Comparative structural analysis of 1-methyladenosine, 7-methylguanosine, ethenoadenosine and their protonated salts IV: ^1H , ^{13}C , and ^{15}N NMR studies at natural isotope abundance

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ABSTRACT

The ^1H , ^{13}C , and ^{15}N NMR spectra of neutral and protonated forms of the nucleosides 1-methyladenosine (m^1A), 7-methylguanosine (m^7G) and ethenoadenosine (EA), as a model compound, have been analyzed in order to assign the site of protonation in m^1A and m^7G . Protonation of these nucleosides occurs in the pyrimidine ring of m^1A and EA and in the imidazole ring of m^7G , with the charge being distributed rather than localized. Structural differences for both m^1A and m^7G were observed in solution and compared with those existing in the crystal state of monomers as well as in tRNA where these nucleosides occur quite often. The protonated nucleoside structures in solution compared favorably in sugar pucker and glycosidic bond conformations with x-ray crystallographic data. Methyl group carbon chemical shifts of the protonated mononucleosides corresponded to those of the methyls of the respective nucleosides in native tRNA structures. Therefore, the tRNA methyl group carbon chemical shifts are indicative of fully protonated nucleosides in the native, three dimensional structure of the nucleic acid.

INTRODUCTION

Previously reported NMR studies of the neutral and protonated forms of the mononucleosides ethenocytidine (EC) and ethenoadenosine (EA), defined their structures, conformations and sites of protonation in solution in comparison to that in the crystal state. (1,2). Carbon NMR chemical shifts were shown to be just as indicative of the site and extent of protonation as were ^{15}N chemical shifts (1), the latter known to elucidate the chemical environment of nitrogen nuclei. These studies of the alkylated nucleosides in their neutral and protonated forms has led us to their use as models for investigating the structure and charge distribution of the most prevalent, naturally occurring methylated and presumably protonated nucleosides of tRNA, 1-methyladenosine, m^1A and 7-methylguanosine, m^7G (3). Approximately 50% of all tRNA species have m^7G at position 46 and 20% have m^1A at position 58 (3). Both nucleosides are involved in tertiary structure base pairing. Protonation plays an important role in determining the base form available for base pairing in macromolecular structures. The keto-enol and enamine-ketamine

tautomerism of DNA and RNA major nucleosides has long been shown important to molecular genetics for specifying base pairs. In the case of m^1A studied here, methylation changes the possibilities of base pairing and site of protonation, viz blocking the N-1 position. Methylation of G to m^7G produces a zwitterion and N-7 is excluded from H-bonding. Physical chemical studies of the nucleoside m^7G as early as 1963 (4) showed it to be a zwitterion.

Natural abundance ^{13}C -NMR spectroscopy of chemically synthesized m^7G and m^1A (5,6) and chemically induced nuclear polarization studies (7), as well as 1H -NMR investigations (4,8) have been performed. However, NMR studies of m^1A and m^7G have not been routinely accomplished under physiological conditions, mostly due to limited solubility of the mononucleosides in aqueous solutions at neutral pH. The structures of both mononucleosides, m^1A and m^7G , are sensitive to pH higher than physiological. Alkaline treatment (pH 11) of m^1A hydroiodide converts the structure to m^6A (9) and at pH 9, m^7G converts to an imidazole ring opened form, 2-6 diamino-4-hydroxy-5N-methyl-formamido-pyrimidine (5,10). Even at pH 7, considerable amounts of the hydroiodide of m^1A can be converted to m^6A (9). NMR results have been variously interpreted with regard to biological relevance without noting the conditions under which the nucleosides were studied in comparison to tRNA investigations. The crystal structure of the complex of m^7G and its hydroiodide has been published (11). An extensive literature search revealed that the crystal structure of m^1A is yet to be reported; however, that of ethenoadenosine and its hydrochloride have been published (12,13). Here we report the 1H , ^{13}C , and ^{15}N NMR and infra-red (IR) spectroscopy of m^1A , and m^7G , and EA as a model compound and their halide salts, define the site of protonation of each purine ring and its characteristic NMR chemical shifts, and compare these chemical shifts to that of the two methylated nucleosides, m^1A and m^7G , in tRNAs under physiological conditions.

