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Reaction of Nitrosoguanidine (N-Methyl-N′-Nitro-N-Nitrosoguanidine) with Tobacco Mosaic Virus and Its RNA

Abstract. *"Nitrosoguanidine"* (N-*methyl*-N′-*nitro*-N-*nitrosoguanidine) *acts on polynucleotides in neutral aqueous solution, methylating guanine in the 7-position, and, to a much lesser extent, adenine. In 67 percent dimethylformamide the effect on the purines decreased, and what seems to be 3-methyl-cytosine appeared. In formamide solution no base changes were detected. Nitrosoguanidine had little mutagenic effect on tobacco mosaic virus RNA in water; it had greater effect in dimethylformamide, and even greater in formamide; it was a strong mutagen only when it acted on intact tobacco mosaic virus particles. Thus, neither the methylation of guanine nor the cytosine modification represents the main mutagenic event.*

N-Methyl-*N′*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) reacts with the guanine residues of polynucleotides occurring in base-stacked array, but not with guanine-containing compounds of small molecular weight (*1*). It was only slightly mutagenic for tobacco mosaic virus RNA (TMV-RNA) under conditions that favored this guanine modification, but it was highly mutagenic under conditions that did not favor the guanine reaction. We now report (i) that the main reaction product is 7-methylguanine, and that adenine is to a lesser extent methylated; (ii) that this reaction proceeds in two steps: the binding of the reagent (possibly between guanine residues), favored by low temperatures; and the transfer of the methyl group, occurring only at elevated temperatures; (iii) that cytosine residues are methylated under conditions which favor mutagenesis and do not favor the G reaction; and (iv) that the most mutagenic action on TMV-RNA occurs under conditions causing minimum base changes even after prolonged reaction periods.

(i) When TMV-RNA was treated with nitrosoguanidine labeled with C^{14} in the methyl group, and the 5′-nucleotides obtained by treatment with snake venom diesterase (SV-DE) were chromatographed on paper, the typical spot showing white fluorescence (W) (*1*) was found to be radioactive (117,000 count/min). Several minor radioactive products were present in amounts too low for detection and characterization by ultraviolet spectrophotometry. Judged by the amounts of C^{14}, these comprised from 4 to 15 percent of the 7-methylguanylic acid fraction. As judged by its location, the strongest was a decomposition product of the reagent. The previously described fluorescent derivative of adenylic acid (*1*), possibly 7-methyladenylic acid, showed 4 percent of the radioactivity of 7-methylguanylic acid. Other products were probably 1,7-dimethylguanylic acid and 7-methylguanosine. A similar assortment of minor reaction products was detected spectrophotometrically when synthetic guanine-containing polynucleotides were treated with nitrosoguanidine and then were degraded with enzyme and chromatographed.

In contrast, when guanidine C^{14}-labeled nitrosoguanidine was used, the fluorescent guanine reaction product (W) was unlabeled, and no other radioactive spots were identified on the chromatogram. Thus, the only carbon that was added to the RNA in the course of the reaction was the methyl carbon.

The ultraviolet absorption maximum (277 nm), as well as the fluorescence excitation and emission maxima (292 to 298 nm, and 380 to 385 nm, respectively, the higher values being the result of slight contaminations with cytidylic acid) of the fluorescent product W corresponded to those of 7-methylguanylic acid. In turn, the spectrum of the product of acid hydrolysis of RNA treated with nitrosoguanidine or of W corresponded to those of 7-methylguanine. When 7-methylguanylic acid was used as marker in the chromatographic isolation of W, the two moved equally in both dimensions. When 7-methylguanine was compared chromatographically to the product of acid hydrolysis of W these also behaved alike (Table 1).

That 7-methylguanylic acid is a fluorescent component of an RNA has not been noted until quite recently (*2*), probably because analyses were usually preceded by acid hydrolysis, which yields the no-longer fluorescent 7-methylguanine.

