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Chemical Modification of Viral Ribonucleic Acid. VII. The Action of Methylating Agents and Nitrosoguanidine on Polynucleotides Including Tobacco Mosaic Virus Ribonucleic Acid*

B. Singer and H. Fraenkel-Conrat

ABSTRACT: *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine methylates the same positions on the guanine, adenine, and cytosine residues as typical alkylating agents, although at a much slower rate. Guanine and adenine react more readily with nitrosoguanidine under conditions favoring base stacking (polymers in aqueous solution), and the opposite is true for cytosine. The action of typical methylating agents is not affected by this conformation. The depressed reactivity of the 1 and 3 positions of complementary base-paired adenine and cytosine, respectively, toward dimethyl sulfate has been confirmed, and observed to be true also for nitrosoguanidine, as far as adenine is concerned (cytosine being poorly reactive in aqueous solutions). Methylation by both types of reagents gives relatively more 7-methyladenine and 3-methyladenine than generally reported, and these reactivities remain unaffected when the formation of 1-methyladenine is depressed by base pairing. Treatment of ribonucleic acid with nitrosoguanidine does not produce detectable levels of nitrosation, but some deamination occurs upon prolonged treatment with the reagent.

During the course of studying the reaction of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (hereafter referred to as nitrosoguanidine) with TMV-RNA it became evident that the primary chemical events were methylation of guanine, adenine, and cytosine residues (Singer and Fraenkel-Conrat, 1967; Singer *et al.*, 1968). It then became desirable to compare the action of typical methylating reagents with that of nitrosoguanidine. Although several investigators have made important contributions to the chemistry of alkylation, each has used a different alkylating agent on a different nucleotide or polynucleotide, and not all possible minor methylation products of adenine have been looked for (Brookes and Lawley, 1960; Fraenkel-Conrat, 1961; Lawley and Brookes, 1963, 1964; Kriek and Emmelot, 1964; Ludlum, 1965; Chen and Davis, 1965; Brimacombe *et al.*, 1965).

The reaction of nitrosoguanidine with RNA has previously been shown to be conformation dependent (Singer and Fraenkel-Conrat, 1967; Singer *et al.*, 1968), a fact which has not been recorded for typical alkylating agents, excepting a recent study of the action of nitrogen mustards (Price *et al.*, 1968). We have now compared the absolute and relative amounts of 7-methylguanine, 1-methyladenine, 3-methyladenine, 7-

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methyladenine, and 3-methylcytosine found after reacting nucleotides, ribopolymers, and RNA with dimethyl sulfate, methyl methanesulfonate, and nitrosoguanidine, generally near neutrality or in slightly acid solution in water and occasionally in the presence of dispersing solvents.

Materials

5′-Ribonucleotides and polynucleotides were commercial samples, dissolved in water to a concentration of 10 mg/ml and kept frozen. TMV-RNA was isolated by standard procedures.

Dimethyl sulfate and methyl methanesulfonate were obtained from Matheson and Eastman Organic Chemicals, respectively. Nitrosoguanidine was obtained from Aldrich Chemical Co. and dissolved in formamide to a concentration of 100 mg/ml immediately before use. ^{14}C-Labeled dimethyl sulfate (2.15 mCi/mmole) was purchased from New England Nuclear Corp. ^{14}C-Labeled methyl methanesulfonate (45 mCi/mmole) was purchased from Nuclear-Chicago. ^{14}C-Labeled nitrosoguanidine (1.5 mCi/mmole) was a gift from Dr. J. Greenberg. [^{3}H]Poly A and [^{3}H]poly C were obtained from Miles. Poly (A,C,G,U) containing ^{14}C-labeled adenine or cytosine was prepared from the triphosphates with DNA-dependent RNA polymerase, and kindly given to us by Dr. J. Krakow. Poly G·poly C, given to us by Dr. M. Chamberlin, was obtained by polymerizing pppG on a poly C template with RNA polymerase.

1-Methyladenine and 7-methyladenine were obtained from Sigma Chemical Co. and K&K Laboratories, respectively. 7-Methylguanine was prepared by acid hydrolysis of 7-methylguanosine obtained from Sigma Chemical Co. 3-Methyladenine was a gift from Dr. P. D. Lawley.

