pH-Dependent Laser Raman Spectroscopic Study of 8-Br-5'AMP

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Abstract: Vibrational assignments for 8-Br-5'AMP are reported. The intensity changes and frequency shifts of the 1456- and 1578-cm^{-1} absorptions in the pH-dependent Raman spectra of adenine and its analogues indicate that the syn conformation inhibits protonation at N_3. Our results suggest evidence for the presence of various resonance forms of the base in solution. Broadening in the 1578-cm^{-1} absorption at pH 9.4 suggests the presence of a deprotonated Schiff base. Ab initio self-consistent-field energies for protonated and deprotonated adenine and 8-chloroadenine indicate that substitution of chlorine decreases the electron density at the N_7 site, thereby reducing the chances of protonation at this site.

Studies of the basic units of nucleic acids have proven to be very useful in relating macromolecular structure and function. In particular, spectroscopic studies are used to determine reaction rates, structural parameters, and reaction mechanisms.[1] Laser Raman spectroscopy[2] is particularly useful since the absorption frequencies and intensities of Raman bands are sensitive to the molecular conformation of biological compounds,[3] and can be carried out in the liquid phase.

Obviously, characterization of relevant spectral absorptions is essential for obtaining useful information from the observed spectra. Normal coordinate analysis[4] has contributed significantly toward spectral characterization. In particular, the phosphate group frequencies[5] have been used to determine the backbone conformation of polynucleotides.[2] The 1100-cm^{-1} absorption has been assigned to the PO_2^- symmetric stretching mode, and the absorption occurring in the 800–880-cm^{-1} region has been used to determine the phosphate–ribose conformation for DNA and RNA.[2] The macromolecule RNA has been observed in the A-type conformation,[2] while DNA has been observed in the A-, B-, and C-type conformations.[2] The A, B, and C forms of DNA have Raman absorptions at 811 ± 4, 835, and in the 870–880 wavenumber regions, respectively.[2] The PO_2^- absorption at 1100 cm^{-1} is used as an internal standard for determining the percent conformation for the various types (A, B, and C) of DNA.[2] The intensity ratio $I(811\ cm^{-1})/I(1100\ cm^{-1})$ for 100% A-type DNA is generally taken to be 1.65 ± 0.05.[2] It has also been reported that the sugar group in nucleotides has either very weak or no Raman absorptions in the region where the absorptions for the base and phosphate groups are observed.[3] Thus, the normal coordinate analysis of the base can be related to the observed Raman spectra of nucleosides and nucleotides.

Protonation, deprotonation, and tautomerization in nucleic acid bases are believed to play a major role in the biochemical initiation mechanisms for translation, mutagenesis, and macromolecular conformation.[6] The substituents on the rings of the bases can alter the stability of various tautomers.[7a] The presence of various tautomers has been shown to alter the normal Watson-Crick base-pairing schemes in nucleic acid helices, which leads to mutations.[7b] O'Connor et al. (OJS)[8] have estimated the pK_a's of the nucleotides from the pH dependence of Raman spectra, while Chinsky et al. (CTD)[9] have studied the changes in the resonance Raman spectra of the bases and nucleosides due to Br substitution at the C_8 position of purines and at the C_5 position of pyrimidines at pH 7. The studies by OJS[8] and CTD[9] show that some of the Raman absorptions are sensitive to both protonation and bromine substitution. Del Bene[10] has calculated the protonation energies using ab initio self-consistent-field (SCF) methods and reports that protonation at the N_1 position of adenine is only slightly preferred over protonation at the N_3 position, which is in agreement with experimental data.[11] The N_7 position of adenine is shown to be the least probable protonation site,[12] but it is reported to be protonated below pH 1.[13]

Substitution of Br at the C_8 position of adenosine (or 5'AMP) causes a conformational change about the N-glycosyl bond from anti in 5'AMP to syn in 8-Br-5'AMP.[14] In the case of 8-bromoadenine nucleosides, the H-bonding interaction between the 5'OH group and N_3 is weakened upon protonation at N_1.[14] This suggests that the proton affinity of the N_3 position is descreased when N_1 is protonated and that steric interactions may hinder protonation at the N_3 position for the molecules in the syn conformation.

