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# Cyanoethylation of DNA *in Vivo* by 3-(Methylnitrosamino)propionitrile, an *Areca*-derived Carcinogen[1]

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## ABSTRACT

**2-Cyanoethyldiazohydroxide is a likely product of metabolic α-hydroxylation of 3-(methylnitrosamino)propionitrile (MNPN). The reaction of 2-(*N*-carbethoxy-*N*-nitrosamino)propionitrile, a stable precursor of 2-cyanoethyldiazohydroxide, with deoxyguanosine, catalyzed by porcine liver esterase, was investigated. Two major deoxyguanosine adducts were produced. They were isolated by high-performance liquid chromatography and characterized by their UV spectra, mass spectra, and proton magnetic resonance spectra. On the basis of these spectral data, the structures of the two adducts were assigned as 7-(2-cyanoethyl)guanine and $O^6$-(2-cyanoethyl)deoxyguanosine.**

**The potential of MNPN to cyanoethylate DNA in F344 rats was evaluated by measuring 7-(2-cyanoethyl)guanine and $O^6$-(2-cyanoethyl)guanine in the liver, nasal mucosa, and esophagus. The highest levels were detected in the nasal cavity, which is one of the major target organs for the carcinogenic effects of MNPN.**

## INTRODUCTION

Betel quid chewing, a habit practiced in India and other Asian countries, has long been known to be associated with cancer of the oral cavity (1–3). In a recent evaluation of this association, the International Agency for Research on Cancer concluded that there is sufficient evidence that the habit of chewing betel quid containing tobacco is carcinogenic to humans; however, there is inadequate evidence that the habit of chewing betel quid without tobacco is carcinogenic to humans (3).

The knowledge that *N*-nitrosamines, formed by nitrosation of tobacco alkaloids, play a significant role in tobacco carcinogenesis led to the hypothesis that nitrosation of arecoline, the major *Areca* alkaloid, might similarly produce nitrosamines with carcinogenic potential (4, 5).

Three *Areca*-derived *N*-nitrosamines, namely *N*-nitrosoguvacoline, *N*-nitrosoguvacine, and MNPN,[3] have indeed been identified in the saliva of betel quid chewers (Fig. 1) (5–8). MNPN proved to be a potent carcinogen in the esophagus and nasal cavity of F344 rats (8, 9). Metabolic α-hydroxylation, which produces electrophilic alkyl diazohydroxides that covalently bind to DNA bases is an accepted activation process for carcinogenic dialkyl-*N*-nitrosamines (10–12). In the case of MNPN, metabolic activation by α-hydroxylation would be expected to lead to the formation of methyl diazohydroxide and 2-cyanoethyldiazohydroxide (Fig. 2), unstable intermediates which should preferentially react at position 7 of guanine (13–18). Compounds with the *N*-nitrosamino group in a position beta to an electron-withdrawing substituent are known to undergo base-catalyzed α,β-elimination. In the case of MNPN, AN, an electrophile capable of alkylating DNA would be formed.

The potential of MNPN to methylate DNA has previously been demonstrated (8). In this study, we have found that *in vitro* the reaction of 2-(*N*-carbethoxy-*N*-nitrosamino)propionitrile with deoxyguanosine leads to the formation of 7-(2-cyanoethyl)guanine (7-CNEGua) and $O^6$-(2-cyanoethyl)deoxyguanosine. More importantly, we determined that MNPN possesses the ability to cyanoethylate liver, nasal mucosa, and esophageal DNA *in vivo* in F344 rats. Cyanoethylated guanines were not detected in the DNA isolated from the liver and brain DNA of F344 rats treated with AN.

## MATERIALS AND METHODS

### Instrumentation

UV spectra of aqueous solutions were obtained with a Hewlett-Packard Model 8452A diode-array spectrophotometer. Mass spectral analyses were performed using a Hewlett-Packard GC-mass spectrophotometer Model 5988A. IR spectra were obtained with a Perkin Elmer Model 267 grating infrared spectrophotometer. $^1$H-NMR spectra were recorded on a Brucker AM 360-MHz FT NMR spectrometer. The basic system for HPLC analyses consisted of a Waters Model W600 single-pump gradient system and a Model U6K injector (Millipore, Waters Chromatography Division, Milford, MA) interfaced with a Model 990 photodiode array, or a Model 440 absorbance, or a Perkin-Elmer Model 650-10S fluorescence detector (Perkin-Elmer Corp., Oak Brook, IL). Melting points were determined on a Fisher-Johns melting point apparatus and were reported uncorrected.