Experimental Methods

Reaction with Dimethyl Sulfate and Methyl Methanesulfonate. Samples (2 mg) of 5′-adenylic acid, 5′-guanylic acid, or 5′-cytidylic acid in 1 ml of 0.01 M phosphate buffer (pH 7) were allowed to react with 60 μl of dimethyl sulfate, dissolved in an equal volume of ethanol, for 5 hr at room temperature. The reagent was extracted with several washings with ether.

Solution of 1–2 mg of the polynucleotides or of poly A·poly U (prepared according to Ludlum, 1965) or poly G·poly C in 0.3 ml of 0.03 M phosphate buffer (pH 7) were allowed to react with 30 μl of dimethyl sulfate (dissolved in an equal volume of ethanol), left at room temperature for 5–8 hr, and then dialyzed against water at 4° for 2 days.

TMV-RNA (1 mg) was dissolved in 0.125 ml of 0.02 M EDTA (pH 7) and reacted with 0.25 μl of dimethyl sulfate or 2.5 μl methyl of methanesulfonate at 0° for 2 hr if retention of about 10% of the infectivity was desired. For much higher levels of alkylation (20% of the G), the reaction was carried out at room temperature for 4 hr using 15 μl of dimethyl sulfate. The RNA was alcohol precipitated three times. More intense treatment of RNA with these reagents, in terms of amount of reagent or time and temperature, rendered the RNA largely alcohol nonprecipitable.[1] Frequently, ^{14}C-labeled dimethyl sulfate or methyl methanesulfonate was used (at about 0.2 mCi/mmole), and occasionally ^{14}C-labeled TMV-RNA (with unlabeled reagents) of about 200 cpm/μg.

[1] Such degradation of RNA upon dimethyl sulfate treatment is not observed upon nitrosoguanidine treatment, nor upon dimethyl sulfate treatment of poly A or poly C. The mechanism of this reaction is under further investigation.

TABLE I: Chromatographic Behavior of Methylation Products from RNA.[a]

	$R_{adenine}$ I	$R_{adenine}$ II	R_F III
1-Methyladenine	0.75	1.2	
3-Methyladenine	0.95	1.4	
7-Methyladenine	0.85	1.2	
5-Aminoimidazole-4-*N*′-methylcarboxamidine	0.60	1.2	
7-Methylguanine	0.51	0.78	
Guanine	0.35	0.59	
3-Methylcytidylic acid			0.69
Cytidylic acid			0.58
Uridylic acid			0.78

[a] Movement relative to adenine, $R_{adenine}$, or the solvent front, R_F, in solvents I, II, and III (see text).

Reaction with Nitrosoguanidine. Solutions of 2 mg of 5′-adenylic acid, 5′-guanylic acid, or 5′-cytidylic acid in 1 ml of water were allowed to react with 50 μl of a 10% solution of nitrosoguanidine in formamide at 37° for 2 days. After evaporation to a small volume, the treated nucleotides were applied to Whatman No. 1 paper and chromatographed in solvent I (see next section) for 2 days to separate reagent from nucleotide. The mixture of reacted and unreacted nucleotides which stayed at the origin was then eluted with water.

The various polynucleotides and the RNA (1–2 mg in 1 ml of water) were allowed to react with 50 μl of a 10% solution of nitrosoguanidine in formamide at 37°, and occasionally at 20°, for periods up to 5 days, then dialyzed against water at 4° for 2 days. Reactions were also performed in 65% dimethylformamide and/or 90% formamide under the same conditions as in water. Frequently ^{14}C-labeled nitrosoguanidine (about 0.2 mCi/mmole) was used.

Identification of Products. The methylated nucleotides, polynucleotides, or RNA was hydrolyzed at 100° for 1 hr in 1 N HCl. Digests of methylated adenylic acid and guanylic acid were two dimensionally chromatographed (descending), on Whatman No. 1 paper. Solvent I was 1-butanol–concentrated NH_4OH–water (85:2:12, v/v) (48 hr). Solvent II was methanol–concentrated HCl–water (70:20:10, v/v) (8 hr). This chromatographic system separates guanine, 7-methylguanine, adenine, 1-methyladenine, 3-methyladenine, 7-methyladenine, and 5-aminoimidazole-4-*N*′-methylcarboxamidine as shown in Table I. Methylated cytidylic acid was chromatographed, descending on Whatman No. 1 in solvent III (isopropyl alcohol–concentrated HCl–water, 68:17:15, v/v) for 18–24 hr.

