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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*

B. Singer and H. Fraenkel-Conrat

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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TABLE I: Relative Yields of Methylated Bases after Reacting TMV or TMV-RNA with Dimethyl Sulfate, Methyl Methanesulfonate, or Nitrosoguanidine.[a]

	TMV[b]			TMV-RNA[c]		
	Dimethyl Sulfate	Methyl Methanesulfonate	Nitrosoguanidine	Dimethyl Sulfate	Methyl Methanesulfonate	Nitrosoguanidine
1-Methyladenine	1	0.7	6.3	17.5	15.5	5
3-Methyladenine	0	0	4.9	2.7	1.3	22
7-Methyladenine	2	0.4	6	3.4	3.5	5
7-Methylguanine	97	97	48	66	73	49
3-Methylcytosine[d]	0.2	1.7	35	10	6.7	19
No. of methyls/lethal events[e]	~50	~30	1–2	~10	~7	1–2

[a] Percentage of total methylation. [b] TMV was reacted with dimethyl sulfate at 0° (24 hr), with methyl methanesulfonate at 37° for 0.25 hr, and with nitrosoguanidine at 37° for 5 days Residual infectivities were 4–35%. [c] TMV-RNA was reacted with dimethyl sulfate and methyl methanesulfonate at 0° for 2 hr, and with nitrosoguanidine at 0° for 15 min. Residual infectivities were 0.03, 20, and 10% (see Singer and Fraenkel-Conrat, 1969b, for details). [d] Determined as the nucleotide. [e] See footnote in text.

pH 4.0) or 0.03 M KH_2PO_4 (final pH 5.5) was allowed to react with 3 mg of [^{14}C]nitrosoguanidine at 37° for 5 days. Isolation of the virus, preparation of RNA, and analytical procedures were the same as for dimethyl sulfate treated virus.

Experiments were also performed using ^{14}C-labeled TMV and unlabeled nitrosoguanidine, for the purpose of detecting nonmethylated reaction products.

Results and Discussion

Table I compares the yields of methylated bases obtained after acid hydrolysis when TMV and TMV-RNA had been reacted with typical methylating agents (dimethyl sulfate or methyl methane sulfonate) or with nitrosoguanidine. In the virus, dimethyl sulfate and methyl methanesulfonate methylate guanine almost exclusively. The methylation of cytosine and adenine is greatly reduced in the virus as compared with the RNA, the reactivity of the 3 and 1 positions of adenine being more depressed than that of the 7 position.

As far as the decrease in reactivity of the 1 position of adenine and the 3 position of cytosine is concerned, the RNA in TMV resembles the double-stranded polymers. However, the decreased reactivity also of the 3 position of A differentiates intraviral from double-stranded RNA, and the reactivity of the intraviral RNA toward nitrosoguanidine further illustrates its unique aspects.

First to be noted about the action of nitrosoguanidine on intact TMV is the very low susceptibility of the RNA in the virus to nitrosoguanidine as compared with dimethyl sulfate. Only about two alkylations per virus particle (per mole of RNA) were obtained during 5 days of treatment with nitrosoguanidine at 37°, whereas 100 alkylations resulted from 24 hr of treatment at 0° with dimethyl sulfate. As regards the comparative reactivity of different groups, nitrosoguanidine alkylated all 3 positions of adenine similarly, which means a relative depression in the reactivity of the 1 position compared with its reactivity in the RNA. However, the most striking effect of nitrosoguanidine acting on intact TMV is its preference for methylation of cytosine (Table I, third column). This reaction is the only instance of an alkylation of a group in RNA approaching in magnitude the alkylation of guanine.

We have previously presented evidence that in free polynucleotides the methylation of guanine and adenine by nitrosoguanidine is favored by base stacking, while that of cytosine is depressed in base-stacked polymers. In the virus rod the bases are not stacked. The slow rate of methylation of guanine and adenine in the virus and the unusually high relative reactivity of cytosine is thus in line with these earlier observations based on the use of either dispersing solvents, or mononucleotides.

Base stacking, however, is not the only factor determining the relative reactivity of different groups of the RNA under different conditions. The unavailability of the 1 and 3 positions of adenine in the virus to dimethyl sulfate, and the lowered reactivity of only the 1 position to nitrosoguanidine requires separate explanations. The lack of reactivity of any group of the RNA inside the virus particle can safely be attributed to protein–RNA interaction. The best-known case of such depressed reactivity is that of the amino group of guanine (and to a lesser degree of adenine), as shown by nitrous acid treatment of the virus (Schuster and Wilhelm, 1963). Thus one may conclude that the "front" of the purines (positions 1, 2, and 6 and substituents) interacts with protein groups and is chemically rather inert in the virus. The pyrimidines are not so tightly packed in protein, so that cytosine which can readily be deaminated can also be methylated by nitrosoguanidine, a reaction particularly favored, compared with dimethyl sulfate, by the lack of base stacking. One must then further postulate that with progressive cytosine methylation during several days at 37° the virus structure gets somewhat loosened up, so that the masking of the "front" of the adenine residues diminishes, and adenine alkylation can proceed further than is possible (at 0° in 24 hr) with dimethyl sulfate which fails to alkylate cytosine.

