Chemical Modification of Ribonucleic Acid. A Direct Study by Carbon-13 Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: Direct study of the methylation of ribonucleic acid with methyl methanesulfonate by carbon-13 nuclear magnetic resonance spectroscopy has demonstrated the usefulness of this method in studying the chemical modification of biomacromolecules and the interaction between nucleic acids and biologically active agents. This direct stable isotope method eliminated all tedious and questionable degradation processes for determining the reactive sites and the product distributions. Six methylated products, 7-methylguanosine, 1-methyladenosine, 3-methylcytidine, 1-methylguanosine, 3-methyluridine, and methyl phosphodiester, were identified by comparison with many model compounds and careful examination of spin–spin coupling and spin–lattice relaxation time. An extensive study of the interaction of phosphate buffer with methyl methanesulfonate accounted for the sharp difference in the ^{13}C spectra of the methylated RNA isolated from the reactions controlled by a pH-stat and phosphate buffer, respectively. The ^{13}C-enriched agent significantly enhances the specificity and sensitivity of the method and provides better quantitative results.

Chemical modification of nucleic acids is one of the promising approaches for studying the structure and function of nucleic acids (Kochetkov & Budowsky, 1969; Brown, 1974; Kearns, 1976; Sattsangi et al., 1980; Eshaghpour et al., 1980). However, the success of this approach certainly relies on the accurate determination of the reactive sites. Although the radioactive labeling method is the most sensitive technique, this approach, at best, can simply indicate the degree of overall modification. In order to gain specific information, it is necessary to undergo a series of enzymatic and acid-catalyzed hydrolyses followed by chromatography or electrophoresis, under acidic or alkaline conditions. This not only is time consuming but also can cause decomposition and/or alteration of the primary products which may thus lead to erroneous conclusions. It becomes apparent that a more sophisticated method is needed for studying the chemical modification of nucleic acids in a more native state. Therefore, in spite of the relatively low sensitivity, the use of stable isotopes has expanded rapidly with their commercial availability and the parallel improvement of instrumental sensitivity, such as the advances in high field pulse Fourier transform nuclear magnetic resonance (NMR) spectroscopy (Müllen & Pregosin, 1976) and selected ion monitoring mass spectrometry (Falkner et al., 1975). Here, we would like to report the application of ^{13}C NMR in the direct study of chemical methylation of RNA by methyl methanesulfonate (MeMS).

Experimental Procedures

Materials

Unfractionated RNA of Torula yeast and poly(U) were obtained from Sigma Chemical Co. Methyl methanesulfonate was purchased from Aldrich Chemical Co. ^{13}C-Enriched (90%) methanol was obtained from Merck. 2-Methylguanosine was purchased from Chemical Dynamic Corp. The methyl phosphate of thymidylyl(3′→5′)thymidine ($dT_{p(Me)}dT$) was kindly provided by Dr. David H. Swenson, National Center for Toxicological Research.

Methods

Purification of RNA. Torula yeast RNA was dialyzed at 4 °C against 0.12 M $NaClO_4$, 0.001 M Na_2EDTA[1] (pH 7.0), and then double-distilled water for three 6-h periods.

Synthesis of [^{13}C]Methyl Methanesulfonate. Methanesulfonic acid (9.6 mL) was refluxed with 41 mL of redistilled thionyl chloride for 3 h and then evaporated to dryness. The dark residue was extracted with boiling dry ether. The extracts were concentrated by removing most of the ether, and the white methanesulfonic anhydride was filtered and recrystallized from dry ether: mp 67–68 °C (lit. mp 70–71 °C) (Owen & Whitelaw, 1953).

Methanesulfonic anhydride (5.87 g) and [^{13}C]methyl alcohol (0.5 mL), which was prepared by reduction of $^{13}CO_2$ (from $Ba^{13}CO_3$) with $LiAlH_4$ (Cox et al., 1950), were refluxed at 150 °C with exclusion of moisture for 1 h by attaching a drying tube to the condensor. The reaction mixture was distilled, and the fraction with bp 97–98 °C (18 mm) [lit. bp 96–98 °C (19 mm)] (Wachtimeister et al., 1966) was collected to give 0.8 mL (98%) of [^{13}C]MeMS. The purity was confirmed by IR, ^1H NMR, ^{13}C NMR, and GC analyses.

