Studies on Carcinogen Chromatin–DNA Interaction: Inhibition of *N*-Methyl-*N*-nitrosourea-Induced Methylation of Chromatin–DNA by Spermine and Distamycin A†

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ABSTRACT: In order to determine whether carcinogens interact with specific regions of DNA, the influence of agents that modulate DNA structure was studied on the in vitro methylation of rat liver chromatin DNA by *N*-methyl-*N*-nitrosourea (MNU). Spermine and distamycin A were used as the modulating agents because, although spermine interacts with phosphate groups and distamycin A with A-T base pairs of the DNA, they both occupy the minor groove of DNA helix and stabilize its structure. The study revealed that both spermine and distamycin A inhibited the MNU-induced methylation of chromatin DNA at the N^7 and O^6 positions of guanine and the N^3 position of adenine and that the inhibition was progressive with increasing concentrations of the inhibitors. Under conditions where the methylation of chromatin DNA was inhibited by 90%, the methylation of 2-deoxy-5-guanylic acid included as an internal control was unaffected. This ruled out the possibility that the inhibition could be due to a suppression of the generation of active methyl groups from MNU during its degradation. Therefore, these results indicated that spermine and distamycin A exerted their inhibitory effect by altering the structure and/or the net charge of DNA. Based on the available knowledge on the interaction of spermine and distamycin A with DNA, it is postulated that MNU alkylates regions of DNA that are readily denaturable, probably A-T rich regions located in the minor groove at or near the DNA binding sites of these two compounds.

Knowledge about the DNA region with which potent carcinogens interact is fundamental for an understanding of the initiation of neoplastic process at molecular level. With this objective in view, we initiated studies on carcinogen-DNA interaction in vitro using *N*-methyl-*N*-nitrosourea (MNU)[1] and chromatin-DNA. MNU was used as the methylating agent since it can act directly requiring no metabolic activation; in addition, its carcinogenic and mutagenic potential is well documented (Montesano & Bartsch, 1976). Chromatin-DNA was employed as the substrate in the methylation reaction because in eukaryotic cell, DNA exists in association with histones and nonhistone chromosomal proteins in a repeated unit structure termed nucleosome (Axel, 1976; Kornberg, 1977; Felsenfeld, 1978). Protein-DNA association seems to direct the carcinogen-DNA interaction as revealed by our recent studies on the preferential methylation in vivo by dimethylnitrosamine of regions of chromatin-DNA accessible to DNase I (Itzhaki, 1976; Ramanathan, 1976; Rao et al., 1977). In order to further elucidate the nature of the DNA region that interacts with MNU, we studied the effect of chemical probes like spermine and distamycin A which bind to DNA in a selective manner leading to an enhancement in the thermal melting of DNA (Zimmer et al., 1971; Tabor & Tabor, 1976) and a stabilization of its structure (Liquori et al., 1967b; Kolchinskii, 1975; Luck et al., 1977).

The study reveals that MNU induced methylation of DNA was arrested by both spermine and distamycin A. The formation of N^7-methylguanine (N^7-MeG), O^6-methylguanine (O^6-MeG), and N^3-methyladenine (N^3-MeA) was equally affected. Based on the facts we know about the binding of these compounds and the effect they exert on DNA structure, it is proposed that MNU methylates DNA regions which are readily denaturable, probably rich in A-T base pairs, at or close to the binding sites of spermine and/or distamycin A located in the minor groove of DNA double helix.

Materials and Methods

[^{14}C]-*N*-Methyl-*N*-nitrosourea (specific activity, 13.3 mCi/mmol) was purchased from New England Nuclear, Boston, Mass. The radiopurity was checked by high pressure liquid chromatography (HPLC) on a μBondapak C-18 column and 0.05 M ammonium formate (pH 5) as the eluting buffer. Spermine was obtained from Sigma Chemicals, St. Louis, Mo., and distamycin A was purchased from Boehringer, Canada. Nonradioactive *N*-methyl-*N*-nitrosourea (MNU) was from K & K Fine Chemicals, Plainview, N.Y., and purified by recrystallization using ethyl acetate and hexane. Protease K was obtained from Beckman Instruments, Palo Alto, Calif.

