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COMMENTARY

Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke

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Introduction

This commentary presents our views on the significance in tobacco carcinogenesis and cancer research of a group of chemicals collectively termed the tobacco-specific nitrosamines. Of the many carcinogens in tobacco and tobacco smoke, only these are specifically associated with tobacco, tobacco smoke and related nicotine-containing products. They are not known to occur in any other product and are present only in environments polluted by tobacco smoke. Thus, they are uniquely suited to studies in tobacco carcinogenesis.

The role of tobacco and tobacco smoke as causative agents for various types of cancer has been frequently reaffirmed, most recently by Working Groups of the International Agency for Research on Cancer (1,2). They concluded that 'the occurrence of malignant tumors of the respiratory tract and of the upper digestive tract in humans is causally related to various forms of tobacco smoking and also that the occurrence of malignant tumors of the bladder, renal pelvis and pancreas are causally related to cigarette smoking'. Cancers of the cervix and nasal cavity may possibly also be related to smoking (1,3). They also concluded that 'there is sufficient evidence that oral use of snuffs of the types commonly used in North America and Western Europe is carcinogenic to humans'. It has been estimated that 30% of all cancer in the USA is associated with tobacco use (4,5).

In view of the widespread knowledge and convincing evidence of the role of tobacco products as causes of cancer, one is sometimes asked why it is necessary to continue to do research in tobacco carcinogenesis. After all, if tobacco use were to cease, the problem would presumably disappear. Although the heightened public awareness of the hazards of tobacco is encouraging, and the goal of a 'smokefree society' by the year 2000 is admirable, statistics on tobacco use are still daunting. In 1985, as an example, ~600 billion cigarettes were sold in the USA and the annual per capita consumption of cigarettes was ~3400 (6).

There are presently ~53 million smokers and over 12 million smokeless tobacco users in the USA and hundreds of millions of tobacco consumers worldwide. Nevertheless, the argument is sometimes advanced that these people know the risks adequately by this time and therefore further research is unnecessary. This position is untenable. First, it is essential that cancer researchers act as sentries to prevent further damage to the public by the tobacco industry. A pertinent example is the recent increase in the USA of snuff-dipping, the practice of placing a pinch of snuff between the cheek and gum. This was widely advertized by segments of the tobacco industry as a safe alternative to smoking. Research has clearly shown that this is not true. Second, the relative risks for many of the cancers associated with tobacco use are readily detected by epidemiology. Therefore, tobacco users are key cohorts for evaluating hypotheses on mechanisms of human carcinogenesis and are a useful base for studying the effects of cancer-modifying factors such as diet, especially when epidemiologic data are combined with appropriate biochemical markers of exposure and uptake. The results of such studies are useful for developing cancer prevention strategies for the general public, regardless of tobacco use.

Origins of tobacco-specific nitrosamines

Dependence on, or addiction to, nicotine is the main reason for the continued use of tobacco products in spite of their well-known adverse health effects (7,8). Nicotine comprises ~1–2% of unburned tobacco, and its levels in mainstream cigarette smoke range typically from 0.5 to 2 mg/cigarette, with a sales-weighted mean value for the USA of ~1 mg/cigarette (9). In smokers, typical plasma levels of nicotine and its major metabolite cotinine are 15 and 275 ng/ml, respectively (10).

Nicotine is known as an alkaloid, which is chemical terminology for any of numerous types of organic bases which contain nitrogen and occur in seed plants. Several alkaloids which are structurally related to nicotine are also found in tobacco; their concentrations in commonly used tobacco blends are less than that of nicotine (11). The structures of nicotine and some minor tobacco alkaloids are illustrated in Figure 1. Nicotine is a tertiary amine, while nornicotine, anabasine and anatabine are secondary amines. Secondary amines and tertiary amines are known to react with nitrosating agents to form stable chemicals known as *N*-nitrosamines (referred to in this paper as nitrosamines). Over 300 different nitrosamines have been shown to be carcinogenic in experimental animals (12). In the case of a secondary amine, the net result of the nitrosation reaction is replacement of N–H by N–N=O. For tertiary amines, the carbon–nitrogen bond is cleaved oxidatively. Thus, nitrosation of nicotine with sodium nitrite between pH 2 and 7 at 20°C gives a nitrosamino ketone (called NNK, for nicotine-derived nitrosamino ketone), a nitrosamino aldehyde, NNA, and *N'*-nitrosonornicotine (NNN) (13). The latter is formed with loss of the methyl group as formaldehyde. Nitrosation of the secondary amines under mild conditions gives NNN, NAB and NAT as illustrated in Figure 1. Collectively, NNK, NNN, NNA, NAB, NAT and related nitrosamines are known as tobacco-specific nitrosamines.