### Chemicals

AN. AN, 99+% grade, was purchased from Aldrich Chemical Company, Milwaukee; WI, and was distilled before application.

MNPN. MNPN was synthesized according to earlier published reports (5, 19). Gas chromatography, high-pressure liquid chromatography, and thin-layer chromatography indicated that the compound was >99% pure.

**2-(*N*-Carbethoxy-*N*-nitrosamino)propionitrile.** This chemical was synthesized from 2-aminopropionitrile and ethyl chloroformate as published elsewhere.[4]

**7-(2-Cyanoethyl)guanine.** 7-(2-Cyanoethyl)guanine was synthesized by modification of Solomon's method (20). The identity of the compound was confirmed by $^1$H-NMR, UV, and MS spectra.

### Reaction of 2-(*N*-carbethoxy-*N*-nitrosamino)propionitrile with Deoxyguanosine

2-(N-Carbethoxy-N-nitrosamino)propionitrile (1.3 g, 7.8 mmol) was mixed with (1.0 g, 3.7 mmol) deoxyguanosine (Sigma Chemical Co., St. Louis, MO) and esterase (1375 units, Porcine Liver, Sigma Chemical Co., St. Louis, MO) in 200 ml of 0.1 M phosphate buffer, pH 7.0. The solution was incubated with constant stirring at 37°C for 3 days. Upon cooling, the mixture was filtered through Whatman filter paper, the filtrate was evaporated to dryness, redissolved in a minimum of water, and separated by HPLC using a 10-μm, Partisil, 10-ODS-3, Magnum-9 (50 cm x 9 mm) column (Whatman Inc., Clifton, NJ). The

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[3] The abbreviations used are: MNPN, 3-(methylnitrosamino)propionitrile; 7-methylguanine; $O^6$-mGua, $O^6$-methylguanine; 7-CNEGua, 7-(2-cyanoethyl)guanine; $O^6$-CNEGua, $O^6$-(2-cyanoethyl)guanine; AN, acrylonitrile; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; NMR, nuclear magnetic resonance.

[4] Prokopczyk, B., Bertinato, P., and Hoffmann, D. Synthesis of 7-(2-cyanoethyl)guanine and $O^6$-(2-cyanoethyl)guanine. Markers for reaction of acrylonitrile (AN) with DNA. Carcinogenesis, in press.

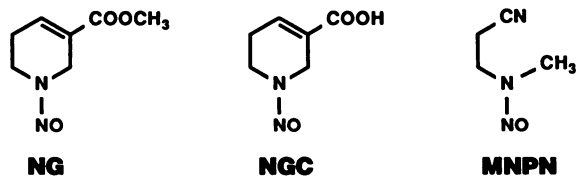


Fig. 1. *Areca*-derived *N*-nitrosamines detected in the saliva and urine of betel quid chewers. *NG*, *N*-nitrosoguvacoline; *NGC*, *N*-nitrosoguvacine.

column was eluted with 0 to 70% methanol in $H_2O$ in 50 min, using the linear curve 6, at a flow rate of 4 ml/min. This method led to the separation of adducts 2 and 3 (Fig. 3). The yields of adduct 2 and 3 were 3.2 and 1.5%, respectively. Unreacted deoxyguanosine (91.6%) was also recovered.

### DNA Alkylation Study

**Animal Treatment.** Groups of 10 male F344 rats each (Charles River Lab., Kingston, NY) (≈250 g b.w.) were given a single s.c. injection of MNPN (45 mg/kg, 0.4 mmol/kg) in saline or AN (50 mg/kg, 0.94 mmol/kg or 100 mg/kg, 1.88 mmol/kg). In another test, AN was administered by i.v. injection (50 mg/kg or 100 mg/kg) to 10 F344 male rats. The rats were sacrificed at various intervals after treatment as shown in Table 1.

**DNA Isolation.** DNA was isolated from the liver, esophagus, and nasal mucosa of animals treated with MNPN and from the liver and brain of rats treated with AN, as previously described (21).

### Analysis of 7-(2-Cyanoethyl)guanine, $O^6$-(2-Cyanoethyl)guanine and Guanine in DNA

A previously developed method for the quantitative analysis of methylated purines in DNA was adapted for quantification of 2-cyanoethylated guanines (8, 22, 23).