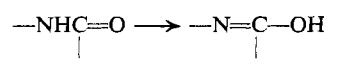
The data in Table I are derived from experiments on TMV and TMV-RNA retaining from 35 to 0.03% of their original

infectivity. This indicates 1–8 inactivating events/molecule. A comparison between the number of lethal events and the number of methyl groups introduced shows that not all methylations caused in TMV-RNA by dimethyl sulfate or methyl methanesulfonate are inactivating events (Table I). In the intact virus the proportion of methylations to inactivating events is even higher. This is in contrast to the action of nitrosoguanidine which causes similar numbers of inactivating and methylating events. The data obtained with both TMV-RNA and TMV could be rationalized on the basis that the 7 methylation of guanine is not inactivating, but that either or both the methylation of adenine and cytosine is inactivating, as would be expected on the basis of present concepts of the replication process.

The extent of methylation as calculated from the specified specific activity of the dimethyl sulfate used in current experiments is only about 40% of that derived from specific activity determinations based on the counts found per micromole of 7-methylguanine or 1-methyladenine after extensive reaction. This appears to be due to the presence of volatile contaminants in the [^{14}C]dimethyl sulfate. If this correction is applicable to the earlier data given in the last line of Table I then the ratios of methylations to lethal events would be higher.

Indications that extensive alkylation causes some degradation of RNA (more than of poly A, see under Methods) make it desirable to distinguish between inactivating events due to the reaction *per se*, and those due to chain breakage. Fractionation by sucrose gradient centrifugation of the alkylated RNA is being used for this purpose. This makes it possible to determine the number of inactivating and methylating events in the undegraded fraction of the RNA. These values are found to be somewhat lower (four methyls per lethal event) than those obtained with unfractionated RNA at low levels of methylation. We concur with Ludlum that ethylation may cause more degradation than methylation (Ludlum, 1969).

The mutagenic efficacy of nitrosoguanidine when acting on intact TMV, as contrasted to its action on TMV-RNA and that of all typical alkylating agents on either TMV or its RNA remains to be accounted for. The low-level mutagenesis of methylation of TMV-RNA has been tentatively attributed to the formation of 7-methylguanine (Singer and Fraenkel-Conrat, 1969), a reaction which may slightly favor the unusual tautomeric form at positions 1 and 6



However, this does not account for the great increase in mutagenicity observed when the intact virus is treated with this reagent.

The main analytical difference in nitrosoguanidine acting on TMV, as contrasted to the free RNA, is the increased formation of 3-methylcytosine. Arguments against this being the mutagenic reaction are, (1) the 3 position of cytosine is involved in base pairing and any addition to it would be expected to be lethal[1] and, (2) 3-methylcytosine represents about 7% of the methylation occurring when nitrosoguanidine acts on RNA. Yet an increase in such methylation to 35%, or 5-fold, increases the mutation rate from 2 to 60 or about 30-fold.

However, one must keep in mind that the level of mutagenesis may represent the result of a balance between mutagenic and inactivating reactions. Thus a high level of mutagenesis can result from suppression of an inactivating reaction. Under the conditions of increased methylation of cytosine, the 1-methylation of adenine and the formation of 7-methylguanine are relatively depressed. If one assumed that one or both of these reactions were lethal, such a decrease, coupled with an increase in the presumed mutagenic event, *e.g.*, methylation of the 3 position of cytosine, could simulate a high mutation rate. All indications are that the methylation of guanine is not lethal, since the amount of 7-methylguanine found after reaction of TMV-RNA and particularly of TMV with dimethyl sulfate or methyl methanesulfonate is in excess of the number of lethal events (Table I). Thus only the balance of 3-methylcytosine to 1-methyladenine remains as potential explanation, in terms of our analytical data, for the high mutagenicity of nitrosoguanidine acting on TMV. Yet, for reasons mentioned above, this interpretation appears theoretically unlikely, though supported by recent data!

There exists the possibility that none of the observed methylation reactions account for the high mutagenicity, but that nitrosoguanidine may cause deamination during the long reaction time required for its action on TMV and that those deaminations represent the mutagenic events. A deaminating action of nitrosoguanidine on adenylic acid has been briefly reported by Rau and Lingens (1967). Also, the finding by Chandra *et al.* (1967) that nitrosoguanidine-treated poly A incorporates glutamic acid when acting as messenger[2] suggests deamination, since the codons for glutamic acid, GAA or GAG, could be simulated by the deamination of adenine to hypoxanthine.

