

## Nicotine-derived *N*-Nitrosamines and Tobacco-related Cancer: Current Status and Future Directions<sup>1</sup>

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The 1982 Report of the Surgeon General of the United States Public Health Service, entitled "The Health Consequences of Smoking," concluded that "cigarette smoking is the major single cause of cancer mortality in the United States. Tobacco's contribution to all cancer deaths is estimated to be 30 percent." The report stated that "85 percent of lung cancer cases are due to smoking and an estimated 50 to 70 percent of oral and laryngeal cancer deaths are associated with smoking." The report noted that "cigarette smoking is estimated to be a factor in over half of esophageal cancer deaths; between 30 and 40 percent of bladder cancers are smoking-related, and up to 30 percent of deaths from pancreatic cancer might be attributable to smoking" (92). Although per capita use of cigarettes among adults (18 years of age and over) has declined from 4345 in 1963 to 3494 in 1983, there are still about 53 million cigarette smokers in the United States (89, 92). Six hundred thirty billion cigarettes were consumed in 1983.

An alarming development is the increasing prevalence of snuff dipping. Snuff dipping is the practice of extracting juices from a pinch of moist fine-cut chewing tobacco, placed between the cheek and the gum. The general popularity of snuff dipping, especially among young people, is a relatively recent trend but women in the southeastern parts of the United States have been snuff dippers for many decades. A recent case-control study showed that the relative risk of oral cavity cancer among white nonsmoking, snuff-dipping women was 4.2. Among chronic users, the relative risk of cancers of the gum and oral cavity approached 50 (96).

In 1983, consumption totaled 21,000 tons of snuff tobacco and 39,000 tons of chewing tobacco (89). Presently, there are an estimated 7 million snuff dippers in the United States (80).

The main reason for the continued use of tobacco, in spite of its well-known adverse health effects, is dependence on nicotine (64). This compound is the major alkaloid<sup>2</sup> in United States tobacco products, typically comprising 1 to 2% of the tobacco. The other tobacco alkaloids are found in significantly lower concentrations than that of nicotine. Important among these are nornicotine, anabasine, and anatabine (82). Their structures are illustrated in Chart 1.

It is well established that both secondary and tertiary amines

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<sup>2</sup> Alkaloid: any of numerous organic bases, containing nitrogen, that occur in seed plants.

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can react with nitrite, yielding nitrosamines (70). More than 300 nitrosamines have been shown to be carcinogenic in one or more of 40 animal species (8,30,66). In the case of secondary amines, nitrosation can be a remarkably rapid reaction, in which the hydrogen attached to nitrogen is replaced by an —NO group in high yields. Tertiary amines react more slowly. Any of the alkyl groups attached to nitrogen is usually detached as a ketone or aldehyde and replaced by the —NO group (65, 88). Thus, nitrosation of the secondary amines nornicotine, anabasine, and anatabine gives the corresponding nitrosamines NNN,<sup>3</sup> NAB, and NAT. Nitrosation of the tertiary amine, nicotine, gives NNN by cleavage of the N—CH<sub>3</sub> bond with loss of formaldehyde or yields NNK or NNA by cleavage of either the 2'-N or 5'-N bond, respectively. The formation of NNN, NNK, and NNA from nicotine and of NNN, NAB, and NAT from nornicotine, anabasine, and anatabine has been confirmed in model studies (43, 71). These alkaloid-derived nitrosamines are called "tobacco-specific nitrosamines."

In agreement with these chemical model studies, all of the tobacco-specific nitrosamines, except NNA, have been detected in cigarette, cigar, and snuff tobacco and in mainstream and sidestream tobacco smoke (54). Their presence in tobacco results from nitrosation of the alkaloids during curing and processing. In cigarette smoke, 25 to 45% of the tobacco-specific nitrosamines originate by transfer from the tobacco and the remainder is pyrosynthesized, probably by reaction of the alkaloids with nitrogen oxides. Since the ribs and stems of the tobacco leaf contain the greatest proportion of nitrate, they have a profound influence on the levels of nitrosamines in tobacco products and in smoke (17, 54). As discussed below, it is likely that tobacco-specific nitrosamines are also endogenously formed in smokers and snuff dippers.

NNN and NNK are strong carcinogens. Thus, they provide a link between nicotine, the habituating factor in tobacco, and tobacco-related cancers.

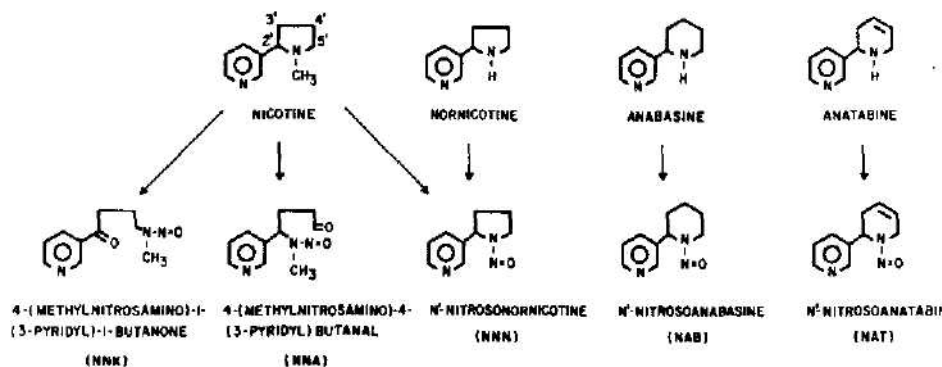
### Occurrence of Tobacco-specific Nitrosamines

There are various ways in which humans may be exposed to tobacco-specific nitrosamines: by inhaling mainstream smoke

<sup>3</sup> The abbreviations used are: NNN, N'-nitrosanonicotine (W refers to the nitrogen of the saturated ring, as opposed to the nitrogen of the pyridine ring which cannot be nitrosated); GC, gas chromatography; NAB, N'-nitrosoanabasine; NAT, W'-nitrosoanatabine; NDEA, N-nitrosodiethylamine; NDELA, N-nitrosodiethanolamine; NDMA, N-nitrosodimethylamine; NNA, 4-(methylnitrosamino)-4-(3-pyridyl)-butanal; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)butan-1-ol; NNK, 4-(methylnitrosamino)-t-(3-pyridyl)-1-butanone (the origin of the term NNK is "nicotine-derived nitrosaminoketone"); TEA, Thermal Energy Analyzer (a highly sensitive detector which is relatively specific to nitrosamines).