In general, for neutral thermal hydrolysis, 3–4 mg DNA were dissolved in 800 μl of 10 mM cacodylate buffer, pH 7.0, and were heated at 100°C for 30 min. After cooling, the samples were divided into two equal portions, and an appropriate amount of HCl was added to bring the solution to 0.1 N HCl. In the aliquot to be used for analysis of 7-methylguanine and 7-CNEGua the precipitation was pelleted by centrifugation. The second portion was analyzed for $O^6$-methylguanine and $O^6$-CNEGua. It was hydrolyzed at 80°C with 0.1 N HCl.

The following solvent elution systems were used:

**System 1.** A 5-μm column, P-SCX, 25 cm x 3.2 mm, (E.S. Industries, Marlton, NJ) was eluted isocratically with 10% methanol, 0.15 mmol ammonium phosphate at a flow of 0.5 ml/min.

**System 2.** A 5-μm column, RP SCX, 25 cm x 4 mm, (E.S. Industries, Marlton, NJ) was eluted with 15% MeOH, 0.2 M ammonium phosphate at a flow of 0.9 ml/min.

**System 3.** For assistance in the structural elucidation of compounds isolated from HPLC we also utilized System 3, a Whatman ODS-3 column (4.6 mm x 25 cm) with a methanol:water gradient from 0 to 70% in 40 min.

## RESULTS

A representative HPLC profile of the mixture obtained upon incubation of 2-(*N*-carbethoxy-*N*-nitrosamino)propionitrile with deoxyguanosine in the presence of esterase is shown in Fig. 3. Peaks 2 and 3 were the major deoxyguanosine adducts. The NMR spectrum (DMSO) of Peak 2 (Fig. 4) showed a —CH group at 8.00 ppm, a $—NH_2$ group at 6.20 ppm, and two triplets at 4.42 and 3.18 ppm which are characteristic of $—N—CH_2$ and $—CH_2—CN$ groups. Irradiation of the triplet at 4.42 ppm resulted in its collapse and caused appearance of a singlet at 3.18 ppm. These data indicated the presence of a 2-cyanoethyl-substituted guanine. The UV spectra of Peak 2 at various pH values are characteristic for 7-substituted guanines (24). On the basis of UV and NMR data, the structure of Peak 2 was tentatively assigned as 7-CNEGua and was confirmed by comparison with data from synthetic 7-CNEGua obtained by a modified Solomon's method.[4] The retention volumes of Peak 2 and the synthetic reference were identical in the HPLC systems 2 and 3.

The NMR spectrum (DMSO) of Peak 3 is illustrated in Fig. 5. The assignment of chemical shifts for Peak 3 is consistent with a (2-cyanoethyl)deoxyguanosine. The proton signals at 6.55, 5.35, and 5.08 ppm all disappeared following the addition of $D_2O$ confirming their assignments as hydroxyl or amine protons. Irradiation of the triplet at 4.58 ppm resulted in its collapse and caused appearance of a singlet at 3.15 ppm confirming the presence of a $—CH_2—CH_2—$ group. UV spectra of peak 3 in $H_2O$ and 0.1 N HCl were almost identical to those of $O^6$-ethyldeoxyguanosine. The difference of the spectrum in 0.1 N NaOH compared to that of $O^6$-deoxyguanosine is due to instability of Peak 3 in alkaline solution. When dissolved in 0.1 N NaOH and then reacidified by 0.1 N HCl, Peak 3 showed a spectrum identical to that taken in 0.1 N NaOH. On the basis of NMR and UV data, Peak 3 was assigned as $O^6$-(2-cyano-