7-Methylguanylic acid is surprisingly resistant to *Escherichia coli* phosphatase. Thus, treatment of SV-DE digests with amounts of the phosphatase that completely dephosphorylated the four typical nucleotides left much of the methylated nucleotide intact. However, with much more enzyme, most of the material was transformed to the still-fluorescent methylnucleoside ("Y"). Because 7-methylguanylic acid is more strongly basic than cytidylic acid it can be separated from all nucleotides by electrophoresis. Although the predominant action of nitrosoguanidine on RNA has now been identified as a methylation, this reagent differs from typical alkylating agents (such as dimethyl sulfate) in its conformational

Table 1. Chromatography of guanine nucleotides, nucleosides, and bases. Solvent I consisted of 75 ml of ethanol and 30 ml of 1*M* ammonium acetate *p*H 7.5. Solvent II was 80 ml of saturated ammonium sulfate, 18 ml water, and 2 ml of isopropanol. Solvent III was 68 ml of isopropanol, and 17.6 ml of concentrated HCl and 14.4 ml of water. Descending chromatography Whatman No. 3 MM paper was used for I and II, and Whatman No. 1 for III.

Compound	Ratio of movement to guanylic acid and guanine, respectively		
	Solvent I	Solvent II	Solvent III
"W"	1.2	1.3	
7-Methylguanylic acid	1.2	1.3	
1-Methylguanylic acid		0.55	
"Y" (nucleoside derived from "W")	2.6	1.3	
7-Methylguanosine	2.6	1.3	
1-Methylguanosine		0.5	
Guanosine	2.6	0.7	
Guanylic acid	1.0	1.0	
"W" hydrolyzed in 1*N* HCl 1 hour 100°C			1.33
7-Methylguanine			1.35
Adenine			1.16
Guanine			1.00

Table 2. Inactivation of tobacco mosaic virus RNA (TMV-RNA) with nitrosoguanidine at various temperatures. One milligram of TMV-RNA in 1 ml of water was treated with 3 mg of nitrosoguanidine. Portions (10 μl) were removed at various intervals and reconstituted with TMV protein. Infectivity was compared with unreacted RNA reconstituted in the same manner.

Temperature (°C)	Infectivity of control (%)		
	15 sec	10 min	30 min
0	89	13*	0.3
10	77	1.5	0
20	67	0.25	0
37	81	25	6†

* At this time, a portion was incubated at 37°C for 30 minutes and the remaining infectivity was then 6 percent of the control. When a portion was incubated at 20°C for 10 minutes, 0.1 percent of the infectivity remained. † At this time, a portion was incubated at 20°C for 10 minutes, and there remained no detectable infectivity.

requirements and in the nature and extent of methylation of adenine.

(ii) The initial rate of inactivation of TMV-RNA is increased at temperatures from 0° to 20°C, but it is greatly decreased at 37°C (Table 2). This effect of temperature can be demonstrated by shifting temperatures. The inactivation rate of the temperature-shifted sample then follows the kinetics characteristic of that temperature. As the reaction periods are lengthened, the extent of inactivation at 37°C becomes more marked. The guanine modification observed after much longer reaction periods is more marked at 37°C than at 23° or 6°C, but it does not significantly increase as temperatures are further raised. The extent of guanine methylation increases for several days at 37°C.

(iii) When TMV-RNA was treated with nitrosoguanidine in 67 percent dimethylformamide for several days at 37°C, somewhat less 7-methylguanylic acid was formed than in water; however, a new product was detected in acid hydrolyzates which, upon chromatography in the isopropanol-HCl system, moved between cytidylic and uridylic acids. This product was also formed under the same conditions from other types of RNA, as well as from polycytidylic acid. It was not detected when the reaction was performed in water or in 67 or 95 percent formamide at various temperatures. Several of the analytical data discussed in the previous two paragraphs are illustrated in Table 3 with soluble RNA (sRNA) which, because of its higher content in G and C, shows greater differences than TMV-RNA.

That the cytosine product, as isolated from TMV-RNA, was derived from cytidylic acid was further supported by separate experiments with TMV-RNA doubly labeled with C^{14} and P^{32}. The ratio of the two labels varies characteristically and consistently from one nucleotide to the other, and in a typical experiment the cytosine product showed a ratio of 0.25, similar to that of cytidylic (0.26), and quite different from that of guanylic acid (0.42), which in turn was characteristically given by the 7-methylguanylic acid (0.41) obtained from the same nitrosoguanidine-treated RNA sample. Adenylic and uridylic acids had ratios of 0.59 and 0.48, respectively.

Table 3. Base ratios of sRNA reacted with nitrosoguanidine (3 mg per milligram of RNA per milliliter) for 3 to 4 days in various solvents. The amount of the cytosine product was calculated with the extinction coefficient assumed to be the same as cytosine. (A, adenine; G, guanine; U, uracil; C, cytosine.)