MATERIAL AND METHODS

Mononucleoside Solutions. Nucleosides m^1A and its hydroiodide ($m^1A \cdot HI$), and m^7G were purchased from Sigma Chemical Company (St. Louis, MO). The nucleosides ethenoadenosine (EA) and its hydrochloride ($EA \cdot HCl$), and two methylated derivatives (10 MeEA; 11 MeEA) were synthesized following the procedure described first by Leonard (14). Solutions of mononucleosides were prepared in phosphate buffered saline (PBS) of pH 7.2 in D_2O (10 mM $MgCl_2$, 15 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 100 mM $NaCl$). In addition, proton and nitrogen NMR spectra were taken of nucleosides in dried, deuterated dimethylsulfoxide

(DMSO- d_6) from Merck Isotopes (Quebec, Canada). Solutions of EA, EA·HCl, and the methylated derivatives also were prepared in DMSO- d_6 . Protonation of m^7G and m^1A in DMSO was accomplished by making the solutions equimolar in trifluoroacetic acid (TFA). DMSO- d_6 was used as a solvent in addition to PBS for the following reasons: 1) The nitrogen and oxygen bound protons of the mononucleosides are not usually observable in PBS- D_2O because they exchange with deuterium; and in PBS- H_2O , C-OH protons are obscured by the solvent peak; 2) We wish to compare our work to that of others who have investigated these compounds in organic solvents; 3) The differences in proton chemical shift between DMSO and D_2O solvents for a particular nucleoside are not significant for non-exchangeable protons (Table 2); 4) It would not be possible to observe the ^{15}N -NMR spectra by a polarization transfer technique (DEPT) in aqueous solvent where the nitrogen bound protons would be exchanged with deuterium; and, 5) The nucleosides are more soluble in DMSO than in PBS.

NMR Spectroscopy. Spectra were obtained with a Nicolet 300 MHz (1H) Fourier transform spectrometer with an Oxford Instruments wide bore magnet and Nicolet 1280 computer. The instrument was equipped with a Nicolet 5mm single frequency (75.5 MHz) ^{13}C probe and a Doty Scientific single frequency ^{13}C pre-amplifier. Proton NMR spectra of the mononucleosides m^1A , m^7G and their salts were recorded at a frequency of 300 MHz with a 5 mm probe. Two dimensional (2D) NOE (15) spectra of EA and EA·HCl were acquired with a 90° pulse of 7.5 sec. Spectra were recorded in 256 blocks of 32 acquisitions (1000 points) per block. Thus, the processed data matrix was $1k \times 1k$ (zero filled twice). Nitrogen NMR spectra of m^1A and A were measured at 30.4 MHz with a 20 mm broadband probe and broadband preamplifier. Both DEPT and BILEV pulse sequences were utilized to obtain ^{15}N spectra. Dioxane (67.4 ppm), DSS (0 ppm) and nitromethane (0 ppm) were the chemical shift reference standards for ^{13}C , 1H and ^{15}N spectra, respectively. TMS served as internal standard (0 ppm) in DMSO- d_6 solutions. Carbon-13 NMR spectra of m^1A , m^7G , EA, and derivatives (10 MeEA; 11 MeEA) were obtained at a frequency of 75.5 MHz. Nucleoside proton resonance assignments were based on: comparison of chemical shifts with that of their respective analogues; literature values (16); and the pattern of spin-spin couplings for ribose protons in different nucleosides. Nucleoside ^{13}C (5,17) and ^{15}N (1) signal assignments were made by comparison to already published data. For assignments of the ^{13}C NMR signals of EA, and EA·HCl, we used a wide range of presently available techniques. To assign quaternary carbons, a recently developed one-dimensional NMR experiment (18) for the determination of long range carbon proton connectivity was

utilized. The pulse sequence is called INAPT (19) and is basically a modified version of the well-known refocused INEPT experiment (20). The pulse width of the 45° ^1H pulse was set to 5 ms (10 dB attenuation). High power decoupling was used during data acquisition. Among the chemical methods for assignment, shifts induced by selective methylation (10 MeEA and 11 MeEA) were found useful.

Infra-red Spectroscopy

Infra-red (IR) spectra of $m^1\text{A}$ and its hydroiodide, and EA and its hydrochloride were taken on a Nicolet FTIR spectrometer. All samples were prepared in KBr pellets.