This study was undertaken to investigate the protonation–deprotonation in 8-bromoadenosine-5'-monophosphate (8-Br-5'AMP (Figure 1) and the effects of conformation on protonation. The Raman spectrum of 8-Br-5'AMP was studied in the 1.1–10.0 pH range and electronic energy calculations were performed for protonated and unprotonated adenine and for C_8-substituted adenine in order to correlate structural and/or electronic energy changes upon N_1-protonation and C_8-substitution with observed changes in the Raman spectra.

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Table I. Observed Vibrational Frequencies of 8-Br-5' AMP at Various pH Values[a]

pH =	1.1	2.0	3.1	4.0	4.9	5.9	6.9	8.1	9.1	10.0
	1570	1555	1598	1552	1578	1578	1576	1578	1572	1580
	1445	1454	1462	1443	1455	1461	1457	1458	1467	1466
	1428	1429	1423							
			1323	1319	1328	1325	1326	1330	1326	1325
	1302	1300	1299	1302	1301	1294	1293	1304	1296	1298
			1231	1222	1226	1238	1223	1233	1230	1231
							980	972	976	980
				787	801		787		800	
	759	764	759	771	761	762	761	758	761	768
	465	478	473	460	444	455	468	454	464	476

[a] The frequencies are in cm^{-1} and are accurate to ± 5 cm^{-1}.

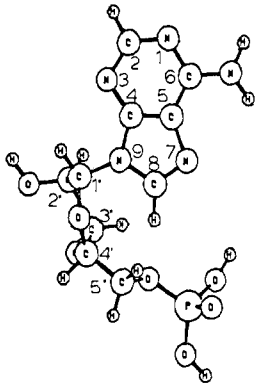

Figure 1. Adenosine-5'-mnophosphate; anti conformation.

Experimental Section

The 8-Br-5'AMP used in this study was synthesized in our laboratory following the method of Muneyama et al.[15a] The purity of the synthesized 8-Br-5'AMP is essentially the same as that of the samples synthesized by Muneyama et al.,[15a] but there is an increase in yield due to modifications.[15b] The samples were dissolved in distilled water, and pH adjustments were made with sodium hydroxide solution (pH 1.1–10.0). The pH was monitored with a Fisher Scientific combination electrode with a Ag/AgCl reference cell and calibrated with Fisher Scientific buffer solutions with the stated accuracy of ±0.01 pH units.

Raman spectra were recorded from 250 to 2000 cm^{-1} with a Spex Ramalog-6 spectrometer, with the argon 514.5-nm line (Coherent Radiation Model 54 Ar ion laser) as the excitation source. The reported frequencies are the average of three independent calibrations and are accurate to within ± 5 cm^{-1}. The vibrational assignments for the 8-Br-5'AMP absorptions were determined by using the experimental results in conjunction with the normal coordinate analysis of Tsuboi et al.[3]

The electronic energies for protonated and unprotonated adenine and the C_8-substituted species were calculated with an STO-3G basis set[16] (GAUSS80, QCPE 421). The optimized geometrical parameters for adenine, its tautomers, protonated adenines, and deprotonated adenines were taken from ref 9. The C_8Cl, N_9C_8, N_7C_8, C_5N_7, and N_9H bond lengths of 8-chloroadenine were optimized with an STO-3G basis set with the GAUSS80 program. We do not presently have a basis set for fourth row atoms; therefore, calculations were performed with Cl at the C_8 position, instead of Br. The 8-Cl-substituted nucleotides are reported to have the same conformation about the N-glycosyl bond as the 8-Br-substituted nucleotides.[17] The geometries for the doubly protonated species were estimated from the optimized geometries of the respective singly protonated species.