With 1 N HCl hydrolysates of RNA, the origin after chromatography in solvent I contains the pyrimidine nucleotides. This area was cut off from the rest of the paper and chromatographed in solvent III while the rest of the paper was chromatographed in solvent II.

TABLE II: Relative Yields of Methylated Bases after Reacting Poly A or TMV-RNA with Dimethyl Sulfate, Methyl Methanesulfonate, or Nitrosoguanidine (Per Cent of Total Methylation).

	Poly A[a]		RNA[a]		
	Dimethyl Sulfate	Nitroso-guanidine	Dimethyl Sulfate	Methyl Methane-sulfonate[c]	Nitroso-guanidine
1-Methyladenine[b]	85	91	11.7	15.6 (20)	9.2
3-Methyladenine	5	2	1.8	1.3 (0.3)	1.3
7-Methyladenine	10	7	3.7	3.5	6.3
7-Methylguanine			77	73 (68)	76
3-Methylcytidylic acid			5.8	6.7 (14)	7.1

[a] The total methylation varied from 0.05 to 50% of the total bases. No significant variation in the proportion of derivatives formed was found over this range, except for the action of nitrosoguanidine on RNA at 20° which led to a relative predominance of 3-methyladenine (1-methyladenine, 5%; 3-methyladenine, 22%; 7-methyladenine, 5%; 7-methylguanine, 49%; and 3-methylcytosine, 19%). [b] The values given include the amount of 5-aminoimidazole-4-*N'*-methylcarboxamidine which Brookes and Lawley (1960) conclude to be derived from 1-methyladenine. [c] Data in parentheses are average values obtained by Lawley and Brookes (1963) using [^{14}C]methyl methanesulfonate on yeast RNA and rat liver RNA. 7-Methyladenine was not determined separately from 1-methyladenine.

In all experiments where ^{14}C-labeled reagents were used, the expected nonradioactive methylated derivatives were added to the digest at the time of chromatography. The marker areas were detected under ultraviolet light, the spots were cut out, and their radioactivity was counted either directly in scintillation vials or on aliquots after elution. If whole paper areas were counted, the scintillation fluid could be washed out by repeated soaking in toluene. The ultraviolet areas could then be eluted in 0.01 N HCl and the spectra of the products determined which also could then be rechromatographed, if desired.

In experiments without ^{14}C label, areas detectable under ultraviolet light were eluted in 0.01 N HCl and the spectra were plotted on a Cary recording spectrophotometer (Model 15). All methylated derivatives found have characteristic spectra as shown in Figure 1.

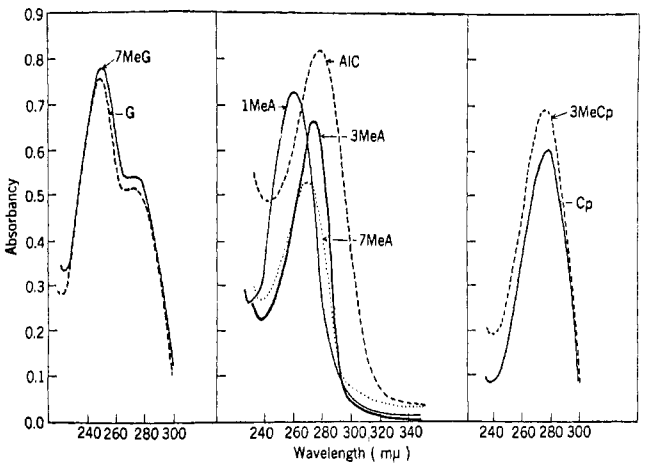

FIGURE 1: Absorption spectra of methylation products (eluted from chromatograms) of guanine, adenine, and cytidylic acid, respectively, in 0.01 N HCl (at unrelated concentrations). AIC stands for 5-aminoimidazole-4-*N'*-methylcarboxamidine (see text). E_{max}: 7MeO, 250 mμ; G, 248 mμ; 1MeA, 260 mμ; A, 262 mμ; 3MeA, 275 mμ; 7MeA, 270 mμ; AIC, 278 mμ; 3MeCp, 276 mμ; Cp, 280 mμ.