Table I: Carbon-13 Chemical Shifts for Neutral and Protonated 1-Methyladenosine ($m^1\text{A}$), 7-Methylguanosine ($m^7\text{G}$), Ethenoadenosine (EA), and Adenosine (A)^a

Carbon-13 Chemical Shifts						
Carbon Atom	$m^1\text{A}$	$m^1\text{A}\cdot\text{HI}$	Protonation Shift ^b	$m^7\text{G}$	$m^7\text{G} + \text{TFA}^c$	Protonation Shift
C-2	149.46	148.66	-0.80	159.90	154.22	-5.68
C-4	144.43	147.64	+3.21	150.20	150.01	-0.19
C-5	122.01	120.20	-1.81	109.87	109.90	+0.03
C-6	154.71	151.87	-2.84	160.43	156.23	-4.20
C-8	141.14	143.96	+2.82	136.02	137.42	+1.40
C-1'	89.09	88.38	+0.71	90.08	90.88	+0.80
C-2'	74.86	75.00	+0.14	74.92	75.01	+0.09
C-3'	71.19	70.93	-0.26	70.47	70.22	-0.25
C-4'	86.45	86.28	-0.17	86.43	86.40	-0.03
C-5'	62.13	61.92	-0.18	61.53	61.40	-0.13
CH_3	37.35	38.73	+1.38	36.58	36.92	+0.34
J^{d}_{CH}	142.3	144.5		145.1	145.2	
CH_3 in tRNA		38.40			36.90	
J_{CH}		145.1			145.0	

Table I: Continued

Carbon-13 Chemical Shifts						
Carbon Atom	EA	EA·HCl	Protonation Shift ^b	A	A·HCl	Protonation Shift
C-2	136.88	137.79	+0.91	152.23	144.52	-7.71
C-4	138.30	142.62	+4.32	148.96	148.11	-0.85
C-5	122.91	119.00	-3.91	119.26	118.35	-0.91
C-6	140.35	136.88	-3.47	156.01	149.88	-6.13
C-8	139.70	143.09	+3.38	139.76	142.17	+2.41
C-10	132.57	122.59	-9.98	-----	-----	----
C-11	112.07	114.56	+2.49	-----	-----	----
C-1'	87.73	88.25	+0.52	87.87	87.86	----
C-2'	74.08	74.54	----	73.37	74.35	+0.98
C-3'	70.10	70.10	+0.46	70.50	69.98	-0.52
C-4'	85.38	85.83	+0.45	85.77	85.77	----
C-5'	61.16	60.83	-0.33	61.56	60.84	-0.72

^aChemical shifts (ppm) for m¹A and m⁷G in phosphate buffered saline (PBS) are relative to dioxane at 67.4 ppm. Carbon chemical shifts (ppm) EA and A are relative to TMS (0 ppm).

^bProtonation shift is the change in chemical shift (- being upfield, + being downfield) between neutral and protonated forms.

^cTFA is trifluoroacetic acid added to m⁷G in equalmolar concentration.

^dCoupling constants (J_{CH}) are in Hz.

RESULTS

We have compared all of the ¹³C-NMR chemical shifts for the neutral and protonated forms of m¹A and m⁷G in physiological solution, phosphate buffered saline (PBS), and of A and ethenoadenosine (EA) in DMSO at 22°C (Table 1). The coupling constants (J_{CH}) for the methyls of the mononucleosides in the neutral and protonated forms are also listed in Table 1. Assignments for A· and EA were aided by comparison to the work of others on adenosine (8) and by the carbon NMR of the two methyl derivatives of EA, 10 MeEA and 11 MeEA (data not shown). Carbon chemical shifts for both forms of m¹A and m⁷G in DMSO had been published previously (5). Our results in aqueous solvent are similar to those in DMSO. The hydroiodide (protonated form) of m¹A had a carbon methyl resonance 1.38 ppm downfield of that of the neutral compound. The methyl

Table II: Proton Chemical Shifts and Coupling Constants for Neutral and Protonated Forms

Proton Number	Proton Chemical Shifts ^a				
	DMSO			PBS ^b	
	m ¹ A	m ¹ A + TFA	m ¹ A·HI	m ¹ A	m ¹ A·HI
COMPOUND					
m ¹ A					
H-2	8.09	8.72	8.72	8.05	8.52
H-8	8.15	8.77	8.77	8.15	8.53
CH ₃ -1	3.44	3.81	3.79	3.55	3.92
H61, H62	7.00	10.00, 9.25	9.18, 10.0		
H1'	5.75	5.96	5.94	5.95	6.14
H2'	4.47	4.52	4.51	4.65	4.74
H3'	4.10	4.18	4.16	4.39	4.44
H4'	3.92	4.00	3.98	4.24	4.26
H5'1	3.65	3.70	3.69	3.88	3.87
H5'2	3.54	3.59	3.57		
O2'-H	5.00	----	5.59		
O3'-H	4.21	----	5.31		
O5'-H	5.10	----	5.08		
Coupling Constants ^c					
1'2'	5.9	5.5	5.5	5.9	5.3
2'3'	5.1	5.2	5.3	5.2	4.7
3'4'	3.6	4.0	3.9	3.6	3.9
4'5'	4.3	3.8	4.6	2.9	3.1
4'5'1	4.0	3.9	4.1	3.8	4.2
5'5'2	12.0	12.0	12.0	12.8	12.7
O2'2'	5.7	----	6.0	----	----
O3'3'	4.7	----	5.1	----	----
O5'5'1	5.5	----	5.4	----	----
O5'5'2	5.7	----	5.5	----	----
J _{1'2'} + J _{3'4'}	9.5	9.5	9.4	9.5	9.2
Proton Chemical Shifts					
COMPOUND	DMSO		PBS		
	m ⁷ G	m ⁷ G + TFA	m ⁷ G	m ⁷ G + TFA	
m ⁷ G					
O6-H	5.35	----	----	----	
H-1	----	11.82	----	10.55	
H-8	9.21	9.35	12.40	11.90	
CH ₃ -7	4.00	4.01	4.07	3.85	
H21, H22	7.50	7.36	8.65, 8.61	9.00	
H1'	5.84	5.84	5.99	5.80	
H2'	4.41	4.37	4.68	4.39	
H3'	4.15	4.16	4.37	4.11	
H4'	3.98	3.90	4.28	4.00	
H5'1	3.71	3.72	3.92	3.68	
H5'2	3.60	3.61	3.84	3.57	