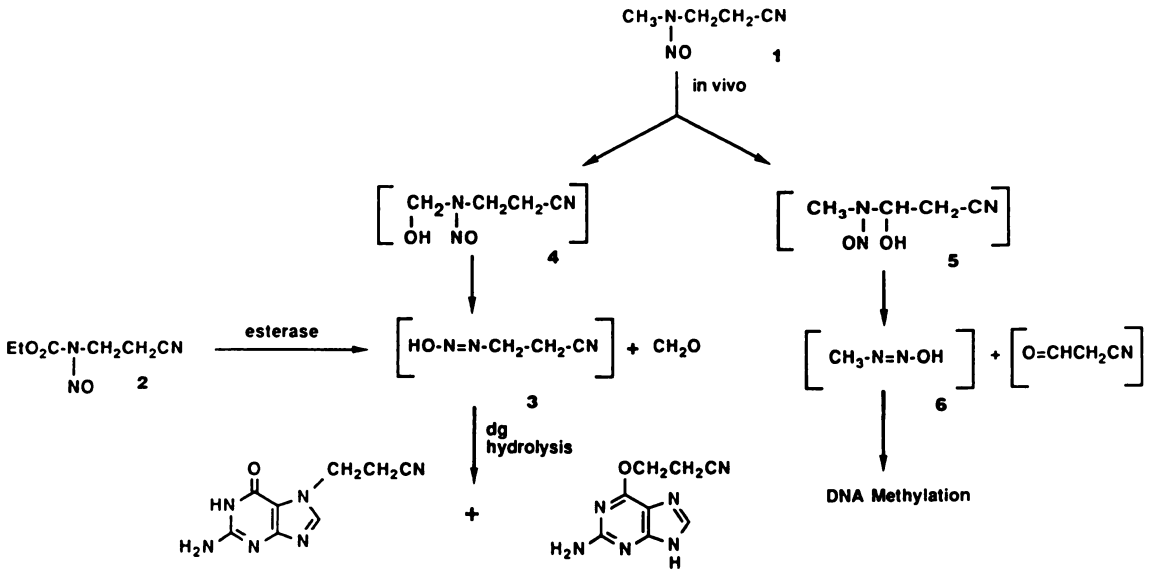


Fig. 2. Intermediates involved in the α-hydroxylation of MNPN and their reaction products with deoxyguanosine (dg) and DNA.

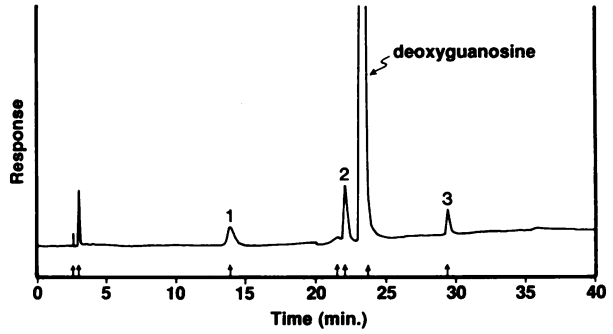


Fig. 3. HPLC trace obtained after reacting 2-(*N*-carbethoxy-*N*-nitrosamino)propionitrile with deoxyguanosine (UV detection, monitored at 254 nm).

ethyl)deoxyguanosine. This structural assignment was further confirmed by converting Peak 3 to the corresponding guanine derivative by hydrolysis in 0.1 N HCl. The 2-cyanoethyl-substitution was retained under the hydrolytic conditions as shown by the NMR spectrum (DMSO) in which the chemical shifts associated with the protons of the —$CH_2CH_2$—CN group and a single proton at position C8 of guanine were essentially the same as those observed at the nucleoside level. The UV spectra (Fig. 6) were identical to those of $O^6$-(2-hydroxyethyl)guanine. Additional structural confirmation was obtained by EI mass spectrometry (Fig. 7). The spectrum exhibited M at *m/e* 204, M + 1 at *m/e* 205, and characteristic fragmentation of a cyanoethyl side to *m/e* 164 (M - $CH_2CN$), *m/e* 151 (M - $CH_2CH_2CN$), *m/e* 151 (M - $CH_2CH_2CN$), and *m/e* 134 (M - $OCH_2CH_2CN$). The molecular weight was also confirmed by fast-atom bombardment MS with M + 1 at *m/e* 205. $O^6$-CNEGua was also found to be unstable in 0.1 N NaOH. On the basis of these data, Peak 3 was identified as $O^6$-(2-cyanoethyl)deoxyguanosine.

DNA samples were analyzed for the presence of 7-CNEGua and $O^6$-CNEGua using HPLC with fluorescence detection. The identity of the adducts was confirmed by injections with synthetic standards. The typical HPLC profiles are shown in Fig. 8.

Levels of 7-CNEGua and $O^6$-CNEGua in DNA of liver, esophagus, and nasal mucosa, measured 2–36 h after treatment with a single s.c. injection of MNPN (0.4 mmol/kg or 250 mg/kg) are listed in Table 1. The highest levels of cyanoethylation were detected in the nasal mucosa. The rats treated with AN were sacrificed 2 h (for those receiving 50 mg/kg), and 6 h after injection (100 mg/kg), and DNA was isolated from the liver and from the brain. Under the experimental conditions described above we could not detect the formation of 7-CNEGua nor of $O^6$-CNEGua. The detection limits, under these experimental conditions, were 20 μmol/mol guanine for 7-CNEGua and 15 μmol/mol guanine for $O^6$-CNEGua, respectively.