Our search for the formation of hypoxanthine and xanthine have indicated the deamination of 1–3% of the purines after several days of nitrosoguanidine treatment of [^{14}C]TMV-RNA at 37°, when the alkylation of guanine is 30–40% (see preceding paper). When the intact virus was treated with nitrosoguanidine at 37° for 6 days at pH 4.0, its RNA contained the same trace amounts of inosinic and xanthylic acid (after T-2 RNase digestion) as the RNA from similarly incubated control virus (0.07–0.2% of the counts of the adenylic and guanylic acid on the same chromatogram, of the order of one to two residues per mole). These results do not support the hypothesis that the mutagenesis of nitrosoguanidine is due to the deamination of adenine (or guanine). However, no means are available to test for the deamination of a few cytosine residues.

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[1] Preliminary data on the use of methylated poly C (containing 4–32% 3-methylcytosine) as messenger in the cell-free amino acid incorporation system support the belief that this reaction is inactivating rather than mutagenic. Yet a recent study of the effect of methylation on the template activity of poly C suggests that 3-methylcytosine acts like adenine and leads to the incorporation of uridylic acid in the poly G product (Ludlum and Wilhelm, 1968).

[2] Preliminary experiments in our laboratory have not confirmed these results.

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Studies of Trinucleotide Conformations. Role of Guanine Residues in an Oligonucleotide Chain*

J. Brahms, A. M. Aubertin, G. Dirheimer, and M. Grunberg-Manago

ABSTRACT: A comparison of spectroscopic (circular dichroism) and thermodynamic properties of various trinucleotides studied at different conditions of ionic strength, nucleotide concentration, and temperature, allows one to detect the conformational characteristics of polynucleotide chains, *i.e.*, the unstacked, single-stranded stacked, and double- or multiple-stranded helical associative forms. Several trinucleotides can form, at low temperatures, single-stranded stacked structures, *e.g.*, ApApUp, ApApCp, and GpApUp. Thermodynamic analysis of the thermal "melting" process gives a relatively small value of ΔF° for the guanine-containing trinucleotide. This suggests a tendency of guanine to unstack. The presence of guanine residues probably has a dual role. In trinucleotide sequences like GpGpC or GpGpU, the formation of intermolecular associations is observed under appropriate conditions.

In other sequences, *e.g.*, ApGpU, UpGpA, and GpUpA, there is no evidence for the formation of dissymmetrical stacked base conformation under conditions favoring the formation of single-stranded structures. Our results suggest the existence of *syn* and *anti* conformers whenever unpaired guanosine residues are next to uridine.

The knowledge of various factors contributing to the conformational stability of ribonucleic acid is of a special importance for the determination of its structure in solution. For the past few years the application of optical methods to the studies of simple oligonucleotides has been very fruitful for the understanding of conformation and of forces contributing to conformational stability. Thus, stacking interactions were shown to be the major source of conformational stability of single-stranded helical structures (Van Holde *et al.*, 1965; Poland *et al.*, 1966; Leng and Felsenfeld, 1966; Brahms *et al.*, 1966; Fasman *et al.*, 1964). Studies of natural 3′→5′ dinucleotides showed that essentially similar thermodynamic stacking interaction parameters were found for various sequences (Brahms *et al.*, 1967; Davies and Tinoco, 1968) and only uridylates at low ionic strength may exist in unstacked conformation (Simpkins and Richards, 1967a,b).

Since the trinucleotides already bear some characteristics of the simplest polynucleotide chain, it was of interest to extend our studies to these compounds, using circular dichroism as a method of conformational investigation. In principle, one could expect that the structural features of a polynucleotide chain could already be detected in simple trinucleotides. These include features such as the base pairing and the intermolecular associations in double or multiple helices, and the base stacking in single-stranded helices or formation of loops. The stacking interaction leading to single-stranded helical structures has been previously observed in various trinucleotides (Cantor and Tinoco, 1965; Zavil'gel'skii and Li, 1967; Inoue *et al.*, 1967). It was shown that in single-stranded ordered structures the optical rotary dispersion mainly depends upon the influence of the nearest residue and can be expressed by a simple semiempirical expression (Cantor and Tinoco, 1965, 1967). The present work was done in an attempt to establish the role of factors contributing to the conformational stability of a polynucleotide helical chain using the temperature dependence of circular dichroism. We have been concerned with the characterization of both intermolecular and intramolecular interactions in trinucleotides. The comparison of optical and thermodynamic properties shows that at high ionic strength intermolecular associations are detectable in some simple trinucleotides when guanine residues are present. This was not observed with other trinucleotides. At low ionic strength and in the absence of complementary bases, guanine residues promote unstacking. This tendency to unstack seems to be particularly pronounced whenever nonpaired guanine residues are next to uridine.

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