DNA Methylation by *N*-Methyl-*N*-nitrosourea, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-Nitroso(1-acetoxyethyl)methylamine, and Diazomethane: Mechanism for the Formation of *N*7-Methylguanine in Sequence-Characterized 5'-^{32}P-End-Labeled DNA

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Abstract: The formation of *N*7-methylguanine (*N*7-MeG) in a sequence-characterized 5'-^{32}P-end-labeled DNA restriction fragment by a series of methylating agents, *N*-methyl-*N*-nitrosourea (MNU), CH_2N_2, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and *N*-nitroso(1-acetoxyethyl)methylamine (NAEMA), is described. These compounds show the same qualitative and quantitative *N*7-MeG pattern, which is consistent with the formation of a common DNA-methylating intermediate, $[CH_3N_2^+]$. A 6-8-fold variation between methylation intensities at different guanines in the DNA fragment suggests the possibility of substantial sequence-dependent changes in nucleophilicity. Salt inhibits the formation of *N*7-MeG by a mechanism that is independent of sequence and the nucleophilicity of the anion but is highly dependent on the DNA affinity binding properties of the cation.

Analysis of the H-ras gene in numerous *N*-methyl-*N*-nitrosourea- (MNU) induced rat mammary tumors has demonstrated the occurrence of a selective point mutation of the 3'-guanine in the 12th codon (G*G*A to G*A*A).[1] The result of this alteration yields a mutated protein product (P-21) that has in vitro[2] and in vivo[3] transformation-inducing properties. It is of interest that neither the other guanine residue in this codon nor the two guanines in the adjacent 13th codon (GGC) are mutated, even though a base transition at these sites would also afford an activated oncogene.[2] It is generally accepted that the alkylation of DNA by MNU at physiological pH involves its base-catalyzed hydrolysis[4] to the corresponding (*E*)-methanediazotic acid, which then ionizes to $CH_3N_2^+$, the "ultimate" electrophilic intermediate responsible for DNA methylation.[5] Recently, an alternative mechanism that does not involve $CH_3N_2^+$ has been proposed to explain the site-specific mutation of the H-ras gene in MNU-induced mammary tumors.[6] It was suggested that the initial step is a H^+ abstraction to yield an *N*-alkyl-*N*-nitrosoimidourea, which reacts at the O^6-position of guanine to form a tetrahedral intermediate. This intermediate subsequently undergoes a concerted intrastrand transfer of the *N*-alkyl group to the N7- and O^6-positions on the adjacent 3'-guanine residue, leaving an O^6-carbamoyl residue on the initially attacked guanine.

To probe further the nature and sequence specificity of the reactive intermediate responsible for DNA alkylation, a 5'-^{32}P-end-labeled sequence-characterized DNA restriction fragment has been reacted in aqueous buffer with a series of methylating agents: MNU, CH_2N_2, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and *N*-nitroso(1-acetoxyethyl)methylamine (NAEMA). By use of piperidine-induced strand cleavage and high-resolution polyacrylamide gel electrophoresis, the sequence specificity for the formation of *N*7-MeG was determined.

Experimental Section

Materials. MNU and MNNG were obtained from Aldrich Chemical Co. and the former was crystallized from CH_3OH. NAEMA was prepared by treating *N*-ethylidenemethylamine with $NaNO_2$ in glacial HOAc saturated with KOAc.[7] Extractive workup afforded product in 54% yield: TLC (hexane–diethyl ether, 1:1) R_f 0.41; UV (CH_2Cl_2) 363 nm (ϵ 70); ^{1}H NMR ([$^2H_6$]DMSO) δ 1.75 (d, 3, $CHCH_3$), 2.07 (s, 3, $COCH_3$), 2.95 (s, 3, NCH_3), 7.09 (q, 1, $CHCH_3$); IR ($CHCl_3$) 3017 cm^{-1}, 1745 (s), 1469 (m). Ethereal solutions of CH_2N_2, prepared by the decomposition of MNNG in strong base, were distilled, and the CH_2N_2 concentration was determined by the conversion of $PhCO_2H$ to $PhCO_2CH_3$, which was quantitated by GLC.

To determine the methylating activity of MNU, MNNG, and NAEMA, they were incubated at 1 mM concentration for 2 h at 37 °C in 10 mM Tris-HCl (pH 8.0). The solvolysis product, CH_3OH, was quantitated by direct analysis of the aqueous reaction mixture on GLC (column, Carbopak B, 4% Carbowax 20M, 0.8% KOH; temperature, 55 °C; detection, flame). The methylating activities of MNU and MNNG were also determined by their base-catalyzed conversion to CH_2N_2.

