remove such possible contaminants in the preparation of our samples. The fact that we do not observe broadening of the H_8 resonance in some of the guanine derivatives as well as several other related compounds, *e.g.*, inosine, 5′-IMP, etc., would seem to rule out the presence of significant concentrations of paramagnetic impurities in the solvent system used in these studies, since the same D_2O was used throughout all the sample preparations. Moreover, with the exception of EDTA, the effects of which we shall comment on later, the addition of efficient chelating agents, such as ethylenediamine and 8-hydroxyquinoline, even at concentration levels of 10^{-3} *M*, does not lead to a reduction of the H_8 line width. Effects arising from scalar coupling of the H_8 proton to the two quadrupolar ^{14}N nuclei of the imidazole ring of the guanine base can also be eliminated, as there is no similar broadening of the H_8 resonances in inosine, 1-methylguanosine, and 6-methoxypurine riboside, all purine derivatives with seemingly rather similar electronic structure insofar as the imidazole ring is concerned.

These considerations leave molecular aggregation and chemical exchange processes as the only possible source of the H_8 line broadening. In the case of 2′-GMP, 2-*N*,*N*-dimethylaminomethyleneguanosine, and ApG, line broadening by molecular aggregation can further be ruled out since the H_8 line width is independent of the concentration. In the case of the other guanine derivatives, with the exception of 1-methylguanosine and 6-methoxypurine riboside, where no H_8 line broadening was observed, molecular aggregation does affect the H_8 line width, but our data here indicate that this intermolecular association leads to a *sharpening* rather than a broadening of the H_8 resonance at temperatures above the phase transition for the gel formation. In fact, under these conditions, the H_8 line width of guanosine, 5′-GMP, etc., approaches that observed for 2′-GMP as the solution is diluted. For all the guanosine derivatives, except 1-methylguanosine and 6-methoxypurine riboside, therefore, there is an intrinsic line broadening of the H_8 resonance at infinite dilution which can only be accounted for by chemical exchange.

Since the H_8 broadening in 2′-GMP is concentration independent, we first discuss this relatively simple case. Here, the observed variation of the H_8 line width with temperature depicted in Figure 4 leaves little doubt that the line broadening is due to chemical exchange of the guanine base between two chemically distinct environments. The three temperature regions cited earlier in the Results correspond to the regions of fast, intermediate, and slow chemical exchange. This conclusion is clearly borne out by the effect of frequency and/or magnetic field on the line broadening, where the nmr time scale has been altered by varying the magnetic field. As expected, a strong field dependence of the H_8 line width was observed in the high-temperature region, where the exchange rate is fast compared to the nmr time scale. At the low-temperature region, where the exchange rate is slow, the observed H_8 line width is expected to be field independent, as observed. In this limit, one might expect to see distinct resonances for the individual species involved in the chemical ex-

change. But if the percentage of the minor species is low, the resonances for the minor species would be much broader than those of the major species and might escape detection because of low signal intensities. However, if the percentage of the minor species is not too low, we might expect some reduction in the integrated intensity of the resonances of the major species at low temperatures from that at higher temperatures, to an extent commensurate with the population of the minor species. We do observe such an intensity reduction. The results of our intensity measurements indicate a reduction of some 12 ± 3% in the intensity of the H_8 resonance of 2′-GMP at −10° compared to that at 20° or higher.

A change in the magnetic environment of the H_8 proton can occur in a number of ways, but the fact that the same line broadening occurs at low concentration in the nucleosides as well as the nucleotides suggests that the sugar–phosphate moiety is not responsible for the chemical-shift difference of this proton between the two species. The magnetic nonequivalence, therefore, most likely arises from some modification localized in the guanine base. In view of our recent findings regarding the tautomerism of the cytosine base, a similar tautomerism of the guanine base immediately comes to mind. For guanine nucleosides and nucleotides there are two possible tautomeric exchange processes which might occur: (i) a lactam–lactim exchange involving proton transfer from N_1 to the C_6 keto oxygen, and (ii) an amino–imino exchange involving proton transfer between the amino group and N_3. However, since the H_8 broadening is still observed in the case of 2-*N*,*N*-dimethylaminomethyleneguanosine, where the amino–imino tautomerism is quenched, but is absent in the case of 1-methylguanosine, where the guanine base is frozen in the lactam structure, tautomeric exchange between the lactam and lactim forms of the guanine base is the most probable source of the observed guanine H_8 line broadening. The absence of H_8 broadening in 6-methoxypurine riboside, where the base is frozen in the lactim structure, supports this conclusion. A comparison of the H_8 chemical shift in guanosine with that in 1-methylguanosine as well as the earlier ir work of Miles[15] indicates that the major tautomer corresponds to the lactam form.