NICOTINIC N-NITROSAMINES AND TOBACCO-RELATED CANCER

Chart 1. Tobacco alkaloids and nitrosamines which can be formed from them. With the exception of NNA, all of these compounds are present in tobacco and tobacco smoke.



and/or environmental tobacco smoke;<sup>4</sup> by chewing tobacco and by snuff dipping; and by endogenous formation of such compounds upon uptake of alkaloids and nitrogen oxides or nitrite. Exposures can be determined in a variety of ways, but the most widely used analytical methods are high-performance liquid chromatography or GC coupled to a nitrosamine-specific detector, the TEA (54). GC-TEA has a detection limit of about 0.3 to 0.5 ng of NNN or NNK per injection. According to preliminary data, the detection limit may be increased 5 to 10 times by using capillary GC-TEA.

Table 1 lists data for levels of tobacco-specific nitrosamines in United States chewing tobacco, snuff, cigarette and cigar mainstream smoke, and cigarette sidestream smoke. Tobacco products also contain volatile nitrosamines and NDELA. However, concentrations of these compounds are at least 1 to 2 orders of magnitude lower. Generally, nitrate content of a tobacco product is proportionally related to the yields of nitrosamines. The mainstream smoke yields of cigarettes are greatly influenced by the efficiency of filter tips, and filtration reduces levels of tobacco-specific nitrosamines in proportion to the reduction of tar (54).

Table 2 lists the estimated daily exposure of United States residents to nitrosamines (18, 75). Clearly, the highest exposure to nitrosamines is that of the tobacco consumer. It must be stressed that the quantities listed in Table 2 reflect exposure by inhalation, ingestion, and skin contact but not uptake. The figures calculated for cigarette smoke exposure are derived from machine smoking data obtained under standard smoking conditions and thus neglect such factors as smoking intensity and depth of inhalation of the smokers. These are primarily regulated by the nicotine delivery of a cigarette (92). Table 2 compares exposure data for the general environment without regard for occupational exposure to nitrosamines. The latter can occur in limited areas in the chemical, rubber, and steel industry and in leather tanneries and may reach several  $\mu\text{g}/\text{worker}/\text{day}$  (75). Finally, it needs to be stated that exposure to nitrosamines from tobacco smoke affects not only the tobacco consumer but, in environments polluted by tobacco smoke, also the nonsmoker (54).

In addition to the relatively high exposures of snuff dippers and cigarette smokers to the nitrosamines present in tobacco and tobacco smoke, endogenous formation of tobacco-specific nitrosamines is possible. The snuff dipper who consumes 10 g

<sup>4</sup> "Passive smoke exposure, or environmental tobacco smoke" are terms relating to all combustion products of tobacco that are not retained by inhalation but released into the environment. The major portion of such environmental air pollutants originates from the burning cone of tobacco products between puffs and is called sidestream smoke.

Table 1  
Tobacco-specific nitrosamines in commercial United States tobacco products (5-)

Tobacco product	NNN	NNK	NAT + NAB
<b>A. Smokeless tobacco</b>			
Chewing tobacco (ppb)	3,500-8,200	100-3,000	500-7,000
Snuff (ppb)	800-89,000	200-8,300	200-4,000
<b>B. Mainstream smoke</b>			
Cigarettes-NF <sup>a</sup> (ng/cigarette)	120-950	80-770	140-990
Cigarettes-F (ng/cigarette)	50-310	30-150	60-370
Little cigar-F (ng/cigar)	5,500	4,200	1,700
Cigar (ng/cigar)	3,200	1,900	1,900
<b>C. Sidestream smoke</b>			
Cigarettes-NF (ng/cigarette)	1,700	410	270
Cigarettes-F (ng/cigarette)	150	190	150

<sup>a</sup> NF, cigarette without filter tip; F, cigarette with filter tip.

Table 2  
Estimated exposure of United States residents to nitrosamines<sup>a</sup>

Source of exposure	Nitrosamines	Primary exposure route	Daily intake ( $\mu\text{g}/\text{person}$ )
Beer	NDMA	Ingestion	0.34
Cosmetics	NDELA	Dermal absorption	0.41
Cured meat; cooked bacon	NPYR <sup>b</sup>	Ingestion	0.17
Scotch whiskey	NDMA	Ingestion	0.03
Cigarette smoking	VNA <sup>c</sup>	Inhalation	0.3
	NDELA	Inhalation	0.5
	NNN	Inhalation	6.1
	NNK	Inhalation	2.9
	NAT + NAB	Inhalation	7.2
Snuff dipping <sup>d</sup>	VNA	Ingestion	3.1
	NDELA	Ingestion	6.6
	NNN	Ingestion	75.0
	NNK	Ingestion	16.1
	NAT + NAB	Ingestion	73.4

<sup>a</sup> From "The Health Effects of Nitrate, Nitrite, and N-Nitroso Compounds, National Research Council, 1981 (75), amended by data for snuff dipping (18). In addition, it has been established that, upon inhalation of the air in cars with new upholstery, one is exposed daily to 0.50  $\mu\text{g}$  of NDMA and 0.20  $\mu\text{g}$  of NDEA (75).

<sup>b</sup> NPYR, N-nitrosopyrrolidine; NEMA, N-nitrosoethylmethylamine; NMOR, N-nitrosomorpholine; VNA, volatile nitrosamines.

<sup>c</sup> VNA, NDMA + NEMA + NDEA + NPYR (75).