Isolation of Chromatin. Hepatic chromatin was prepared from male Fischer rats (120–150 g) by the method of McConaughy & McCarthy (1972), which is a combination of procedures described by Smith et al. (1969) and the glycine extraction procedure of Kongsvik & Messineo (1970). This chromatin has the same spectral characteristics at 260/240, 260/280 nm, protein/DNA ratio and melting behavior found for chromatin prepared by pelleting the nuclei through sucrose, lysing the nuclei, and centrifugation through sucrose (McConaughy & McCarthy, 1972). The procedure primarily consists of (i) homogenization of the nuclei in 0.075 M NaCl, 0.025 M EDTA (pH 8), and 0.1% Triton X-100 using Potter-Elvehjem homogenizer fitted with a Teflon pestle, (ii) centrifugation of the homogenate after an interval of 15 min at 3000 rpm for 10 min in an International Model PR6 refrigerated centrifuge, (iii) washing three times with 0.05 M Tris-HCl (pH 7.5) containing 0.1% Triton X-100 followed by three more times with 0.05 M Tris-HCl (pH 7.5) and finally

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[1] Abbreviations used: MNU, *N*-methyl-*N*-nitrosourea; N^7-MeG, N^7-methylguanine; O^6-MeG, O^6-methylguanine; N^3-MeA, N^3-methyladenine.

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once with 0.01 M glycine (pH 6.0), and (iv) solubilization of chromatin in 0.01 M glycine, 16 mL/g liver, overnight. A clear chromatin solution is obtained by gentle homogenization before use.

Isolation of Nuclease Nondigestible DNA. Rat liver chromatin-DNA (300 μg) was treated with either 5 μg/mL of DNase I in the presence of 1 mM $MgCl_2$ and 5 mM sodium phosphate (pH 6.8), or 1 μg/mL micrococcal nuclease in the presence of 0.1 mM $CaCl_2$ and 1 mM Tris (pH 8.0). The incubation was carried out for 30 min at 37 °C (Ramanathan et al., 1976). The nondigestible DNA was precipitated with perchloric acid (PCA) (0.5 N final concentration), deproteinized with protease K (200 μg/mL) in the presence of 0.1 M Tris-HCl (pH 7.2) and 0.5% sodium dodecyl sulfate at 37 °C for 15 h, and further purified by Marmur's procedure (Marmur, 1961). DNA was precipitated with ethanol and dialyzed against 0.01 M glycine (pH 6.8) overnight.

Methylation of Chromatin. Chromatin or free DNA (150–200 μg of DNA/mL) was incubated in a total volume of 4 mL containing 0.01 M Tris-HCl (pH 7.8) with [^{14}C]-MNU (2 μmol/mL, sp act. 1.7×10^6 dpm/μmol) for 1 h at 37 °C. The pH of the incubation mixture was maintained constant throughout the reaction at 7.8 using dilute sodium hydroxide. The A_{260nm} and A_{390nm} at the end of the incubation revealed complete degradation of MNU. In experiments wherein the effect of spermine and distamycin A on the methylation of DNA was studied, freshly prepared aqueous solutions of spermine hydrochloride (pH 7.8) and distamycin A were used.

Isolation of Methylated DNA. At the end of 1 h incubation, the nucleoprotein was precipitated with cold PCA (0.5 N final concentration) and washed repeatedly with 0.25 N PCA until no radioactivity was found in the wash. The washed nucleoprotein was suspended in 2 mL of 0.1 M Tris-HCl (pH 7.2) and 0.5% $NaDodSO_4$ and deproteinized with protease K (200 μg/mL) at 37 °C for 15 h. The DNA was precipitated with 0.5 N PCA in the cold and recovered by centrifugation.

Isolation and Fractionation of Methylated Bases. The methylated bases were released by hydrolysis with 0.1 N PCA at 70 °C for 45 min or at 37 °C for 16 h. The absorption at 260 nm was measured in a Beckman spectrophotometer and the radioactivity was determined in an Intertechnique scintillation spectrometer. The alkylated bases were fractionated on Sephadex G-10 columns with 0.05 M ammonium formate (pH 6.8) as the eluting buffer, or by HPLC on μBondapak C-18 column with 0.05 M ammonium formate (pH 5) as the eluting buffer. The individual methylated bases, N^7-MeG, O^6-MeG, and N^3-MeA were identified using authentic standards as markers. The purity of the methylated bases was checked by paper chromatography using different solvent systems (Rao et al., 1978).