Preparation of 5'-^{32}P-End-Labeled DNA Restriction Fragment. By use of established methods[8] a 5'-^{32}P-end-labeled 576 base pair (bp) fragment was isolated from a 3220-bp clone from the promotor region of the coat protein gene of the canine parvovirus[9] by sequential restriction with *Nco*I endonuclease, removal of the 5'-phosphate terminus with alkaline phosphatase, rephosphorylation with [γ-^{32}P]ATP in the presence of T4 kinase, restriction with *Hin*dIII endonuclease, and purification by electrophoresis on a 5% polyacrylamide gel.

Methylation at N7-G. MNU, MNNG, or NAEMA was incubated for 2 h at 37 °C with the 5'-^{32}P-end-labeled 576-bp restriction fragment (80 000–100 000 cpm) in 10 mM Tris-HCl buffer (pH 8.0) containing the desired concentrations of salt. The reactions were terminated by precipitation of the DNA with NaOAc followed by repeated washing with cold EtOH. The dried DNA was then treated with 1 M piperidine for 20 min at 90 °C to convert the *N*7-MeG lesions into strand breaks.[8,10] The piperidine was then removed in vacuo. The biphasic reaction of distilled ethereal CH_2N_2 and DNA in aqueous buffer was stirred for 1 h at 0 °C. The ether was removed and the DNA worked up as described above.

(1) For review see: Barbacid, M. *Annu. Rev. Biochem.* **1987**, *56*, 779–827.
(2) (a) Stacey, D. W.; Kung, H. F. *Nature* **1984**, *310*, 508–511. (b) Feramisco, J. R.; Gross, M.; Kamata, T.; Rosenberg, M.; Sweet, R. W. *Cell* **1984**, *38*, 109–117. (c) Hyland, J. K.; Rogers, C. M.; Scolnick, E. M.; Stein, R. B.; Ellis, R.; Baserga, R. *Virology* **1985**, *141*, 333–336.
(3) Quaife, C. J.; Pinkert, C. A.; Ornitz, D. M.; Palmiter, R. D.; Brinster, R. L. *Cell* **1987**, *48*, 1023–1034.
(4) (a) Garrett, E. R.; Goto, S.; Stubbins, J. F. *J. Pharm. Sci.* **1965**, *54*, 119–123. (b) Snyder, J. K.; Stock, L. M. *J. Org. Chem.* **1980**, *45*, 1990–1999.
(5) (a) Kriek, E.; Emmelot, P. *Biochim. Biophys. Acta* **1964**, *91*, 59–66. (b) O'Connor, P. J.; Capps, M. J.; Craig, A. W.; Lawley, P. D.; Shah, S. A. *Biochem. J.* **1972**, *129*, 519–528. (c) Magee, P. N.; Nicoll, J. W.; Pegg, A. E.; Swann, P. F. *Biochem. Soc. Trans.* **1975**, 62–65. (d) White, E. H.; Woodcock, D. J. In *The Chemistry of the Amino Group*; Patai, S., Ed.; Wiley-Interscience: New York, 1968; pp 409–497. (e) Gold, B.; Linder, W. B. *J. Am. Chem. Soc.* **1979**, *101*, 6772–6773. (f) Smith, R. H., Jr.; Koepke, S. R.; Tondeur, Y.; Denlinger, C. L.; Michejda, C. J. *J. Chem. Soc., Chem. Commun.* **1985**, 936–937. (g) Wurdeman, R. L.; Gold, B. *Chem. Res. Toxicol.* **1988**, *1*, 146–147.
(6) (a) Buckley, N. *J. Am. Chem. Soc.* **1987**, *109*, 7918–7920. (b) Buckley, N.; Brent, T. P. *J. Am. Chem. Soc.* **1988**, *110*, 7520–7529.
(7) Roller, P. P.; Shimp, D. R.; Keefer, L. K. *Tetrahedron Lett.* **1975**, *25*, 2065–2068.
(8) Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499–560.
(9) Rhode, S. L., III *J. Virol.* **1985**, *54*, 630–633.
(10) Mattes, W. B.; Hartley, J. A.; Kohn, K. W. *Biochim. Biophys. Acta* **1986**, *868*, 71–76.