<sup>d</sup> Brunnmann et al. (18); average values from the leading 5 United States fine-cut tobaccos used for snuff dipping in 1981; assumed daily consumption, 10 g/day; VNA = NDMA + NPYR + NMOR.

of fine-cut tobacco per day ingests 10 to 20 mg of nitrite, 100 to 200 mg of nitrate, and 100 to 200 mg of nicotine (54). Microorganisms in the oral cavity can reduce nitrate to nitrite (75). Smoking of 20 cigarettes daily provides up to 12 mg of NO<sub>x</sub> and 30 mg of nicotine as well as other nitrosatable amines. The nitrosation potential of cigarette smoke has been demonstrated

by the fact that urinary excretion of N-nitrosoproline by cigarette smokers is increased (5.9  $\mu$ g/24 hr) compared to that of nonsmokers (3.6  $\mu$ g/24 hr) who were on an identical diet. The addition to the diet of the nitrosation inhibitor ascorbic acid (1000 mg/day) diminished W-nitrosoproline formation in cigarette smokers (53). These results suggest that tobacco-specific nitrosamines can form endogenously. Indeed, tobacco-specific nitrosamines are present in the saliva of snuff dippers (52). Further studies on the endogenous formation of tobacco-specific nitrosamines in smokers are in progress.

**Carcinogenicity of Tobacco-specific Nitrosamines**

NNN and NNK induce benign and malignant tumors in mice, rats, and hamsters (Table 3). NAB appears to be weakly carcinogenic in rats, whereas NAT is inactive when tested in doses of 2.8 mmol/rat and below. Upon s.c. injection, NNN induces primarily papilloma and carcinoma of the nasal cavity but also some esophageal tumors at doses down to 0.2 mmol/rat {1 mmol/kg). Administration of NNN in the drinking water to rats causes benign and malignant tumors of the esophagus in addition to nasal cavity tumors. These results indicate that the organospecificity of NNN depends on the route of administration.

NNK is the most potent carcinogen among the tobacco-specific nitrosamines. It induces lung tumors in mice; nasal cavity, tracheal, and lung tumors in hamsters; and nasal cavity, lung, and liver tumors in rats. Perhaps the most important observation is the induction of lung adenomas and adenocarcinomas in hamsters in response to doses as low as 0.005 mmol/hamster, and squamous carcinoma and adenocarcinoma of the lung in rats at doses of 0.2 mmol/rat (1 mmol/kg). In both hamsters and rats, lower doses must be tested before the possible contribution of NNK to the lung cancer risk of cigarette smokers can be estimated. (A cigarette smoker inhales on the average about 0.07  $\mu$ mol NNK/kg per year.) Additional bioassays should elucidate possible additive or synergistic carcinogenic effects among the tobacco-specific nitrosamines.

Several aspects of the carcinogenicity of tobacco-specific nitrosamines are currently being studied, and others remain to

be explored. An extensive body of epidemiological evidence has indicated that the combination of alcohol and tobacco consumption represents a major composite risk factor for cancer of the upper digestive tract (92). One working hypothesis presumes that tobacco smoke is the source of the carcinogenic stimuli and that alcohol facilitates the activation of tobacco-associated carcinogens. Since NNN is the most abundant carcinogen in tobacco and tobacco smoke, it has been chosen for model studies. In hamsters on an alcohol-containing liquid diet, NNN did not induce a higher incidence of upper respiratory tract tumors than in hamsters of the same strain on a control liquid diet (67). In rats on an alcohol-containing liquid diet, the induction of esophageal tumors by NNN was inhibited and that of nasal cavity tumors was increased relative to tumors in the control group. Biochemical studies with rats given alcohol showed that the  $\alpha$ -carbon hydroxylation of NNN was increased in the nasal mucosa but not in other tissues (23). This finding supports the hypothesis that alcohol facilitates the activation of tobacco-associated nitrosamines. However, the effects of alcohol on the pharmacokinetics and distribution of these nitrosamines are probably also important.

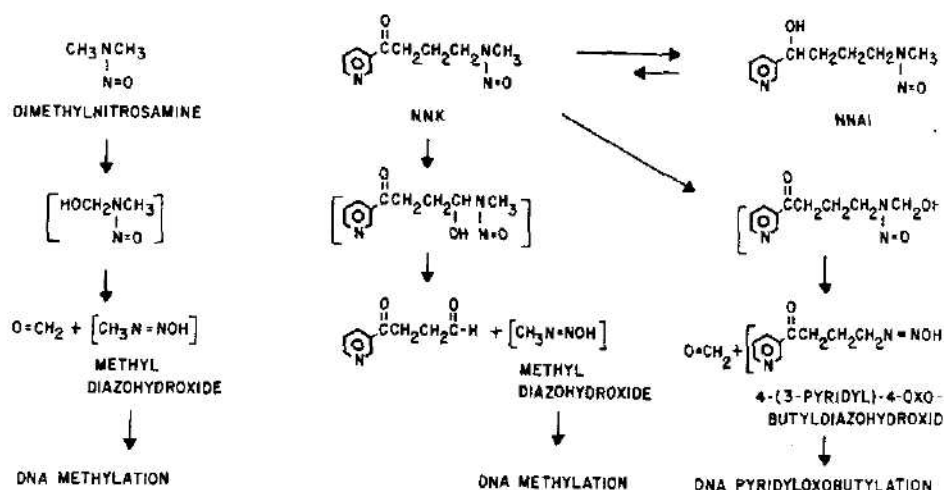
Several epidemiological studies have held that the induction of lung cancer in cigarette smokers who worked within uranium mines or who were asbestos workers is a synergistic effect (92). We do not know whether NNN or NNK play a role in such synergistic effects of cigarette smoke and occupational respiratory carcinogens. However, *in vitro* studies have shown that the surface of asbestos particles enhances nitrosamine formation from nicotine and nitrogen dioxide (3). This observation appears to support the working hypothesis that the endogenous formation of tobacco-specific nitrosamines in the lungs of cigarette smokers is enhanced in the presence of asbestos particles.

We are not aware of studies specifically aimed at exploring the effects of nutrients on the carcinogenic potencies of NNN or NNK. This would be important in view of epidemiological findings which have indicated that the lack of certain vitamins, zinc, and possibly selenium may play a contributing role in the risk for cancer of the upper digestive tract, larynx, and lung of tobacco consumers (20, 33, 97).