Results

In Table I is presented the effect of 0.0125, 0.025, 0.05, and 0.1 μmol/mL of spermine on the methylation of rat liver chromatin DNA. The results indicate a progressive inhibition with increasing concentration of spermine from 0 to 0.1 μmol/mL.

In order to ascertain whether free spermine is required for the suppression of methylation reaction, the following experiment was conducted. The chromatin-DNA or free DNA was incubated with spermine (0.05 μmol/mL of incubation mixture) for 10 min in ice and centrifuged to separate the complex from free spermine, if any, and the pelleted complex was washed with 0.01 M glycine (pH 6.8) and resuspended in the reaction mixture. The results obtained indicated that under these conditions the alkylation was inhibited by 86%, thereby suggesting that free spermine is not required for the inhibition of methylation reaction.

TABLE I: Effect of Spermine on the Methylation of Rat Liver Chromatin DNA in Vitro by [^{14}C]MNU.[a]

spermine (μmol/mL)	sp act. (dpm/A_{260nm})	% inhibition
0.00	3321 ± 89	0
0.0125	2485 ± 26	25
0.025	1527 ± 184	55
0.05	836 ± 11	75
0.10	330 ± 8	90

[a] Values are the average of six experiments using four different preparations of rat liver chromatin ± SE.

To explain the inhibition by spermine of MNU-induced methylation of chromatin-DNA, we considered the following four possibilities: (i) inability of MNU to methylate spermine-DNA complex, particularly when present in an insoluble form; (ii) an interference by spermine with the release of active methyl groups from MNU during its degradation; (iii) an interaction beween spermine and MNU; (iv) spermine induced alterations of structure and/or net charge of DNA, such that the alkylatable sites are rendered unavailable for MNU interaction.

The next series of experiments was designed to test which of these possibilities were operative under the conditions of our experimentation. Chromatin-DNA or free DNA was incubated with spermine (0.025 or 0.05 μmol/mL of incubation mixture) for 1 h. At the end of the alkylation reaction, the alkylated chromatin-DNA or free DNA was fractionated into sedimentable and nonsedimentable fractions by centrifugation at 4000 rpm for 20 min in an International refrigerated centrifuge, Model PR6, at 4 °C. The DNA of these fractions was analyzed for its radioactivity. The presence of spermine at 0.025 μmol/mL reaction mixture produced 62 and 51% and 0.05 μmol/mL gave 84 and 68% inhibition in the sedimentable and nonsedimentable fractions of chromatin-DNA, respectively. With free DNA as the substrate in the methylation reaction, spermine at 0.05 μmol/mL inhibited the sedimentable and nonsedimentable fraction by 60 and 64%, respectively. No attempt was made to fractionate the DNA into sedimentable and nonsedimentable fractions when 0.025 μmol of spermine per mL was used, since at this concentration spermine caused no obvious precipitation. However, the reaction was inhibited by 50%.

Additional supporting evidence was obtained by carrying out the methylation under conditions where the spermine-DNA complex remains in solution. DNA isolated from the limit digest of rat liver chromatin-DNA, using either DNase I or micrococcal nuclease, does not form insoluble complexes with spermine (S. Rajalakshmi, unpublished data). The data shown in Table II illustrate the effect of spermine on the methylation of rat liver DNA and DNA isolated from the nuclease nondigestible fraction of chromatin. The results show that the methylation of all three preparations of DNA was inhibited, independent of their ability to form soluble or insoluble complexes with spermine. These two experiments together rule out the possibility that the inhibition of methylation reaction by spermine is due to the formation of an insoluble complex.

