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Evidence for 4-(3-pyridyl)-4-oxobutylation of DNA in F344 rats treated with the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine

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DNA was isolated from tissues of F344 rats 24 h after treatment by s.c. injection with [5-^{3}H]4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone ([5-^{3}H]NNK) or [5-^{3}H]*N'*-nitrosonornicotine ([5-^{3}H]NNN). It was hydrolyzed with acid or at pH 7, 100°C, and the hydrolysates were analyzed by HPLC. The major product in each case was identified as 4-hydroxy-1-(3-pyridyl)-1-butanone, formed by hydrolysis of a DNA adduct. It was detected in DNA from the livers of rats treated with [5-^{3}H]NNK or [5-^{3}H]NNN, and in DNA from lungs of rats treated with [5-^{3}H]NNK. These results demonstrate that 4-(3-pyridyl)-4-oxobutylation of DNA occurs in rats treated with NNK or NNN, and are consistent with the hypothesis that these nitrosamines are metabolically activated by α-hydroxylation.

Introduction

The major tobacco alkaloid nicotine is the precursor to two carcinogenic nitrosamines found in tobacco and tobacco smoke—4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) and *N'*-nitrosonornicotine (NNN) (1). Their structures are shown in Figure 1. These nitrosamines are believed to be important in tobacco-induced cancer because they are relatively strong carcinogens. NNK induces tumors of the liver, lung and nasal cavity in rats, and the lung and nasal cavity in Syrian golden hamsters (1). It also causes tumors of the skin and lung in mice (1,2). NNN gives tumors of the esophagus and nasal cavity in rats, and trachea and nasal cavity in hamsters (1). A mixture of the two nitrosamines induces oral tumors in rats when applied locally (3). The levels of NNK and NNN in tobacco and tobacco smoke are sufficiently high that the estimated exposure of a long-term smoker or snuff-dipper would be similar to the total doses of these compounds required to produce tumors in experimental animals.

Studies of the metabolism of NNK and NNN in experimental animals and in human tissues and subcellular fractions have shown the existence of several enzymatic pathways (1,5). These include hydroxylation of the methylene and methyl carbons of NNK adjacent to the *N*-nitroso nitrogen (α-hydroxylation), reduction of the carbonyl group and oxidation of the pyridine nitrogen. For NNN, hydroxylation of all the pyrrolidine ring carbons has been observed, along with denitrosation and pyridine *N*-oxidation (1,5). Based on mutagenicity experiments with stable precursors to the intermediates formed upon α-hydroxylation of NNK and NNN, and by analogy to studies on structurally less complex analogues such as *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine, we have proposed that α-hydroxylation is a major pathway of metabolic activation of NNK and NNN (6,7). This hypothesis is supported by the detection of 7-methylguanine, O^6-methylguanine and O^4-methylthymidine in the DNA of target tissues of rats treated with NNK (4,8). These DNA modifications arise at least partially from α-hydroxylation of NNK at the methylene carbon. As illustrated in Figure 1, hydroxylation of NNK at the methyl carbon, or 2'-hydroxylation of NNN, should give 4-(3-pyridyl)-4-oxobutyl diazohydroxide (Structure 3), via the unstable α-hydroxynitrosamines, Structures 1 and 2. The diazohydroxide (Structure 3) would be expected to cause 4-(3-pyridyl)-4-oxobutylation of DNA. Although 4-(3-pyridyl)-4-oxobutylation of hemoglobin by NNK and NNN has recently been demonstrated (9), no information is available on the occurrence of this specific alkylation pathway in DNA of animals treated with these nitrosamines. Previous studies have demonstrated the presence of radioactivity in DNA of tissues from rats treated with [2'-^{14}C]NNN, but the structure of the incorporated material was not determined (10).

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosonornicotine.

Materials and methods

Chemicals

[5-^{3}H]NNK (0.95 Ci/mmol; purity 98%), [5-^{3}H]NNN (4.9 Ci/mmol; purity, 98%) and [5-^{3}H]4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone (0.82 Ci/mmol; purity, 98%) were purchased from Chemsyn Science Laboratories, Lenexa, KS, USA. The purities of [5-^{3}H]NNK and [5-^{3}H]NNN were confirmed by HPLC. [5-^{3}H]4-hydroxy-1-(3-pyridyl)-1-butanone was prepared by hydrolysis of [5-^{3}H]4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone as described (11). NNN, NNK, 4-hydroxy-1-(3-pyridyl)-1-butanone, 3-hydroxy-1-(3-pyridyl)-1-butanone, 4-oxo-1-(3-pyridyl)-1-butanone, 1-(3-pyridyl)-1,4-butanediol, 5-(3-pyridyl)-2-hydroxytetrahydrofuran and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol were synthesized (6,7,12–14).