Table 3  
Carcinogenicity of tobacco-specific nitrosamines

Nitrosamine	Species and strains	Route of application	Principal target organs	Dose	Ref.
NNN	A/J mouse F344 rat	i.p.	Lung	0.12 mmol/mouse	9, 22, 41
		s.c.	Nasal cavity, esophagus	0.2-3.4 mmol/rat	23, 42, 59
		p.o.	Esophagus, nasal cavity	1.0-3.6 mmol/rat	23, 47, 58
	Sprague-Dawley rat Syrian golden hamster	p.o.	Nasal cavity	8.8 mmol/rat	86
		s.c.	Trachea, nasal cavity	0.9-2.1 mmol/ hamster	49, 55, 67
NNK	A/J mouse F344 rat	i.p.	Lung	0.12 mmol/mouse	22, 41
		s.c.	Nasal cavity, lung, liver	0.2-2.8 mmol/rat	42, 59
	Syrian golden hamster	s.c.	Trachea, lung, nasal cavity	0.9 mmol/hamster 0.005 mmol/hamster	38, 55
NAT	F344 rat	s.c.	None	0.2-2.8 mmol/rat	59
NAB	F344 rat Syrian golden hamster	p.o.	Esophagus	3-12 mmol/rat	10, 59
		s.c.	None	2 mmol/hamster	49
NNA	A/J mouse	i.p.	None	0.12 mmol/mouse	41

Chart 2. Metabolic activation of dimethylnitrosamine and NNK. Structures in brackets are likely intermediates that have not been isolated. For clarity, several metabolic products of NNK and NNAL have been omitted. See Refs. 22, 27, and 46 for details.



### Metabolic Activation of Tobacco-specific Nitrosamines

Nitrosamines are enzymatically converted to unstable electrophilic intermediates which can react with nucleophilic centers in cellular macromolecules. While the structures of these electrophilic intermediates, or ultimate carcinogens, have not been unambiguously characterized for any nitrosamine, evidence indicates that they may be alkyl diazohydroxides (69). A generally accepted scheme for metabolic activation of NDMA is illustrated in Chart 2. Enzymatic hydroxylation of the carbon attached to the nitroso nitrogen, the  $\alpha$ -carbon, leads to the unstable intermediate *W*-methyl-*W*-hydroxymethylnitrosamine. This has been synthesized and has a half-life of approximately 10 sec at pH 7 (72). It spontaneously decomposes to a methylating species, probably methyl diazohydroxide. This reacts with DNA yielding, after hydrolysis, 7-methylguanine,  $O^6$ -methylguanine, 3-methyladenine, and a spectrum of other products (76, 84). These are the same products formed by the nonenzymatic reaction with DNA of such methylating agents as *N*-methyl-*N*-nitrosourea, methyl methanesulfonate, and dimethyl sulfate, although the relative yields of the various products depend upon the nature of the methylating agent and the reaction conditions (76, 84).

Extensive studies have shown that  $O^6$ -methylguanine is one important product relating to tumor initiation.  $O^6$ -Methylguanine causes miscoding, leading to incorporation of thymidine during DNA replication. Its presence in a particular cell type during replication depends on the extent of its formation by metabolism of NDMA or other methylating agents in that cell type and on the activity of the repair enzyme,  $O^6$ -methylguanine-DNA methyltransferase. In certain cases, there is reasonably good agreement between levels of  $O^6$ -methylguanine in DNA and susceptibility to tumor induction in tissues of animals treated with agents such as NDMA or *N*-methyl-*N*-nitrosourea (76, 86).

The structural relationship of NDMA and NNK is evident from Chart 2; and by analogy to the metabolic pathway illustrated for NDMA, hydroxylation of the methylene carbon  $\alpha$  to the nitroso nitrogen of NNK should yield a methylating intermediate. This expectation, which was supported by metabolism studies (46), has been confirmed recently. Treatment of rats with NNK led to formation of 7-methylguanine and  $O^6$ -methylguanine in DNA of lung, liver, and nasal mucosa<sup>5,6</sup> (25). The level of methylation

<sup>5</sup> P. G. Foiles, N. Trushin, and A. Castonguay. Development of a biotin-avidin enzyme-linked immunosorbent assay for  $O^6$ -methyldeoxyguanosine and its application to measurement of DNA methylation by the tobacco-specific carcinogen 4-(methylnitrosamino)-3-(3-pyridyl)-1-butanone, submitted for publication.

<sup>6</sup> F.-L. Chung, M. Wang, and S. S. Hecht. Effects of dietary indoles and isothiocyanates on *W*-nitrosodimethylamine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone  $\alpha$ -hydroxylation and DNA methylation in rat liver, submitted for publication.

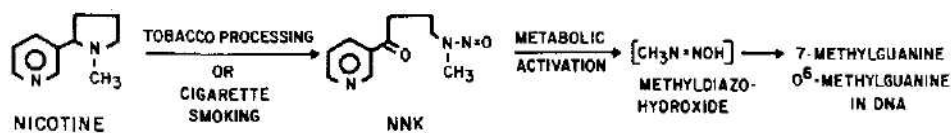
was substantially lower than that induced by NDMA, and the kinetics of methylation was apparently quite different from that observed with NDMA. These differences between NDMA and NNK probably result from the relative structural complexity of NNK. For example, a major metabolic reaction is reduction of the carbonyl group to the alcohol, NNAL, which may act as a slow release form of NNK (1, 26).

Independent of the mechanism, it is significant that  $O^6$ -methylguanine is formed in DNA upon treatment of rats with NNK. The possible relationship of this miscoding base to carcinogenesis, by various methylating agents has been indicated. It is an important consideration that human exposure to NNK, through smoking or snuff dipping, greatly exceeds any known exposure to other methylating agents including NDMA. Studies with cultured human buccal mucosa, trachea, esophagus, bronchus, peripheral lung, and bladder have shown that these tissues can metabolize NNAL by  $\alpha$ -carbon hydroxylation (24). Therefore, it is reasonable to expect that snuff dippers or smokers will have  $O^6$ -methylguanine in the DNA of their oral or bronchial tissues. The reported inhibition of repair of  $O^6$ -methylguanine by cigarette smoke may be significant in this respect (83). Thus, the formation of NNK from nicotine during tobacco processing and cigarette smoking and its metabolic conversion to a methylating agent provide a pathway by which the *W*-methyl group of nicotine can methylate DNA, as illustrated in Chart 3. This demonstrates the mechanistic link between nicotine and tobacco-related cancer.