Table II: Continued

Coupling Constants ^c				
1'2'	4.2	3.9	4.3	4.2
2'3'	4.5	4.3	4.6	4.7
3'4'	4.7	4.9	4.8	5.0
4'5'	3.2	3.3	2.3	2.5
4'5'1	3.6	3.6	3.2	3.4
5'5'2	12.2	12.2	13.0	12.8
J _{1'2'} + J _{3'4'}	8.9	8.8	9.1	9.2
Proton Chemical Shifts				
COMPOUND	EA	EA*HCl	Protonation Shift ^d	
EA				
H-2	9.31	9.66	+0.35	
H-8	8.58	8.96	+0.38	
H-10	7.57	8.12	+0.55	
H-11	8.08	8.49	+0.41	
H-1'	6.06	6.12	+0.07	
H-2'	4.59	4.58		
H-3'	4.22	4.22		
H-4'	3.99	4.03		
H'5'1	3.72	3.72		
H'5'2	3.60	3.60		
02'-H	5.55	----		
03'-H	5.25	----		
05'-H	5.08	----		
Coupling Constants				
Couplings	EA	EA*HCl		
1'2'	5.5	5.5		
2'3'	4.0	5.0		
3'4'	4.0	4.5		
4'5'1	4.0	4.0		
4'5'2	5.0	4.0		
5'OH	4.0	---		
5'1'OH	5.5	---		
5'15'2	12.0	12.0		
02'H	6.0	---		
03'H	5.0	---		
H-2	2.0	2.0		
H-8	3.0	2.0		
H-10	2.0	2.0		
H-11	2.0	2.0		

^aChemical shifts in ppm were referenced to TMS in DMSO and DSS in PBS. Chemical Shifts accuracy was ± 0.55 ppm.

^bPBS is phosphate buffered saline; TFA is trifluoroacetic acid added in equal molar concentration.

^cCoupling constants (J_{HH}) are reported in Hz.

^dProtonation shift is the difference in chemical shift (- being upfield, and +, downfield) between the neutral and protonated forms.

resonance of m^7G in the presence of equimolar trifluoroacetic acid (TFA) was 0.4 ppm downfield of that of the neutral form (zwitterion). Methyl carbon chemical shifts of $m^1A \cdot HI$ and m^7G plus TFA are found to be the same as those of the corresponding methyls in native tRNA in PBS (Table 1 and reference 21).

Also the proton-carbon coupling constants (J_{CH}) for the protonated forms of the mononucleosides are similar, in the range of accuracy, to the couplings of the corresponding methyls in tRNA^{Phe} (Table 1).

To confirm that the methyl carbon chemical shifts of $m^1A \cdot HI$ and m^7G plus TFA, were truly attributable to the protonated forms of the mononucleosides, we characterized both the neutral and protonated forms of these mononucleosides and EA as a model compound for m^1A , by 1H and ^{15}N -NMR and by IR spectroscopy. This investigation also revealed the sites of protonation. Proton NMR

Fig. 1. Proton NMR of neutral and protonated forms of m^1A and m^7G . Proton NMR spectra of the nucleosides in DMSO were taken at 300-MHz at 22°C. The spectra of the neutral and protonated forms of m^1A and m^7G are shown in A and B, respectively. Protonation of m^7G was achieved by addition of equimolar TFA. Only the downfield signals are shown.

spectra of the free and protonated mononucleosides of m^1A and m^7G in DMSO are shown in Figure 1. Proton chemical shifts from these spectra, and spectra taken of EA and EA·HCl in DMSO are listed in Table 2. Proton signals for EA and EA·HCl were assigned according to 1H -NMR studies by others (14,22) and are used here for comparison to m^1A and m^7G . The proton chemical shifts of m^1A and m^7G in PBS listed in Table 2 have never before been reported. Some of them differ enough from those in DMSO to be of significance. Most likely the small differences in chemical shift for the methyl protons are due to the change in solvent character and partial protonation in PBS at pH 7.2.