The formation of tobacco-specific nitrosamines from alkaloids under mild conditions suggested that they should be present in tobacco and tobacco smoke. Analytical studies confirmed this hypothesis. Since nitrite is present in saliva and nitrogen oxides are present in inhaled mainstream tobacco smoke, additional amounts of tobacco-specific nitrosamines should be formed *in vivo* by reaction of these nitrosating agents with nicotine and the other alkaloids. Presently available data favor this view, although the quantitation of endogenous nitrosation of nicotine has not yet been accomplished.

Analysis of tobacco products for tobacco-specific nitrosamines

The first analysis of tobacco smoke for a tobacco-specific nitrosamine, NNN, was reported in 1974 (14). The presence of NNN in unburned tobacco was also documented in 1974 (15) and the

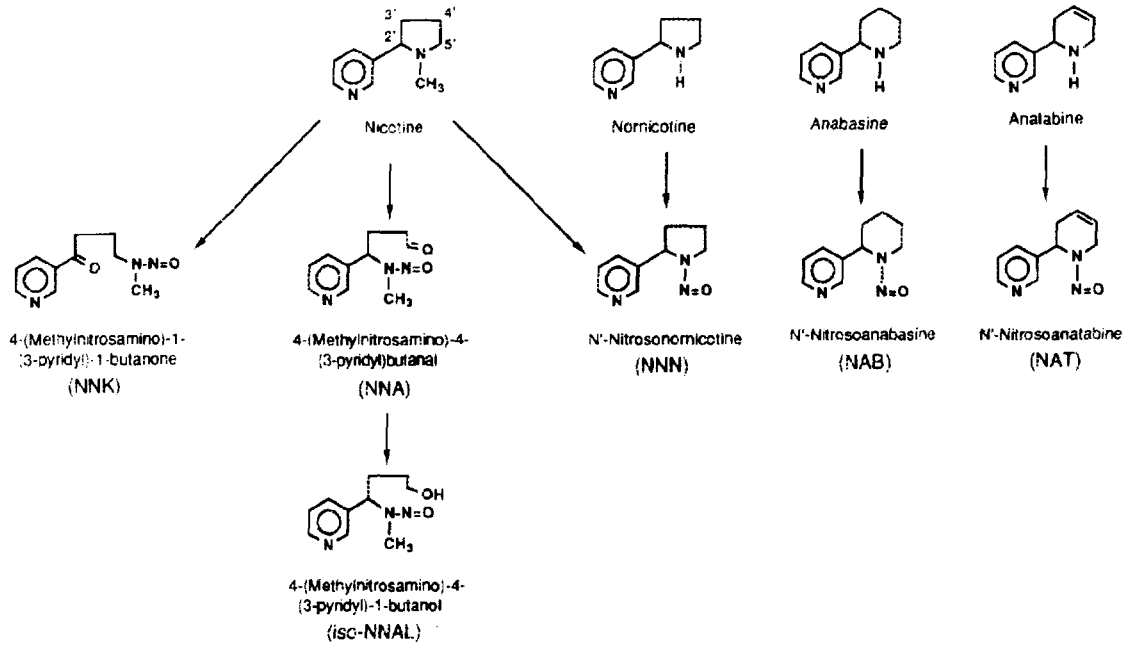

Fig. 1. Structures of tobacco-specific nitrosamines formed by nitrosation of tobacco alkaloids. With the exception of NNA, all have been detected in tobacco or tobacco smoke (see Table I).