Much less is known about the properties of 4-(3-pyridyl)-4-oxobutyl diazohydroxide, the likely electrophilic intermediate formed upon methyl hydroxylation of NNK as illustrated in Chart 2. It is well established that this process occurs *in vitro* and *in vivo*, since the products formed upon reaction with  $H_2O$  of 4-(3-pyridyl)-4-oxobutyl diazohydroxide are metabolites of NNK (26, 46). 4-(3-Pyridyl)-4-oxobutyl diazohydroxide can be generated chemically by hydrolysis of 4-(carboxynitrosamino)-*t*-(3-pyridyl)-1-butanone (see structure in Table 4), a reaction which is analogous to the formation of methyl diazohydroxide from methylnitrosourea (40). The mutagenicity data summarized in Table 4 clearly show that 4-(3-pyridyl)-4-oxobutyl diazohydroxide can damage DNA and is more effective in causing mutations in *Salmonella typhimurium* than is methyl diazohydroxide (44). Thus it is possible that methyl hydroxylation of NNK, yielding 4-(3-pyridyl)-4-oxobutyl diazohydroxide may be more important in the expression of NNK carcinogenesis than is methylene hydroxylation, which results in DNA methylation. The structures of the DNA adducts formed by methyl hydroxylation of NNK are not known at present.

$\alpha$ -Carbon hydroxylation of NNK would yield the diazohydrox-

Chart 3. Scheme linking nicotine, the major tobacco alkaloid and habituating factor in tobacco, to formation of the promutagenic DNA adduct, O<sup>6</sup>-methylguanine.



ides illustrated in Chart 4. Extensive metabolism studies have shown that these reactions do occur *in vitro* and *in vivo* and, as with NNK, the products expected upon reaction of the diazohydroxides with H<sub>2</sub>O have been isolated as major metabolites of NNN (39). One of the diazohydroxides formed from NNN, by 2'-hydroxylation, is 4-(3-pyridyl)-4-oxobutyl diazohydroxide which is the same compound produced by methyl hydroxylation of NNK. The other, formed by 5'-hydroxylation, is 1-(3-pyridyl)-3-formylpropyl diazohydroxide which has 2 electrophilic centers, the diazohydroxide and aldehyde groups. Such diazohydroxides can react *in vitro* with deoxyguanosine mainly at positions 1 and W<sup>2</sup> to give cyclic propanodeoxyguanosine adducts (28). This reactivity contrasts to that of methyl diazohydroxide, which reacts mainly at position 7 of deoxyguanosine. The extent to which 1-(3-pyridyl)-3-formylpropyl diazohydroxide might form cyclic 1, N<sup>2</sup>-propanodeoxyguanosine adducts or related adducts in DNA is presently under investigation. Data on the structure and persistence of the DNA adducts formed from NNN and NNK will enhance our understanding of their mechanisms of carcinogenesis. Based on presently available data from studies of metabolism and from structure-mutagenicity and structure-carcinogenicity experiments,  $\alpha$ -carbon hydroxylation appears to be the major pathway of metabolic activation of NNK and NNN (22, 39, 44-46).

NNK induces tumors in the nasal cavity, lung, and liver of rats, while NNN causes tumor formation in the nasal cavity and esophagus (Table 3). If the mechanism of metabolic activation of NNK and NNN is  $\alpha$ -carbon hydroxylation, then this reaction probably must occur in the target tissues because the  $\alpha$ -hydroxynitrosamines and diazohydroxides formed are most probably too reactive to be transported from one tissue to another, and there is presently no evidence that they are conjugated. Studies of target tissue metabolism of NNK and NNN have clearly shown that they do contain substantial enzyme activity for  $\alpha$ -carbon hydroxylation of both nitrosamines. The rat nasal mucosa appears to have the highest enzymatic activity for metabolic activation of NNK and NNN, of the various tissues studied (13, 15, 23). The rat esophagus has high activity for  $\alpha$ -hydroxylation of NNN and shows a remarkable degree of selectivity, in that 2'-hydroxylation of NNN is favored over 5'-hydroxylation, in contrast to several other tissues (23, 39, 45). The high capacity of the rat nasal mucosa to metabolize NNK and NNN by  $\alpha$ -carbon hydroxylation is in agreement with the hypothesis that it is an activation pathway, since the nasal cavity is a major site of tumor development. While it is clear that metabolism by  $\alpha$ -carbon hydroxylation may be necessary for tumor initiation, it is not sufficient since tissues such as rat liver can catalyze this reaction but do not develop tumors. Other factors- which will probably influence the susceptibility of a given tissue to tumor initiation by NNK and NNN are the extents of the various competing metabolic processes [e.g., carbonyl reduction *versus* N-oxidation *versus* methylene or methyl hydroxylation of NNK], the nature of the DNA adducts formed [e.g., methylation *versus* 4-(3-pyridyl)oxobutylation], the repair of these adducts, and their presence during replication.

A practical consequence of the reactivity of the intermediates

Table 4  
Mutagenic activities of methyl nitrosourea and 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone in *S. typhimurium* TA 1535 and TA 100<sup>a</sup>

Dose ( $\mu\text{mol}/\text{plate}$ )	His <sup>+</sup> revertants/plate			
	Methylnitrosourea		4-Carbethoxynitrosamino-1-(3-pyridyl)-1-butanone	
	TA 1535	TA 100	TA 1535	TA 100
$4 \times 10^{-1}$	2270	2071	2107	2260
$3 \times 10^{-1}$	2142	1916	2101	2104
$2 \times 10^{-1}$	2406	2122	1890	1984
$1 \times 10^{-1}$	2226	2146	1751	1878
$4 \times 10^{-2}$	1230	797	1225	1379
$4 \times 10^{-3}$	19	7	571	551
$4 \times 10^{-4}$	20	7	136	56
$4 \times 10^{-5}$	17	3	32	0

<sup>a</sup> For details, see Ref. 44.