Method of hydrolysis	Base (ratio)*					
	A	G	U	C	7-Methyl G†	3-Methyl C (6)
			Water 37°C			
HCl	1.0	1.04	0.79	1.31	0.70	
SV-DE	1.0	1.15	0.83	1.33	0.65	
			Water + TMV protein 37°C			
HCl	1.0	1.34	0.81	1.41	0.40	
			Water 20° to 25°C			
HCl	1.0	1.33	0.75	1.36	0.18	
SV-DE	1.0	1.13	0.90	1.41	0.41	
			67 percent dimethylformamide 37°C			
HCl	1.0	1.52	0.80	1.19		0.18
SV-DE	1.0	1.44	0.83	1.27	0.29	
			Formamide 37°C			
HCl	1.0	1.63	0.70	1.34		
SV-DE	1.0	1.72	0.87	1.36	Trace	
			Untreated			
HCl	1.0	1.65	0.80	1.30		

*After acid hydrolysis A, G, and 7-methyl G represent the purines, and U and C represent the 3′-nucleotides; after snake venom digestion all are the 5′-nucleotides. † The customary solvent used to separate the products of HCl hydrolysis (68 ml of isopropanol + 16 ml of concentrated HCl + 16 ml of water) did not give as good separation of 7-methylguanine, guanine, and adenine as the composition used in Table 1.

Table 4. Mutagenicity of nitrosoguanidine treatment of tobacco mosaic virus RNA under various conditions. Mutagenicity is defined as the ratio of necrotic lesions on *Nicotiana sylvestris* produced by the experimental sample, divided by those of an untreated control when tested at the same level of infectivity, as determined on *N. tabaccum*, var. *Xanthi nc* (residual infectivities of samples tested 1 to 50 percent, that is, five to one lethal events). Each sample was tested two to six times on nine leaves of *N. sylvestris*, representing three plants.

Reaction medium	Samples tested (No.)	Average mutagenicity
Water	6	2
67 percent dimethylformamide	10	4
Formamide	6	7
Reacted in the virus (in water)		
Tested as virus	6	22
Tested as RNA	6	24

To ascertain the reactivity of the RNA in the virus particle, doubly labeled TMV was treated with nitrosoguanidine, and its RNA was then isolated and analyzed with added unlabeled nitrosoguanidine-treated RNA as carrier, to ascertain the presence of 7-methylguanylic acid and of the cytosine derivative in the treated virus. The radioactivity found in the 7-methylguanylic acid area (about 2 percent of that in guanylic acid) appeared not free from contaminants, since the ratio of C^{14} to P^{32} was not that of the guanylic acid. The radioactivity found in the cytosine product area (also about 2 percent of that of cytidylic acid) was shown by rechromatography to behave like the reaction product obtained from polycytidylic acid upon nitrosoguanidine treatment in 67 percent dimethylformamide. This experiment thus supported the concept that the guanine reaction, but not the cytosine reaction, was suppressed in the virus particle. When sRNA was treated with nitrosoguanidine in the presence of added TMV protein, it behaved typically, an indication that the unusual reactivity of the RNA in the virus is not due to the presence of protein, but to the structure of the virus particles (Table 3).

(iv) Treatment of TMV-RNA with nitrosoguanidine causes little mutagenesis (*1*). This is of the order caused by dimethyl sulfate (*3*) and may in both cases be attributed to the formation of 7-methylguanine, of 1- or 3-methyladenine, or to other unknown side reactions. That the RNA in the virus particle is highly susceptible to nitrosoguanidine mutagenesis when the guanine reaction is inhibited and a cytosine modification reaction could be detected

would suggest that cytosine modification represents the mutagenic event. However, when TMV-RNA was treated with nitrosoguanidine in the presence of dispersing solvents, the cytosine reaction was observed only in dimethylformamide, while the greatest numbers of mutants was obtained in formamide where, even after prolonged reaction periods, no base changes were detected (Tables 3 and 4). Thus, the high mutagenic action of nitrosoguanidine appears not to be attributable to either the observed guanine methylation or to the recently identified cytosine 3-methylation. The possibility that the mutagenesis of nitroguanidine acting on intact TMV is due to deamination has not yet been investigated. The nature of the amino acid replacements observed in the coat proteins of nitrosoguanidine mutants of TMV is not in conflict with such a mechanism. The chemical nature and biological significance of the observed adenine modification also require further study.