Synthesis of 5′-(Methyl phosphate) of Guanosine, Adenosine, Cytidine, and Uridine (Smith et al., 1958). Free acids of ribonucleotides (0.43 mmol) were dissolved in 12.5 mL of methanol containing 2.5 mmol of dicyclohexylcarbodiimide (DCC) and 0.86 mmol of tri-*n*-butylamine. After stirring for 4 days at room temperature, the solvent was evaporated, and the residue was chromatographed on Cellulose-300 [2-propanol–NH_4OH–H_2O (6:3:1)]. The residue was dissolved in a small amount of methanol and precipitated with acetone. The white precipitate was washed with acetone and dried in vacuo overnight at room temperature. TLC showed one spot, and ^1H NMR gave a three-proton doublet at 3.6 ppm from Me_4Si with a coupling constant of 10.7 Hz.

Reaction of Methyl Methanesulfonate with Phosphate Buffer. Methyl methanesulfonate (50 μL) was added to each

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[1] Abbreviations used: Na_2EDTA, disodium ethylenediaminetetraacetate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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of the solutions (0.4 mL) in a 5-mm NMR tube: (i) 0.05 M phosphate buffer, pD 7.8 (Rhaese & Boetker, 1973); (ii) 0.01 M phosphate buffer, pD 7.6 (Lawley & Brooks, 1963); (iii) 0.001 M Tris-HCl buffer, pD 7.4 (Rutman et al., 1969). The ^{1}H NMR spectra of these solutions were taken at some period of time and carefully integrated. When the reaction of MeMS (10 μL) in 0.25 M phosphate buffer (0.46 mL), pD 7.8 (Bannon & Verly, 1972), was reexamined by ^{1}H NMR, a doublet at δ 3.58 with a coupling constant of 11 Hz was observed. The hydrolysis products of MeMS, methanol (δ 3.34), and methanesulfonate (δ 2.80) were also observed in this spectrum. Furthermore, the reaction of MeMS (0.65 mL) in 1.3 M phosphate buffer (13 mL), pD 10.5, at 37 °C (Strauss & Hill, 1970) was also examined.

Methylation of RNA with Methyl Methanesulfonate in the Absence of Phosphate Buffer. Unfractionated RNA of Torula yeast (1.6 g) in 6 mL of H_2O was treated with 400 μL (4.7 mmol) of MeMS at room temperature and at pH 7.00 ± 0.01, controlled by titrating with 0.1 N NaOH through a pH-stat titrator. After 12 h, the reaction mixture was dialyzed 3 times at 4 °C against a total of 4 L of 0.1 M $NaClO_4$ (pH 7.0) and then 3 times against double-distilled water. The resulting RNA solution was lyophilized.

Methylation of RNA with Methyl Methanesulfonate in the Presence of Phosphate Buffer. Unfractionated RNA (0.8 g) in 5 mL of 0.01 M phosphate buffer (pH 7.0) was incubated with 20 μL of MeMS. After 12 h, the reaction mixture was dialyzed and lyophilized as described previously.

Proton-decoupled ^{13}C natural abundance spectra of these methylated RNAs were measured in deuterium oxide solutions (400 mg/1.5 mL) with about 8 Mg^{2+} ions per RNA molecule at pD 7.4 and 25 °C.

Methylation of RNA with [^{13}C]Methyl Methanesulfonate: A solution of 1.5 g of unfractionated RNA of Torula yeast in 25.5 mL of H_2O was treated with 390 μL (4.57 mmol) of [^{13}C]MeMS at room temperature and pH 7.00 ± 0.01, controlled by titrating 0.098 N NaOH through a pH-stat titrator. An aliquot of this solution was withdrawn at a given period of time (Table III), dialyzed against 75 mL of H_2O 3 times and 50 mL of H_2O 6 times, each for 20 min each time, and then lyophilized. ^{13}C NMR samples were prepared in the same manner as described previously.

Methylation of Poly(U) with Methyl Methanesulfonate. Poly(U) (50 mg) in 1.2 mL of H_2O was treated with 50 μL of MeMS at room temperature and pH 7.00 ± 0.01 for 67 h and with another 50 μL of MeMS for 69 h, then dialyzed and lyophilized. Only N-3 methylation was detected by ^{1}H NMR (δ (3-NCH_3) 3.22) and ^{13}C NMR (δ(3-NCH_3) 28.0 ppm).