0002-7863/89/1511-6408$01.50/0

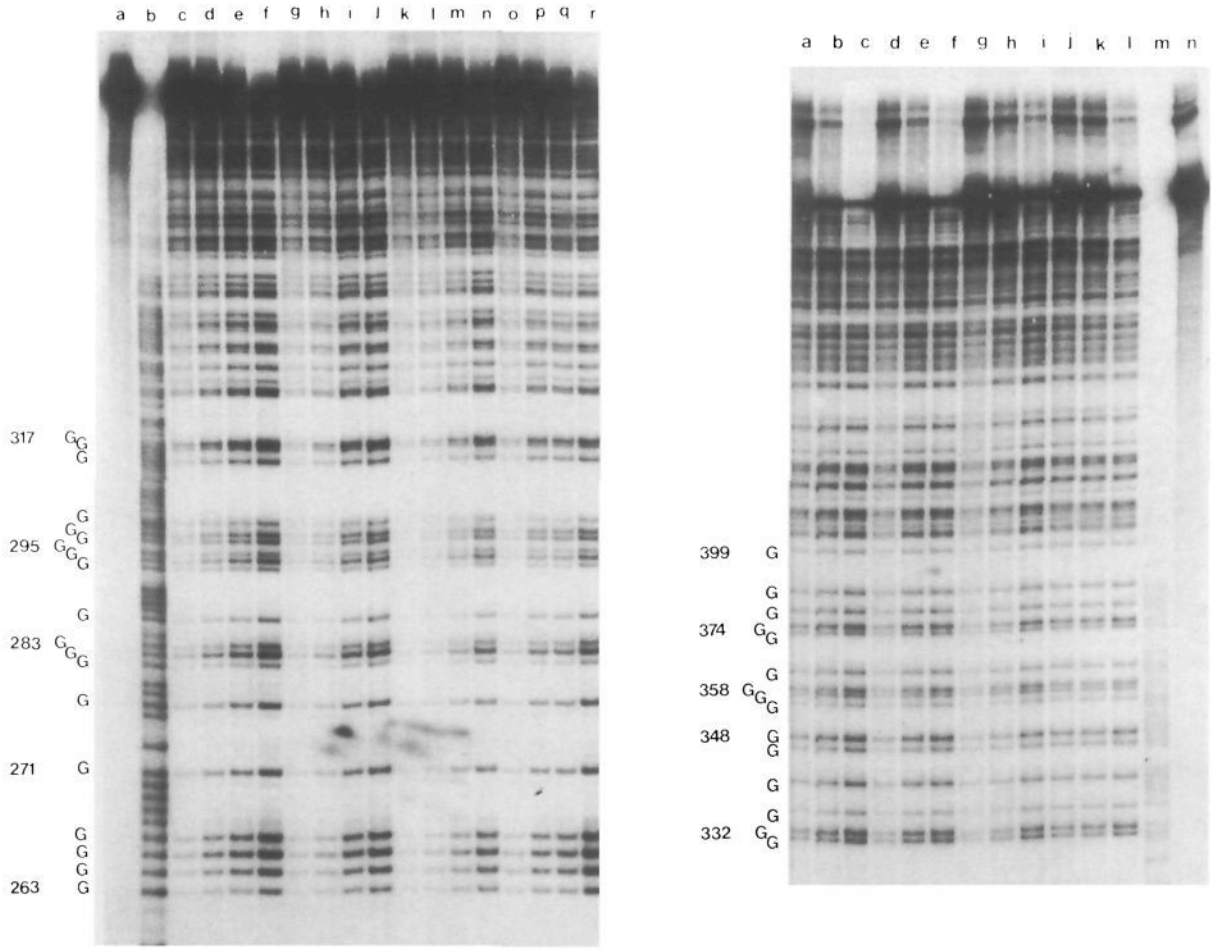

Figure 1. (a) Methylation of DNA at N7-G position: (lane a) control; (lane b) Maxam–Gilbert G+A; (lanes c–f) 50, 100, 250, and 500 μM MNU; (lanes g–j) 50, 100, 250, and 500 μM NAEMA; (lanes k–n) 50, 100, 250, and 500 μM MNNG; (lanes o–r) 15.5, 31.1, 77.6, and 155.2 μL of an ethereal solution of CH_2N_2 (483 μM) incubated with an aqueous solution (30 μL) of DNA fragment. Approximately the first 100 bp from the 5′-terminus (bp 239) of the fragment are resolved in the autoradiogram. (b) (Lanes a–c) 100, 250, and 500 μM MNU; (lanes d–f) 100, 250, and 500 μM NAEMA; (lanes g–i) 100, 250, and 500 μM MNNG; (lanes j–l) 31.1, 77.6, and 155.2 μL of an ethereal solution of CH_2N_2 (483 μM) incubated with an aqueous solution (30 μL) of DNA fragment; (lane m) Maxam–Gilbert G+A; (lane n) control. Bases 331–420 of the fragment are resolved in the autoradiogram. (c) Sequence of the 576-bp restriction fragment.

Sequencing Gels. The DNA was suspended in loading buffer (containing 80% deionized formamide, 50 mM Tris–borate, pH 8.3, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue), denatured at 90 °C for 1 min, and cooled in ice. The DNA was placed into wells on top of a 12% polyacrylamide (7.8 M urea) denaturing gel, which was run at 65 W. The standard Maxam–Gilbert G and/or G+A reaction lanes[8] were included as sequence markers. The gel was then exposed to Kodak X-OMAT AR film at −70 °C and the resulting autoradiogram analyzed on a Shimadzu CS-9000 scanning densitometer.