After this paper had been prepared for publication, we learned that Chandra *et al.* (*4*) had studied the template activity of nitrosoguanidine-treated polynucleotides and the transfer activity of treated sRNA, and had offered tentative conclusions that agree with our data. Other data on the action of nitrosoguanidine on DNA (*5*) gives evidence of the formation of small amounts (no quantitative data given) of 7-methylguanine.

B. SINGER
H. FRAENKEL-CONRAT
Department of Molecular Biology and Virus Laboratory, and *Space Sciences Laboratory, University of California, Berkeley 94720*

J. GREENBERG
Palo Alto Medical Research Foundation, Palo Alto, California

A. M. MICHELSON
Institut de Biologie Physico-Chimique, Paris, France

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Motor Effects of Copper in the Caudate Nucleus: Reversible Lesions with Ion-Exchange Resin Beads

Abstract. *A method employing the use of ion-exchange resin beads is described for the punctate introduction of discrete amounts of various anions, cations, or zwitterions into given brain regions. A series of experiments utilizing the method to introduce ionic copper into the caudate nucleus with the resulting motor manifestations are discussed.*

The concentrations of ionic copper in the basal ganglia, substantia nigra, and nucleus ruber are, in general, higher than those in other areas of the brain (*1*). For this reason, there is considerable interest in the function of this metal in the extrapyramidal system (*2*). Ionic copper has particular relevance to the neuropathology of Wilson's disease, in which abnormally high concentrations of Cu^{2+} are found in the lenticular nucleus, and experimental methods have been developed to systematically alter the copper metabolism of various regions of the nervous system. For example, intraperitoneal injection of various concentrations of a copper sulfate solution resulted in an increase in the Cu^{2+} concentrations in the caudate and lenticular nuclei of guinea pigs. Highest uptake was observed in the caudate nucleus (*3*). No motor involvement has been reported, however. In cats, intraventricular injection of a complex of copper and albumin produces marked motor manifestations ranging from clonic and tonic seizures to rigidity and quadriplegia (*4*). Changes in copper metabolism in the nervous system have also been attempted through modification of the diet (*5*). Pregnant ewes maintained on a diet supplemented with ammonium molybdate bore lambs with ataxia or incoordination of the hind limbs, although no deficit was observed in the ewes. Direct and discrete application of copper complexes to substantia nigra, nucleus ruber, or targets in the basal ganglia has not been attempted.

The disadvantages of the techniques used in the earlier studies are several. Intraperitoneal introduction of chemical agents does not permit study of their metabolism in discrete areas, since the compounds can diffusely affect a number of neural and nonneural structures not under experimental control. The special problem of passage across the blood-brain barrier is also encountered. Intraventricular injection is slightly more specific, the agents introduced affecting only those structures which border the ventricles. Heavy-metal salts directly applied to particular targets can be localized, but the presence of both the anions and the cations makes difficult the assessment of their relative or interactive effects if both are biochemically active. Introduction of compounds in solution for the purpose of chemical localization in brain has a special disadvantage. Even if punctate application of the solution is successful, diffusion as a consequence of capillary action around the guide cannula is not uncommon, not only making the localization of effect difficult, but making more uncertain the amount of chemical applied at a given site.

Punctate application of a given ion-exchange resin bead loaded with the particular anion, cation, or zwitterion for which it is selective offers some advantages for the study of certain aspects of nervous system metabolism. Accurate amounts of the charged particle can be stereotaxically deposited through cannulas surgically placed at the desired location in brain. The resin portion of the complex is inert and will not enter into biochemical reaction. The ion-resin complex is solid, and it can be accurately placed without loss by capillary action of effective material from the site of application. As a solid, it is also relatively easy to handle. Of course, the disadvantage of tissue displacement is common to all methods.

Our interest in an effective method for the introduction of ionic copper into the brain originates from studies on the effects of caudate lesions on a variety of behaviors (*6*). In these earlier studies, bilateral caudate ablations in cats effected a loss of the ability to inhibit a previously learned instrumental feeding response. Others have also found that ablations and electrical and chemical stimulation of the caudate result in distinct behaviors similar to those we observed. Ablation of the caudate brings about ipsilateral turning if the lesion is unilateral and obstinate progression if the lesion is bilateral (*6–8*). However, electrical stimulation of the caudate evokes contralateral turning (*8*), as does application of acetylcholine or diisopropyl fluorophosphate (*9, 10*) whereas injection of alumina cream into