Table I. Some recent analyses of tobacco-specific nitrosamines in smokeless tobacco and mainstream and sidestream cigarette smoke[a]

	NNN	NNK	NAB	NAT	iso-NNAL
Smokeless tobacco (p.p.m.)					
Chewing tobacco	0.67 – 4.1	0.03 – 0.38	0.03–0.14	0.33– 2.3	NDe[b]
Moist snuff	5.8 – 64	0.1 – 3.1	0.2 –6.5	3.5 –214	0.08–2.5
Dry snuff	9.4 –55	1.9 –14	0.7 –1.2	1.9 –40	0.07–0.14
Tobacco used in betel quid	0.47 – 0.85	0.13 – 0.23	0.03 – 0.07	0.30– 0.45	NDe
Cigarette smoke (μg/cigarette)					
Mainstream	0.066– 1.01	0.017– 0.43	0.10–0.74[c]		ND[d]
Sidestream	0.19 – 0.86	0.39 – 1.44	0.13–0.78		ND

[a]References 24 – 26.
[b]NDe, not determined.
[c]Combined NAB and NAT.
[d]ND, not detected.

first report of NNK in tobacco was published in 1978 (16). Subsequent studies established the presence of all the tobacco-specific nitrosamines illustrated in Figure 1, with the exception of NNA, in tobacco or tobacco smoke (17). However, the reduced form of NNA, iso-NNAL, has recently been detected (18). The identities of the tobacco-specific nitrosamines in tobacco and tobacco smoke have been established by mass spectrometry. Although the initial analyses were carried out by classical gas chromatographic and high-performance liquid chromatographic methods, the present method of choice for the analysis of tobacco-specific nitrosamines is a nitrosamine-specific detector, the Thermal Energy Analyzer, interfaced with a gas chromatograph (19). Numerous reports of the analysis of tobacco-specific nitrosamines have appeared from various laboratories (20–23). The reported values are similar to those summarized in Table I for some recently marketed products.

The levels of tobacco-specific nitrosamines in tobacco products, as illustrated in Table I, are remarkably higher than those of nitrosamines in other consumer products. For example, in the USA, nitrosamine levels in bacon and beer are regulated by the US Department of Agriculture and US Food and Drug Administration. They cannot exceed 5 p.p.b. (27,28). However, there is no regulation of nitrosamine levels in tobacco products. Tobacco-specific nitrosamines are typically found in tobacco products in the p.p.m. range. Their total level in 1 g of moist snuff, of the types used by millions of snuff dippers in the USA, is up to 30 000 times higher than the regulated levels of nitrosamines in other products. The amounts of tobacco-specific nitrosamines in the mainstream smoke of a single typical USA cigarette are up to 250 times higher than the nitrosamine levels regulated for 1 g of cooked bacon or cosmetics or 1 ml of beer. Looked at in another way, one would have to consume $\sim 3 \times 10^3$ liters of beer or 3×10^6 g of bacon in order to have the same estimated nitrosamine exposure which would be obtained by using 10 g of smokeless tobacco. (This calculation takes into account only the tobacco-specific nitrosamines and ignores the

numerous other nitrosamines in tobacco.) The permission of such high levels of nitrosamines in tobacco products is a major legislative failure.

The above cited levels of exposure are only estimates. The actual uptake of nitrosamines may be influenced by many factors, and the possibility of endogenous formation has not been taken into account. For these reasons and others discussed below, the development of methods to determine human dosimetry of tobacco-specific nitrosamines is important.

It is also worthwhile to consider the levels of tobacco-specific nitrosamines in tobacco products compared to those of other carcinogens. Tobacco-specific nitrosamines are typically present in concentrations > 1000 times greater than those of benzo[*a*]pyrene in moist and dry snuff (26). These products also contain up to 0.2 p.p.m. of volatile nitrosamines and 1 pCi/g of ^{210}Po (26). The levels of the carcinogens formaldehyde, acetaldehyde and crotonaldehyde in snuff are in the range of 1 – 10 p.p.m. (26). In cigarette mainstream smoke, total levels of tobacco-specific nitrosamines typically range from 200 to 1600 ng/cigarette (25). The total levels of volatile nitrosamines such as *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine are typically 15 – 100 ng/cigarette (25), whereas those of total four-ring and higher polynuclear aromatic hydrocarbons are typically 60 – 200 ng/cigarette (29). Levels of carcinogenic aromatic amines range from 1 to 20 ng/cigarette for 2-naphthylamine and 4-aminobiphenyl, and 30 – 160 ng/cigarette for *o*-toluidine (29). The highest concentrations of carcinogenic agents in tobacco smoke are those of acetaldehyde (18 – 1400 μg/cigarette), crotonaldehyde (10 – 20 μg/cigarette), formaldehyde (70 – 100 μg) and benzene (20 – 50 μg) (29). The complex nature of cigarette smoke precludes the assignment of its carcinogenic activities to any one component or group of components. However, the comparatively high concentrations of tobacco-specific nitrosamines in snuff make them prime candidates for explaining its carcinogenic activity in snuff-dippers.