Results

From the intensities of the bands (Figure 1), it appears that MNU, NAEMA, and MNNG do not yield *N*7-MeG with equal efficiency. To determine the origin of this phenomenon, the methylating activities of the three compounds were determined by the quantitation of the CH_3OH that is formed from their hydrolysis with the same buffer, temperature, and reaction time employed in the DNA-methylation studies. The results of this study show that the relative yields of CH_3OH from MNU, MNNG, and NAEMA are 1.00, 0.42, and 0.88, respectively. The absolute yield of CH_3OH from MNU was 91%. Furthermore, the samples of MNU and MNNG were analyzed by their conversion into CH_2N_2, which was quantitated by its reaction with benzoic acid. On the basis of this method, MNNG yielded 45% of the methyl benzoate generated from MNU. MNU and MNNG have $t_{1/2}$ of 5 and 150 min, respectively, in Tris buffer (pH 8.0) at 37 °C. Since the methylating agents were incubated with the DNA for 2 h, only 75% of the MNNG will have hydrolyzed. Correction of the diazomethane generated for the 75% hydrolysis predicts that the theoretical yield of *N*7-MeG from MNNG relative to that of MNU should be 35%. These determined methylating activities agree reasonably well with the gel data (Figure 1), which show band intensities for MNNG that range between 31 and 39% of that for MNU. The band intensity of MNU relative to that of NAEMA is 1:0.8. Thus, it is evident that the quantitative difference (Figure 1) between the three nitroso compounds reflects the purity (methylating activity) and

the hydrolytic stability of the compounds and is not related to specific differences in their reactions with DNA.

The CH_2N_2 used in the DNA methylation experiments was prepared by the base-catalyzed hydrolysis of MNNG in the presence of diethyl ether and distillation of the ethereal layer. The CH_2N_2 concentration was determined by reaction of an aliquot with a solution of $PhCO_2H$ and quantitation of the $PhCO_2CH_3$ by GLC. The reaction of CH_2N_2 with DNA was performed in a stirred two-phase system (ether–aqueous buffer), and therefore, it is not possible to know the concentration of the methylating agent in the aqueous phase. Despite this shortcoming, which prevents absolute quantitative comparison, it is still evident that the methylation pattern for CH_2N_2 is identical with those of the other three nitroso compounds.

DNA Methylation. The *N*7-MeG profiles generated by the reaction of the different methylating agents with the [5′-^{32}P]DNA restriction fragment are qualitatively identical (Figures 1 and 2). There is an obvious concentration-dependent enhancement in DNA cleavage without any concomitant change in the relative intensities of the bands. It is important to note that most of the radioactivity is associated with the unmodified DNA that remains near the top of the lanes. This reflects a low level of DNA modification (<1 methylation/DNA duplex) and ensures that a potentially efficient methylation pathway is not masked by less competitive processes. Although the G sites in the fragment are not modified with a high sequence selectivity, there are discernible patterns in the dG_{3-4} stretches. For example, the relative intensities are as follows: G_{281}–G_{283}, 1.0:6.4:3.6; G_{293}–G_{295}, 1.0:7.9:3.6; G_{263}–G_{266}, 1.0:1.8:2.8:2.2.

In addition to the effect within the poly(dG) runs, there is a relationship between methylation intensity and the identity of the flanking bases as is observed at $AG_{271}T$ or $AG_{277}A$ relative to $TG_{286}A$. The relative methylation intensities at G*G*G, A*G*T, A*G*A, T*G*A, A*G*C, and T*G*C are 5.0:3.5:2.5:1.7:1.5:1.0. As previously noted for (chloroethyl)nitrosoureas,[11] the base 5′ to the N7-G site plays the dominant role in determining the extent of methylation. In general, the 3′-base has less of an effect on the alkylation, although a 3′-C does seem to consistently decrease the intensity of the cleavage ($AG_{300}C$ and $AG_{355}C$). Although the neighboring bases have a major impact on G methylation, more global factors must also be at play since methylation at $AG_{346}A$ is only 50% of that at $AG_{348}A$. Similarly, in both the G_{281}–G_{283} and G_{263}–G_{266} runs, there is a 5′-T, yet *N*7-MeG production is 2 times higher at G_{263} than at G_{281}.

Salt Effect. It has been previously noted that DNA alkylation by MNU and *N*-ethyl-*N*-nitrosourea (ENU) is markedly inhibited by the addition of inorganic salts[5g,12] and cationic DNA affinity binding molecules.[5g,13] The effect of different salts on DNA methylation shows that the inhibition of *N*7-MeG is not related to DNA sequence but is quantitatively dependent on the cation structure; the doubly charged cation Mg^{2+} is a far more effective inhibitor than either Na^+ or K^+ (Figure 3). The nucleophilicity of the anion appears to have only a very weak effect on the level of inhibition with $I^- > Cl^-$ (Figure 4).