Direct estimation of MNU at the beginning and end of the reaction by measuring the absorption at 260 and 390 nm revealed complete degradation of MNU in the presence of

TABLE II: Effect of Spermine on the Methylation of Different DNAs by [^{14}C]MNU.[a]

DNA source		% inhibition
total liver chromatin		75 ± 4[b]
nuclease nondigestible	DNase I	73 ± 2[c]
	micrococcal nuclease	67 ± 5[c]

[a] Spermine (0.05 μmol per mL) was used. The sp act. (dpm/A_{260nm}) of DNA was 6542 for total DNA, 7982 for DNase I nondigestible, and 6496 for micrococcal nuclease nondigestible DNA. [b] Values are the average of six experiments. [c] Values are the average of three experiments.

TABLE III: Effect of Spermine on the Methylation of 2-Deoxy-5-guanylic Acid by [^{14}C]MNU.[a]

spermine (μmol/mL)	sp act. (dpm/A_{260nm})	
	N^7-MeG	O^6-MeG
0.00	942 ± 53	93 ± 10
0.05	971 ± 47	121 ± 16
0.1	920 ± 36	110 ± 12

[a] The experimental set-up was similar to the one described in Table I and in addition the incubation mixture contained 150 to 200 μg/mL of 2-deoxy-5-guanylic acid. At the end of the incubation, the chromatin-DNA was precipitated with PCA (0.5 N final concentration). The supernatant containing methylated d-GMP was processed for N^7-MeG and O^6-MeG and the pelleted DNA for N^7-MeG, O^6-MeG, and N^3-MeA as described in the text. Under these experimental conditions, spermine at 0.05 and 0.1 μmol/mL inhibited the methylation of chromatin-DNA by 75 and 90%. The values are the average of four experiments ± SE.

TABLE IV: Effect of Spermine on the Methylation of Chromatin-DNA by [^{14}C]MNU at the N^7 and O^6 Positions of Guanine and the N^3 Position of Adenine.[a]

spermine (μmol/mL)	% inhibition of sp radioact.			
	total methylation	N^7-MeG	O^6-MeG	N^3-MeA[b]
0.00	0	0	0	0
0.025	55	58	50	60
0.05	80	80	80	100

[a] Values are the average of two experiments. The specific radioactivity (dpm/A_{260nm}) for N^7-MeG was 3838, for O^6-MeG 360, and for N^3-MeA 96. [b] Some percentage of N^3-MeA formed might have been eliminated by 0.5 N PCA used during the preparation of DNA.

TABLE V: Effect of Spermine on the Methylation of Free DNA by [^{14}C]MNU at the N^7 and O^6 Positions of Guanine and the N^3 Position of Adenine.[a]

spermine (μmol/mL)	% inhibition of sp radioact.			
	total methylation	N^7-MeG	O^6-MeG	N^3-MeA[b]
0.00	0	0	0	0
0.05	65	59	54	51
0.1	92	93	77	100

[a] Values are the average of two experiments. Sp radioact. (dpm/A_{260nm}) for N^7-MeG was 2112, for O^6-MeG 223, and for N^3-MeA 91. [b] Some percentage of N^3-MeA formed might have been eliminated by 0.5 N PCA used during the precipitation of methylated DNA.

TABLE VI: Effect of Distamycin A on the Methylation of Chromatin-DNA by [^{14}C]MNU.[a]

distamycin A (μmol/mL)	sp. radioact. (dpm/A_{260nm})	% inhibition
0	5416 ± 306	0
0.050	3900 ± 56	28
0.100	2708 ± 110	50
0.225	975 ± 22	82
0.450	379 ± 17	93

[a] Values are the mean of three experiments ± SE.

spermine. Furthermore, the kinetics of degradation of MNU during the reaction remained the same, whether spermine was present or absent.

The data shown in Table III reveal that spermine did not inhibit the methylation of 2-deoxy-5-guanylic acid (d-GMP), included in the reaction mixture as an internal control, under conditions where DNA methylation was inhibited by 75 and 90%. These data eliminate the possibility that the arrest of methylation reaction is due either to an inhibition of MNU degradation or a suppression of the release of active methyl groups.

An interaction between spermine and MNU leading to a reduction of methylation reaction can be eliminated on the basis of the following:

(a) The ratio of MNU (2 μmol/mL) to spermine (0.025 or 0.05 μmol/mL) is very high and excess MNU is available for methylation of DNA even if all the spermine interacts with MNU.