The pD dependence of the H_8 line width of 2′-GMP can be explained as follows. At high pD values, the chemical exchange is expected to be base catalyzed, as the presence of base will aid the removal of a proton from an N–H or OH group of the guanine base. If this is the case, both the forward and backward rates will be accelerated with increasing pD and correspondingly the H_8 line width will be reduced. At low pD values, the protonation of the guanine base occurs for both tautomeric species around pD 2–3. (pD titration of model compounds suggests similar pK_a values for both structures.) It should be noted, however, that this protonation process does not occur at the sites involved in the lactam–lactim exchange, since the sites of protonation on the guanine base are generally thought to be N_3 or N_7. Nevertheless, the H_8 line width should decrease precipitously below pD ~3.0, since we expect the tautomeric exchange to be grossly accelerated when the guanine base becomes positively charged. Consideration of the H_8 chemical shifts for the two tautomers and their respective protonated species also suggests that the effective chemical-shift difference between the lactam and lactim structures decreases with lowering pD. This would lengthen the nmr time scale of observation and contribute to decreasing the observed H_8 line width. Finally, it is interesting to note the "plateau" in the H_8 line width *vs.* pD profile over the pD range 4–6. This behavior can be contrasted with the rather abrupt pD dependence over the same pD region in the case of cytosine. We believe that this difference merely reflects the higher pK_a of the cytosine base compared to the guanine base. We shall later argue that the flat maximum in the 2′-GMP pD profile corresponds to the solvent contribution to the kinetics of the tautomeric exchange.

Some discussion of the possible origin of the chemical-shift difference of the H_8 proton between the two tautomeric structures would be appropriate at this point. Although the subject of substituent effects on proton chemical shifts in heterocyclic systems is a complicated one and it would be rather difficult to predict accurately the effect of the lactam–lactim tautomerism on the H_8 chemical shift, there is at least one contribution to this shift which may be ascertained with some reliability. It has been argued that since the pyrimidine base is not aromatic when the guanine base is in the keto structure, the induced motions of the π electrons of the pyrimidine ring in a magnetic field are rather localized so that the ring current magnetic anisotropy of the guanine base arises principally from the ring current of the π-electron cloud of the imidazole ring. Evidence for this is apparent from the unusual high-field position of the H_8 resonance in the pmr spectrum of guanosine compared with such completely conjugated systems as adenine nucleosides and nucleotides. However, upon transfer of a proton from N_1 to the C_6 keto oxygen to form the lactim structure, the pyrimidine ring becomes conjugated and its π electrons should contribute additional ring current magnetic anisotropy to the guanine base and lead to a downfield shift of the H_8 proton. Estimation of this ring current effect[16] as well as consideration of the H_8 chemical shifts in model compounds where the guanine base can exist in one or the other tautomeric structures suggests that this additional ring current effect can amount to ~30 Hz at 100 MHz.

We now turn to the more complicated case of 5′-GMP. Here the observed pD dependence of the H_8 line width at infinite dilution can also be attributed to tautomeric exchange. At higher concentrations, however, and particularly in the pD region 3–6, it is clear that some intermolecular process is affecting the tautomeric equilibrium and hence the H_8 line width. In view of the strong tendency of 5′-GMP toward gel formation, this molecular aggregation is to be expected, even though our experiments are undertaken at temperatures above the phase transition of the gel and at concentrations significantly lower than those where gel formation is supposed to prevail. Presumably this intermolecular association is also taking place in solutions of guanosine, 3′-GMP, 2′-deoxyguanosine, 5′-dGMP, and 5′-GTP, albeit to a lesser

(15) H. T. Miles, *Proc. Nat. Acad. Sci. U. S.*, 47, 791 (1961).

(16) J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High Resolution Nuclear Magnetic Resonance Spectroscopy," McGraw-Hill, New York, N. Y., 1959.

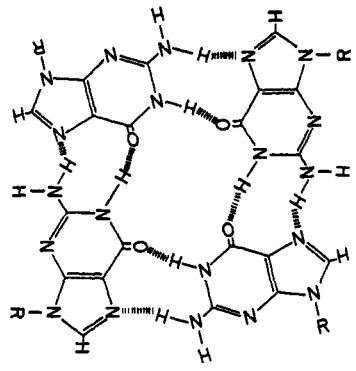


Figure 6. Proposed structure of the hydrogen-bonded tetramer of guanine derivatives (see ref 12 of text).

extent, judging from the effect of concentration on the H_8 line widths in these guanine derivatives. It has been proposed, at least in the case of 3′-GMP, that the gel formation proceeds *via* hydrogen bonding among four guanine bases to form a tetramer (Figure 6), and that these tetramers in turn stack to form higher aggregates. In the case of 5′-GMP, X-ray studies of fibers obtained from dehydration of the gels have also suggested the possibility of an alternate structure,[12] wherein one of the hydrogen bonds between the guanine bases in the tetramer is broken to form similar hydrogen bonds between contiguous tetramers in the "stack," followed by some tilting of the guanine bases to yield a more or less continuous helix. Although the structure of the molecular aggregates for 5′-GMP may indeed differ somewhat from that postulated for 3′-GMP in the gel or fiber state, it is not clear whether these structural differences can also be extended to their structures in solution. Moreover, if the guanine species in equilibrium in solution are principally those of the monomer, the tetramer, and aggregates of the tetramer, it is unnecessary to concern ourselves with these structural differences, as we have no way of ascertaining them in the thermodynamic considerations which we shall present later. The work of Gellert, *et al.*,[12] has suggested that the tetramer and its aggregated species are only stable in the intermediate pD region 3–6, where the phosphate group of a nucleotide would be protonated (pD <6.5) and when the guanine base is neutral (3.0 < pD < 9.0). Should this be the case, and if in addition the lactam tautomeric structure predominates[17] in the tetramer and its higher molecular aggregates, the intermolecular aggregation would shift the equilibrium away from the lactim species. In solution, we expect the equilibrium between the monomer, the tetramer, and its aggregated species to be rapid, *i.e.*, the time scale of breakup of the aggregates should be fast compared with their nmr transverse relaxation times. If this condition is satisfied, then the molecular association should lead to a narrowing of the H_8 resonance, as observed.