Discussion

The reactions of MNU, MNNG, NAEMA, and CH_2N_2 with a ^{32}P-end-labeled restriction fragment were investigated to determine if these compounds, which yield methylating intermediates by divergent routes, would afford similar sequence-selective methylation patterns. The hydrolysis of MNU ($t_{1/2}$ = ~5 min, pH 8.0, 37 °C) in aqueous solution proceeds by specific-base catalysis and the formation of a tetrahedral intermediate at the carbonyl carbon.[4] Collapse of this intermediate yields methanediazotic acid and a carbamic acid derivative. This reaction is not subject to salt effects.[4b] In contrast, the hydrolysis of MNNG ($t_{1/2}$ = ~150 min, pH 8.0, 37 °C) is subject to general-base catalysis, although the same diazotic acid is formed.[14] In pH 7.0 buffer at 37 °C, the hydrolysis of the secondary α-acetoxy nitrosamine, NAEMA, is approximately 400 times faster than that of the primary α-acetate, *N*-nitroso(acetoxymethyl)-methylamine.[15] The marked rate acceleration and the isolation of *N*-nitrosoimmonium salts are evidence that the hydrolysis of NAEMA proceeds by a $B_{AL}1$ mechanism.[15] Subsequent hydrolysis of the immonium compound, possibly via an α-hydroxy

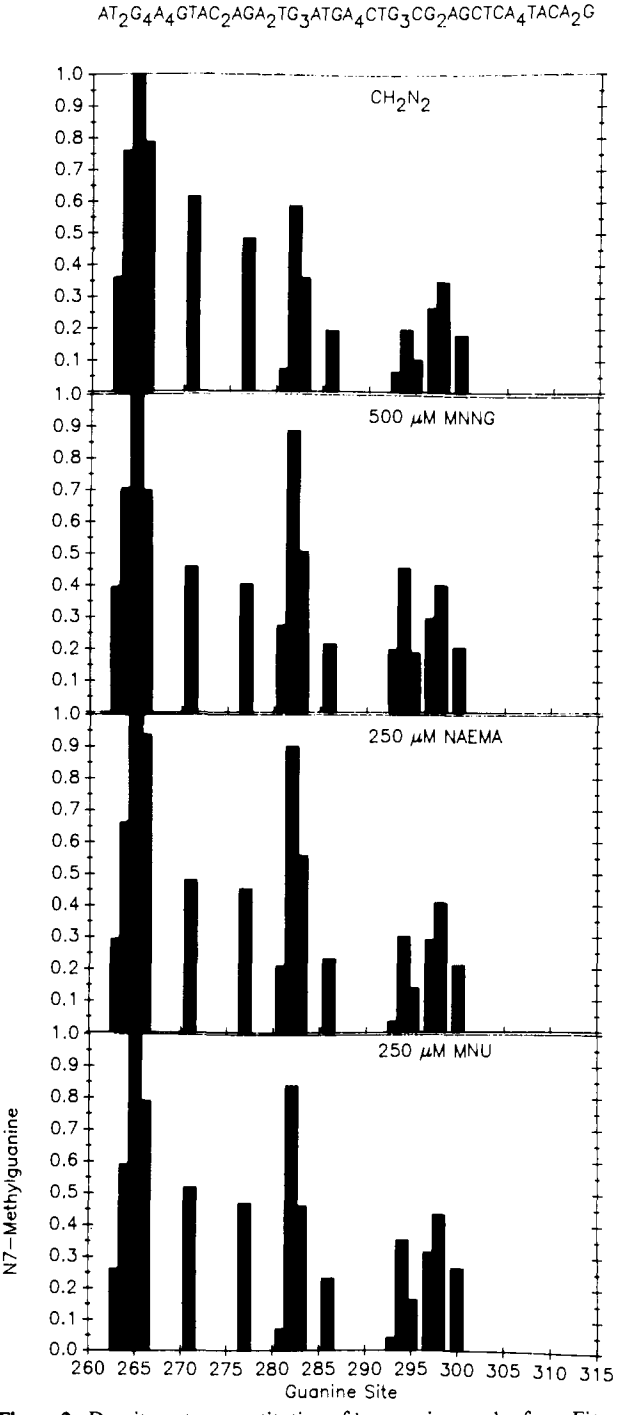

Figure 2. Densitometry quantitation of lanes e, i, n, and q from Figure 1a. Data are normalized for the most intense band (G_{265}) in each lane.

(11) Hartley, J. A.; Gibson, N. W.; Kohn, K. W.; Mattes, W. B. *Cancer Res.* **1986**, *46*, 1943–1947.

(12) (a) Kriek, E.; Emmelot, P. *Biochemistry* **1963**, *2*, 733–740. (b) McCalla, D. R. *Biochim. Biophys. Acta* **1968**, *155*, 114–120. (c) Jensen, D. E.; Reed, D. J. *Biochemistry* **1978**, *17*, 5098–5107. (d) Briscoe, W. T.; Cotter, L.-E. *Chem.-Biol. Interact.* **1985**, *56*, 321–331.

(13) Rajalakshmi, S.; Rao, P. M.; Sarma, D. S. R. *Biochemistry* **1978**, *17*, 4515–4518.