Results

Band Assignments. The spectra of 8-Br-5'AMP as a function of pH are shown in Figure 2. The observed vibrational frequencies are listed in Table I. At pH <2.7, the 1200 to 1500 cm^{-1} region has absorptions at 1300, 1429, and 1454 cm^{-1}, but at pH >3.1, absorptions are observed at 1231, 1300, 1323, and 1462 cm^{-1}. The absorptions at 1231, 1302, 1323, and 1423 cm^{-1} shift and/or change intensity with pH variation. Therefore, the observed changes in these absorptions must be due to deprotonation. The 761-, 1223-, 1326-, 1423-, and 1457-cm^{-1} absorptions are affected by Br substitution at C_8 (Table II) and may be due to the imidazole ring of adenine. The absorptions that vary with protonation

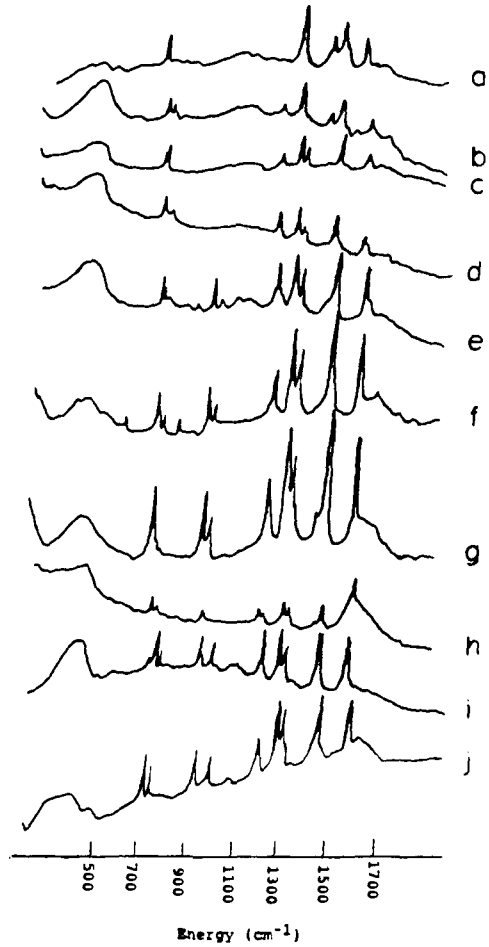

Figure 2. Raman spectra of 8-Br-5'AMP at various pH values: (a) pH 1.5; (b) pH 2.7; (c) pH 3.1; (d) pH 4.9; (e) pH 6.9; (f) pH 8.1; (g) pH 9.1; (h) pH 9.4; (i) pH 9.6; and (j) pH 10.0.

Table II. Changes in the Vibrational Spectra of 5'AMP and 8-Br-5'AMP Due to pH Variation

5'AMP[a]	8-Br-5'AMP[b]	changes due to pH[a,b]
1581[c]	1578	no change on deprotonation
1560[c,d]	*e*	disappears above pH 3.95
1509	1457	decreases in intensity upon deprotonation
1483[c]	*e*	disapperas below pH 3.01
1407[c,d]	1428[d]	decreases in intensity upon deprotonation
1378	1326	appears above pH 3.01
1340	1293	changes shape at pH 3.01
1290	1223	appears above pH 3.01
1253[c]	*e*	appears above pH 3.01

[a] Results of T. O'Connor et al., ref 11. [b] This work. [c] Found diagnostic for pK_a determination. [d] Not present at pH 7.0. [e] Does not appear in the spectra.