Kinetic Analysis. In order to satisfy our contention that the observed line broadening of the H_8 resonance of guanosine and its related derivatives arises from tautomeric exchange between the lactam and lactim tautomers of the guanine base, we have carried out a

(17) Although it is possible to write down a tetramer structure with each of the four guanine bases in the lactim form, this structure can be shown to be much less stable from thermodynamic arguments.

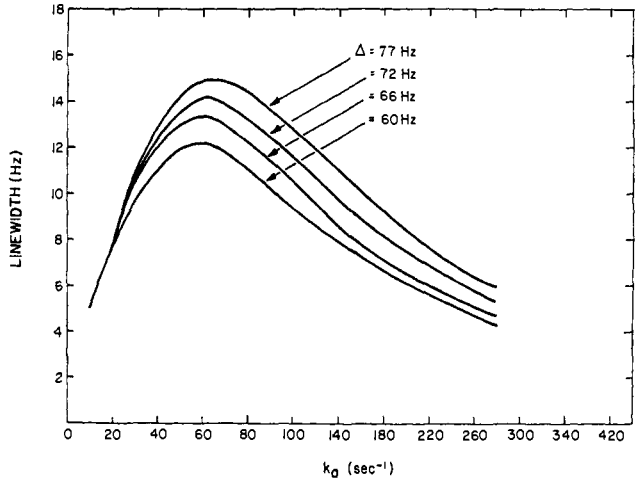


Figure 7. Calculated dependence of the H_8 line width at 220 MHz on the exchange rate constant $k_a(T)$ for a range of chemical-shift difference (Δ) of the H_8 proton between the two tautomers assuming 16% minor tautomer.

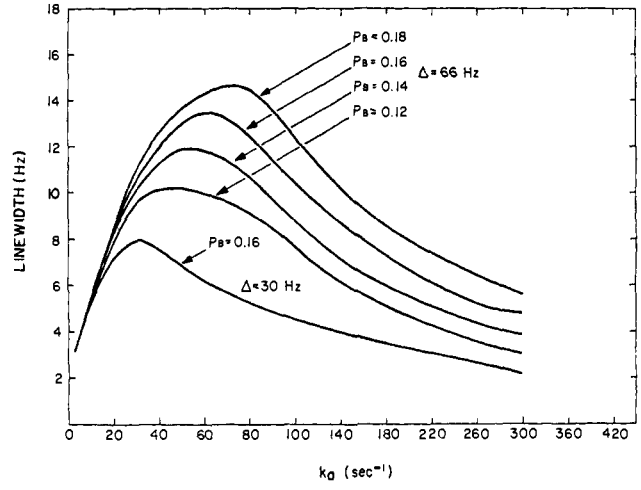
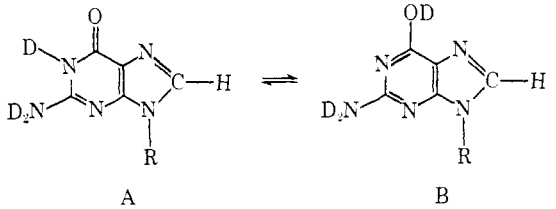


Figure 8. Calculated dependence of the H_8 line width on the exchange rate constant $k_a(T)$ for a range of populations of the minor tautomer assuming a Δ of 0.30 ppm.

detailed kinetic analysis of this problem similar to that which we have undertaken for the cytosine system.

We consider the relatively simple case of 2′-GMP, where molecular aggregation has been shown to be unimportant and therefore may be neglected, and where the H_8 line broadening would be predominantly determined by the chemical exchange between the lactam and lactim tautomeric species. Except at low pD values where the guanine base becomes protonated, our observation should then be adequately interpreted in terms of the following equilibrium



A B

Assuming this equilibrium, the line width of the H_8 resonance can be calculated for a given k_a (the pre-exchange rate constant for A), if the relative popula-

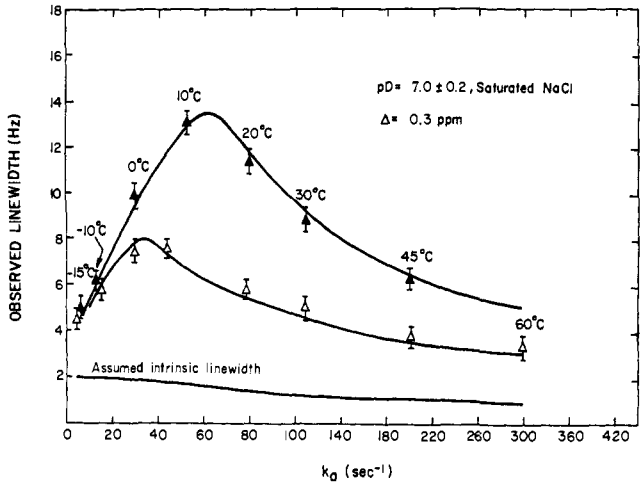


Figure 9. A comparison of the calculated and observed H_8 line width at 100 and 220 MHz for 2′-GMP near pD ~7.0 in saturated NaCl.