(14) (a) Schultz, U.; McCalla, D. R. *Can. J. Chem.* **1969**, *47*, 2021–2027. (b) Lawley, P. D.; Thatcher, C. J. *Biochem. J.* **1970**, *116*, 693–707. (c) Lawley, P. D.; Shah, S. A. *Biochem. J.* **1972**, *128*, 117–132.

(15) Weissler, M. In *N-Nitrosamines*; Anselme, J.-P., Ed.; ACS Symposium Series 101; American Chemical Society: Washington, DC, 1979; pp 57–75.

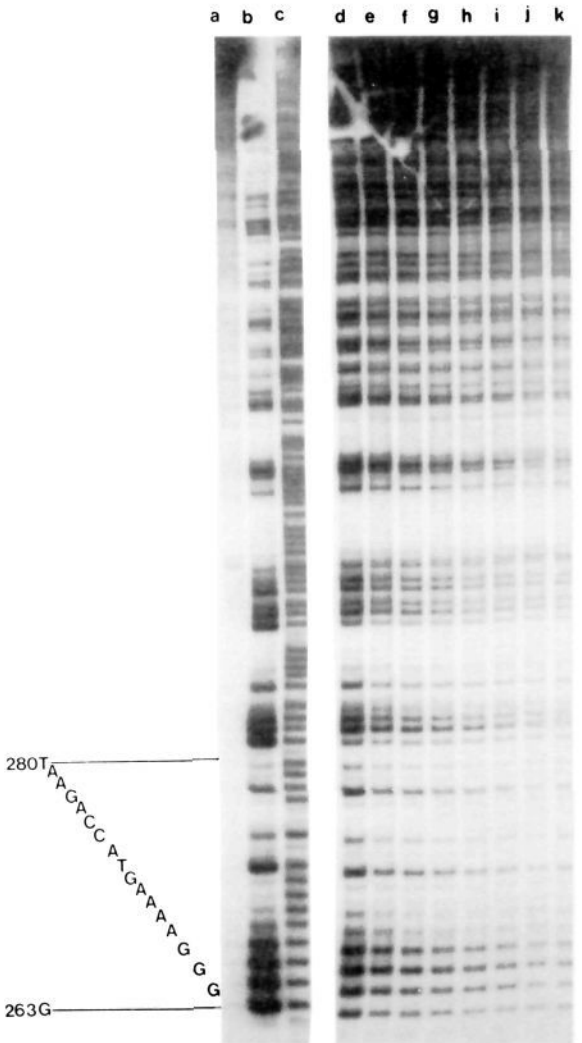

Figure 3. Effect of salt on *N*7-MeG formation: (lane a) control; (lane b) Maxam–Gilbert G lane; (lane c) Maxam–Gilbert G+A lane; (lanes d–k) 500 μM MNU, (lanes e, g, and i) 50, 100, and 200 mM NaCl, respectively; (lanes f, h, and j) 50, 100, and 200 mM NaI, respectively; (lane k) 10 mM $MgCl_2$.

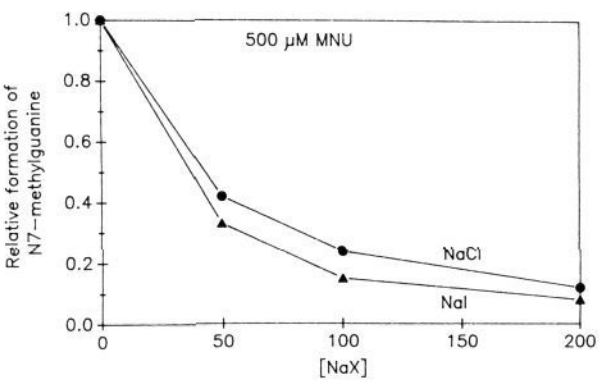

Figure 4. Plot of relative formation of total *N*7-MeG (Figure 3) at 500 μM MNU vs [NaCl] and [NaI].

nitrosamine, affords methanediazotic acid. Regardless of the precursor, it appears that methylation activity results from the formation of methanediazotic acid which is nonisolable and ionizes to $CH_3N_2^+$. The interconversion of CH_2N_2 and $CH_3N_2^+$ in aqueous solution is well documented.[5f,16] It has been shown that the hydrolyses of MNU, trimethyltriazene, ethyl *N*-methyl-*N*-nitrosocarbamate, and *N*-nitroso(acetoxymethyl)methylamine in deuterated phosphate buffer yield the same distribution of CH_3OD, CH_2DOD, CHD_2OD, and CD_3OD solvolysis products via an equilibrium between $CH_3N_2^+$ and CH_2N_2.[5f] Again, the solvolytic chemistry of the methylating agent is independent of its precursor.