Results

Methylations of Base-Stacked Polynucleotudes. Tables II–IV summarize averaged data obtained upon acid hydrolysis of the products of the reactions of dimethyl sulfate, methyl methanesulfonate, and nitrosoguanidine with polynucleotides and RNA in aqueous solution.

To avoid confusion, attention is drawn to the fact that the data are expressed in part in terms of relative reactivity (per cent total alkylation), and in part in absolute terms (per cent based reacted), and that these values are obviously not directly comparable. The majority of the data was obtained with radioactive reagents. Reactions with unlabeled reagent are also included where the methylated bases were identified by R_F and spectra, but minor products can be detected in this manner only when large quantities of material are being chromatographed.

Both dimethyl sulfate and nitrosoguanidine methylate poly A to a similar extent (Table II), although at quite different rates, as illustrated by the fact that similar levels of methylation were attained by the much more intense reaction conditions used with nitrosoguanidine, and by the mild ones used with dimethyl sulfate (see Materials and Experimental Methods). Lawley and Brookes (1964) discuss the formation of 3-methyladenine and 7-methyladenine, as well as 1-methyladenine, by the action of dimethyl sulfate on deoxyadenylic acid or DNA. However, later papers by Brimacombe *et al.* (1965), Ludlum (1965), and Chen and Davis (1965), working on the alkylation of poly A by dimethyl sulfate and methyl methanesulfonate, indicate that only 1-methyl A is formed. The above data clearly show that both dimethyl sulfate and nitrosoguanidine methylate adenine also in the 3 and 7 positions. Quite in contrast to the reactivity of poly A to both types

TABLE III: The Effect of Base Stacking on Alkylation by Nitrosoguanidine.[a]

	pA H_2O (% of original base)	pG H_2O (% of original base)	pC H_2O (% of original base)	Poly A (% of original base[b]) H_2O	Poly A (% of original base[b]) DMF and FA	Poly (A,G) (% of original base) H_2O	Poly (A,G) (% of original base) DMF	Poly C (% of original base) H_2O	Poly C (% of original base) DMF	RNA H_2O[c] (% of total methylation)	RNA DMF[d] (% of total methylation)
1-Methyladenine	<1.5			30	17	1.5				9.2	12.2
3-Methyladenine				2.5	1.5	1.0				1.3	1.8
7-Methyladenine				6	5	0.5				6.3	5.1
7-Methylguanine		<5				15	<1			76	59
3-Methylcytidylic acid			20					<1.5	9	7.1	22

[a] All reactions were at 37° for 2–5 days with 2.5 mg of nitrosoguanidine/mg of nucleotide, in either H_2O, 67% dimethylformamide (DMF), or 90% formamide (FA). At 20°, poly A and poly (A,G) reacted to only a small extent. [b] In ammonium bicarbonate (pH 8) in the presence or absence of 90% ethylene glycol as dispersing agent, the proportion of the methylated adenines differed (1-methyl:3-methyl:7-methyl, 50:21:29). [c] The absolute amount of methylation found in similar experiments where optical density was measured was about 25% of the guanine as 7-methylguanine and 5% of the adenine as 1-methyladenine. [d] Total methylation in dimethylformamide was about 50% of that in H_2O and similar to that obtained in 90% formamide which also yielded a similar methylation pattern.

TABLE IV: Extent of Methylation of Nucleotides and Polynucleotides by Dimethyl Sulfate in Aqueous Solution.[a]

	pA	pG	pC	Poly A	Poly G	Poly C	RNA
Methyladenine[b]	~60			40			3
Methylguanine		25			12		19
Methylcytydilic acid			20			50	<1

[a] All reactions were at room temperature for 5–8 hr, using 30 μl of dimethyl sulfate/mg of nucleotide. All are per cent of original base. [b] Summation of methylation on 1, 3, and 7 positions.

of reagents, poly C was readily methylated by dimethyl sulfate, but not by nitrosoguanidine in aqueous solution (Tables III and IV).