The addition of TFA in equalmolar concentration to that of m^1A and m^7G in DMSO solutions of the neutral mononucleosides produced dramatic and important changes in proton chemical shifts of the heteroaromatic ring protons. The proton chemical shifts of m^1A ·HI (hydroiodide salt) in DMSO are also different from those of the neutral nucleoside, but are essentially identical to that of m^1A plus TFA (Table 2). Thus, the hydroiodide corresponds to the protonated form of the nucleoside. Differences in proton chemical shifts of the non-exchangeable protons for neutral versus protonated nucleosides in PBS corresponded in direction and size to those in DMSO (Table 2).

The broad proton resonance at 7.00 ppm in the spectrum of neutral m^1A in DMSO (Figure 1) integrated to a single proton (relative to H-8, or H-2). It was a fully exchangeable proton not seen in deuterated aqueous solutions of m^1A (Table 2). Therefore, it was assigned to N-6. This N6-imino proton signal of m^1A was replaced in spectra of m^1A plus TFA and m^1A ·HI with two downfield signals (9.25 and 10.00 ppm). Each of these two peaks integrated to a single proton, and was not visible in spectra of D_2O solutions. The two signals are attributable to the two protons of a fully protonated amino group with slow chemical exchange. In the case of m^7G in the neutral form we observed an OH proton at 5.35 ppm; whereas in the protonated form this signal disappeared and a new signal was observed at 11.82 ppm and is assigned to the proton on nitrogen-1 (Figure 1 and Table 2). Previously reported chemical shifts for m^7G (23) corresponded to those we have listed under DMSO plus TFA in Table 2. The chemical shift changes resulting from acid addition prompt us to attribute the chemical shifts in DMSO alone to the neutral form (zwitter-ion) and that with TFA in DMSO to the protonated nucleoside.

Coupling constants (J_{HH}) for neutral and protonated forms of m^1A , m^7G , and EA in physiological and organic solvents are also shown in Table 2. Conformational parameters calculated according to the review of Davies (16),

Fig. 2. Structures and conformational parameters for neutral and protonated forms of m¹A, A and EA. The figure compares the neutral and protonated (+) structures of A (Figure 2A) and EA (Figure 2B) from the x-ray crystallographic data of the nucleosides with that of m¹A (Figure 2C) from yeast tRNA Phe. Figures (2 and 3) were produced from the Cartesian coordinates of the respective x-ray crystallographic data by means of the molecular graphics computer program, PCMODEL (Academic Press, Inc.). X-ray crystallographic data and solution conformational parameters of the neutral and protonated forms are shown in the accompanying tables. Evaluation of syn/anti conformations was based on the relationship between vicinal $J_{CH} = (C8-H1') - (C4-H1')$, and the prevalent conformer which is anti if $J_{CH} > 0$, and syn if $J_{CH} < 0$ (34).

are shown in Figure 2 for m^1A and Figure 3 for m^7G and indicate that the neutral form of m^1A is somewhat preferentially in the 2'-endo conformation (62%); whereas that of m^7G is comprised of equal proportions of conformers (53% 3'-exo). The extent of these conformers in solution was analysed by a statistical comparison of J_{HH} values for a variety of nucleosides of known conformation (16) as well as from 2D NOE spectra. Both nucleosides primarily take the gauche conformation of the exomethylene group. Their conformations around the glycosidic bond under physiological conditions are primarily syn for m^7G and anti for m^1A (Figures 3 and 2, respectively). However, with the protonated forms m^7G is a mixture of syn and anti conformations; whereas m^1A is anti. Conformational parameters for the neutral and protonated forms of EA were found to be comparable to those of m^1A and $m^1A \cdot HCl$, respectively (Figure 2).