Although the role for a tetrahedral intermediate in the hydrolysis of MNU is well established at near neutral pH and is the dominant nucleophilic substitution product producing pathway, a priori it is not possible to rule out an alternative reaction that is specific for the generation of DNA adducts. Recently, it has been proposed that MNU, as well as other *N*-alkylnitrosoureas, alkylates DNA via a reaction that is initiated by the attack of an imidourea on the O^6-position of guanine.[6] This tetrahedral intermediate then can transfer the methyl group regioselectively to the N7- and O^6-positions of a 3′-guanine. The results (Figures 1 and 2) show that this prediction is not observed at the following G_2 sites; $G_{297}G_{298}$, $G_{301}G_{302}$, $G_{331}G_{332}$, and $G_{357}G_{358}$. There is also no preferential methylation of a 3′-G within any of the G_3 or G_4 stretches.

In addition to the sequence selectivity, the inhibition of DNA methylation by inorganic and DNA affinity binding cations provides an important insight into the reaction mechanism. The greater effectiveness of Mg^{2+} relative to the monocations in inhibiting methylation, without a change in the sequence selectivity (Figure 3), is consistent with entropy-driven binding of the cations to polyanionic DNA and the screening of the electrostatic charge of the polymer's nucleophilic sites.[5g,12c] The use of the affinity binding constants for Na^+ and Mg^{2+} of $\sim 2.5 \times 10^1$ and 3.5×10^2 M^{-1}, respectively,[17] would predict the observed similarity in *N*7-MeG formation by MNU in the presence of 200 mM NaCl and 10 mM $MgCl_2$ (Figure 3). Also consistent with this account is the inhibition of methylation at micromolar concentrations of ethidium bromide, spermine, and distamycin A.[5g,13] While these organic cations can intercalate or groove bind to DNA, there is extensive electrostatic association of these compounds with the phosphate backbone at low salt concentrations. It is of significance that neither the inorganic nor organic salts have any effect on the methylation of DNA by the uncharged alkylating agent dimethyl sulfate (DMS).[5g] A similar inhibitory salt effect has been reported for nitrogen mustards that alkylate DNA via a positively charged aziridinium ion.[18]

The contribution of the salt anion to the inhibition of *N*7-MeG formation from MNU is relatively weak considering the overall decrease in DNA cleavage upon the addition of salt (Figure 4). Clearly, the inhibitory salt effect does not result from the scavenging of reactive methylating intermediate by the added nucleophile, since scavenging efficiency would be related to anion nucleophilicity (S_{NMeI} of Cl^- and I^- are 4.37 and 7.42, respectively[19]).

It is improbable that steric effects are responsible for the *N*7-MeG pattern produced by $CH_3N_2^+$ because in the same DNA restriction fragment the 5′-G in the dG_3 and dG_4 stretches are accessible to the bulkier DMS.[20] The methylation patterns observed on the sequencing gels for both $CH_3N_2^+$ and DMS require opening of the imidazole ring of *N*7-MeG[21] and subsequent piperidine-induced strand cleavage.[8,10] Therefore, the chemistry associated with elaboration of the single-strand break also cannot be responsible for the *N*7-MeG pattern induced by $CH_3N_2^+$.

Visual inspection of the sequencing gels may give the impression that the entire region of a poly(dG) stretch is a hot spot for methylation. However, if the methylation at all four guanines within the G_4 run is averaged, the resulting value is only 1.3 times

(16) McGarrity, J. F.; Smyth, T. *J. Am. Chem. Soc.* **1980**, *102*, 7303–7308.

(17) See Zimmerman, H. W. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 115–130.

(18) Kohn, K. W.; Hartley, J. A.; Mattes, W. B. *Nucleic Acids Res.* **1987**, 10531–10549.

(19) Pearson, R. G.; Sobel, H.; Songstad, J. *J. Am. Chem. Soc.* **1968**, *90*, 319–326.

(20) The documented use of DMS to generate the Maxam–Gilbert G lane (ref 8) is extensive. For an example of the sequence selectivity of DMS in the same 576-bp restriction fragment, see ref 5g.

(21) Brookes, P.; Lawley, P. D. *J. Chem. Soc.* **1961**, 3923–3928.

larger than that of the N7-MeG formed at an isolated AGT or AGA site. For the G_3 run the average value of N7-MeG is essentially the same for an isolated base. Therefore, the methylating intermediate is not specifically attracted to the dG_3 or dG_4 runs, but rather, the reaction with DNA is focused within these regions to select guanines. Furthermore, the 6-8-fold difference between the strongest and weakest methylation site, G_{281} vs G_{282} and G_{293} vs G_{294}, indicates a substantial difference in the nucleophilicity of the guanine residues as a function of local sequence, assuming a minimal role for steric effects. This interpretation requires electrophilic selectivity by $CH_3N_2^+$. Previous reports shown k_{LiN_3}/k_{MeOH} ratios of 17.6 and 18.4 for 1-butanediazonium ion and 1-hexanediazonium ion, respectively, in aqueous CH_3OH.[22] These are values close to that found with *t*-BuBr.[22] We have found a k_{NaI}/k_{NaBr} ratio of 4.5 for the reaction of 1-propanediazonium ion in phosphate buffer.[23] On the basis of the calculated dissociation enthalpies of $CH_3N_2^+$ and 1-propanediazonium ion of 38 and 10 kcal/mol, respectively,[24] the former species should be capable of the same selectivity as the higher homologues.