Dimethyl sulfate, methyl methanesulfonate, and nitrosoguanidine show similar methylation patterns with RNA upon extensive reaction (Table II). In agreement with Lawley and Brookes (1963), guanine methylation predominates, with lesser amounts of adenine and cytosine becoming methylated. Again the data show the formation of substantial amounts of 3-methyladenine and more of 7-methyladenine, in addition to 1-methyladenine. A fourth methylated adenine derivative was usually found in quite varying amounts. This product corresponds to the 5-aminoimidazole-4-*N'*-methylcarboxamidine discussed by Brookes and Lawley (1960) as related to 1-methyladenine, but not generally commented on in the literature. In the tables, this product is included in the 1-methyladenine data.

The conformation dependence of the reaction of nitrosoguanidine is illustrated by the comparison of its action at 20° with that noted customarily at 37° (5 days) (Table II). For it appears that at 20° the adenine in RNA (but not in poly A) is methylated predominantly at the 3 position, rather than at the 1 and 7 positions. It has previously been reported that nitrosoguanidine causes loss of infectivity more rapidly at 20° than at 37°. Dimethyl sulfate and methyl methanesulfonate show no clear differences in methylation pattern at these two temperatures.

Methylations of Noninteracting Nucleotides. We have reported in recent papers (Singer and Fraenkel-Conrat, 1967; Singer *et al.*, 1968) that alkylation of guanine by nitrosoguanidine was detectable only in polynucleotides and that this alkylation could be depressed by performing the reaction in 67% dimethylformamide or 90% formamide. In contrast, the alkylation of cytosine in polynucleotides by nitrosoguanidine was favored by dimethylformamide. It was concluded from these data that the base-stacked conformation was favorable for alkylation of guanine by nitrosoguanidine, and unfavorable for the alkylation of cytosine. The present comparative study was therefore extended to 5'-mononucleotides, on the one hand (pA, pG, pC), and to poly A, poly C, poly G, poly (G,U), poly (A,G), and RNA in such unstacking solvents, on the other. The results in Table III show that little

TABLE V: Effect of Double Strandedness on Methylation of Poly A by Dimethyl Sulfate and Nitrosoguanidine.

	Dimethyl Sulfate		Nitrosoguanidine	
	Poly A[a,b]	Poly A·Poly U[b]	Poly A[a]	Poly A·Poly U[b]
1-Methyladenine[c]	81	34 (8)	92	15 (1.2)
3-Methyladenine	5	38 (142)	2	14 (45)
7-Methyladenine	13	29 (44)	7	71 (65)

[a] Approximately 20% of the A residues were methylated in a typical experiment. [b] Per cent of total alkylation. Figures in parentheses represent percentages of the alkylation observed with poly A. [c] Includes 5-aminoimidazole-4-*N'*-methylcarboxamidine.

methylation results from nitrosoguanidine acting on pA and pG and extensive methylation of pC. In contrast, in polymers, adenine and guanine[2] reacted in aqueous solution, but cytosine appreciably only in dimethylformamide. Thus the favorable effect of base stacking on the nitrosoguanidine–guanine reaction appeared to hold also for adenine, and the unfavorable effect of stacking on the methylation of cytosine was further substantiated. In contrast, dimethyl sulfate gives comparable levels of reaction for all nucleotides and polynucleotides in aqueous media (Table IV). A comparison of the methylation pattern achieved with nitrosoguanidine acting on TMV-RNA in 67% dimethylformamide, and 90% formamide showed no marked difference between these two dispersing solvents, in contrast to earlier results (Singer *et al.*, 1968). The absolute and relative depression in the reactivity of guanine, and the greater reactivity of cytosine was again observed in these solvents. The adenine residues were alkylated to a slightly decreased extent with typical proportions of the three products (1-methyladenine > 7-methyladenine > 3-methyladenine). One experiment at alkaline pH (0.05 M ammonium bicarbonate) and in 80% ethylene glycol gave results which differed in various of these aspects, and such conditions require further study.