Animal treatments

Male F344 rats weighing 250–270 g were obtained from Charles River Breeding Laboratories, Kingston, NY, USA. They were housed under standard conditions as described (3). Each of three rats was given an s.c. injection of [5-^{3}H]NNK (2 mCi/2 μmol) or [5-^{3}H]NNN (1.7 mCi/0.35 μmol) in 0.6 ml of saline, and killed 24 h later by decapitation.

Isolation and hydrolysis of DNA

DNA was isolated from liver, kidney and lung of each rat, and from esophagus and nasal mucosa of pools of three rats. The procedure used for DNA isolation was the same as that described previously (4), except that after treatment with RNase the samples were incubated with 1 mg Protease (Type XIV, Sigma Chemical Co., St Louis, MO, USA) for 30 min, then extracted as usual. The purity of the DNA was assessed by its OD_{260}/OD_{280} ratio, and by analysis for protein and RNA contamination. The protein analysis was done by the method of Lowry (15). The detection limit was approximately 1 μg/100 μg DNA sample. RNA contamination was assessed by hydrolyzing the samples with nuclease P_1 and alkaline phosphatase as described (16), and analyzing the hydrolysate for uridine by HPLC using the conditions of Hartwick *et al.* (17), except that elution was isocratic until uridine eluted.

For acid hydrolysis of the DNA, 0.5–1 ml of 0.8 N HCl was added to 2–3 mg of DNA. The samples were heated at 80°C for 6 h. After cooling, the pH was adjusted to 6–8 with 1 N NaOH. The samples were filtered through Gelman acrodiscs LC13, 0.45 μm (Gelman Sciences, Inc., Ann Arbor, MI, USA) and analyzed by HPLC. For neutral thermal hydrolysis, 3–4 mg DNA were dissolved in 1 ml of 10 mM sodium cacodylate buffer, pH 7.0, and heated at 100°C for 35 min. After cooling, an appropriate amount of HCl was added to bring the solution to 0.1 N HCl. The samples were centrifuged and the supernatant removed. The pH of the supernatant was adjusted to 6–8 with 1 N NaOH. It was filtered as above and analyzed by HPLC.

HPLC analysis

The HPLC apparatus has been described previously (9). Reverse phase analyses were carried out using two μBondapak C_{18} columns (Millipore, Waters Division, Milford, MA, USA) with elution by 0–50% methanol in 0.02 M sodium phosphate buffer, pH 7, in 100 min at 1 ml/min. Normal phase analyses were carried out using two Lichrosorb Si50 5 μ columns (E.Merck, Darmstadt, FRG) eluted with solvent A for 10 min, then a gradient of 0–100% solvent B in solvent A in 40 min at 1 ml/min. Solvent A was 50:50 $CHCl_3$:hexane and solvent B was 10% methanol in solvent A. Radioactivity was measured with a Flo-one/Beta radioactive flow detector (Radiomatic Instruments, Tampa, FL, USA) when reverse phase conditions were used. When normal phase conditions were used, 1 ml fractions were collected with an Isco Retriever III fraction collector (Isco, Lincoln, NE, USA). They were concentrated to dryness, and the radioactivity was determined by scintillation counting with a Beckman Instruments Model LS 8900 scintillation counter (Beckman Instruments, Inc., Fullerton, CA, USA).