NMR Spectroscopy. The NMR spectra were recorded at ambient temperature by using a deuterium lock on a Jeol PFT-100 spectrometer, operating at 23.5 KG, interfaced with a Joel EC-100 Fourier-transform computer with 20K memory. The ^{1}H NMR spectra were obtained by applying 90° pulses (25.5 μs) with a repetition time of 5.0 s (between pulses). The proton-decoupled ^{13}C NMR spectra were measured while the protons were decoupled by using a broad-band (2.5 kHz) incoherent radio-frequency source (99.99 MHz). For polynucleotides and monomers, 2- and 4-s repetitions were used, respectively. The ^{13}C–^{1}H coupling constants of mononucleosides or nucleotides were determined from the proton-coupled spectra obtained by a gated decoupling technique. The kinetic studies performed by using ^{1}H NMR were done with a Varian TA-60 and EM-360 spectrometers. NMR samples were prepared in D_2O solution and the pDs were adjusted to

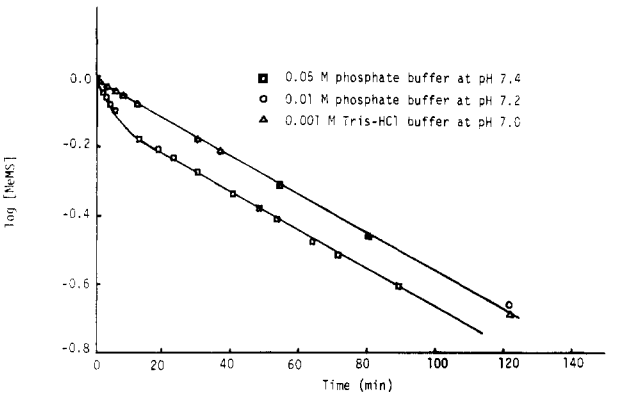

FIGURE 1: Disintegration of methyl methanesulfonate in various buffer solutions.

7.40 ± 0.02 with NaOD. In addition, all the polynucleotide samples had Mg^{2+} added, with the molar ratio of $Mg(ClO_4)_2$ to polynucleotide adjusted to 1:8. Sample tubes had outside diameters of 5 mm for ^{1}H NMR and 10 mm for ^{13}C NMR. 4,4-Dimethyl-4-silapentane-1-sulfonate (DSS) or acetone was used as an internal reference for ^{1}H NMR measurement, where δ (Me_4Si) = δ (Me_2CO) + 2.22 ppm. Methanol was added as an internal reference for ^{13}C NMR, where δ (Me_4Si) = δ (MeOH) + 49.5 ppm. In the case of RNA, either dioxane was used as an internal reference (at 66.6 ppm downfield from Me_4Si) or no reference material was added and the RNA peak at 165.8 ppm was used as reference. The spin–lattice relaxation times (T_1) of the methyl carbons of RNA methylated by [^{13}C]methyl methanesulfonate for 62 h were meaured by using a 180°–τ–90° pusle sequence (Müllen & Pregosin, 1976).

Results

Hydrolysis of Methyl Methanesulfonate in Buffer Solutions. Methyl methanesulfonate can undergo hydrolysis, releasing methanol and methanesulfonic acid, which results in a change of the pH value. The pH changes due to the hydrolysis of MeMS in various buffer systems have been observed. For phosphate buffer, the lower the concentration of the buffer the larger the pH changes. In 0.01 M phosphate buffer used by Lawley & Brookes (1963), the pH changes from 7.1 to 6.0 within 1 h. If a higher concentration of buffer is used, the disappearance of MeMS does not follow a simple pseudo-first-order kinetic relationship at the beginning (Figure 1). When MeMS was incubated in 0.25 M phosphate buffer, a doublet at δ 3.58 with a coupling constant of 11 Hz [^3J(^{1}H,^{31}P)] was observed by ^{1}H NMR, suggesting the formation of methyl phosphate. At high concentration of phosphate buffer (1.3 M) and high initial pH (10.5), the ^{1}H NMR spectrum displayed two doublets at δ 3.58 and 3.48 due to the formation of dimethyl phosphate and monomethyl phosphate, with coupling constants ^3J(^{31}P, H) = 11 and 10 Hz, respectively. These results indicate that MeMS reacts with not only water but also the phosphate buffer. The reactions of MeMS in phosphate buffer can be shown as a set of parallel reactions.