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Table III. Vibrational Assignments for 8-Br-5'AMP

ν (cm^{-1})	assignments[a,b]
468	$-NH_2$ out-of-plane deformation
761	in-phase ring breathing
980	PO_3^{2-} sym s
1223	N_1C_2 s + C_2H b + N_9C_8 s + C_8N_7 s
1293	C_5N_7 s - C_8N_7 s + N_1C_6 s + C_6NH_2 s
1326	N_1C_6 s - N_7C_5 s + C_8N_7 s
1423	C_6NH_2 s - N_1C_6 s + C_8Br b
1457	C_4N_9 s - C_5C_6 s + C_8Br b + N_3C_2 s
1576	C_4C_5 s + C_5C_6 s - C_4N_3 s - C_5N_7 s
1652	$-NH_2$ scissor

[a] s = stretch, b = bend; + = in phase, - = out of phase, sym = symetric; values are at pH 7 except the 1652- and 1428-cm^{-1} bands. [b] Assignments were made based on the results of T. O'Connor et al. (ref 8), the results of this study, and the normal coordinate analysis by Tsuboi et al. (ref 3).

and Br substitution at the C_8 position are the 1223, 1326, and 1423 cm^{-1} absorptions. Therefore, the absorptions at 1223, 1326, and 1423 cm^{-1} are attributed to vibrational modes arising from the pyrimidine and imidazole rings of adenine.

At pH >3.1, the 1423-cm^{-1} absorption disappears, while absorptions at 1231 and 1323 cm^{-1} are present. OJS[8] have observed similar behavior for the 1290-, 1378-, and 1407-cm^{-1} absorptions of 5'AMP, suggesting that the 1290-, 1378-, and 1407-cm^{-1} absorptions of 5'AMP and the 1231-, 1323-, and 1423-cm^{-1} absorptions of 8-Br-5'AMP arise from the same vibrational modes. CTD[9] associate the 1340-cm^{-1} absorption of adenine with the 1332-cm^{-1} absorption in 8-bromoadenosine. Our results indicate that it is the 1378-cm^{-1} absorption of 5'AMP that shifts to 1323 cm^{-1} in 8-Br-5'AMP, while the 1340-cm^{-1} absorption of 5'AMP is associated with the 1299-cm^{-1} absorption of 8-Br-5'AMP. The complete vibrational assignments are listed in Table III.

A comparison of intensity and frequency changes in the 1340-cm^{-1} absorption of 5'AMP (OJS)[8] and the 1299-cm^{-1} absorption in the spectra of 8-Br-5'AMP due to pH variation suggests that the 1340-cm^{-1} absorption in the 5'AMP spectra shifts to 1299 cm^{-1} in the 8-Br-5'AMP spectra. CTD[9] report that it is the 1310-cm^{-1} absorption in the adenine spectra that shifts to 1307 cm^{-1} in the 8-bromoadenosine spectra, and they assign this absorption to the N_7C_8 stretching mode. The relative intensity of the 1299-cm^{-1} absorption of 8-Br-5'AMP varies with pH, but it is not reported to be diagnostic for pK_a determination.[8] Therefore, we assign the 1299-cm^{-1} absorption to the C_6–NH_2 + N_7C_8 stretching vibration (where the + indicates in phase).

The 1236-cm^{-1} absorption of 8-bromoadenine is associated only with imidazole ring vibrations due to the -20-cm^{-1} shift (from 1256 to 1236 cm^{-1}) upon Br substitution at C_8.[9] On the other hand, OJS[8] report that the 1256-cm^{-1} absorption of 5'AMP is diagnostic for pK_a determination. Since the observed behavior of the intensities and frequencies as a function of pH indicates that the 1290-cm^{-1} absorption of 5'AMP shifts to 1223 cm^{-1} in 8-Br-5'AMP, it is possible that the 1256- and 1290-cm^{-1} absorptions of 5'AMP overlap at 1223 cm^{-1} in 8-Br-5'AMP. Therefore, the 1223-cm^{-1} absorption of 8-Br-5'AMP is assigned to the N_1C_2 s + C_2H b + N_9C_8 s + C_8N_7 s vibration (s = stretch; b = bend; + = in phase; - = out of phase).