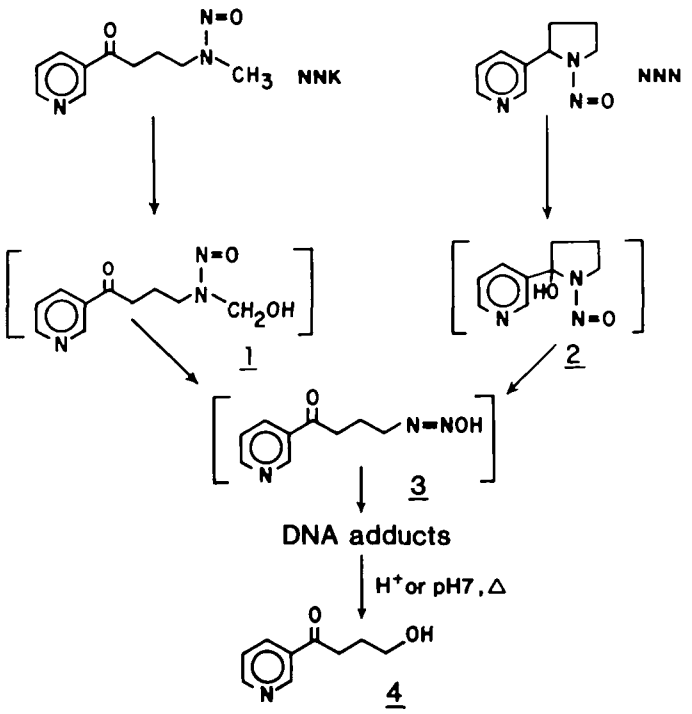


Fig. 1. Intermediates and products involved in 4-(3-pyridyl)-4-oxobutylation of DNA by NNK and NNN.

Results

Figure 2 illustrates a chromatogram obtained upon HPLC analysis of the acid hydrolysate of DNA isolated from the liver of a rat treated with [5-^{3}H]NNK. The radioactive peak which eluted at 50 min had the same retention time as reference 4-hydroxy-1-(3-pyridyl)-1-butanone (Structure 4 of Figure 1). Further evidence for the identity of the radioactive peak was obtained by analysis using normal phase HPLC, under conditions which fail to elute related modified DNA bases or nucleosides, such as 2′-deoxy-*N*-[1-methyl-3-oxo-3-(3-pyridyl)propyl]guanosine (Structure 6). As illustrated in Figure 3, the radioactivity again coeluted with reference 4-hydroxy-1-(3-pyridyl)-1-butanone. Treatment of the radioactive peak and unlabelled Structure 4 with $NaBH_4$, followed by normal phase HPLC analysis, gave a chromatogram in which the radioactivity coeluted with reference 1-(3-pyridyl)-1,4-butanediol (retention time, 62 min), the expected product of reduction. These results confirm the identity of the radioactive unknown as 4-hydroxy-1-(3-pyridyl)-1-butanone, Structure 4. The amount released from DNA upon acid hydrolysis was 0.67 ± 0.1 pmol/mg DNA.

Since Structure 4 is a metabolite of NNK, we considered the possibility that it might be non-covalently bound to DNA. Two experiments indicated that this was not the case. In one, an aliquot of DNA was hydrolyzed and analyzed for Structure 4. A second aliquot was redissolved in H_2O, reprecipitated, and washed with ethanol and acetone. It was hydrolyzed and analyzed for Structure 4. The result was the same as that obtained for the first aliquot, indicating that the additional purification did not remove any Structure 4 from the DNA. In a second experiment, 0.5 μCi of [5-^{3}H]4-hydroxy-1-(3-pyridyl)-1-butanone was added to an aqueous solution of 3 mg DNA. The mixture was incubated at room temperature for 1 h and the H_2O was removed by rotary evaporation. The DNA was then redissolved in H_2O, precipitated, washed with ethanol and acetone and hydrolyzed. HPLC analysis of the DNA hydrolysate showed the absence of Structure 4; all the radioactivity was found in the aqueous supernatant obtained upon precipitation of the DNA. These results demon-