$$CH_3SO_2OCH_3 + H_2O \xrightarrow{k_{H_2O}} CH_3OH + CH_3SO_3H$$

$$CH_3SO_2OCH_3 + HPO_4^{2-} \xrightarrow{k_p} (CH_3O)HPO_3^- + CH_3SO_3^-$$

$$CH_3SO_2OCH_3 + (CH_3O)HPO_3^- \xrightarrow{k_p'} (CH_3O)_2HPO_2 + CH_3SO_3^-$$

The rate equations for these reactions are

$$d[CH_3OH]/dt = k_{H_2O}[H_2O][MeMS] \quad (1)$$

$$d[(CH_3O)HPO_3^-]/dt = k_p[MeMS][HPO_4^{2-}] - k_p'[MeMS][(CH_3O)HPO_3^-] \quad (2)$$

$$d[(CH_3O)_2HPO_2]/dt = k_p'[MeMS][(CH_3O)HPO_3^-] \quad (3)$$

$$[HPO_4^{2-}]_0 = [HPO_4^{2-}] + [(CH_3O)HPO_3^-] + [(CH_3)_2HPO_2] \quad (4)$$

From eq 1–4, it can be seen that

$$-d \ln \{[HPO_4^{2-}]_0 - ([(CH_3O)HPO_3^-] + [(CH_3O)_2HPO_2])\}/\{d[CH_3OH]/[H_2O]\} = k_p/k_{H_2O} \quad (5)$$

Thus the rate constant of methylation of phosphate relative to the rate of hydrolysis of MeMS can be calculated from a plot of $\ln \{[HPO_4^{2-}]_0 - ([(CH_3O)HPO_3^-] + [(CH_3O)_2HPO_2])\}$ vs. $[CH_3OH]$ (Figure 2). The phosphate methylation is about 500 times farther than the hydrolysis. Within the first hour about 60% of MeMS reacted with phosphate buffer, and after 10 h as much as 90% is converted to monomethyl phosphate (61%) and dimethyl phosphate (29%). The hydrolysis of MeMS at constant pH 7.0, controlled by the pH-stat instead of buffer, has been investigated by titration and ^{1}H NMR. The pseudo-first-order rate constants obtained from the titration method and the ^{1}H NMR study are 3.14×10^{-2} h^{-1} and 3.06×10^{-2} h^{-1}, respectively.

Model Studies. To establish the foundation of the relative chemical reactivity of different nucleotides toward methylation and for the spectral interpretation of methylated RNA, we studied the reactions of MeMS with four basic mononucleotides (Lee & Chang, 1978; Chang & Lee, 1980) and prepared methylated nucleosides (m^1A, m^6A, Am, m^1G, m^6G, m^7G, m^3C, m^4C, m_2^4C, Cm, and m^4U) (C.-G. Lee and C.-j. Chang, unpublished results). The methyl carbon chemical shifts of these model compounds are summarized in Table I.

Reactions of Methyl Methanesulfonate with RNA. When the ^{13}C natural abundance spectra of native RNA before and after reacting with methyl methanesulfonate are compared, five methyl carbon signals at 53.3, 38.1, 36.2, 30.8, and 28.7 ppm are clearly observed (Chang & Lee, 1976). A similar reaction was also performed in phosphate buffer solution, instead of controlling the pH with the pH stat. The ^{13}C spectrum of this RNA revealed only three methyl carbon peaks at 38.1, 36.2, and 30.8 ppm (Chang & Lee, 1976). For improvement of the signal-to-noise ratio and detection of the chemical modification at an early stage, RNA was reacted with 90% ^{13}C-enriched MeMS. The spectrum of this product displayed six signals at 53.3, 38.1, 36.2, 30.8, 28.7, and 28.0 ppm (Chang & Lee, 1978).