The 1485-cm^{-1} absorption of adenine is reported to shift to 1465 cm^{-1} in the 8-bromoadenosine spectra (CTD).[9] The 1465-cm^{-1} absorption is the strongest absorption in the 8-bromoadenine spectrum while the 1340-cm^{-1} absorption is the strongest absorption in the adenine spectrum at neutral pH.[9] However, below pH 4.9, the 1299-cm^{-1} absorption of 8-Br-5'AMP is the most intense peak in the spectrum, but this change in intensity of the 1480- and 1340-cm^{-1} absorptions due to pH is not observed in the 5'AMP spectra. At this time, it is not clear whether intensity changes in one or both the 1299- and 1457-cm^{-1} absorptions due to pH variation causes the most intense peak to change. In 5'AMP, at pH 5.99, absorptions are present at 1483 and 1509 cm^{-1}; however, at pH 3.46, the 1483-cm^{-1} absorption is not detected, and the 1509-cm^{-1} absorption has gained intensity. OJS[8] report that the 1480-cm^{-1} absorption of 5'AMP is diagnostic for pK_a determination, although it is uncertain whether the 1457-cm^{-1} absorption in the spectra of 8-Br-5'AMP varies with pH. Since nitrogen protonation causes an increase in bond angle,[12] and bond angle changes affect the coupling of Raman active vibrations, N_3 protonation may cause the 1483-cm^{-1} absorption of 5'AMP to shift to 1509 cm^{-1}. Therefore, we conclude that the 1509-cm^{-1} absorption of 5'AMP (or the 1511-cm^{-1} absorption of adenine, CTD[9]) shifts to 1457 cm^{-1} in 8-Br-5'AMP, and we assign the 1457-cm^{-1} absorption to imidazole ring vibrations of 8-Br-5'AMP.

The 1578-cm^{-1} absorption does not vary with pH nor Br substitution, therefore we assign this absorption to the C_4C_5 double bond stretching vibrations. OJS[8] reports that in the 5'AMP spectra, the 1581-cm^{-1} absorption shifts to 1576 cm^{-1} upon deprotonation and is diagnostic for estimating pK_a values for deprotonation at N_1.

The 468-cm^{-1} absorption changes shape at pH 9.4 (Figure 2) and is assigned to NH_2 out-of-plane wagging.[3] At pH 8.1, a broad absorption is present at 1652 cm^{-1}, but at pH 9.4, the 1652-cm^{-1} absorption has merged with the 1578-cm^{-1} absorption (Figure 2). The 1652-cm^{-1} absorption occurs in the NH_2 scissoring frequency range.[18] The resonance Raman spectra of visual pigments show an absorption at 1620 cm^{-1} that is assigned to the unprotonated Schiff base.[2] Therefore, it is quite possible that the broadening observed in the 1578-cm^{-1} absorption at pH 9.4 is due to deprotonation of the external NH_2 group in basic solution. This fact further suggests that different resonance forms of adenine are present at different solution pH values. A similar behavior has been demonstrated for guanine.[19]

The 761-cm^{-1} absorption is assigned to a ring breathing mode where all atoms stretch in phase. In unsubstituted adenine, this absorption is observed at 729 cm^{-1},[3] but it is not affected by changes in pH.

The appearance of the 980-cm^{-1} phosphate symmetric stretching vibration at pH 6.9 indicates that the phosphate group is ionized.[18] The absorptions occurring in the 811 to 880 cm^{-1} region have been assigned to the ribose–phosphate diester linkage and are diagnostic for various forms of DNA and the A form of RNA.[2] So the appearance of a very weak absorption in the 787 to 805 cm^{-1} region for 8-Br-5'AMP suggests that an intramolecular interaction involving the phosphate group is present at pH $\geq$3.7.[2]

Discussion

The optimized geometries of adenine indicate an increase in the N_1C_2 bond length upon N_1 protonation accompanied by bond angle changes in the pyrimidine ring.[10] The Cl substitution does not significantly change the bond lengths in the imidazole portion of adenine, but Mulliken population analysis shows a small increase in the electronic charge in the 2p orbitals of the C_8 atom upon halogenation.[20] These results suggest that the disappearance of the 1223-cm^{-1} absorption below pH 3.1 is due to N_1-protonation (Table II), while the -20-cm^{-1} shift[9] is due to mass and force constant variations at the C_8 position.