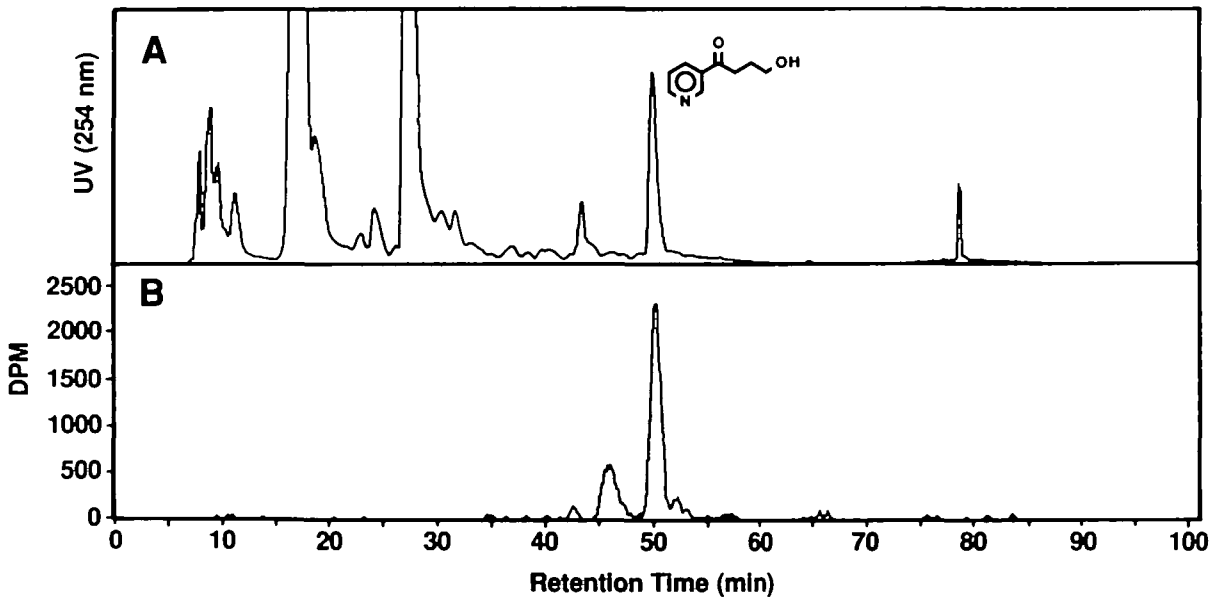


Fig. 2. Chromatograms obtained upon reverse phase HPLC analysis of an acid hydrolysate of hepatic DNA from a rat treated with [5-^{3}H]NNK. (A) Reference 4-hydroxy-1-(3-pyridyl)-1-butanone, (B) detection of radioactivity.