Initial studies on the formation of NNN in tobacco demonstrated that it was produced during curing (30). Subsequent detailed investigations have shown that the type of post-harvest processing employed greatly influences the levels of tobacco-specific nitrosamines in tobacco (22,31). Other factors such as tobacco genotype, field conditions and length and conditions of storage can also influence the eventual levels of tobacco-specific nitrosamines (22,23,31,32). Consistent correlations of tobacco-specific nitrosamine content in tobacco with alkaloid and nitrate or nitrite levels have not been observed, although the levels of tobacco-specific nitrosamines in tobacco blends made with stems, which have high nitrate content, did correlate with nitrate (22,23,31,33,34). International comparisons of levels of tobacco-specific nitrosamines in chewing tobacco have demonstrated that the processes used in some countries such as Sweden yield lower levels than those used for the most popular USA brands (24). This indicates that the levels of tobacco-specific nitrosamines in commercial products can be reduced by the use of favorable curing and processing conditions. However, there is no commercially employed process which is known to decrease the total levels of tobacco-specific nitrosamines to < 1000 p.p.b. (24).

Thirty to fifty percent of the NNN and NNK present in mainstream cigarette smoke is transferred from the tobacco (35,36). The rest is formed during smoking by reactions of alkaloids with nitrogen oxides. Nitrate content of tobacco is correlated with tobacco-specific nitrosamine levels in mainstream smoke (33,37). Relatively high levels of NNK and NNN are present in sidestream cigarette smoke and these levels are not generally decreased in lower tar cigarettes (25). The generation of up to 1.5 μg per cigarette of NNK in sidestream smoke is significant with respect to the question of cancer induction by environmental tobacco smoke.

Table II. Organospecificity of NNK for lung

Species/strain	Route	Total dose (mmol/kg)	Percentage of animals with lung tumors	Reference
F344 rat	s.c.	9	77	41
		3	67	41
		1	57	41
		0.3	48	40
	Oral swabbing (with NNN)	0.2	17	42
Syrian golden hamster	s.c.	8	80	43
		0.04	20	39
	Oral swabbing	13	87	Unpublished
SENCAR mouse	Topical	1	63	44
A/J mouse	i.p.	4	100	38

Recent observations on the carcinogenicity of tobacco-specific nitrosamines

Bioassays of the tobacco-specific nitrosamines for carcinogenicity have been carried out in mice, rats and hamsters. The results of many of those assays have been summarized previously (17). These studies showed that NNK and NNN are the strongest carcinogens among the tobacco-specific nitrosamines. NNK induces tumors of the lung, nasal cavity and liver in F344 rats; lung, trachea and nasal cavity in Syrian golden hamsters; and lung in mice. NNN gives tumors of the esophagus and nasal cavity in rats; trachea and nasal cavity in Syrian golden hamsters; and lung in mice. NAB is a weak esophageal carcinogen in the rat, and was inactive in the Syrian golden hamster. NNAL induces lung tumors in A/J mice (38). NAT was inactive in the rat (total doses up to 9 mmol/kg) and NNA was inactive in A/J mice (4 mmol/kg).