Methylation of Base-Paired Nucleotides. While base-stacking affects the reactivity of nucleotides to nitrosoguanidine but not to typical methylating agents, the alkylation of base-paired adenine and cytosine is affected by both types of reagents. In regard to the reaction of poly A·poly U with dimethyl sulfate, our data indicate that double strandedness depresses the reaction of the 1 position of adenine with nitrosoguanidine more than that with dimethyl sulfate (Table V). In contrast to Ludlum (1965) and Chen and Davis (1965) we find evidence for the alkylation of the 3 and 7 position of adenine in both poly A and the double-stranded complex, the reactivity of these groups to both reagents not being greatly affected by double strandedness. In poly C·poly G only the alkylation of the cytosine is depressed in agreement with expectation (Pochon and Michelson, 1967) (from about 40 to 2% of the cytosine being methylated by dimethyl sulfate). The alkylation of guanine occurs normally with both types of reagents.

An inspection of the data obtained with both reagents acting on TMV-RNA indicates the customary predominance of alkylation of guanine. This is in contrast to the data obtained with nucleotides and homopolymers which indicate that adenine and cytosine tend to be more reactive than guanine. It appears probable that this altered reactivity of the bases in RNA is a result of random base-pairing interactions depressing the comparative reactivity of cytosine and of the 1 position of adenine, thus producing a predominance of guanine alkylations as well as a pattern of 1, 3, and 7 alkylations of adenine halfway between that obtained with poly A and poly A·poly U. However, the alkylation pattern of RNA by methyl methanesulfonate was not found affected by the level of hypo- or hyperchromicity of the RNA as effected by the use of EDTA or $MgCl_2$ in the reaction medium, excepting a depressed reactivity (to 2.5% of total methylation) of the C in the hypochromed sample.

It nevertheless appears that [^{14}C]dimethyl sulfate could be used as a probe for the existence and location of single-stranded segments in double-stranded nucleic acids, by analyzing the pattern of base methylations.

Unidentified Methylated Products. In reporting the identification of 7-methylguanylic acid as a brightly fluorescent spot on two-dimensional chromatograms of snake venom digests of nitrosoguanidine-treated RNA, a second spot showing much less intense white fluorescence was reported between adenylic acid and adenosine (Singer and Faenkel-Conrat, 1967). This material was shown to be a methylation product since it was labeled when [^{14}C]methylnitrosonitroguanidine was used, and it was believed to be derived from adenine on the basis of its ratio of ^{14}C to ^{32}P counts, if double-labeled TMV-RNA was used. Subsequent experiments with poly A and nitrosoguanidine on the one hand, and with RNA and dimethyl sulfate on the other, have failed to reveal the presence of this product detectable by either its fluorescence or its radioactivity (less than 1% of the 1-methyladenine formed). Counts found in the fluorescent area on chromatograms of digests of nitrosoguanidine-treated RNA did not, after acid hydrolysis, chromatograph with any of the four known methyl derivatives of adenine. Finally, using poly (A,C,G,U) prepared with ATP as the only ^{14}C-labeled building block, the white fluorescent area in question (due to treated carrier RNA) contained no more radioactivity than adjacent areas (about 0.25% of adenine). It thus seems now doubtful that this material is derived from adenine, and it is certainly of much lesser quantitative significance than the four nonfluorescent methylated bases since identified as minor reaction products in the treated RNA.

[2] Poly G was peculiarly resistant to nitrosoguanidine alkylation, but in poly (G,U) and poly (A,G) methylation of the guanine occurred readily.

The Occurrence of Other Reactions. In the hope of identifying this and other minor nitrosoguanidine–RNA reaction products, it seemed indicated to test for the presence of nitroso (methyl) derivatives of the nucleotides. To this end a snake venom digest of nitrosoguanidine-treated RNA was subjected to the Griess test for any N–NO compounds and the diphenylamine test for O–NO_2, O–NO, N–NO_2, and NNO groups (Feigl, 1966). These tests indicated the absence of such nitroso compounds, to the extent of less than 0.03 and 0.07% of nitrosoguanidine tested in parallel or upon addition to the RNA digest.

The possibility that nitrosoguanidine could cause deamination of nucleotides was indicated by the studies of Rau and Lingens (1967) and of Chandra *et al.* (1967). To test for the occurrence of deamination, we treated [^{14}C]TMV-RNA and other polynucleotides containing labeled adenine or cytosine with nitrosoguanidine for 4 or 5 days at 37°.