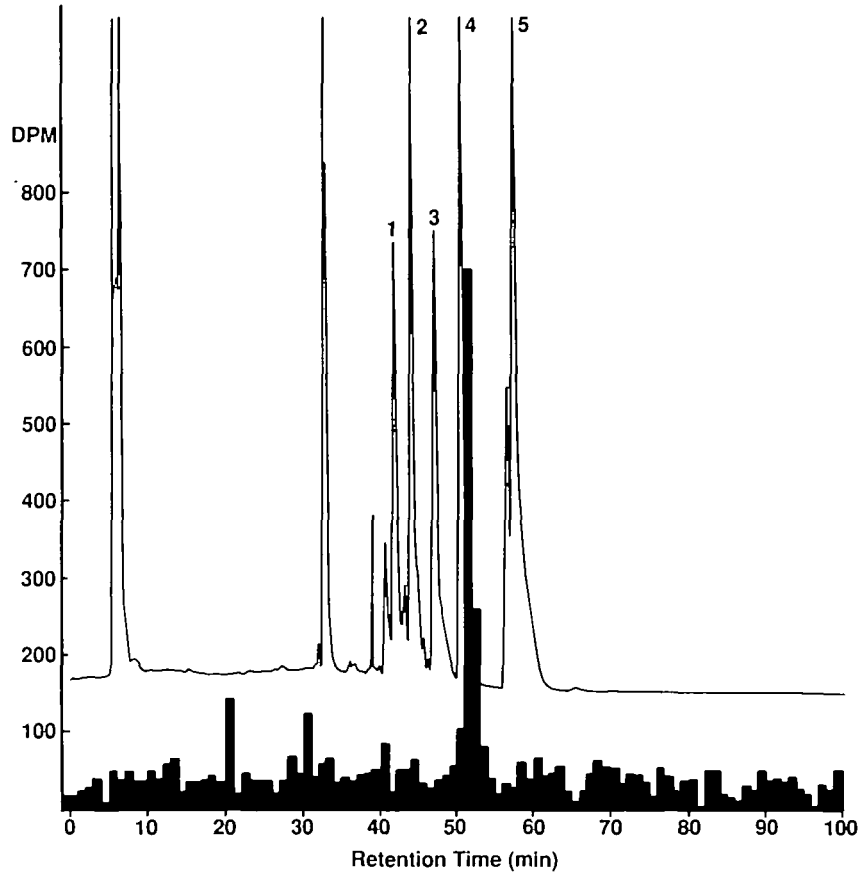


Fig. 3. Chromatogram obtained upon normal phase HPLC analysis of an acid hydrolysate of hepatic DNA from a rat treated with [5-^{3}H]NNK, mixed with the following reference standards: **peak 1**, NNK; **peak 2**, 3-hydroxy-1-(3-pyridyl)-1-butanone; **peak 3**, 5-(3-pyridyl)-2-hydroxytetrahydrofuran; **peak 4**, 4-hydroxy-1-(3-pyridyl)-1-butanone; **peak 5**, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol. Under these conditions, 4-oxo-1-(3-pyridyl)-1-butanone elutes at 42 min. The reference standards were detected by UV at 254 nm. The histogram is radioactivity from the DNA hydrolysates.

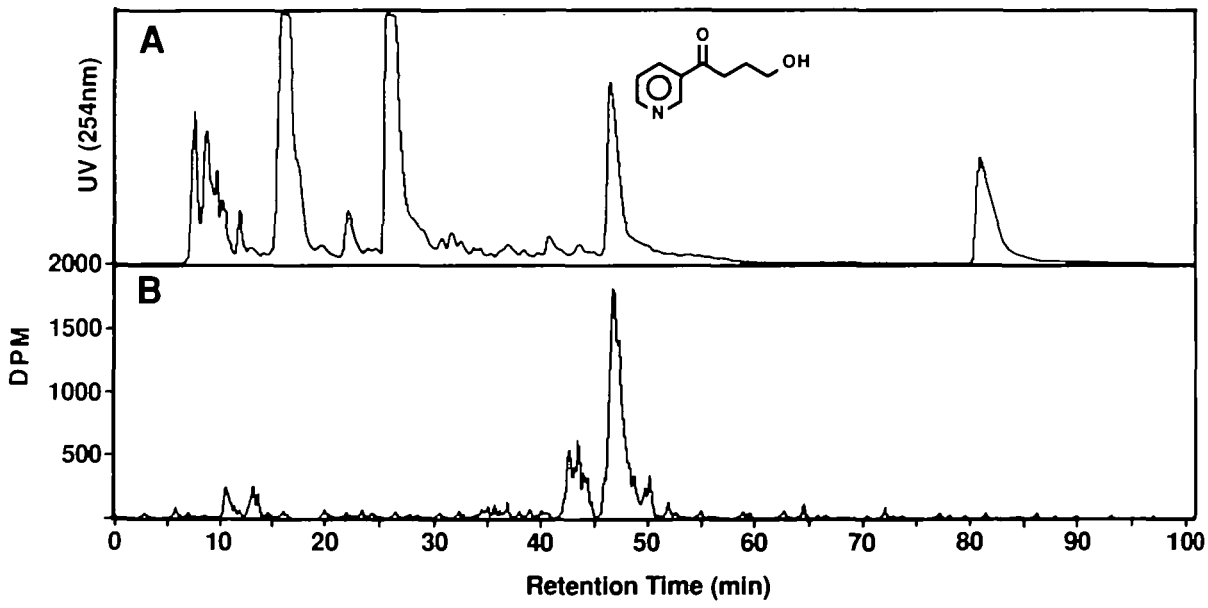
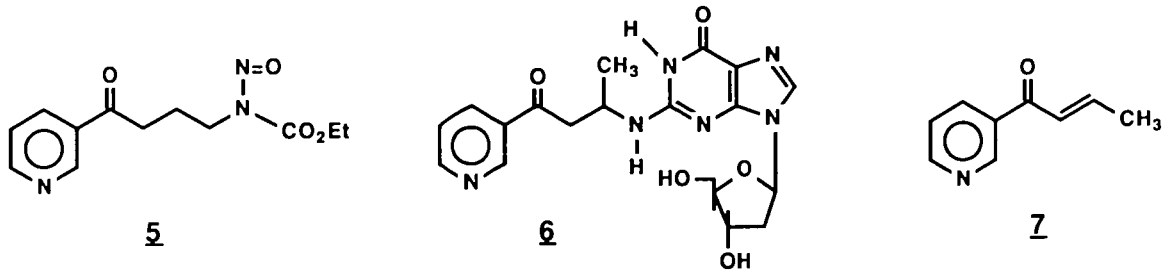


Fig. 4. Chromatograms obtained upon reverse phase HPLC analysis of an acid hydrolysate of hepatic DNA from a rat treated with [5-^{3}H]NNN. (A) Reference 4-hydroxy-1-(3-pyridyl)-1-butanone, (B) detection of radioactivity.

strate that 4-hydroxy-1-(3-pyridyl)-1-butanone does not bind to DNA non-covalently.