The observed changes in the behavior of the absorptions in the 1475 to 1515 cm^{-1} region of 8-Br-5'AMP and 5'AMP due to pH variation and Br substitution at C_8 may be explained by assuming that, in the syn conformation, N_3 is not protonated, but an intramolecular interction with the ribose or phosphate group may be present. This will explain why there is only one absorption associated with the 1457-cm^{-1} vibration for 8-Br-5'AMP in neutral solution.

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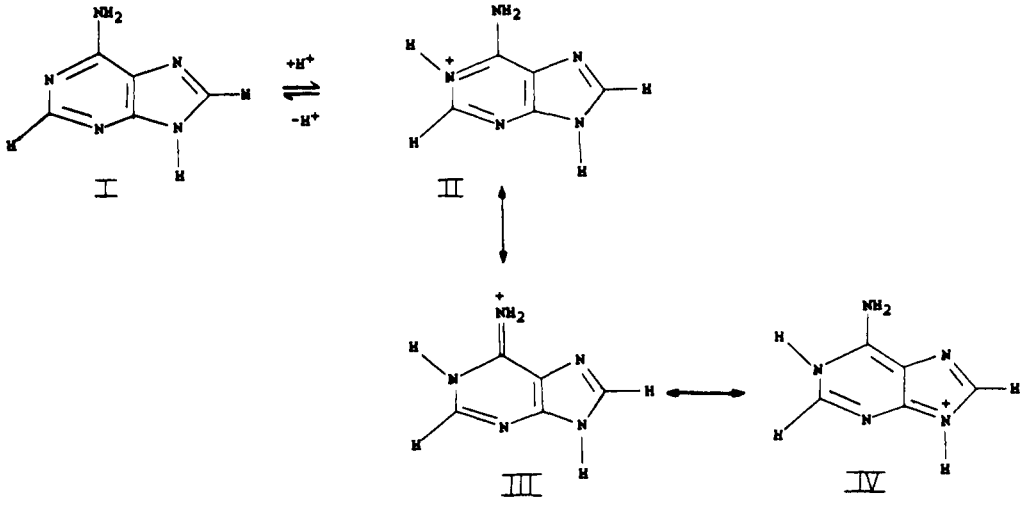
Scheme I



Scheme II

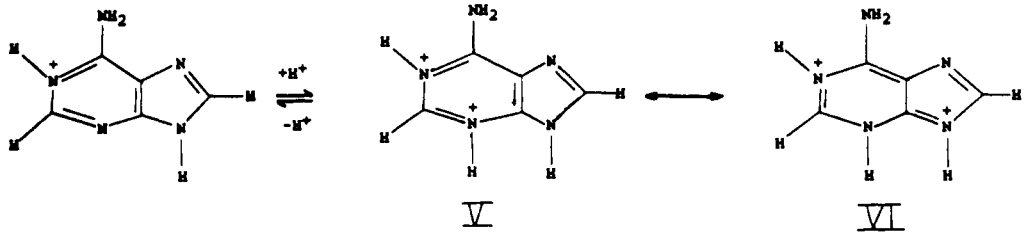


Figure 3. Protonation/deprotonation of adenine and its resonance forms.

Table IV. Electronic Energies and Relative Stability of Adenine and Protonated Adenine

	deprot[a]	N_1 prot[b]	N_3 prot[b]	N_7 prot[b]
		Electronic Energies (au)		
adenine	−458.61250	−459.08127	−459.07459	−459.05689
8-Cl-ade[c]	−912.61988	−913.06957	−913.06259	−913.03826
		Relative Stability (kcal/mol)		
adenine	0	294.15	289.96	278.85
8-Cl-ade	0	282.18	277.80	262.52
		Differences in Relative Stability (kcal/mol)		
	$N_1 - N_3$	$N_1 - N_7$		
adenine	4.19	15.30		
8-Cl-ade	4.38	19.65		

[a] Deprot = deprotonated. [b] Prot = protonated. [c] 8-Cl-ade = 8-Cl-adenine.