The carcinogenic potency of NNK is particularly notable. In Syrian golden hamsters, a single dose of 1 mg (5 μmol) was sufficient to induce respiratory tract tumors in 6/20 animals (39). Ongoing studies indicate that single doses of 1 – 2 mg amounts of NNK will induce multiple tumors in A/J mouse lung which is particularly sensitive to its tumorigenic effects. A comparative study of NNK and the extensively assayed carcinogen *N*-nitrosodimethylamine (NDMA) in rats demonstrated that NNK was more potent than NDMA (40). Each compound was administered by s.c. injection to F344 rats, in total doses of 0.33 mmol/kg. Both nitrosamines induced liver tumors, but only NNK gave a significant incidence of lung tumors (13/27 rats) and nasal cavity tumors (6/27 rats). Ongoing studies indicate that 0.5 – 1 p.p.m. NNK administered in the drinking water to F344 rats over a 2-year period is sufficient to induce a significant incidence of lung tumors.

The organospecificity of NNK for the lung is striking, as summarized in Table II. Lung is the main target organ for NNK administered s.c. to rats; adenomas and carcinomas are induced. Significant incidences of lung tumors also result upon oral administration of NNK to rats or upon swabbing of a mixture of NNK and NNN in the oral cavity of rats. In Syrian golden hamsters, lung tumors have been induced by either s.c. injec-

tion or oral swabbing of NNK, and in mice, topical application of NNK resulted in the induction of lung tumors as well as skin tumors. Organospecificity is a general property of nitrosamines and various tissues are common targets depending on the structure of the nitrosamine and the animal species (12). Tumors can typically be induced in the esophagus, lung, liver, pancreas or bladder of rats. The organospecificity of NNK for the lung supports its potential role as an important factor in tobacco carcinogenesis.

Whereas both NNK and NNN have the properties of organospecificity in that the former induces lung tumors in rats and the latter nasal tumors, independent of the route of administration, these compounds are also effective local carcinogens, which is only known for a few other nitrosamines (12). In three separate assays, NNN induced tumors of the esophagus and nasal cavity when administered in the drinking water or in a liquid diet, but only tumors of the nasal cavity when given by s.c. injection (45–47). On this basis, we expected that a mixture of NNN and NNK would give oral cavity tumors when applied to the rat oral mucosa. This expectation was confirmed in a recent bioassay, in which oral tumors were induced in 8/30 rats by NNN and NNK (42). (Lung tumors were also observed, Table II.) This result is important with respect to the potential role of NNN and NNK as causative agents in oral cancer induced by snuff. A subsequent bioassay demonstrated that NNK can also induce local tumors. Topical application of NNK to the skin of SENCAR mice resulted in the induction of skin tumors in 60–80% of the animals (44). (Lung tumors were also observed.) NNN was inactive on mouse skin (44). Although the ability of NNK to induce tumors on mouse skin is significant because it demonstrates its versatility, it should be noted that benzo[*a*]pyrene is at least 200 times more potent in the skin model than is NNK.

Whereas a mixture of NNN and NNK applied to the rat oral cavity induced local tumors in 8/30 animals, NNN and NNK applied together with an aqueous extract of snuff induced local tumors in only 3/30 rats (42). An aqueous extract of snuff, which contains one tenth the amount of NNN and NNK used in the other two groups, was inactive (42). These results indicate that there are synergisms in NNN and NNK oral carcinogenesis and suggest that tumor inhibitors are present in the aqueous extract of snuff. Biochemical studies support this concept. DNA methylation by NNK was lower in the oral tissues of rats pre-treated with snuff extract than in control rats (48). Preliminary experiments indicate that these inhibitors may be polyphenols or related compounds. Since whole snuff, in contrast to its extract, can induce local tumors in the rat oral cavity, there are probably potentiating factors for carcinogenesis by NNN and NNK (42,49). One possible factor is physical irritation. This has been shown to enhance *N*-nitrosomethylurea tumorigenesis in the Syrian golden hamster oral cavity (50). Another is Herpes simplex virus which appears to enhance the tumorigenicity of snuff and is inactivated by snuff extracts (51,52).

The identification of agents which could inhibit tumorigenesis by NNN and NNK is important in gaining insights into the practicality of cancer chemoprevention, since smokers are a high risk group in which such agents could presumably be evaluated. Recent studies have shown that isothiocyanates are broad spectrum inhibitors of the metabolic activation and DNA binding of NNN and NNK (53,54). Some of these compounds, as well as the related glucosinolates, occur in cruciferous vegetables. Bioassays to test their efficacy in prevention of NNK tumorigenesis are currently in progress.