(b) The pH of the reaction renders interaction between spermine and carbenium ion unlikely [we use this term for tricoordinated carbocations (classical carbonium ions) in conformity with Olah's nomenclature (Olah, 1972)].

(c) The direct analysis of the acid soluble fraction after the reaction by three methods (HPLC, Sephadex G-10 and paper chromatography) revealed the presence of only spermine and MNU degradation products. These results suggest that the inhibition of MNU-induced methylation in the presence of spermine may be due to its specific binding to DNA, which probably renders the methylatable sites unavailable for reaction with MNU either by altering the structure and/or net charge of DNA.

The next obvious question that arises is whether spermine by virtue of its specific binding to DNA also exhibits a selectivity in inhibiting the alkylation at the N^7 and O^6 positions of guanine and N^3 position of adenine.

In Tables IV and V are given the data on the effect of spermine on the methylation at N^7 and O^6 positions of guanine and N^3 position of adenine in chromatin and free DNA. The results show that at the concentrations tested the alkylation at all the three positions is affected.

Since the inhibitory effect of spermine might be related to its known adaptation in the minor groove and the subsequent stabilization of the DNA double helix, another compound distamycin A, which also binds to the minor groove and stabilizes the DNA structure, was tested for its effect on the methylation of chromatin-DNA by MNU. The data presented in Table VI clearly show that distamycin A inhibits the methylation of chromatin-DNA by MNU in a manner similar to that exhibited by spermine. Furthermore, the base analysis shows that the methylation at N^7 and O^6 positions of guanine, as well as N^3 position of adenine, is inhibited (data not shown).

Discussion

The study has revealed that spermine and distamycin A inhibit the methylation of DNA at N^7 and O^6 positions of guanine and N^3 position of adenine. Furthermore, the inhibition is attributable neither to an interference with the generation of active methyl groups from MNU during its degradation, nor to an interaction between spermine and MNU. The inhibitory effect exerted by spermine and distamycin A appears to be due to the specific binding of these compounds to DNA, thereby rendering the three methylatable sites studied unavailable for interaction with MNU.

Spermine, by virtue of its basic nature, binds to phosphate and its structure permits adaptation in the minor groove of DNA (Liquori et al., 1967b). The neutralization of the phosphate groups leads to a regular conformation in DNA–spermine complex with the elimination of denatured regions which in aqueous solution are likely to be concentrated in the more solvated A-T rich regions of DNA. However, the neutralization of charge by spermine in itself cannot be responsible, although it may contribute to the inhibition of methylation reaction. The complete absence of inhibition of methylation of d-GMP included as an internal control in the reaction argues against the implication of charge neutralization alone. Furthermore, distamycin A, which interacts by electrostatic forces with the carbonyl groups of thymine and/or N^3 atom of adenine in the minor groove of DNA (Wartell et al., 1974; Melnikova et al., 1975; Zimmer, 1975) also inhibits the methylation. Thus, it is likely that the the binding of spermine (or distamycin A) to the minor groove and/or the subsequent stabilization of DNA structure is responsibile for the inhibitory effect of these compounds on the methylation of DNA by MNU.

It is of interest to note that the structural alteration(s) induced by spermine at these concentrations is not inhibitory to the synthesis of macromolecules such as RNA, DNA, and protein, mediated by enzymes; in fact, spermine is stimulatory to several enzymatic processes (Janne et al., 1975; Tabor & Tabor, 1976). Thus, whatever structure DNA assumes in the presence of spermine, while permitting enzymatic processes, inhibits chemical methylation by MNU. This raises a provocative question, whether MNU interaction with DNA is stereospecific and selective. It may be pointed out in this context that spermine also prevents the intercalation of benz[*a*]-pyrene into DNA (Liquori et al., 1967a).

In conclusion, the study reveals that spermine and distamycin A inhibit the methylation of both chromatin DNA and free DNA. Based on the following facts, viz., (a) the specific interaction of these compounds with minor groove, (b) their ability to increase thermal melting of DNA, and (c) stabilization of helical structure in the A-T rich DNA region, it may be concluded tentatively that MNU alkylates regions of DNA that are readily denaturable, probably rich in A-T base pairs located at or close to the binding site of spermine and distamycin A, possibly in the minor groove.

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