It was also possible that the 4-hydroxy-1-(3-pyridyl)-1-butanone which we detected might have been bound to protein or RNA impurities in our DNA preparations, despite our use of protease and RNase in the DNA isolation and purification protocol. The OD_{260}/OD_{280} ratios of our DNA preparations were consistently 1.8 or higher. To further assess purity, DNA preparations were analyzed for protein and RNA contamination. Protein contamination was $<1\%$. RNA contamination was assessed by analysis



5 6 7

Scheme 1

of uridine—the maximum amount detected was <4 μg/mg DNA.

The amount of Structure 4 released upon acid hydrolysis of hepatic DNA from [5-^{3}H]NNK treated rats was approximately 50% of the total radioactivity detected in the DNA. A peak released upon acid hydrolysis and eluting just prior to Structure 4, as illustrated in Figure 2, accounted for approximately 20% of the radioactivity. The structure of this material is unknown; it was not eluted from normal phase HPLC under the conditions illustrated in Figure 3.

Neutral thermal hydrolysis of the DNA isolated from the livers of rats treated with [5-^{3}H]NNK also yielded Structure 4 as a major product. Its identity was confirmed by normal phase HPLC analysis, and reduction with $NaBH_4$ as described above. The amount released was approximately the same as the amount released upon acid hydrolysis. Structure 4 was not released upon treatment of the DNA with 0.2 M NaOH at room temperature.

DNA was also isolated from lung, esophagus, kidney and nasal mucosa of rats treated with [5-^{3}H]NNK, and hydrolyzed with acid. Analysis by reverse phase and normal phase HPLC demonstrated the presence of Structure 4 in lung DNA only.

As illustrated in Figure 1, metabolism of NNN by 2′-hydroxylation should yield the same intermediate, Structure 3, as produced by methyl hydroxylation of NNK, and ultimately the same DNA adduct(s). To test this hypothesis, rats were treated with [5-^{3}H]-NNN, and DNA was isolated from liver, lung, esophagus, nasal mucosa and kidney. The DNA was hydrolyzed with acid and the hydrolysates were analyzed by HPLC. A typical chromatogram obtained upon analysis of a liver DNA hydrolysate is illustrated in Figure 4. Structure 4 was detected in DNA hydrolysates from liver, 0.08 ± 0.01 pmol/mg DNA, but not in any of the other tissues. The identity of Structure 4 was confirmed by normal phase HPLC analysis, and by reduction with $NaBH_4$, as described above. It accounted for approximately 35% of the radioactivity in hepatic DNA. The peak eluting prior to Structure 4 accounted for approximately 15%. Structure 4 was also released by neutral thermal hydrolysis.

Discussion

The results of this study demonstrate that 4-hydroxy-1-(3-pyridyl)-1-butanone is formed upon hydrolysis of DNA isolated from tissues of rats treated with NNK or NNN. This product was most likely produced by hydrolysis of a DNA adduct containing the 4-(3-pyridyl)-4-oxobutyl group. These results provide strong evidence that 4-(3-pyridyl)-4-oxobutylation of DNA occurs in rats treated with NNK and NNN. These observations are consistent with the activation pathway illustrated in Figure 1. The likely alkylating agent is 4-(3-pyridyl)-4-oxobutyl diazohydroxide (Structure 3).