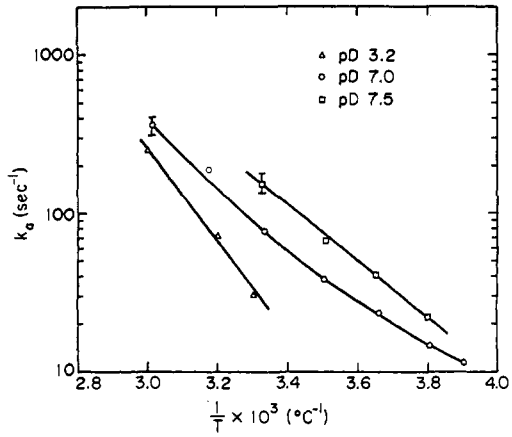


Figure 10. Log k_a *vs.* the inverse of the absolute temperature for 2′-GMP.

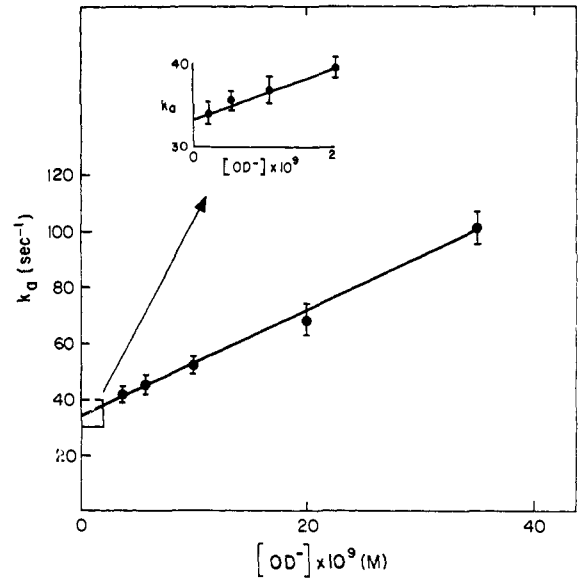


Figure 11. Dependence of the preexchange rate constant (k_a) on the base (OD^-) concentration.

tions between the two tautomers as well as the chemical-shift difference, Δ, are known. We have carried out such calculations for the H_8 resonance using the dynamic nmr program developed by G. Binsch and D. A. Kleier.[18] Typical results of this calculation are summarized in Figures 7 and 8. In Figure 7 the calculated line width is given as a function of k_a for a range of the chemical-shift difference Δ assuming 16% minor lactim tautomer. Similarly, in Figure 8, the H_8 line width is calculated as a function of k_a for several populations of the minor tautomer, p_B, ranging from 0.12 to 0.18 assuming a Δ of 0.30 ppm.

From plots of this type, we may extract the rate constant from the experimental H_8 line widths for a given assumed Δ and $\Delta G°$, where $\Delta G°$ is the standard free-energy difference between the two tautomers. At a given temperature, the observed line widths at 100 and 220 MHz must, however, yield the same k_a and this correspondence must apply over the whole temperature range, if Δ and $\Delta G°$ are to be unique. (We are assuming here that Δ and $\Delta G°$ are invariant over this temperature range.) As a result of a number of iterations, we have found that $\Delta \sim 66$ Hz at 220 MHz and $\Delta G° \sim 1.0$ kcal/mole gave the best fit to our data. A comparison of the observed H_8 line width at both 100 and 220 MHz with the theoretically predicted line width is depicted in Figure 9 for 2′-GMP at pD 7.0 ± 0.2 in saturated NaCl. We recall that our intensity measurements of the H_8 resonance had previously suggested that the percentage of the minor tautomer is 12 ± 3% at −10°, which corresponds to a $\Delta G°$ of ~1.0 ± 0.2 kcal/mol, and we had suggested that $\Delta \sim 0.3$ ppm. These values agree well with the results of our kinetic analysis and also indicate that to a good approximation $\Delta S° \approx 0$, $\Delta H° \approx \Delta G° \approx 1.0$ kcal/mol.

In Figure 10, we have plotted the log of the rate constant, log k_a, extracted from the above analysis against the reciprocal of the absolute temperature for 2′-GMP at pD 7.0 ± 0.2 in the presence of saturated NaCl. For comparison, we have included similar data at pD 3.2 and 7.5. Two exchange paths are indicated by the *non*linearity of the plot for pD 7.0 as well as the linearity of the plots at pD 3.2 and 7.5. In view of the strong pD dependence of the H_8 line width at high pD values (pD >6), and the lack of dependence of the line width on the solution pD over the pD range 4–6, we suggest that the tautomeric exchange under consideration is catalyzed by both the solvent (D_2O) and base (OD^-), *i.e.*

$$k_a(T) = \frac{1}{\tau_{ab}} = -\left(\frac{1}{[A]}\frac{d[A]}{dt}\right)_f = k_B[OD^-] + k_S'[D_2O] = k_B[OD^-] + k_S \quad (1)$$

and that the pD dependence of the log k_a *vs.* $1/T$ plots merely reflects the relative contribution of the two mechanisms at various pD values. At pD 7.5, base catalysis should dominate, whereas this mechanism should be insignificant at pD 3.2 for any reasonable value of k_B. Under these conditions, log k_a *vs.* $1/T$ should be linear, as observed.