Spectral Interpretation. The assignments of the methyl carbon signals are mainly based on the chemical shifts of the methylated nucleosides and nucleotides (Table I) and on the relative chemical reactivities of different reactive sites (Lee & Chang, 1978; Chang & Lee, 1980). The strongest methyl carbon signal at 36.2 ppm can be assigned to the methyl carbon resonance of 7-methylguanosine (36.3 ppm). This is verified by detecting the new signal at 108.3 ppm corresponding to the C-5 signal of 7-methylguanosine (Chang & Lee, 1976). The next strongest peak at 38.1 ppm can be designated to the methyl carbon signal of 1-methyladenosine (38.0 ppm). The signal at 30.8 ppm is assigned to the methyl carbon resonance of 3-methylcytidine (30.7 ppm). The distinct chemical shift change resulted from the N-7 methylation of guanosine (116.2 ppm → 108.3 ppm) furnishes a direct way to calculate the degree of the N-7 methylation of guanosine (Table II). It increases from 14% to 52% when the reaction time changes from 0.5 to 12 h. However, the ratio of the major products remains nearly constant (Table II), probably suggesting that this RNA may assume a random coil conformation after 14% of the N-7 position of guanosine is methylated. Methylation at the 3 position of cytidine also resulted in the shift of the C-4 signal of cytidine (165.8 ppm → 159.4 ppm). Two weak peaks at 28.7 and 28.0 ppm are attributed to the methyl carbon signals of 1-methylguanosine (28.6 ppm) and 3-methyluridine (28.4 ppm), respectively. The differentiation of these two resonance signals relied upon the methylation study of poly(U) which yielded only 3-methyluridine (28.0 ppm). These signals might also be designated to the methyl peaks of N^2-methylguanosine (27.9 ppm), N^6-methyladenosine (27.3 ppm), and/or N^4-methylcytidine (27.5 ppm). However, the treatments of pG, pA, and pC with MeMS do not produce a detectable amount of these methylated compounds (Lee & Chang, 1978; Chang & Lee, 1980). The contribution from these methylated products may therefore be insignificant. The most downfield signal at 53.3 ppm, with a splitting of 6 Hz, is assigned to the methyl carbon resonance of methyl phosphate (m^p). The methyl carbon of methyl phosphate of mononucleotides (m^p-pA, m^p-pG, m^p-pC and m^p-pU; ~53.4 ppm) couples with phosphorus-31 to give a doublet [^2J (^{13}C, ^{31}P) ≈ 6 Hz] whereas the methyl carbon resonance of O^6-methylguanosine (53.7 ppm) appears as a singlet.

Calculation of Product Distribution and Spin–Lattice Relaxation Time (T_1). All the product distributions are calculated from the integration curves. The calculation depends on the assumption that the difference in spin–lattice relaxation time (T_1) for those methyl carbon resonances is negligible under the measuring conditions (repetition time 2 s for 90° pulses). When a nucleus is allowed to wait for a time t after a 180° pulse, the magnetization along the z axis, M_z, will decay back to its equilibrium state M_0 at a rate of $M_z = M_0(1 - e^{-t/T_1})$ by spin–lattice relaxation. Our initial as-

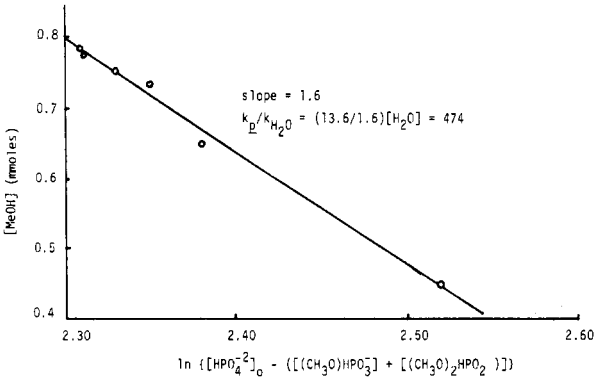

FIGURE 2: Reaction of methyl methanesulfonate (7.6 mmol) with 1.3 M phosphate buffer (16.9 mmol) at pH 10.5.

Table I: Methyl Carbon Chemical Shifts of Model Compounds

purine	δ (^{13}C)	pyrimidine	δ (^{13}C)
m^1A	38.0	m^3C	30.7
m^6A	27.3	m^4C	27.5
Am	58.4	m_2^4C	38.2, 37.4
m^1pA	38.4	Cm	58.4
m^p-pA	53.5	m^3pC	30.8
m^1m^p-pA	53.5, 38.4	m^p-pC	53.5
		m^3m^p-pC	53.5, 31.0
m^1G	28.6		
m^6G	53.7		
m^7G	36.3	m^4U	55.1
m^2G	27.9	m^3pU	28.4
m^p-pG	53.2	m^3m^p-pU	53.5, 28.3
$m^1m^7m^p$-pG	53.5, 36.5, 29.1		