## DISCUSSION

MNPN is activated *in vivo* by α-hydroxylation leading to the formation of two electrophiles (Fig. 2). Methyldiazohydroxide (*6*) resulting from the methylene-carbon oxidation, can methylate nucleophilic centers in DNA. If α-hydroxylation occurs on the methyl group of MNPN, 2-cyanoethyldiazohydroxide can form. This unstable metabolite would be expected to react with nucleophilic centers of DNA (Fig. 2). The involvement of intermediate *3* in the DNA alkylation was investigated by employing a stable precursor, 2-(*N*-carbethoxynitrosamino)propionitrile (*2*). The formation of 7-CNEGua and $O^6$-(2-cyanoethyl)deoxyguanosine, respectively, was detected *in vitro*. Most importantly, the present study clearly shows that MNPN gives rise to both methylation and cyanoethylation of cellular DNA *in vivo*, supporting the concept that α-carbon hydroxylation indeed occurs at both the methyl and cyanoethyl moieties. Theoretically, the cyanoethylation may be partially due to the formation of acrylonitrile by α,β-elimination. However, since we were unable to detect the formation of cyanoethyl-substituted guanines in our study with AN, it is strongly indicated that the prevailing pathway for the metabolic activation of MNPN, which leads to the formation of cyanoethyl-substituted guanines, is *via* electrophile *3*. This agrees with the previously published work (25, 26) where direct alkylation of DNA by AN was not detected.

In rat liver DNA the levels of $m^7$-Gua were 3.3–7.5 times higher than those of 7-CNEGua. In contrast, the extent of formation of $O^6$-CNEGua was similar to that of $O^6$-mGua (Table 1). However, this ratio does not directly reflect the rate of metabolism of MNPN to a methylating or cyanoethylating intermediate since both the extent of alkylation and

Table 1 *Levels of 7-methylguanine, $O^6$-methylguanine, 7-(2-cyanoethyl)guanine, and $O^6$-(2-cyanoethyl)guanine in F344 rat tissues at intervals after s.c. injection of MNPN*

F344 rats were given a s.c. injection of MNPN in saline (0.4 mmol/kg). Values are the mean ± SD of determinations of DNA from liver of five rats and the mean of duplicate determinations from DNA (five rats each) from nasal mucosa and esophagus.

| Survival interval (h) | μmol/mol Guanine | | $m^7$-Gua: 7-CNEGua | μmol/mol Guanine | | $O^6$-mGua: $O^6$-CNEGua | $O^6$-mGua: $m^7$-Gua | $O^6$-CNEGua: 7-CNEGua |
|---|---|---|---|---|---|---|---|---|
| | $m^7$-Gua[a] | 7-CNEGua[a] | | $O^6$-mGua[a] | $O^6$-CNEGua[a] | | | |
| Liver | | | | | | | | |
| 2 | 339 ± 68 | 101 ± 29 | 3.4 | 75 ± 7 | 78 ± 5 | 0.96 | 0.22 | 0.77 |
| 6 | 1207 ± 75 | 253 ± 53 | 4.8 | 146 ± 16 | 118 ± 7 | 1.23 | 0.12 | 0.47 |
| 24 | 1342 ± 178 | 180 ± 40 | 7.5 | 144 ± 27 | 202 ± 18 | 0.71 | 0.11 | 1.12 |
| 36 | 912 ± 260 | 128 ± 28 | 7.1 | 29 ± 7 | 59 ± 15 | 0.49 | 0.03 | 0.46 |
| Nasal mucosa | | | | | | | | |
| 2 | 523 | 307 | 1.7 | 83 | 53 | 1.57 | 0.16 | 0.17 |
| 6 | 1845 | 1225 | 1.5 | 291 | 97 | 3.00 | 0.16 | 0.08 |
| 24 | 1669 | 768 | 2.2 | 189 | 201 | 0.94 | 0.11 | 0.26 |
| 36 | 890 | 580 | 1.5 | 110 | 121 | 0.91 | 0.12 | 0.21 |
| Esophagus | | | | | | | | |
| 2 | 150 | 90.5 | 1.7 | ND[b] | ND | | | |
| 6 | 253 | 260 | 1.0 | 10 | ND | | 0.04 | |
| 24 | 152 | 200 | 0.8 | 4 | ND | | 0.03 | |
| 36 | 127 | 123 | 1.0 | 8 | ND | | 0.06 | |

[a] μmol/mol; [b] ND, not detected.