In Figure 11, we have plotted the rate constant extracted from the line-width data *vs.* the OD^- concentration. (For reasons to be discussed below, we have neglected the line-width data below pD ~3.0.) This

(18) G. Binsch and D. A. Kleier, "The Computation of Complex Exchange Broadened NMR Spectra Computer Program DNMR," Quantum Chemistry Program Exchange, 1969.

plot clearly depicts the involvement of two kinetic pathways and enables us to determine the relative contribution of the solvent and base-catalyzed steps. The following rate law at 30° is deduced from this analysis

$$-\left(\frac{d[A]}{dt}\right)_f = 1.7 \times 10^9[OD^-][A] + 0.7[D_2O][A]\ M\ sec^{-1}$$

Both k_B and k_S' are of the proper order of magnitude for proton abstraction from an ND group by OD^- and D_2O.

The Arrhenius plot in Figure 10 yielded activation energies of 7 ± 1 kcal/mol and 13 ± 1 kcal/mol. A careful study of the temperature dependence of the H_8 line width at several pD values indicates that the lower activation energy component is the pD-dependent pathway.

As we discussed earlier, the abrupt sharpening of the H_8 line width below pD ~3.0 is most probably due to the protonation of the guanine base ($pK_a \sim 2.5$). Although the presence of acid can catalyze the exchange between the two tautomeric forms, our line-width data do not support this. Such an acid-catalyzed mechanism ought to be diffusion controlled, and would lead to a precipitous sharpening of the H_8 resonance at much higher pD values, say pD ~6.0. In fact, analysis of the low pD data in terms of an acid-catalyzed mechanism yielded a bimolecular rate constant of $2.5 \times 10^4\ M^{-1}$ sec^{-1}, a factor of 10^4 lower than that usually accepted for acid-catalyzed processes.[19] We therefore rule out this possibility.

Effect of Intermolecular Association. With the exception of 2′-GMP, 2-*N*,*N*-dimethylaminomethyleneguanosine, and ApG, the H_8 line width is concentration dependent over the pD range 3–7. The effect of increasing the concentration is one of line narrowing both in the case of 2′-deoxyguanosine derivatives, which are known not to form gels, and for guanosine, 5′-GMP, and 3′-GMP above the melting temperature of the gels. In the case of the latter compounds, gel formation leads to discontinuous broadening of the H_8 resonance, as expected.

In this section we shall be concerned with the equilibrium processes which lead to the observed sharpening of the H_8 resonance with increasing concentration. We shall assume that the line narrowing arises merely from the effect of intermolecular association on the tautomeric equilibrium, that the association proceeds *via* formation of the hydrogen-bonded tetramer proposed by Gellert, *et al.*,[12] and that under favorable conditions, the tetramer can undergo further aggregation by vertical stacking or other interaction. We shall further assume that tetramer formation is only important when the guanine base is in the normal keto structure, when the guanine base is neutral, and when the phosphate group is monoprotonated in the case of the nucleotides. Finally, it will be assumed in this analysis that the chemical exchange of a guanine base among the various species is rapid compared with the inverse of the shortest nmr transverse relaxation time of the largest molecular aggregate, and that these molecular aggregates do not contribute much broadening to the weighted averaged intrinsic line width. As it turned out, this latter approximation is not a serious one, since even at the concentration of 0.01 *M* 5′-GMP, the highest concentration employed in these studies, the largest molecular aggregates of any significant concentration involve no more than eight guanine bases and this associated dimer of the tetramer accounts for no more than 13% of the total G molecules in solution.

With these assumptions, the observed H_8 line width at a given stoichiometric concentration C_0 would be determined by $[G_{lactlm}]/C_0$, the fraction of molecules existing in solution in the lactim form irrespective of the degree of ionization of the phosphate group, and whether the guanine base is protonated or not. In the absence of extensive intermolecular association, the lactam tautomer should exist predominantly in solution as the monomer. We shall designate this species by G when the guanine base is neutral and its phosphate group is singly ionized. $[G_{lactlm}]$ can be related to the equilibrium concentration of G *via* the following multiple equilibria (eq 2–8). In these expressions, the

$$G \overset{K_T}{\rightleftharpoons} G^* \quad (2)$$

$$G_- \overset{K_T}{\rightleftharpoons} G_-^* \quad (3)$$

$$G_+ \overset{K_T}{\rightleftharpoons} G_+^* \quad (4)$$

$$G \overset{K_a'}{\rightleftharpoons} G_- + D^+ \quad (5)$$

$$G^* \overset{K_a'}{\rightleftharpoons} G_-^* + D^+ \quad (6)$$

$$G_+ \overset{K_a}{\rightleftharpoons} G + D^+ \quad (7)$$

$$G_+^* \overset{K_a}{\rightleftharpoons} G^* + D^+ \quad (8)$$

minor lactim tautomer is distinguished from the lactam species by a superscript asterisk, and the subscripts + and − are used to designate the species when the base becomes protonated and when the phosphate group is doubly ionized, respectively. Expressions 2–4 denote the tautomeric equilibria between the various lactam and lactim species. Ionization of the secondary hydrogen of the phosphate group is given by expressions 5 and 6; expressions 7 and 8 account for protonation of the guanine base. In these equilibria, we have assumed that the pK_a values of both the guanine base and the phosphate group are not affected by the tautomerism of the base as there is no experimental evidence to suggest the contrary. pH titration of model compounds indicates that the pK_a of the guanine base differs by no more than 0.1 or 0.2 p*K* unit between the lactam and lactim structures. The tautomeric equilibrium is therefore not grossly shifted upon protonation of the base. Ionization of the phosphate group should not affect the lactam–lactim equilibrium.