It is informative to compare the estimated levels of exposure of tobacco users to NNN and NNK with their carcinogenic activities in experimental animals. Assuming a total amount of NNN and NNK in mainstream cigarette smoke of 440 ng/cigarette, a one-pack-a-day smoker would be exposed to ~8 μg, or 0.1 μg/kg. The total dose in 40 years would be 117 mg or ~1.6 mg/kg (0.01 mmol/kg). A total dose of 0.05 mmol/kg of NNK alone was sufficient to induce a significant incidence of respiratory tract tumors in hamsters. Snuff-dippers who use the most popular products presently marketed in the USA would be exposed to ~67 μg/g of NNK and NNN. A user of 10 g/day would be exposed to 670 μg or 3.5 μmol. In 40 years of snuff-dipping, total estimated exposure would be ~9.8 g or 130 mg/kg (~0.7 mmol/kg). A total dose of 1.6 mmol/kg of NNK and NNN applied to the rat oral cavity was sufficient to induce a significant incidence of oral tumors. These estimates, which ignore the possibility of endogenous nitrosamine formation, and disregard the amounts of the numerous nitrosamines in tobacco and tobacco smoke other than NNK and NNN, clearly demonstrate an unacceptable risk to the tobacco consumer. The estimated levels of exposure to NNK and NNN are close to the doses shown to cause cancer in animals. Any product other than tobacco which presented an unacceptable risk of such magnitude to the consumer would be banned by regulatory agencies in the USA. There is no regulation of carcinogen exposure through tobacco.

Recent studies on the metabolic activation of tobacco-specific nitrosamines

Nitrosamines require metabolic activation for binding to DNA and other cellular macromolecules (12). Therefore it is necessary to understand their metabolism as a prerequisite for determining the types of DNA modifications that are induced and their possible roles in the initiation of carcinogenesis. Metabolic transformations of NNK and NNN have been investigated *in vivo* in experimental animals and in subcellular fractions and cultured cells and tissues of experimental animals and humans. The results of many of these studies have been previously summarized (17,55–57). Identified metabolites of NNK result from initial reduction of the carbonyl group to NNAL, oxidation of the pyridine nitrogen and hydroxylation of the methylene and methyl carbons adjacent to the *N*-nitroso nitrogen (α-hydroxylation). For NNN, major metabolic pathways are initiated by α-hydroxylation at the 2′- and 5′-carbons, and by oxidation of the pyridine nitrogen. Hydroxylations of the 3′- and 4′-carbons, as well as denitrosation, have also been observed. The α-hydroxylation pathways appear to be catalyzed at least in part by cytochrome P-450 enzymes. Various lines of evidence strongly suggest that different isozymes are involved in the 2′- and 5′-hydroxylation of NNN, as well as in the metabolism by α-hydroxylation of NNK in different cell types. Further studies on the identification of the specific cytochrome P-450 isozymes involved in these transformations, and their distributions in various tissues, would be likely to produce major insights into the molecular basis of the organospecificity of NNK and NNN carcinogenesis.

α-Hydroxylation of nitrosamines is believed to be important in their metabolic activation to intermediates which bind to DNA (12). The most extensively studied example is NDMA, which undergoes metabolic α-hydroxylation yielding α-hydroxymethylmethyl nitrosamine, an unstable compound which has a half-life of ~10 s at physiological pH (58). It spontaneously decomposes to formaldehyde and a methylating agent, probably methyldiazohydroxide. The latter is electrophilic and reacts with nucleophilic