Fluctuations in the nucleophilicity at N7-G sites as a consequence of subtle sequence-dependent conformational changes that alter base stacking could account for the methylation pattern. In this context, the relationship between base-stacking geometries and N7-G nucleophilicity may be related to the sequence-related electrostatic component of the stacking energy.[25] Effective intrastrand base-base electrostatic neutralization,[26] which is dependent on the helix twist angle and base roll, tilt, and propeller twist angles, would be expected to diminish nucleophilicity. The dominant effect of the 5'-base on N7-G methylation is consistent with the proposed influence of base stacking, since CPK models show that the N7-G site in B-DNA sits directly over the C4 or N1 of the 5'-purine or -pyrimidine, respectively. The influence of C on lowering the alkylation intensities at a 5'-G is also consistent with the importance of stacking since the 4-amino protons of C, which protrude into the major groove and bear a positive electrostatic charge, are situated over the N7 site of the 5'-G. The influence of a 3'-C on the alkylation of DNA by nitrogen mustards has been noted.[18] The role of base stacking in DNA and RNA alkylation, as it affects the overall proportion of adducts, has also been previously recognized. The absolute amount of N7-MeG is approximately 2-fold higher in poly(dG)·poly(dC) than in any dG-containing alternating heteropolymer.[12d] The enhanced methylation of guanine and adenine in RNA by MNNG under conditions favoring base stacking has also been reported.[27] Under the same conditions, DMS methylated both 5'-ribonucleotides and polyribonucleotides to a similar extent.[27]

Conclusions

The common sequence selectivity of the different methylating precursors and the salt-induced inhibition of DNA methylation, although restricted to events at N7-G, are not in agreement with the recently proposed regioselective methylation mechanism. They are congruous with the involvement of a common $CH_3N_2^+$ species that is formed external to the double helix. The origin of the sequence-dependent selectivity for certain G sites remains a topic for further investigation.

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(22) Kirmse, W.; Schnurr, O.; Jendralla, H. *Chem. Ber.* **1979**, *112*, 2120-2144.
(23) Church, K.; Gold, B. *Chem. Res. Toxicol.* **1988**, *1*, 269-273.
(24) Ford, G. P. *J. Am. Chem. Soc.* **1986**, *108*, 5104-5108.
(25) Aida, M.; Nagata, C. *Int. J. Quantum Chem.* **1986**, *29*, 1253-1261.
(26) Sarai, A.; Mazur, J.; Nussinov, R.; Jernigan, R. L. *Biochemistry* **1988**, *27*, 8498-8502.
(27) Singer, B.; Fraenkel-Conrat, H. *Biochemistry* **1969**, *8*, 3260-3266.

Carboxylate-Histidine-Zinc Interactions in Protein Structure and Function

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Abstract: The three-dimensional structures of proteins contained in the Brookhaven Protein Data Bank were analyzed for bound metal ions. Well over 150 unique protein structures are available which contain seven different types of bound metal ions. Iron, calcium, and zinc are most commonly observed, and the extended coordination polyhedra of biological zinc are the subject of this study. In particular, histidine residues ligating zinc ions are often found to bridge both the zinc ion and the carboxylate side chain of a nearby aspartate (sometimes glutamate) residue. We refer to the carboxylate-histidine-zinc interaction as *indirect* carboxylate-metal coordination, and we observe this feature in all zinc enzymes of reported three-dimensional structure. Additionally, we also observe a related carbonyl-histidine-zinc interaction in some metalloproteins. We observe some *direct* carboxylate-zinc interactions, and their coordination stereochemistry is exclusively syn with respect to the carboxylate. On the basis of available protein structures and known homologues thereof, more than 30 examples of indirect carboxylate-zinc coordination across bridging histidine can be identified. The carboxylate-histidine-zinc triad may be important in the function of many zinc-containing proteins and enzymes, e.g., by strengthening metal complexation or modulating the nucleophilicity of zinc-bound water. The presence of an uncomplexed carboxylate-histidine couple (a grouping more basic than histidine alone) in a native protein can also signal a regulatory metal binding site. Indeed, the Asp^----His couple of the serine protease active site may comprise a structural, evolutionary link to the Asp^----His of the zinc protease metal coordination polyhedron.

Among the first-row transition metals, zinc is second only to iron in terms of abundance and functional importance in biological systems.[1] From an inorganic perspective, the coordination chemistry of divalent zinc cation (Zn^{2+}, hereafter zinc) might be

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