From the above multiple equilibria, it is readily deduced that

$$[G_{lactlm}] = [G_+^*] + [G^*] + [G_-^*] = K_T\left(1 + \frac{[D^+]}{K_a} + \frac{K_a'}{[D^+]}\right)[G] \quad (9)$$

This equation can be used to ascertain $[G]/C_0$ from the experimentally observed line width, if the kinetics of the tautomeric exchange deduced for 2′-GMP can be assumed to be applicable to all the guanine derivatives. Conversely, the pD and concentration dependence of the H_8 line width can be calculated if [G] is known as a

(19) M. Eigen and L. De. Maeyer, *Tech. Org. Chem.*, 8, 895 (1963).

function of pD and concentration at a given temperature. Note that in deducing $[G_{lactam}]/C_0$ and hence $[G]/C_0$ from the experimental line widths, proper considerations must be made to account for changes in the chemical-shift difference between the lactam and lactim species with pD and concentration, *i.e.*, changes in the effective nmr time scale. In the discussion to follow, for example, the chemical shift of the G molecules in the molecular aggregates may be somewhat different from that in the unassociated G species, in which case the association process will alter somewhat the effective nmr time scale which determines the chemical exchange contribution to the H_8 line width, assuming of course that the interconversion between the lactam and lactim species remains the rate-limiting step in the overall equilibrium. However, since the H_8 chemical shift was observed to be independent of the concentration over the complete range of concentration investigated (0.001–0.01 *M*), the chemical-shift difference between G* and the weight-averaged G species is changed at most a few hertz over this concentration range, and we may ignore this complication in our analysis.

In our model, intermolecular association decreases the equilibrium concentration of G. Accordingly, $[G_{lactim}]/C_0$ decreases, and the exchange contribution to the H_8 line width is reduced. The effect of this molecular aggregation can be accounted for by including the following processes (eq 10–13). Equation

$$4G \overset{K_1}{\rightleftharpoons} G_4 \tag{10}$$

$$2G_4 \overset{K_2}{\rightleftharpoons} (G_4)_2 \tag{11}$$

$$G_4 + (G_4)_2 \overset{K_3}{\rightleftharpoons} (G_4)_3 \tag{12}$$

$$\vdots \qquad \vdots \qquad \vdots$$

$$G_4 + (G_4)_{n-1} \overset{K_n}{\rightleftharpoons} (G_4)_n \tag{13}$$

10 gives the equilibrium for tetramer formation. The vertical stacking or association of the tetramer to form higher aggregates is depicted by eq 11–13. G_4, $(G_4)_2$, $(G_4)_3$ ... $(G_4)_n$ denote the hydrogen-bonded tetramer and its stacked or associated dimer, trimer, and *n*-mer, respectively.

For a given stoichiometric concentration C_0, [G] can be obtained from mass balance considerations. By definition

$$C_0 = [G] + [G_+] + [G_-] + [G^*] + [G_+^*] + [G_-^*] + 4[G_4] + 8[(G_4)_2] + \ldots 4n[(G_4)_n] + \ldots \tag{14}$$

from which it follows that

$$C_0 = (1 + K_T)\left(1 + \frac{K_a'}{[D^+]} + \frac{[D^+]}{K_a}\right)[G] + 4K_1[G]^4\{1 + 2[G]^4K_1K_2 + \ldots + n[G]^{4(n-1)}K_1^{(n-1)}K_2K_3\ldots K_n\} \tag{15}$$

If we now make the reasonable simplifying assumption that the stacking constants for each successive association step are equal, *i.e.*, $K_2 = K_3 = \ldots K_n = K$, then eq 15 simplifies to

$$C_0 - \eta[G] = \frac{4K_1[G]^4}{(1 - K_1K[G]^4)^2} = f([G]) \tag{16}$$

where

$$\eta = (1 + K_T)\left(1 + \frac{K_a'}{[D^+]} + \frac{[D^+]}{K_a}\right) \tag{17}$$

and

$$K_1K[G]^4 < 1 \tag{18}$$

It is not difficult to show that the above considerations do reproduce the two-maxima line width *vs.* pD profile experimentally observed for guanosine, 5′-GMP, etc. We shall omit the details here, except to point out that the theory does correctly predict the pD value corresponding to the line-width minimum. Over the pD range 3–6, the kinetics of the tautomerism is essentially independent of the pD, so that the minimum line width should occur at a pD for which $[G_{lactim}]/C_0$ is a minimum. Simple differentiation shows that $[D^+]_{min} = \sqrt{(K_aK_a')}$ or $(pD)_{min} = {}^1/_2(pK_a' + pK_a) \cong {}^1/_2(3 + 7) = 5$, in agreement with experiment.