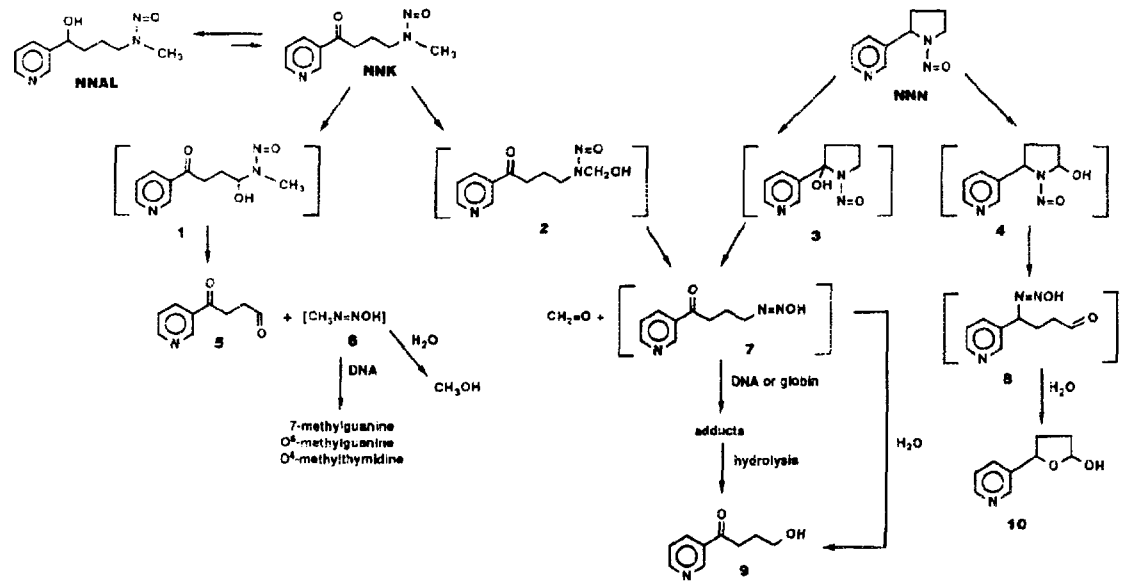

Fig. 2. Metabolic activation of NNK and NNN to hypothetical intermediates (shown in brackets) which bind to DNA and protein. Structures of aldehydes and alcohols resulting from decomposition or reaction with H_2O of the intermediates are also indicated. Further metabolic transformations of these compounds as well as other metabolic pathways of NNK and NNN are summarized in references 55–57.

centers in DNA to yield a variety of products including 7-methylguanine, O^6-methylguanine and O^4-methylthymine. Among these, O^6-methylguanine has been conclusively shown to cause miscoding (59). The resulting point mutation can lead to oncogene activation (60). In a number of studies in which animals have been treated with methylating carcinogens, including nitrosamines, the persistence of O^6-methylguanine in DNA of various tissues correlates with the susceptibility of those tissues for tumor development. One result of such studies has been the conclusion that, for methylating carcinogens, persistent O^6-methylguanine in replicating cells may be necessary but not sufficient for tumor development (61,62).

Based on these observations and on metabolism studies of NNK, α-hydroxylation seemed to be a likely metabolic activation pathway. As illustrated in Figure 2, α-hydroxylation of NNK on the methylene group would be expected to give methyl diazohydroxide (Compound 6), resulting in methylation of DNA. *In vitro* metabolism studies confirmed that the keto aldehyde (Compound 5) was a metabolite of NNK (63), and *in vivo* DNA binding experiments demonstrated the presence of 7-methylguanine, O^6-methylguanine and O^4-methylthymidine in lung, liver and nasal mucosa of F344 rats, but not in esophagus, spleen, heart and brain (40,64–66). Methylation in kidney DNA was detected only after 12 days of 100 mg/kg NNK, but not after 40 mg/kg (66). It is interesting and perhaps significant that DNA methylation has been consistently detected only in target tissues of NNK-treated rats.