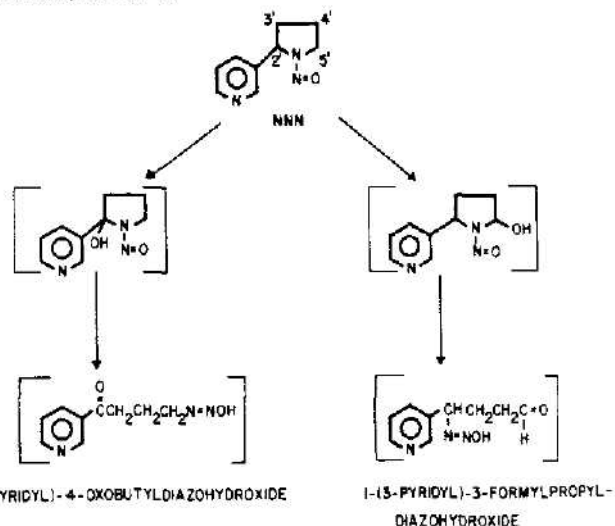


Chart 4. Formation of diazohydroxides from NNN. Metabolic studies have indicated that these intermediates are produced *in vitro* and *in vivo*. See Refs. 22 and 39 for details.

formed by  $\alpha$ -carbon hydroxylation of NNK and NNN is that whole-body autoradiography can be used to identify the tissues that have the capacity to activate these nitrosamines (14, 93). Whole-body autoradiography has been performed with [2'-<sup>14</sup>C]NNN and [carbonyl-<sup>14</sup>C]NNK (14, 27, 93). Sections are washed with acid to remove unbound metabolites which are all acid soluble. In this way, bound radioactivity can easily be detected. The nasal mucosa, mucosa of the trachea and bronchi, and liver tissues of the rat showed the highest bound label 4 hr after treatment with [carbonyl-<sup>14</sup>C]NNK (26). Four hr after treatment of rats with [2'-<sup>14</sup>C]NNN, bound label was present in the nasal mucosa, tracheobronchial mucosa, and esophagus (15). These results agree remarkably well with the tumor data. The whole-body autoradiography technique has recently been applied to pregnant C57BL mice treated with [carbonyl-<sup>14</sup>C]NNK (27). An important finding was that bound radioactivity was present in the tissues of the nose, lung, and liver of 18-day-old fetuses. Parallel meta-

bolic studies showed that these tissues can  $\alpha$ -carbon hydroxylate NNK. These results suggest that NNK could act as a transplacental carcinogen, as has been observed with related compounds such as NDEA (73). These findings have relevance to the problem of cigarette smoking by pregnant women.

An important question is whether or not the target tissues of NNK and NNN carcinogenicity are likely to be the same in laboratory animals and humans. Available data on the metabolism of NNK and NNN in cultured rat and human tissues suggest that the target tissues may not be the same. For example, cultured rat esophagus preferentially hydroxylates NNN at the 2'-position and apparently does not carry out pyridine W-oxidation to a significant extent (39). Cultured human esophagus, obtained at autopsy, has much lower enzyme activity for NNN metabolism than does rat esophagus and preferentially carries out pyridine W-oxidation and 5'-hydroxylation (24). The problem is further complicated by the wide variation in the ability of human tissues to metabolize NNK and NNN, as has been observed in studies of human tissue metabolism of other carcinogens (4, 24, 36). DNA adduct studies in human tissues will be necessary to provide further information on potential susceptibility to NNK and NNN carcinogenesis. It is notable, however, that human tissues which are exposed to NNK and NNN in tobacco chewers and smokers fulfill the necessary condition of being able to catalyze  $\alpha$ -carbon hydroxylation. These data provide a further connection between exposure to nicotine-derived nitrosamines and potential for cancer development.

#### **Possible Role of Tobacco-specific Nitrosamines in Tobacco-related Cancer**

Tobacco contains more than 2500 compounds and tobacco smoke more than 3800 (31) including tumor initiators such as the polynuclear aromatic hydrocarbons (98), tumor promoters, cocarcinogens, and organ-specific carcinogens (92, 98). These large numbers of tobacco and tobacco smoke constituents make it unlikely that the total carcinogenic activities of tobacco products can be explained by individual compounds or groups of compounds. Thus, research has focused on major groups of tumorigenic agents, especially those carcinogens that are unique for tobacco and its smoke. Among them, the tobacco-specific nitrosamines play a prominent role. They derive from the habituating compound nicotine, they are present in high concentrations, and they are powerful carcinogens. However, despite our ever-increasing knowledge on the carcinogenic effects of tobacco-specific nitrosamines, we can at this time only assume that these agents play a major role for the increased cancer risk of tobacco chewers and smokers.

Evidence for an association of nicotine-derived carcinogens and increased human cancer risk is perhaps most strongly supported by the observation of oral cancer rates among long-term snuff dippers and by the finding of high levels of tobacco-specific nitrosamines in this tobacco product as well as in the saliva of snuff dippers. The tobacco-specific nitrosamines are the only known carcinogens in snuff, although one may assume that snuff contains trace amounts of  $^{210}\text{Po}$  (35). Specific areas of the snuff dippers' gums are exposed daily and for many hr to the carcinogenic stimuli of NNN and NNK. Consumption of 10 g of fine-cut tobacco per day entails exposure to about 0.15 to 0.17 mmol of NNN and 0.025 to 0.034 mmol of NNK per year

(Table 2). In the rat, snuff induces neoplastic changes in the oral mucosa and, upon infection with herpes simplex virus 1, squamous carcinoma (51).

Case control studies have shown that chronic users of snuff have an about 50-fold increased risk for cancer of the gum and buccal mucosa compared to controls and that the risk increases with the duration, *i.e.*, the number of years of snuff dipping (S 68,92,96). Low intake or lack of fruits and vegetables increase the risk for oral cancer among snuff dippers (97). This observation is consistent with the hypothesis that reduced intake of certain micronutrients, especially of vitamin C and carotenoids, increases the susceptibility of epithelial tissues to the insults of carcinogenic stimuli (20, 33). Lastly, abstinence from snuff dipping (and smoking) reduces the occurrence of neoplastic changes in the oral mucosa of snuff dippers (78).

A recent case-control study from the National Cancer Institute reported an elevated risk for squamous cell tumors and adenocarcinoma of the nasal cavity in snuff dippers and smokers (12). Despite the fact that the histological types of most nasal cavity tumors induced experimentally by NNN and NNK (Table 3) are not identical to the malignant tumors occurring in tobacco users, we contend that the malignant potential of NNN and NNK in laboratory animals has significant implications.