Nitrogen NMR chemical shifts of neutral and protonated adenosine (A) and m^1A in DMSO are compared in Table 3. An ^{15}N -NMR spectrum of m^1A is shown in Figure 4. Signal assignments for m^1A were based on published ^{15}N studies of adenosine (24) and the proton coupled, nitrogen spectrum of m^1A . Published assignments for ethenoadenosine (EA) were also useful (1). Note that the most significant ring nitrogen chemical shift alterations were strong upfield changes occurring with protonation at N-1 in adenosine and NH_2 -6 in m^1A , as well as N-6 of EA. The ^{15}N spectra of m^1A confirm the protonation of NH_2 -6 to NH_2 -6 seen in 1H -NMR studies. In all three adenosines the most dramatically changed nitrogen is that which becomes fully protonated. However, we also observed smaller changes in chemical shifts on other nitrogens as expected of protonated heterocyclic ring systems (Table 3).

Infra-red (IR) spectra were taken of m^1A , $m^1A \cdot HCl$, and the corresponding model compounds EA and $EA \cdot HCl$ to obtain additional information on the nature of double bonds in these two pairs of nucleosides, particularly the bond of C-6 to N-6. IR spectroscopic data on the forms of EA will directly reflect the known crystallographic structures of EA. IR spectra of the neutral and protonated forms of EA and m^1A are compared in Table 4. The table shows assignments, and a comparison of scissoring and stretching vibrations of the double bonds in m^1A and EA. Assignments were made by comparison to the work of Tsuboi on adenosine (25, 26). Spectra of the neutral forms of EA and m^1A had scissoring bands for double-bonded C6=N6 (1648.5 and 1650 cm^{-1} , respectively) and stretching bands for C6=N6 (1299.6 and 1298.2 cm^{-1} , respectively), which disappeared in the protonated forms. In the protonated forms these lines were replaced with scissoring and stretching bands of C6-NH₂ for

m^1A^+HI (1685 and 1239 cm^{-1} , respectively) and of C6-N(H)6- for EA^+HCl (1679.7 and 1238 cm^{-1} , respectively). This is consistent with the crystal data for EA and EA^+HCl (12,13), and confirmed the ^{15}N , 1H and ^{13}C NMR data that showed protonation occurring at the N-6 position of both EA and m^1A . As a band of reference, the C2-H bending vibration for both forms of A and EA was located at 1384 cm^{-1} .

DISCUSSION

In this paper we have concentrated on detailed investigations of neutral and protonated forms of m^1A , m^7G and the model compound EA in order to better understand the role of protonation in molecular biology. Our goals also included elucidating the biological function of two common, methylated

	P	S π /N π	γ	X		
m^7G	$2T_1$	g $^+$	syn	(224 $^\circ$)	x-rays	
			47/53	g $^+$	syn	solution
			45/55	g $^+$	anti	solution
$m^7G^+H^+$	$3T_4$	g $^+$	anti	(180 $^\circ$)	x-rays

Fig. 3. Structures and conformational parameters for neutral and protonated forms of m¹G. The figure compares the neutral and protonated (+) structures of m¹G from the x-ray crystallographic data of the nucleoside (Figure 3A) with that in yeast tRNA Phe (Figure 3B). X-ray crystallographic data and solution conformational parameters of the neutral and protonated forms are shown in the accompanying table.

Fig. 4. Natural abundance nitrogen-15 NMR spectrum of the neutral form of m¹A. N-15 spectrum of m¹A was taken at a concentration of 0.3M in DMSO-d₆ (0.6 ml).

Table III: Nitrogen Chemical Shifts for the Nucleosides 1-Methyladenosine, Adenosine and Ethenoadenosine^a

		N-1	N-3	N-7	N-9	N-6
¹ mAdenosine		-231.07	-175.54	-135.65	-206.9	-196.64
Protonated ¹ mAdenosine		-227.36	-155.27	-136.84	-203.33	-285.64
Protonation Shift ^b		+ 3.71	+ 20.27	- 1.19	+ 3.57	- 89.0
Adenosine		-139.6	-152.7	-134.7	-205.6	-293.8
Protonated Adenosine						
Mole. Equiv. of Acid ^c	0.06	-147.0	-153.0	-135.4	-205.0	-293.4
	0.31	-155.6	-152.9	-135.3	-204.4	-292.2
	1.60	-211.3	-150.8	-131.4	-297.4	-284.9
Protonation Shift		- 71.7	+ 1.9	+ 3.3	+ 8.2	+ 8.9
Ethenoadenosine ^d		-178.5	-152.2	-139.1	-207.3	----- -150.0
Protonated Ethenoadenosine		-182.0	-147.6	-139.9	-205.3	----- -190.4
Protonation Shift		- 3.5	+ 4.6	- 0.8	+ 2.0	----- - 40.4

^aChemical shifts in DMSO are in ppm relative to nitromethane (0 ppm).