Previous studies of NNK and NNN metabolic activation have suggested the existence of a pathway leading to 4-(3-pyridyl)-4-oxobutylation of DNA. 4-Hydroxy-1-(3-pyridyl)-1-butanone and products of its further metabolism have been detected as metabolites of NNK and NNN *in vitro* and *in vivo* (6,7). Metabolically formed 4-hydroxy-1-(3-pyridyl)-1-butanone originates, at least in part, from hydrolysis of 4-(3-pyridyl)-4-oxobutyl diazohydroxide (Structure 3) or a related intermediate (11,13). The present results are also consistent with recent studies on the alkylation of hemoglobin in rats treated with NNK and NNN. Base hydrolysis of globin yielded 4-hydroxy-1-(3-pyridyl)-1-butanone (9).

Under the conditions used in this study, the highest levels of Structure 4 were released upon hydrolysis of hepatic DNA. Among the other tissues examined, Structure 4 was detected only in lung DNA from rats treated with NNK. The failure to detect Structure 4 in the hydrolysates of nasal mucosa DNA from NNN and NNK treated rats, and in esophageal DNA from NNN treated rats, is probably only a reflection of the sensitivity of the analytical method. Previous studies of the metabolism of NNN and NNK in cultured rat esophagus and nasal mucosa have demonstrated the formation of 4-hydroxy-1-(3-pyridyl)-1-butanone, indicating that the alkylating intermediate, Structure 3, is likely to be produced in these tissues (18,19). Although comparative studies of methylation and 4-(3-pyridyl)-4-oxobutylation of DNA by NNK have not been carried out, it is interesting to note that the highest initial levels of methylation have been observed in nasal mucosa DNA, followed by liver and lung (4,8).

Although this study has demonstrated the binding of the 4-(3-pyridyl)-4-oxobutyl residue to DNA, its point of attachment has not been determined. The release of Structure 4 upon acid hydrolysis, but not base hydrolysis, suggests that the alkylation has occurred on one of the nucleoside residues rather than on phosphate. Among the nucleosides, deoxyguanosine is generally the most reactive toward diazohydroxides, and reaction at the 7-, O^6- or N^2-positions might be anticipated based on previous results (13,20). The release of Structure 4 upon neutral thermal hydrolysis suggests that an adduct at position 7 of deoxyguanosine may constitute a major portion of the bound radioactivity, although other types of adducts also exhibit thermal instability. The acid hydrolysis procedure would be expected to hydrolyze adducts at positions 7-, O^6- or N^2- of deoxyguanosine, as well as modifications of other nucleosides.

In an earlier study of the reaction of 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone (Structure 5, see Scheme 1) with deoxyguanosine, we characterized one of the products as 2′-deoxy-*N*-[1-methyl-3-oxo-3-(3-pyridyl)propyl]guanosine (Structure 6) (13). This was produced at least in part by reaction with deoxyguanosine of the α,β-unsaturated ketone, Structure 7, which is one of the products of solvolysis of Structure 5 (13). Hydrolysis of Structure 6 does not produce Structure 4. Thus Structure 6 would appear to be a relatively minor adduct, if formed, in DNA from rats treated with NNK and NNN. Cellular thiols may partially protect DNA from reaction with Structure 7 that could be generated *in vivo* from NNK or NNN.

As far as we are aware, there have been no previous reports on the structural characterization of the DNA alkylating moiety formed *in vivo* from a cyclic nitrosamine (21,22). The results re-

ported here for NNN support the hypothesis that α-hydroxylation is one mechanism by which cyclic nitrosamines are activated to intermediates that react with DNA. Previous studies have shown that α-acetoxy-*N*-nitrosopyrrolidine gives rise to cyclic 1,N^2-deoxyguanosine adducts, but their presence in DNA of animals treated with *N*-nitrosopyrrolidine has not been established (23). A fluorescent adduct has been detected in the livers of *N*-nitrosopyrrolidine-treated rats but its structure has not been elucidated (24). Tentative evidence for 7-(2-hydroxyethyl)guanine in the liver DNA of *N*-nitrosomorpholine treated rats has been presented, and 1,6-hexanediol has been identified in hydrolysates of liver RNA of rats treated with *N*-nitrosohexamethyleneimine (25,26). Although the present study supports the view that α-hydroxylation of cyclic nitrosamines leads to DNA modification, it is important to note that 4-(3-pyridyl)-4-oxobutylation of DNA was detected for NNN only in hepatic DNA. Nevertheless, NNN rarely induces liver tumors in rats (27–31). Thus the significance of this alkylation product in carcinogenesis by NNN is unclear.

Acknowledgements

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