When the customary acid hydrolysis procedure was used to detect hypoxanthine and xanthine in untreated [^{14}C]TMV-RNA, it became evident that 2–4% of each of these bases arose from adenine and guanine during hydrolysis. Degradation by snake venom diesterase with or without alkaline phosphatase treatment, or with T-2 ribonuclease, yielded only about 0.4% of the adenine and guanine of TMV-RNA as inosine and xanthosine (or the corresponding nucleotides). The nitrosoguanidine-treated samples (5 days at 37°), in contrast, contained 1–3% of each of the deaminated bases. With poly (A,C,G,U) containing ^{14}C only in the adenine, the corresponding figures were 0.05% hypoxanthine in the incubated control and 1.0% in the nitrosoguanidine-treated sample. With [^{3}H]poly A, the corresponding figures were 0.03 and 0.16% deamination. Snake venom digestion of nitrosoguanidine-treated [^{3}H]poly C (2 days, 37°) showed it contains 0.4% of its radioactivity in 5′-uridylic acid, as compared with 0.2% in the untreated poly C. The possible role of these reactions in accounting for the high mutagenesis of nitrosoguanidine acting on TMV will be discussed in the following paper (Singer and Fraenkel-Conrat, 1969).

Discussion

The three major aspects to consider in regard to the alkylating action of the reagents are: (a) the products of methylation of RNA by dimethyl sulfate, methyl methanesulfonate, and nitrosoguanidine; (b) the effect of base stacking on methylation; and (c) the effect of hydrogen bonding on methylation. These different aspects are interrelated and will be discussed together.

All reagents give stable methyl derivatives at the same sites, namely, the 7 position of guanine, 3 position of cytosine, and 1, 3, and 7 positions of adenine in both homopolymers and RNA. However, the fact that in RNA the 7 positions of adenine are generally methylated more than the 3 position and to almost the same extent as the well-recognized alkylation of cytosine has been frequently overlooked. The amounts of 3-methyladenine and 7-methyladenine, relative to 1-methyladenine, are increased when the adenine is in a more or less hydrogen-bonded form as in RNA and particularly in poly A·poly U (Tables II and V) and, therefore, these alkylations become important in any discussion of the role of alkylation in mutation. In the experiments given in Table V it is also to be noted that double strandedness does not make the 1 position of adenine as unavailable for methylation by dimethyl sulfate as by nitrosoguanidine. It is possible that the higher reactivity of dimethyl sulfate leading to more extensive methylation in the 3 and 7 position causes sufficient disruption of the secondary structure to make the 1 position of some adenine residues available for reaction. Another expected effect of hydrogen bonding is the relative decrease in methylation of C by dimethyl sulfate in the RNA and particularly in poly C·poly G.

A comparison of the relative amounts of methylation products of adenine, guanine, and cytosine in RNA by dimethyl sulfate, methyl methanesulfonate, and nitrosoguanidine (the latter at 37°) generally supports the data given by Lawley and Brookes (1963) for methylation of yeast RNA and rat liver RNA by methyl methanesulfonate (Table II). Thus the sites in RNA reactive toward alkyl and nitrosoalkyl compounds are in order of decreasing reactivity: N-7 of guanine, N-1 of adenine, N-3 of cytosine, N-7 of adenine, and N-3 of adenine. The sites in poly A reactive toward these reagents are in order of decreasing reactivity: N-1, N-7, and N-3. The same comparison for the adenine in poly A·poly U is N-7 and N-3 greater than N-1.

Another conformation dependence seems to be a special property of alkylation by nitrosoguanidine, possibly because this reagent may need to intercalate between bases as a first step in alkylating adenine and guanine, neither of which react well as mononucleotides (Tables III) or as dinucleotides (Singer and Fraenkel-Conrat, 1967). On the other hand, cytosine is alkylated as the nucleotide but not appreciably in poly C except when the reaction is carried out under conditions which disrupt stacking such as in dimethylformamide. When RNA is reacted with nitrosoguanidine in dimethylformamide or formamide, the total methylation is decreased by about 50%, the relative amounts of adenine, guanine, and cytosine methylation reflecting each base's dependence upon conformation and secondary structure. The most striking effect is that in RNA, as in poly C, the amount of 3-methylcytosine increases in dimethylformamide to about 50% of the 7-methylguanine formed whereas in aqueous solution 3-methylcytosine represents only about 10% of 7-methylguanine.