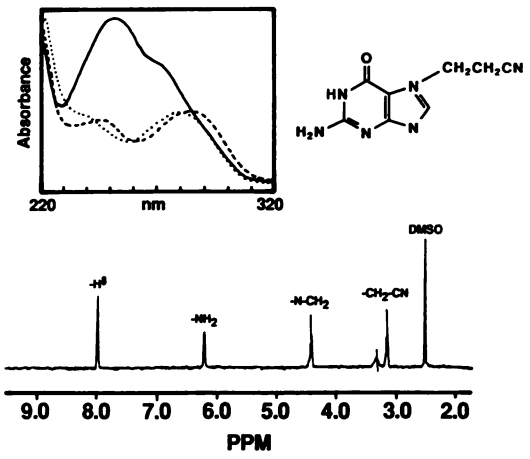


Fig. 4. NMR and UV spectra of Peak 2 (Fig. 3). UV spectrum in 0.1 N HCl (——), in $H_2O$ (– – –), and in 0.1 N NaOH (·····).

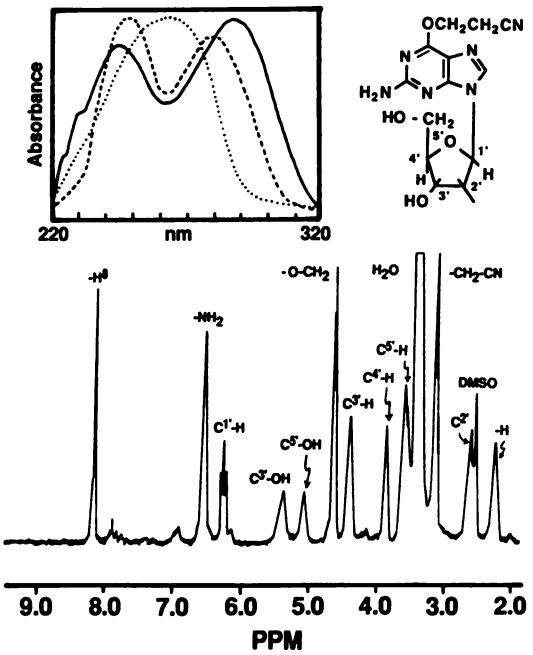


Fig. 5. NMR and UV spectra of Peak 3 (Fig. 3). UV spectrum in 0.1 N HCl (——), in $H_2O$ ((– – –), and in 0.1 N NaOH (·····).

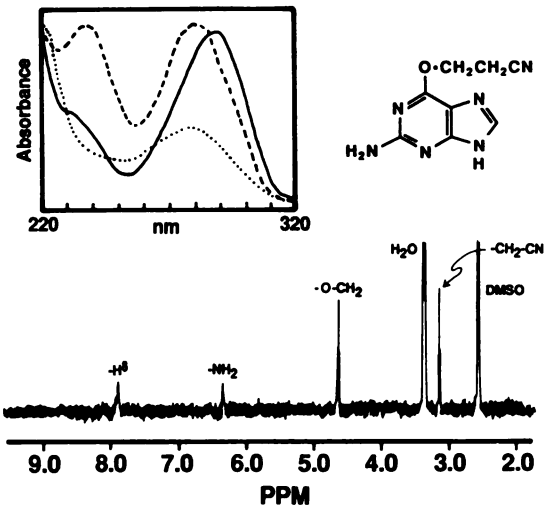


Fig. 6. NMR and UV spectra of the derivative obtained by the acid hydrolysis of the material eluting as Peak 3. (Fig. 3). UV spectrum in 0.1 N HCl (——), in $H_2O$ (– – –), and in 0.1 N NaOH (·····).