We shall now attempt to extract the intermolecular association constants K_1 and K from the line-width data in the case of 5′-GMP, whose concentration dependence we have studied extensively. To do this, we have resorted to the following graphical method. From eq 16, we note that if $C_0 - \eta[G]$ and $f([G]) = 4K_1[G]^4/(1 - K_1K[G]^4)^2$ are plotted *vs.* [G], the curves should intersect at the experimental [G] values determined from the observed line widths for one unique value of K_1 and K. Our attempts to fit the experimental data are summarized in Figures 12 and 13. In Figure 12, only the data at low 5′-GMP concentrations have been used. Under these conditions $KK_1[G]^4 \ll 1$, so that $f(G) \approx 4K_1[G]^4$, and the correct value of K_1 may be determined by comparison of the experimental points with a family of curves of the function $f([G])$ corresponding to different K_1 values. In Figure 12, $f([G])$ is given for K_1 values of $10^7 M^{-3}$, $2.5 \times 10^7 M^{-3}$, and $10^8 M^{-3}$. The curve for $K_1 = 2.5 \times 10^7 M^{-3}$ can be seen to fit the low concentration data of 5′-GMP. After K_1 has been determined, the tetramer stacking or association constant can be obtained by fitting the high concentration data in a similar manner using the full expression for $f([G])$, *i.e.*, without making the $K_1K[G]^4 \ll 1$ approximation. This graphical determination of K is given in Figure 13, from which we ascertained that $K = 40 \pm 10 M^{-1}$ from the quality of the fit of the high concentration data. The above analysis yields the following distribution of species in solution for 0.01 *M* 5′-GMP: [G], 27%; [G*], 5%; [G_4], 55%; [G_4]$_n$ ($n \geq 2$), 13%.

The analysis contained in this section illustrates the subtle factors which determine the molecular aggregation properties of the guanine nucleosides and nucleotides. Presumably K and K_1 are both small in the case of 2′-GMP, 2-*N*,*N*-dimethylaminomethyleneguanosine, and ApG. In the case of 2′-deoxyguanosine, 5′-dGMP, and 5′-GTP, the value of K_1 is probably similar to that for guanosine and 5′-GMP, but K is apparently much smaller. Clearly gel formation is possible only when the tetramer stacking constant is large.

EDTA as a Catalyst for Tautomeric Exchange. As in the case of the H_5 resonance of cytosine and its derivatives, we have observed that the addition of 10^{-6} *M* EDTA is sufficient to sharpen the broad H_8 resonance in the guanine derivatives. There are two pos-

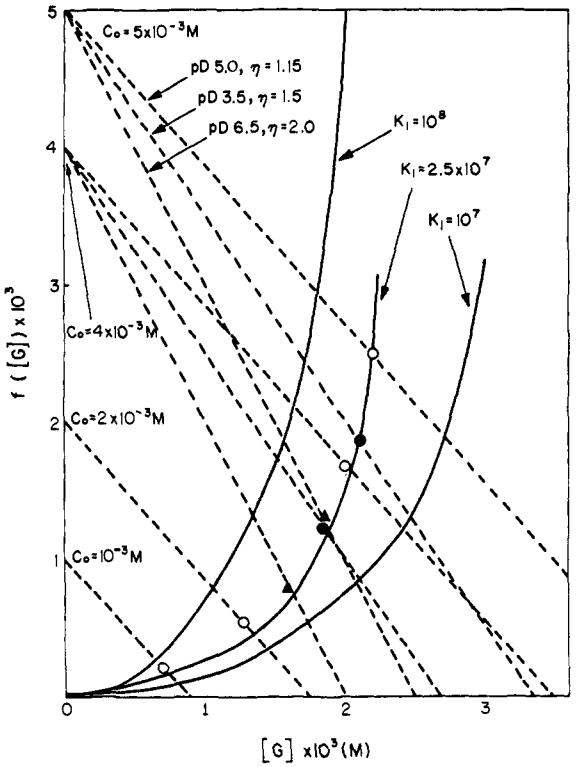


Figure 12. Graphical determination of the tetramer formation constant (K_1) for 5′-GMP.

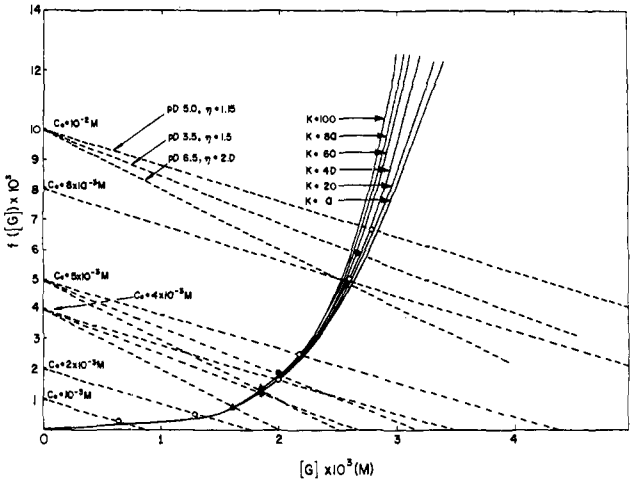


Figure 13. Graphical determination of the tetramer stacking constant (K) for 5′-GMP.

sible explanations for this remarkable phenomenon. The first of these is that the broadening is due to paramagnetic ion contaminants present at the concentration level of 10^{-6} M and the added EDTA is merely serving as a chelating agent. We can, however, argue that it is unlikely that paramagnetic ion contaminants at this concentration level should lead to the line broadening observed. Moreover if this were the case, other efficient chelating agents such as 8-hydroxyquinoline and ethylenediamine should be effective in collapsing the broadened H_8 resonance as well. The fact that these chelating agents do not sharpen the H_8 resonance even at concentrations as high as 10^{-3} M would seem to rule out this possibility. This leaves us with the other only alternative, which is that EDTA serves as a catalyst for the tautomeric exchange between the lactam and lactim tautomers of the guanine base. The manner in which EDTA can promote the tautomeric exchange is not exactly clear, but near neutral pD, EDTA is both a proton acceptor as well as a proton donor,[20] and hence we suspect it acts through a concerted mechanism involving simultaneous proton abstraction from the N_1–D by one of the four carboxylate anions and proton transfer from one of two protonated EDTA nitrogen sites to the keto group of the guanine base. If this mechanism is correct, then the efficiency with which EDTA catalyzes the tautomeric exchange indicates that the kinetics is almost diffusion controlled.