^bProtonation shift is the change in chemical shift (- for upfield and + for downfield) between the neutral and protonated forms of the nucleoside.

^cMole equivalence of acid added to titrate adenosine protonation. data is from Sierzputowska-Gracz et al. (1984).

^dEthenoadenosine data from Sierzputowska-Gracz et al. (1984).

nucleosides, ¹m¹A and ⁷m⁷G, in tRNA by first establishing the presence or absence of charge in these nucleosides. To do this we sought solution conditions under which the methyls of the mononucleosides displayed carbon chemical shifts equivalent to those for the nucleosides in the tRNAs (Table 1). The carbon chemical shifts of the methyl carbons of the neutral ¹m¹A and zwitterionic ⁷m⁷G were found to be upfield of those in the tRNAs. In contrast, the

Table IV: Infra-red Bands Observed for C6-N6 of m^1a and EA^a

Band No. ^b	m^1A	$m^1A \cdot HI$	EA	$EA \cdot HCl$	Assignment
1.	-----	1685.0 vs ^c		1679.7 vs	C6=NH-
2.	1650.0 vs	-----	1648.5 vs		C6=N-
3.	1384.0 s	1384.4 s	1384.3 s	1384.0 s	C2-H
4.	1298.2 m	-----	1299.6 s	-----	C6=N-
5.	-----	1239.0 s	-----	1238.0 s	C6-NH-

^aInfra-red bands are reported in cm^{-1} .^bBand numbers refer to lines in Figure 5.^cThe strength of a band is reported as: vs, very strong; s, strong; m, medium.

¹³C NMR studies of the halide salts of the mononucleosides suggested a similarity in electronic configuration to these two nucleosides in native tRNA structure.

What are the electron distributions in the mononucleosides under these conditions and do they constitute positively charged nucleosides?

7-Methylguanosine. The neutral and protonated forms of m^7G were found to coexist in a crystal structure (11). The protonated form has an additional proton at position N-1. Electronic structures and conformational parameters of both the neutral and protonated forms of m^7G in the crystal state and in solution are presented in Figure 3. Protonation at N-1 essentially removes the negative charge at O-6 of the zwitterion leaving the positive charge in the imidazole ring. Neutral and protonated m^7G in the dual-crystal take opposite conformations about the N-glycosidic linkage. The neutral form is syn; while the protonated form is anti. In solution the neutral form is typically syn; whereas the protonated form is a mixture of syn and anti conformations in DMSO, and in PBS. The ¹H and ¹³C NMR chemical shifts for both the neutral and fully protonated forms in DMSO and PBS, verify the existence of these structures in solution. The methyl carbon chemical shift of m^7G in tRNA^{Phe} under native conditions (in PBS) (27) is the same as that for the fully protonated nucleoside in PBS (Table 1).

1-Methyladenosine and Ethenoadenosine. Because of the absence of any crystallographic data on m^1A our reference has been made to the x-ray crystallographic structures of adenosine and adenosine hydrochloride (28,29), and ethenoadenosine and ethenoadenosine hydrochloride (12,13), as model compounds for m^1A and $m^1A \cdot HCl$. The structures of the neutral and protonated

forms of m^1A , A and EA are depicted in Figure 2. Neutral and protonated forms of A and EA in solution take the same anti conformation about the N-glycosidic bond. However, EA·HCl is syn in the crystal. The conformation of m^1A and $m^1A\cdot HI$ about N9-C1' is comparable to that of EA and A, i.e., anti.

The electron distributions of EA and EA·HCl are known from the x-ray crystallographic investigations (12,13). (Structure of EA is shown in Figure 2.) The 1H , ^{13}C and ^{15}N -NMR spectra will reflect these distributions. Nitrogen-15 NMR spectra and signal assignments of EA and EA·HCl have been published (1) and are presented in Table 3 for comparison to that of m^1A and adenosine. The significant change in nitrogen chemical shift upfield ($\Delta=-40.4$ ppm) for N-6 of EA·HCl in comparison to N-6 of EA reflects the protonation of this nitrogen as seen in the crystal. IR studies supported the NMR data with regard to the protonation of C6=N6- of EA to C6-N(H)6- of EA·HCl. The change in EA ^{15}N -6 chemical shift is qualitatively comparable to the ^{15}N -spectral change for N-6 of $m^1A\cdot HI$ versus m^1A ($\Delta=-89.0$ ppm). Ethenocytidine, the pyrimidine model for m^1A , has a corresponding change in the ^{15}N -4 chemical shift ($\Delta=-41.8$ ppm) (1) and has been shown to experience protonation at N-4 in the crystal of the hydrochloride (2).