If the 1578-cm^{-1} absorption is assigned to the C_4C_5 double bond stretching motion, then it seems unusual for this absorption to be diagnostic for pK_a determination. One explanation for the observed facts may be that in the anti conformation (such as in 5'AMP) N_3 is not sterically restricted from being protonated. The difference in protonation energies between the N_1 and N_3 positions of adenine is only 4.1 kcal/mol (Table IV). This suggests that both N_1 and N_3 can be protonated in adenine nucleotides and nucleosides in the anti conformation. If both N_1 and N_3 are protonated at the same time, then the possibility exists for two positive charges to be in close proximity in the six-member ring of adenine (Figure 3; structure V). In order to minimize the electrostatic repulsion in the six-member ring, some of the charge must be delocalized to the imidazole ring (Figure 3; structure VI). The delocalization process should involve a shift in the C_4C_5 double bond to double bonds at C_5C_6 and C_4N_9 and may explain the splitting of the 1485- and 1583-cm^{-1} absorptions (due to pH changes) in the 5'AMP spectra. Thus resonance form VI (Figure 3) must contribute to the delocalization of the positive charges generated in the doubly protonated species of 5'AMP while resonance form III (Figure 3) must be the predominant species present in the protonated form of 8-Br-5'AMP. The observed changes in the 1457-cm^{-1} absorption of 8-Br-5'AMP also support the view that electron density shifts from the imidazole portion of adenine to the six-member ring of adenine when N_3 is protonated or is intramolecularly H-bonded. Our results suggest that different resonance forms of adenine are present depending upon which sites are protonated and that protonation at N_3 is dependent upon the conformation about the N–glycosyl bond. In another paper,[21] we report the localized molecular structures from the localized molecular orbitals (LMOs) for adenine and its protonated tautomers using the Partial Retention of Diatomic Differential Overlap (PRDDO) method to see what bonding patterns are predicted. The LMOs (Figure 4) show that when only N_1 is protonated (Figure 4; structure II) a partial double bond is present between C_4 and N_9, but when N_1 and N_3 are protonated (Figure 4; structure V) a full bond must be drawn between C_4 and N_9. The results do not indicate that a complete shift in electron density occurs with the "π" electrons in the C_4C_5 double bond, but they do indicate that the positive charge in the six-member ring of doubly protonated adenine draws electron density from the imidazole ring of adenine.

Protonation and Tautomerization Energies. The ab initio SCF calculations at the STO-3G level for adenine and 8-chloroadenine indicate that the order of protonation is $N_1 > N_3 > N_7$ and that Cl substitution at C_8 does not affect the order of protonation (Table IV). Tautomeric stability has been shown to shift upon geometry optimization,[22] as well as with the use of an extended basis set.[23] Despite the fact that solvation effects seem to play the dominant role in determining tautomer stability,[6a] the relative tautomeric stability ordering for adenine and protonated adenines does not change upon geometry optimization.[24]

The Cl atom substitution does, however, affect N_7-protonation. A comparison of the energy differences between the N_1 and N_7 positions for substituted and unsubstituted adenine indicates that the N_7-protonated form is less stable in 8-chloroadenine than in adenine by 4.4 kcal/mol (Table IV). Mulliken population analysis shows that the electron density in the $2p_z$ orbital of C_8 is larger