Table II: Distribution (%) of Methyl Products from Methylation of Torula Yeast RNA by [^{13}C]Methyl Methanesulfonate at pH 7.0[a]

time (h)	m^P (53.4)	(39.8)[b]	m^1A (38.1)	m^7G (36.2)	(34.7)[b]	(34.1)[b]	m^3C (30.8)	m^1G (28.7)	m^3U (28.0)	m^7G[c]
0.5	5.0		23.4	63.1			8.5			14
1	4.7	1.4	22.6	61.7			9.6			18
3	5.0	1.8	23.3	59.5			9.3	0.6	0.4	30
6	4.9	1.1	22.6	59.5			9.9	1.1	0.9	
12	5.2	1.2	24.7	56.0			9.5	1.9	0.9	52
62	5.2	1.2	22.2	48.7	2.2	1.2	11.0	5.3	3.0	

[a] The value in the parentheses is the chemical shift (ppm) of the methyl carbon of the corresponding product. [b] Unidentified product. [c] The percentage of guanosine which reacted at the N-7 position.

Table III: Spin-Lattice Relaxation Time (T_1) of the Methyl Carbons of RNA Methylated by [^{13}C]MeMS for 62 h

	m^P		m^1A	m^7G	m^3C	m^1G	m^3U
ppm	53.5	53.2	38.1	35.2	30.8	28.7	28.0
T_1 (ms)	570	546	206	162	239	217	242

sumption is based on the measured "average" T_1 values of RNA (≤100 ms) (Komoroski & Allerhand, 1974; Bolton & James, 1980). Usually $5T_1$ is required for a "full" relaxation ($t = 5T_1$, $M_z = 0.993\ M_0$). The T_1 relaxation times for all methyl carbon resonances of RNA methylated by [^{13}C]MeMS for 62 h were measured by using a 180°–τ–90° pulse sequence (Table III). The extraordinarily long T_1 (570 ms) for the methyl carbon of methyl phosphate in RNA seems to suggest that M_0 may not be completely restored ($M_z = 0.97\ M_0$). We then measured the integration curves by using three different repetition times (2, 5, and 10 s) to evaluate the actual experimental error. No significant deviation was observed.

Discussion

Alkylating agents such as alkyl methanesulfonates produce a wide range of biological effects (Hallaender, 1971; Montesano & Batsch, 1976; Vogel & Natarajan, 1979; Pratt & Ruddon, 1979), exhibiting mutagenic, carcinogenic, and antitumor activities. Nucleic acids generally have been considered as prime target molecules for mutagenic and carcinogenic agents (Singer, 1975; Searle, 1978; Grover, 1979). For simulation of physiological pH conditions, most alkylation reactions were conducted in buffer solution. We found that methyl methanesulfonate reacted with inorganic phosphate very readily to produce methyl phosphate, reducing the buffer function of the phosphate solution. As a consequence, the solution became acidic due to the hydrolysis of MeMS to methanesulfonic acid, which could deactivate nucleotides or nucleic acids by protonation. In potassium phosphate solution used by Lawley & Brookes (1963), the pH changed from 7.1 to 6 within 1 h.

In order to overcome this problem, Strauss & Hill (1970) adjusted the ratio of MeMS and phosphate and set the initial pH at 10.5, which would keep the final pH of the overnight reaction at pH 6.5. In these conditions, about 60% of MeMS reacted with phosphate instantaneously. Within 10 h, 90% of MeMS reacted with phosphate. Similarly, Uhlenhopp & Krasna (1971) observed that dimethyl sulfate alkylated phosphate, citrate, and chloride but is unreactive toward perchlorate and cacodylate. Thus, some workers recently have tried to use cacodylate buffer instead of phosphate buffer to control the reaction. However, in a typical experiment, 10 mg of tRNA in 2 mL of cacodylate buffer (0.01 M cacodylic acid, 0.07 M sodium cacodylate, 0.001 M Na_2EDTA, and 0.12 M $NaClO_4$) was treated with dimethyl sulfate (4–40 μL) at pH 7. After the reaction was completed (~4 h), the pH had decreased to 5.5 (Trifunac & Krasna, 1974). This is probably due to the low buffer capacity of cacodylate. The conformation and the reactivity of each functional group of nucleic acid undoubtedly depend on the acidity of the solution. Thus the outcome of the alkylation reaction depends on the nucleophilicity of the buffer component and the pH variation during the entire period of reaction. It is very difficult to compare all the results obtained from reactions with different alkylating agents in different solutions. We have clearly demonstrated the sharp difference in the ^{13}C spectra of the two methylated RNA samples isolated from the reactions controlled by a pH-stat and phosphate buffer, respectively. Two of the methylated products, 1-methylguanosine and methyl phosphate, are not detectable when the reaction was conducted in phosphate buffer. The disappearance of the methyl phosphate peak also substantiates the assignment of the signal at 53.3 ppm to the methyl carbon resonance of methyl phosphate.