Nitrogen-15 NMR chemical shift is considered one of the best spectroscopic criterium for assessing protonation of the purine ring (1). The protonated nitrogen has a very large chemical shift 30-45 ppm upfield from the corresponding unprotonated atom. The positive charge is probably not concentrated at the quaternary nitrogen N-1, but displaced about the heterocyclic ring in the direction of C-6, C-5 and C-4. We have found that the ^{13}C chemical shift is also a very useful parameter for analysis of the purine ring protonation, and in this case demonstrated the spread of positive charge about the pyrimidine ring.

The altered electron distribution in protonated m^1A and EA in comparison to the neutral forms was reflected qualitatively and quantitatively, in changes in carbon chemical shifts (Table 1). We have observed that protonation significantly changes the carbon chemical shifts either upfield ($\Delta=-ppm$) or downfield ($\Delta=+ppm$) for certain ring carbons of these purine nucleosides (2). Carbon chemical shift changes with protonation of EA and A are related to the position of the carbon versus the site of protonation, i.e., N-6, or N-1, respectively. Thus, carbons α to N-6 protonation site in EA (C-6 and C-10) exhibit large upfield shifts with protonation; whereas with A, it is those carbons α to N-1 (Table 1). Carbons β to the protonation site also move upfield; while those γ , move downfield. These changes in chemical shift

have been observed previously for the purine and pyrimidine rings of etheno-adenosine, cytidine and ethenocytidine in non-physiological solution (2).

The ^1H , ^{13}C and ^{15}N -NMR data and IR spectra for m^1A and $\text{m}^1\text{A}\cdot\text{HI}$ were all completely consistent with the respective neutral and protonated structures found for EA. Thus, the electron distribution and positive charge on $\text{m}^1\text{A}\cdot\text{HI}$, also depicted in Figure 2, is comparable to that for $\text{EA}\cdot\text{HCl}$ in solution and in the crystal state.

Others (30) have shown that the vicinal and geminal coupling constants (J_{CH}) may also be used to discern the purine ring protonation. The influence of protonation on the geometry of the x-ray crystallographic structure of purines has been studied in detail (31). For example, an angle of C6-N1-C2 larger than 121.3° is found for protonated purines; whereas angles smaller than 121.3° are found for the non-protonated forms. Thus, consistent with the crystallographic data of $\text{EA}\cdot\text{HCl}$, the amino groups at position 6 of $\text{EA}\cdot\text{HCl}$ and $\text{m}^1\text{A}\cdot\text{HI}$ were found by ^1H and ^{15}N NMR and IR spectroscopy to be the first site of protonation of these purine rings.

Of particular interest to our work are the changes in methyl carbon chemical shifts for m^1A and m^7G which can be compared to those in tRNAs (21). The downfield chemical shift of the methyl carbon of protonated m^1A in PBS (38.7 ppm) was similar to that for the methyl carbon of m^1A in native tRNA^{Phe} in PBS (38.4 ppm). This strongly suggests that m^1A is fully protonated, and positively charged in native tRNA^{Phe} as is m^7G . How then are the protons provided under physiological conditions?

Methylation of G to m^7G produces a zwitterion with a positive charge in the imidazole ring. We suggest that the m^7G positive charge in tRNA is localized in the imidazole ring by tertiary structure base pairing that eliminates the zwitterion negative charge on the pyrimidine ring. Full protonation of m^1A would also be achieved through tertiary structure base pairing in the tRNA molecule.

Protonation in solution and base pairing in tRNA ensures the stability of the m^1A and m^7G ring structures. Methylation of A-58 and G-46 to m^1A and m^7G , respectively, in tRNA would normally produce ring structures labile to some degree under physiological conditions, but primarily under alkaline conditions ($>\text{pH } 8$). Now we are able to solve problems mentioned in a paper (11), whose authors were expecting the m^7G residue of tRNA to be deprotonated in position N(1) under physiological conditions and in our own observations (32,33) of unexplained carbon chemical shift changes for m^1A and m^7G in tRNA. We know now that protonated forms of m^1A and m^7G are present in native, ter-

tiary structure tRNA, by virtue of tertiary structure hydrogen bonds, and that neutral forms are present only when the tRNA molecule is thermally denatured (Table 1) or when Mg^{2+} ions are removed (21). The integrities of the purine ring structures are supported by protonation either at low pH (<pH 6) for the mononucleosides, or tertiary structure base pairing in tRNA.

This paper is fourth in the series: Comparative structural analysis of mononucleosides and their salts. We would like to publish in the near future the last paper in this series demonstrating the role of different counter ions in the structure of protonated nucleoside salts.

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