It is of interest to note that the effect of a group of alkylating agents larger than those used by us (the nitrogen mustards) on the purines resembles nitrosoguanidine in being favored by base stacking (Price *et al.*, 1968). Double strandedness shows the usual protecting action on the N-1 and N-3, of adenine and cytosine, respectively.

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Chemical Modification of Viral Ribonucleic Acid. VIII. The Chemical and Biological Effects of Methylating Agents and Nitrosoguanidine on Tobacco Mosaic Virus*

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ABSTRACT: The ribonucleic acid in the tobacco mosaic virus particle reacts very sluggishly with nitrosoguanidine, as compared with dimethyl sulfate, and with the free ribonucleic acid reacting with either reagent. Yet, the nitrosoguanidine tobacco mosaic virus reaction is the only one of these reactions which is highly mutagenic. The distribution of the few methyl groups that could be introduced into the intraviral ribonucleic acid (two per mole, 2×10^6 daltons, during 5 days at 37°) was unusual in that the extent of methylation of cytosine approached that of guanine, and that the 1-methylation of adenine was somewhat depressed. In contrast, dimethyl sulfate caused (in 24 hr at 0°) about 97 guanine methylations and a total of 3 other methylations at a similar level of inactivation (2–3 inactivating events), associated with little mutagenesis. Deamination of adenine was found not to account for the high mutagenic action of nitrosoguanidine on tobacco mosaic virus but the possibility that mutagenesis is a consequence of cytosine deaminations has not been excluded. Alternatively, the mutagenicity of this reaction may be due to the increased methylation of cytosine.

The previous paper in this series dealt with the effects of typical methylating agents and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (hereafter referred to as nitrosoguanidine) on polynucleotides and TMV-RNA. It was concluded that in RNA both types of reagents methylated the same sites in relatively the same proportions, but that the mechanism of methylation by the two reagents was not identical. Methylation with dimethyl sulfate or methyl methanesulfonate was affected mainly by hydrogen bonding which reduced the availability of N-1 of adenine and N-3 of cytosine. In contrast with nitrosoguanidine, in addition to the effect of hydrogen bonding, methylation of guanine and adenine was favored by base stacking, while methylation of cytosine occurred more readily when bases were unstacked.

In this paper, we report on the extension of such studies to the methylation of the RNA in the TMV particle. The comparative study of the action of dimethyl sulfate and nitrosoguanidine on TMV is of interest since nitrosoguanidine is highly mutagenic for the virus but not for the isolated RNA (Singer and Fraenkel-Conrat, 1967; Singer *et al.*, 1968), while typical methylating agents act as similarly low-level mutagens on both the RNA and the intact virus (Fraenkel-Conrat, 1961; Singer and Fraenkel-Conrat, 1969a).

Materials and Methods

Tobacco mosaic virus and [^{14}C]TMV were isolated by standard procedures. The reagents and methylated bases were the same as described in the preceding paper (Singer and Fraenkel-Conrat, 1969b).

Methylation with dimethyl sulfate was performed as follows. TMV (40 mg) in 1–2 ml of 0.005 M EDTA (pH 7) was allowed to react with 2 μl of [^{14}C]dimethyl sulfate at 0° for 24 hr, then centrifuged at 40,000 rpm for 2 hr, resuspended in water, and again centrifuged 40,000 rpm for 2 hr. H_2O (2 ml) was added to dissolve the pellet which was then treated with phenol to isolate the RNA. An aliquot of the RNA, after three alcohol precipitations, was reconstituted with TMV protein and infectivity was determined. RNA (1 mg) was hydrolyzed with 1 N HCl for 1 hr at 100°. Chromatography and isolation of added methylated bases was as previously described. The technique of methylation with methyl methanesulfonate was the same as for dimethyl sulfate except that 10 μl of [^{14}C]-methyl methanesulfonate was reacted with TMV at 37° for 15 min.

Reaction with nitrosoguanidine was performed as follows. TMV (40 mg) in either 1–2 ml of 0.5 M acetate (pH 4 and final

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