Conclusions

We have presented experimental evidence to show that the unusual H_8 broadening observed in guanosine and its related derivatives at low concentrations is due to chemical exchange between the lactam and lactim tautomers of the guanine base. We have studied this line broadening as a function of temperature, concentration, solution pD, and external magnetic field, as well as for guanosine derivatives capable of existing in only the normal and abnormal tautomeric structure. On the basis of a quantitative analysis of line-width data, we have ascertained the kinetics of the tautomerism, and have demonstrated that the tautomeric exchange between the lactam and lactim species is both base (OD^-) and solvent (D_2O) catalyzed. In the case of 2′-GMP, it was shown that the minor lactim tautomer is present to the extent of 16 ± 3% at room temperature in neutral aqueous solution. With the other guanine nucleosides and nucleotides, although the tautomeric equilibrium was found to be shifted to the lactam form by intermolecular association, all the evidence indicates that the thermodynamics of the tautomerism at infinite dilution is similar to that for the 2′-GMP case.

The results of this pmr study indicate that the guanine base exists in significant percentages in the abnormal lactim tautomeric form. This conclusion is contrary to what is generally accepted as all previous investigations of the guanine tautomerism had provided estimates which are significantly lower than our present indications. Miles has earlier concluded, on the basis of ir studies, that the population of the minor lactim tautomer of guanine is negligible under ordinary conditions.[15] Although a recent theoretical calculation by Bodor, *et al.*,[21] correctly predicted the lactam structure of the guanine base to be more stable than the lactim structure, the calculated energy difference between the two forms is again significantly larger than that indicated by our present study.

We need not overemphasize the biological significance of our present findings. On the one hand, the high percentages of the abnormal tautomers of guanine and cytosine can provide an important source of spontaneous mutation.[8-10] On the other hand, tautomerism of nucleic acid bases can allow for alternate base-pairing schemes which may be important in stabilizing RNA

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structures[22,23] as well as enzymatic and nonenzymatic codon–anticodon recognition.[24] The odd tautomers of cytosine and guanine, for example, may well provide a molecular rationale for the widespread occurrence of A–C and G–U base pairs thought to be present in tRNA structures.[25]

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Studies on Transfer Ribonucleic Acids and Related Compounds. V.[1] Synthesis of Ribonucleotides with Phosphomonoester End Groups on a Polymer Support

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Abstract: Protected ribooligonucleotides with 3′-phosphate end groups have been synthesized on a 4′-aminophenoxymethylpolystyrene support. The dinucleotide, Bz-C^{Bz}-(OBz)-*p*-U(OBz)-*p*, which was prepared by condensation of Bz-C^{Bz}(OBz)-*p* with the amidate resin of U(OBz)-*p* and liberated with isoamyl nitrite, was isolated by gel filtration on Sephadex LH-20 in 38% yield. The synthesis of four dinucleotides containing monomethoxytrityl groups and the trinucleotide ApUpGp is described.

The concept of polymer support synthesis developed in the polypeptide field[2,3] has been introduced by Letsinger into polynucleotide synthesis.[4] Several approaches have been devised for the synthesis of deoxyribopolynucleotides without phosphate end groups.[5–7] Blackburn has synthesized pTpTpT[8] on an insoluble resin containing aminophenoxymethylstyrene and released the product with acid treatment.[10] The acid treatment is not applicable to purine deoxyribonucleotides. The glycosidic linkage of N-acylated deoxyadenosine is especially labile to acid. The 2-(α-pyridyl)-ethanol function which was used to link phosphomonoesters to polystyrene required alkali to liberate thymidine oligonucleotides.[11] Recently Yip and Tsou reported a synthesis of uridine trinucleotide without a phosphomonoester end group using succinylated polystyrene which was cleaved with ammonia from the product.[12] Neither the acidic nor the alkaline condition is compatible with the oligonucleotides bearing specific protecting groups suitable for the fragment condensation. Polymer support synthesis of protected oligonucleotides has yet to be achieved.

The present paper reports the synthesis of protected ribooligonucleotides bearing 3′-phosphate end groups using an insoluble polymer of 4′-aminophenoxymethylstyrene and isoamyl nitrite treatment to release the protected oligonucleotides. Previously we described that aromatic phosphoramidates of protected ribonucleotides were key intermediates for ribooligonucleotide synthesis and could be selectively converted to the corresponding phosphate by the treatment with isoamyl nitrite.[9] The aromatic amidate group of protected deoxyribooligonucleotides also has been removed by this method without damage to glycosidic linkages or N protecting groups.[13] In this paper charging and release of protected deoxyribo- and ribomononucleotides are described first. Detritylation of ribonucleotides linked to the polymer allows condensation with protected ribonucleoside 3′-phosphates to yield protected ribooligonucleotides.

Kinetics of Charging and Release of the Mononucleotide

The formation of the phosphoramidate linkage and the recovery of nucleotides were studied first using 3′-*O*-acetylthymidine 5′-phosphate (I) and 4′-aminophenoxy-

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