Studies from Asia have reported a significantly increased risk<sup>1</sup> for cancer of the mouth, oropharynx, and esophagus in chewers who consume tobacco alone or in combination with betel quic (96, 97). Extracts of Indian chewing tobacco preparations are carcinogenic in laboratory animals (7), and the tobacco-specific nitrosamines are the only carcinogens which have been detected unambiguously in these products (54). NNN, NNK, and NAT have also been found in the saliva of tobacco chewers in India (87, 95).

Although polynuclear aromatic hydrocarbons are undoubtedly important in tobacco smoke carcinogenesis, several observations lead to the assumption that NNN and NNK also play a role in the increased risk for cancer of cigarette smokers. It has been calculated that the "average cigarette smoker" inhales per year about 0.012 mmol of NNN and 0.005 mmol of NNK (Table 2). The tobacco-specific nitrosamines come in direct contact with the tissues of the upper alimentary tract, trachea, and lung. Studies have shown that these specific human tissues can metabolically activate NNN and NNK (24). These observations support the concept that the tobacco-specific nitrosamines contribute to the smokers' increased risk of cancer of the oral cavity, pharynx, larynx, lung, and esophagus. These carcinogens have not been detected in the blood of smokers because of the limited sensitivity of presently available methods, but their existence and that of their metabolites in circulating blood can be assumed. Thus, these compounds will be transported to liver, pancreas, kidney, and bladder, organs at increased risk of cancer in smokers (91,92, 99). This consideration is important since, in addition to certain aromatic amines, the tobacco-specific nitrosamines are the only known organ-specific carcinogens in cigarette smoke (92).

A recent epidemiological study reported an increased risk of bladder cancer among individuals with a history of urinary infections, and especially among cigarette smokers (61). Since it is known that, during bladder infection, urinary nitrate is reduced to nitrite and since the urine of patients contains volatile nitrosamines (16, 37, 48), it is also likely that tobacco-specific nitro-

samines are formed in the urinary bladder during infection. This concept is supported by the fact that urine contains not only nicotine but also nicotine N'-oxide and that the alkaloid but, even more so the N'-oxide, can be nitrosated to NNN (63). Furthermore, thiocyanate, which is present in the urine of smokers in elevated concentrations (92), can catalyze N-nitrosamine formation (11). Confirmation of this concept would further enhance the hypothesis that NNN and NNK contribute to the increased risk of cigarette smokers for cancer of the urinary tract.

Since 1981, several prospective and case-control studies have indicated an association between passive smoke exposure<sup>4</sup> and lung cancer (29, 50, 90, 92). A number of other investigations have failed to confirm such an association (60, 62, 92). This lack of accord is not surprising due to the many sources of bias and confounders inherent in cases of weak associations. The analyses of saliva, serum, and urine of "passive smokers" support the concept of a weak association because levels of nicotine and cotinine in these physiological fluids amount to merely a few (not exceeding 3) % of the levels recorded for active cigarette smokers (56, 91, 93, 94). If an association of passive smoke exposure and lung cancer were to be established, nitrosamines should be considered suspect etiological agents. It is of importance to note that the sidestream smoke of tobacco products contains higher amounts of volatile nitrosamines (NDMA, 0.35 to 1.0  $\mu$ g/cigarette) and of NNN and NNK (Table 1) than does mainstream smoke [0.001 to 0.02  $\mu$ g NDMA/cigarette (54)]. Although sidestream smoke is quickly diluted by air, nitrosamines can reach measurable concentrations in certain enclosed environments (54). According to one prospective study, "passive smokers" have an increased risk of nasal sinus cancer (50). Also, one case-control study has incriminated the uptake of sidestream smoke nitrosamines by women as a possible risk factor for childhood brain tumors in their offsprings (79). In summary, an association between cancer and nitrosamines as tobacco-derived air pollutants should at this time be considered only as a working hypothesis requiring further studies.

### Prospects for Reducing Exposure to Tobacco-specific Nitrosamines

The only certain way of eliminating the risk associated with exposure to tobacco or tobacco smoke and thus to tobacco-specific nitrosamines is the cessation of tobacco usage. However, despite extensive educational programs, there are today in the United States alone 53 million cigarette smokers, 8 million pipe and cigar smokers, and 7 million snuff dippers. Therefore, product modifications aimed at reducing levels of tobacco-specific nitrosamines are mandatory. Precautions must also be taken to minimize the potential precursors for endogenous nitrosamine formation. In the case of chewing tobacco and fine-cut tobacco for snuff dipping, it appears that one effective method to reduce NNN and NNK is to utilize tobaccos that are low in nitrate (2, 17). For commercial fine-cut tobaccos, it has been shown that changes in processing, including modification of the fermentation procedure, lead to a very substantial reduction of tobacco-specific nitrosamine concentrations (18, 19). It has also been reported that the packaging of snuff in individual small airtight aluminum pouches reduces the formation of tobacco-specific nitrosamines during storage (52). While it seems feasible to use ascorbic acid or other nitrosation inhibitors during the processing

of chewing tobacco and of snuff, we are not aware of studies or processes which have explored this concept.

Tobacco-specific nitrosamine reduction in mainstream cigarette smoke by filter tips is concomitant with reduction of tar but not selective as it is for volatile nitrosamines (54). A major reduction of nicotine in tobacco smoke will lead either to products of very limited consumer acceptability or to increased smoking intensity (92). Thus, this approach appears to be impractical as a method for reducing tobacco-specific nitrosamines. A promising method for selectively reducing nitrosamines in cigarette smoke is the use of tobaccos that are low in nitrate (2) and the exclusion of nitrate-rich stems and ribs (17). Addition of nitrosation inhibitors to smoking tobacco is not recommended unless it can be clearly demonstrated that the combustion products of such additives do not increase the toxicity and/or tumorigenicity of the resulting smoke.

### Prospects for Assays in Humans

The 68 million tobacco users in the United States are a unique group for assessing nitrosamine carcinogenesis in humans. With the possible exception of workers in certain occupational situations, no other population group is chronically exposed to such high levels of nitrosamines. While data on the carcinogenicity and metabolism of nitrosamines in laboratory animals are extensive, little is known about the effects of these compounds in humans. Important aspects include their metabolic activation and detoxification, pharmacokinetics, macromolecular binding, and organ specificity. Studies of nitrosamine metabolism and carcinogenesis in humans require the development of reliable ultra-sensitive analytical methods which can be routinely applied by clinical chemists to large numbers of tobacco users for appropriate risk assessment.