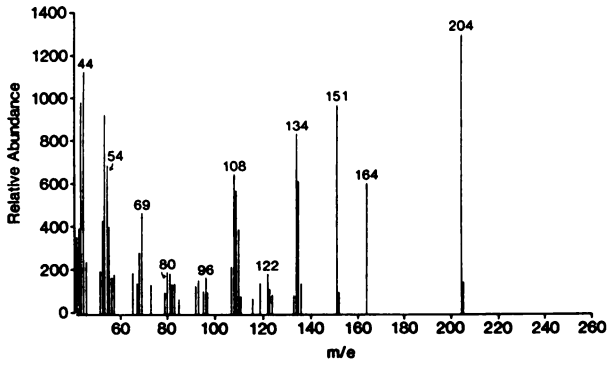


Fig. 7. EI mass spectrum of $O^6$-(2-cyanoethyl)guanine.

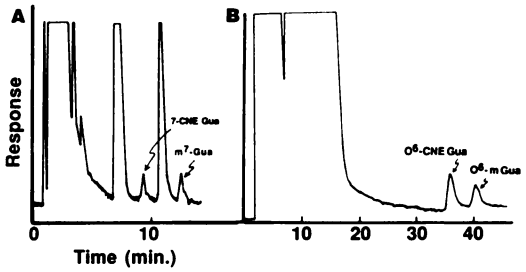


Fig. 8. HPLC elution profiles of rat DNA hydrolysate from rats treated with 0.4 mmol/kg MNPN. *A*, neutral thermal hydrolysis; *B*, acid hydrolysis (fluorescence detector; excitation 286 nm, emission 366 nm).

the profile of alkylation products can differ considerably. This was illustrated with *N*-nitrosomethylethylamine by von Hofe *et al.* (27) who calculated the ratio of 7-methylguanine:7-ethylguanine to be approximately 68 when equimolar amounts of methyl and ethyl carbonium ions reacted concurrently with DNA in the absence of other competitive reactions. The alkylation patterns for MNPN are currently being studied. In DNA isolated from the nasal mucosa the ratios of $m^7$-Gua:7-CNEGua were considerably lower (1.5–2.2) and the extent of formation of the cyanoethylated adduct was considerably higher, suggesting that MNPN is more extensively metabolized in this organ to the cyanoethylating intermediate. Interestingly, the nasal mucosa was found to be the primary target tissue in our carcinogenicity assays (8, 9). The involvement of cyanoethylated adducts in MNPN carcinogenicity needs further elucidation.

The miscoding properties of $O^6$-mGua and $O^4$-methylthymidine are known to be involved in the initiation of carcinogenesis (28). Furthermore, as reported by Singer, not only the extent of the alkylation at the $O^6$ position of guanine but also the persistence of the adducts are of significance to the initiation phase of carcinogenesis (29). Some of the $O^6$ guanine adducts are not enzymatically removed from the given cell population and can thus cause mispairing during transcription. This may actually correlate with carcinogenesis data (30). $O^6$-Ethylguanine, for example, was not rapidly removed from the DNA of the brain, which is the target organ for cancer induction by diethylnitrosourea and diethylnitrosamine in rats, but, relative to 7-ethylguanine, $O^6$-ethylguanine was rapidly removed from other tissues, such as liver (31). A similar observation applies to the relative persistence of $O^6$-CNEGua in the nasal mucosa and in the liver of MNPN-treated rats. The fact that the $O^6$-mGua:$m^7$-Gua ratio in the rat liver (observed 2–24 h) and nasal mucosa (2–36 h) was very close to the "theoretical" value of 0.1 observed upon reaction of methylated nitroso compounds with DNA (31), suggests that repair of $O^6$-mGua had not

occurred to any significant extent. The same ratio for liver DNA 36 h after treatment was 0.03 indicating that significant repair had taken place. The similar ratios for $O^6$-CNEGua *versus* 7-CNEGua remained almost constant between 2–36 h which indicates that the repair mechanism for $O^6$-CNEGua in the nasal mucosa is slower than that in the liver. A similar "theoretical" ratio for *in vitro* cyanoethylation of DNA at the 7 and $O^6$ positions of guanine was not established in our studies. However, the reaction of 2-(*N*-carbethoxy-*N*-nitrosamino)propionitrile with deoxyguanosine resulted in the formation of $O^6$-CNEGua:7-CNEGua at a ratio of 0.53, which is again close to that observed in the liver in our *in vivo* experiment with MNPN, and that reported by Singer and Grunberger for *in vitro* DNA ethylation (24).

The present results provide the first evidence for DNA cyanoethylation *in vivo* by the *Areca*-derived carcinogen MNPN. However, additional studies are needed to determine the involvement of cyanoethyl adducts in MNPN carcinogenicity. These studies are now in progress.

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