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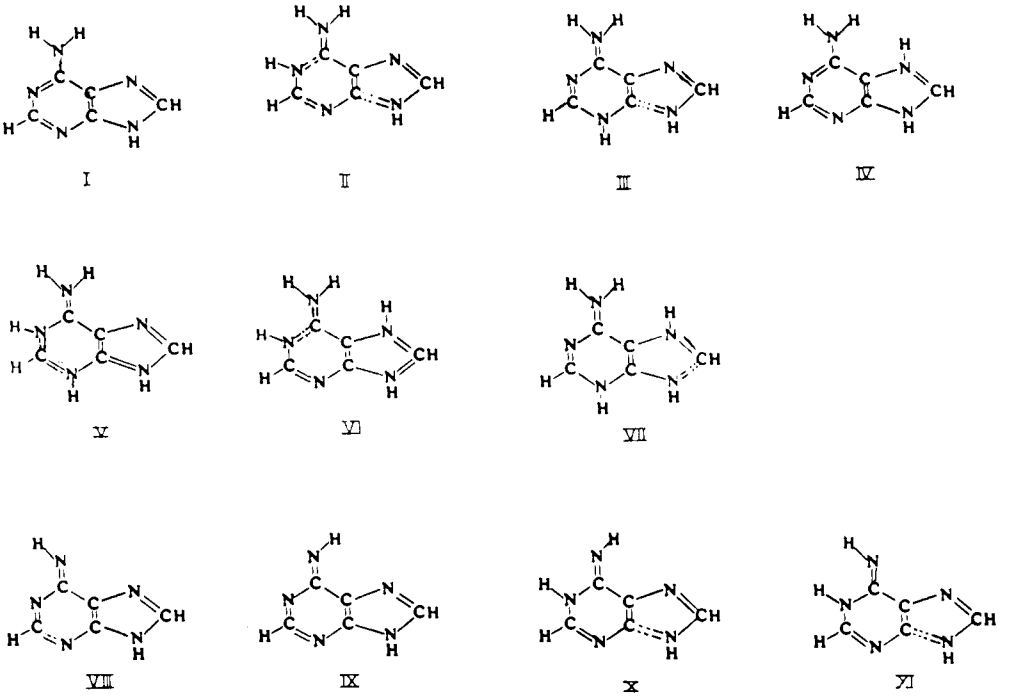

Figure 4. Localized molecular structures of adenine and its protonated species.

for substituted adenine than for the unsubstituted species and that the $2p_z$ orbital of N_7 shows a decrease in electron density upon 8-Cl substitution.[20] The change in electron density may be due to the electron-withdrawing effect of the Cl atom. Since the N_7 site is protonated below pH 1.1,[13] studies in the pH region below 1.1 should reveal whether Raman spectroscopy can monitor N_7 protonation and whether Br substitution at C_8 will affect protonation at N_7.

Conclusions

The only absorption not affected by Br substitution or pH variation is the 1576-cm^{-1} absorption of 8-Br-5′AMP. This absorption has been assigned to the C_4C_5 double bond stretch. The absorption due to the NH_2 scissoring motion is observed at high pH values and appears to overlap with the 1578-cm^{-1} absorption at pH 9.4. This may represent the formation of an unprotonated Schiff base. The 761- and 1457-cm^{-1} absorptions are sensitive to the substituent at C_8 and are assigned to the in-phase ring breathing mode and imidazole ring vibrations, respectively.

Our results demonstrate that different tautomers of adenine exist in solution at various pH values and that the presence of a given tautomer is dependent upon the protonated site. The observed protonation sites in 5′AMP and 8-Br-5′AMP are also dependent upon the conformation about the base ribose bond. It appears that when N_3 is protonated, resonance form III is suppressed (Figure 3) and that N_3 is protonated only when the anti conformation is present. Thus, the syn conformation in 8-Br-5′AMP does seem to sterically inhibit protonation at N_3. It must be kept in mind that the sugar and phosphate backbone conformation is also dependent upon the conformation at the ribosyl linkage (i.e., the N–glycosyl conformation), and these factors may contribute to the biological activity of 8-Br-substituted adenosine and its analogues.

The electronegativity of the Cl atom causes the N_7 position to be a less stable protonation site, relative to N_1, in 8-Cl-substituted adenine than in adenine by 4.4 kcal/mol. Since the N_7 site of adenine has been shown to be an important metal ion binding site,[25] the substitution of an electronegative atom at C_8 may affect the biological activity of these compounds due to the change in electron density at N_7.

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Registry No. 8-Br-5′AMP, 23567-96-6; adenine, 73-24-5; 8-chloroadenine, 28128-28-1.

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