Elmore et al. (1948) first described the possible formation of phosphotriester from the DNA alkylation by mustard gas by using electrometric titration. Then various controversial conclusions on the alkylation of the phosphate groups of nucleic acids were evoked (Lawley, 1966). The existence of alkyl phosphotriester was not assayed until 1972 (Bannon & Verly, 1972) using tedious enzymatic degradation methods, and then it was verified by direct comparison with authentic samples (Jensen & Reed, 1978; Jensen, 1978) prepared from dinucleotides by a synthetic scheme described by Miller et al. (1974). We have found that the phosphate alkylation can be measured directly by ^{13}C NMR method on the basis of two-bond coupling with ^{31}P. Since the methyl chemical shifts of O^6-methylguanosine and methyl phosphate are very close, we cannt completely rule out the possibility that part of the 53.3-ppm signal may arise from the signal of O^6-methylguanosine. Furthermore, the terminal and/or the internucleotide phosphate groups of RNA can be attacked by alkylating agents to form phosphodiester and phosphotriester, respectively. From the chemical shifts of the model compounds (53.4 ± 0.2 ppm for p(Me)X; 55.8 ppm for $dT_{p(Me)}dT$) we may suggest that MeMS reacts only with the terminal phosphate group. This is in agreement with our observation on DNA methylation (C.-j. Chang and J. DaSilva Gomes, unpublished results). The phosphate methylation of salmon sperm DNA could be similarly determined by ^{13}C NMR. However, this phosphomethyl peak was absent after the DNA was treated with alkaline phosphatase prior to reacting with MeMS. A direct ^{31}P NMR study of the DNA samples further substantiated this conclusion.

The determination of different sites of modification is mainly based on comparison with the chemical shifts of the model compounds. Some of the signals may not be unambiguously distinguished by this approach. We have shown that a specific heteronuclear spin–spin coupling such as ^{13}C–^{31}P two-bond coupling can be useful in differentiating O^6-methylguanosine and terminal methyl phosphate. However, the direct observation of small coupling may often be limited by the broad

signals of macromolecules. This problem can probably be overcome by increasing the probe temperature and/or breaking into smaller components by enzymatic degradation. A triple resonance experiment, irradiating ^{31}P and ^{1}H simultaneously, may be another complementary method to confirm the ^{31}P–^{13}C interaction. In addition to the parameters of chemical shift and spin–spin coupling, relaxation time can also be utilized to distinguish different products. Both N-1 and N-7 of guanosine can be attacked by MeMS. Nevertheless, the spin–lattice relaxation time of the methyl carbon of 7-methylguanosine (162 ms) is shorter than those of 1-methylguanosine and other methylated products (>200 ms) presumably because of the peri interaction between the 7-methyl and 6-oxygen groups (Table III).

The low natural abundance of carbon-13 (1.1%) makes the direct measurement of chemical modification of RNA become impracticable when the degree of modification is low. This difficulty can be resolved by using ^{13}C-enriched modifying agent as shown in this study. This approach also eliminates the interference of ^{13}C natural abundance signal and thus enhances the specificity. Other laboratories have taken an alternative approach to improve the sensitivity and specificity (Agris & Schmidt, 1980; Agris et al., 1975; Schweizer et al., 1980) by using a nutritional auxotroph to specifically incorporate ^{13}C-enriched precursors into RNA. In principle, ^{2}H NMR in conjunction with a high-field spectrometer may provide better specificity to detect the low degree of the reactions, such as an in vivo system, than ^{13}C NMR since the natural abundance of deuterium (0.015%) is much lower. However, it requires the degradation of the ^{2}H-enriched RNA into small components by enzymatic hydrolysis to reduce the line width of ^{2}H resonance signals. The success of this method remains to be investigated.

In conclusion, we have demonstrated the potential application of ^{13}C NMR in the direct study of the chemical modification of biological macromolecules. It should provide an important approach to the determination of the reactive sites and mechanism of action of mutagens, carcinogens, and other bioactive substances.

Acknowledgments

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