Our initial studies in this area involved the attempted detection in smokers' urine of NNN and one of its metabolites, N'-nitrosonornicotine 1-N-oxide. These experiments were not successful because NNN is extensively metabolized and, although we did detect traces of NNN in smokers' urine, we could not rule out the possibility of artifact formation. Methods for detection of the NNN metabolite, N'-nitrosonornicotine 1-N-oxide, were not sufficiently sensitive. The other major metabolites of NNN can also be formed metabolically from nicotine and would therefore not be useful for studies in smokers. However, the observation that NNAL is a major metabolite of NNK, particularly in human tissues, has suggested new approaches. Quantitation of NNAL as its monophosphate, using postlabeling with <sup>32</sup>P, might be feasible. This approach is currently under investigation. Accurate measurement of circulating levels of NNAL in smokers' blood would allow us to quantify an effective dose of NNK. Presently, such an estimate is based on the measured levels of NNK in cigarette smoke or snuff. This disregards 2 major factors: the tendency of smokers to compensate for low nicotine delivery by changing inhalation patterns; and the *in vivo* formation of nitrosamines in smokers (57). There is little doubt that nicotine is subject to endogenous nitrosation in view of the findings of elevated levels of N-nitrosoproline in smokers' urine. Dosimetry studies in humans will allow realistic risk assessment by providing data which can be compared with the relative carcinogenic potential of NNK in various animals and tissues.

The quantitation of nicotine-derived nitrosamine-macromole-

cule adducts in smokers or chewers is a potentially exciting area of research because levels of these adducts could provide an index of an individual's capacity to metabolically activate these carcinogens. Such data might eventually lead to an estimate of susceptibility to tobacco-related cancer. Nicotine-derived nitrosamines are better candidates for this experimental approach than are other carcinogens such as benzo(a)pyrene, because they occur only in tobacco and tobacco smoke. Ultrasensitive methods using either immunochemical techniques or postlabeling with  $^{32}\text{P}$  are presently available for measurement of carcinogen-DNA adducts and, in some cases, have already been applied to humans (32, 34, 74, 77). A sensitive biotin-avidin enzyme-linked immunosorbent assay for measurement of  $\text{O}^6$ -methyldeoxyguanosine in DNA has been developed in our laboratory and will be applied to human tissues exposed to NNK.<sup>5</sup> The detection of hemoglobin adducts of NNK or NNN is also under investigation. This approach has been used previously for monitoring human exposure to ethylene oxide and for experiments with methylating agents (6). An advantage of this strategy is the relative stability of circulating hemoglobin in humans (120 days) which can provide a cumulative index of exposure and activation.

#### Postscript

In the past decade, extensive studies have conclusively demonstrated that the nicotine-derived nitrosamines NNK and NNN are important in tobacco carcinogenesis. These nitrosamines, as well as NAB and NAT, are present in relatively high concentrations in unburned tobacco and in mainstream and sidestream tobacco smoke. In addition, there is strongly suggestive evidence that they are formed endogenously in snuff dippers and smokers. NNK and NNN are potent carcinogens in laboratory animals. It is particularly notable that NNK induces lung tumors in rats and hamsters. The carcinogenic properties of NNK and NNN are partially due to their metabolic conversion to electrophilic intermediates. Among these, methyl diazohydroxide formed from NNK leads to  $\text{O}^6$ -methylguanine in DNA. The metabolic activation of NNK and NNN occurs in tissues of laboratory animals and humans. These data on the occurrence, carcinogenicity, and metabolic activation of nicotine-derived nitrosamines strongly suggest that these compounds are important in the development of tobacco-related cancers in humans.

As long as society condones tobacco usage, millions of people will be voluntarily or involuntarily exposed to tobacco carcinogens including the tobacco-specific nitrosamines. Whereas progress has been achieved in reducing toxic agents and carcinogens in tobacco products, the prognosis for substantial reduction of nicotine is disappointing. Nicotine, the precursor for the highly carcinogenic NNN and NNK, is considered to be the leading factor for the tobacco habituation (64). The majority of smokers of low-yield cigarettes will compensate for the low nicotine delivery by intensifying their smoking habit (57, 92). Based on this realization, it has been suggested that the delivery of tar be reduced but that the nicotine delivery be kept at a medium level and the smokers' compensation be thereby inhibited (81). This strategy, however, disregards the importance of nicotine as a major precursor for tobacco-specific nitrosamines and as the major habituating agent in cigarette products. In view of the major role and potencies of these carcinogens, the strategy of reducing tar without reducing nicotine is not acceptable as a

practical solution.

Although the reduction of nitrate in tobacco is a promising measure for the reduction of tobacco-specific nitrosamine formation, we place major emphasis on further elucidation of the mode of action of these carcinogens including their metabolic activation, DNA binding, and DNA repair. The delineation of the processes is expected to lead to the development of methods for assessing levels of nitrosamine metabolites in smokers and for early detection of chemical lesions in cells from oral, lung and bladder epithelia.

The modifying effects of dietary constituents on tobacco-specific nitrosamine carcinogenicity also require mechanistic studies. Epidemiological investigations have indicated that sufficient intake of nutrients, especially of green vegetables and fruits may be an important inhibitor of the development of oral and oropharyngeal cancer in snuff dippers and of lung cancer due to smoke exposure (20,33, 50,97). Biochemical studies and bioassays are needed to evaluate the effects of dietary constituent on the carcinogenicity of NNN and NNK and to allow determination of susceptibility to carcinogenic insults as a function of nutritional status. These studies would also be expected to lead to a scientific basis for chemoprevention of cancer development in tobacco chewers and smokers who are not willing to give up the habit.

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#### Note Added in Proof

The results of a recently completed bioassay in the authors' laboratory demonstrated that NNK is more carcinogenic than NDMA in F344 rats. After administration of total doses of 0.33 mmol/kg, NNK induced liver tumors in 4 of 29 rats, nasal cavity tumors in 6 of 29 rats, and lung tumors in 12 of 29 rats, whereas NDM/ induced liver tumors in 5 of 26 rats and nasal cavity tumors in 1 of 26 rats. NDM<sup>A</sup> did not induce lung tumors.

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