Several studies have examined the relationship of dose and levels of methylated bases in DNA following treatment of rats with NNK. Following a single dose of NNK, levels of DNA methylation were highest in nasal mucosa, followed by liver and lung (40). The rates of removal of O^6-methylguanine from lung and nasal mucosa were slower than from liver (40). During treatment with 100 mg/kg NNK for 12 days, O^6-methylguanine accumulated and persisted in lung, whereas it was repaired in hepatocytes (66). The accumulation and persistence of O^6-methylguanine in lung correlated with inhibition by NNK treatment of the repair enzyme, O^6-methylguanine-DNA methyltransferase in lung (66). In contrast, O^4-methylthymidine repair in lung and liver was not inhibited by NNK treatment (66). A striking non-linear relationship of dose and O^6-methylguanine levels in lung was observed; efficiency of methylation was greater at lower doses than at higher doses of NNK during treatment over a 4–12 day period (67). Methylation efficiency was particularly high in Clara cells (67). These results may be due to the presence in Clara cells of a cytochrome P-450 isozyme with high affinity for the metabolism of NNK by α-hydroxylation (67). The efficient formation of O^6-methylguanine in lung following low doses of NNK, and its accumulation and persistence, are highly relevant to the question of the causative agents for lung cancer induced by smoking. These results, taken together with earlier studies that demonstrated the ability of human lung tissue to carry out α-hydroxylation of NNK (68), strongly suggest that O^6-methylguanine is formed in the lungs of smokers. These observations create a mechanistic link between nicotine and a change in DNA associated with the initiation of cancer. As more sensitive methods are developed for the analysis of DNA adducts in human tissues, it is likely that methylated bases will be detected in the lung tissue of smokers and in the oral tissues of snuff-dippers.

Differences in extents and efficiencies of O^6-methylguanine formation from NNK have been observed in comparative studies of the respiratory and olfactory portions of the rat nasal mucosa (69). At low doses, the extent of methylation as well as the methylation efficiency were greater in the respiratory than in the olfactory mucosa. However, NNK toxicity was greater in the olfactory portions of the nose, with Bowman's glands and Steno's gland being the most sensitive sites. Interestingly, relatively high binding of [carbonyl-^{14}C]NNK to Bowman's glands and Steno's glands has been observed, indicating that 4-(3-pyridyl)-4-oxobutylation of these tissues occurs (70). It has been proposed that a combination of DNA methylation and toxicity is related to the development of nasal tumors originating in the olfactory portions of the rat nose upon treatment with NNK (69). This hypothesis

requires further evaluation, particularly in view of the high nasal tumorigenicity of NNN, which is not known to methylate DNA.

The studies described above strongly suggest that O^6-methylguanine is one important DNA modification leading to cancer induction by NNK. As illustrated in Figure 2, a second pathway of NNK α-hydroxylation is observed, resulting initially in the formation of the proposed unstable Compound 2. Its decomposition would give the putative alkylating species, 4-(3-pyridyl)-4-oxobutyl diazohydroxide, Compound 7. Although the existence of this metabolic pathway was established in earlier studies by the identification of the keto alcohol, Compound 9, as a metabolite of NNK (63), the ability of Compound 7 to form macromolecular adducts *in vivo* has only recently been demonstrated. Acid hydrolysis or neutral thermal hydrolysis of DNA isolated from the liver or lung of rats treated with [5-^{3}H]NNK resulted in the liberation of Compound 9, consistent with 4-(3-pyridyl)-4-oxobutylation of DNA by Compound 7 (71). Similar results were obtained upon acid or base hydrolysis of globin from NNK-treated rats, as discussed in more detail below (72). The points of attachment of the 4-(3-pyridyl)-4-oxobutyl residue to DNA and globin have not yet been determined, and the significance of this adduct in DNA, with respect to initiation of cancer, is presently unknown. However, a potentially important consequence of this finding is the development of methods to analyze human tissues or blood cells for Compound 9, which unlike products of methylation should result only from exposure to nicotine-related compounds and thus could provide a specific index of macromolecular binding resulting from exposure to tobacco products.

Figure 2 shows that NNK and NNN have at least one metabolic pathway in common. The intermediate, Compound 7, is formed from NNN upon 2′-hydroxylation and from NNK upon methyl hydroxylation. On this basis, it was expected that NNK and NNN should form some similar DNA and globin adducts. Recent studies have confirmed this by demonstrating the presence of the keto alcohol, Compound 9, in hydrolysates of DNA and globin isolated from NNN-treated rats (71,72). Adducts resulting from the interaction of the diazohydroxide, Compound 8, with cellular macromolecules have not yet been observed. Comparative mutagenicity studies of stable precursors to Compounds 7 and 8 showed that the former was highly mutagenic toward *Salmonella typhimurium* while the latter was inactive at equimolar doses, suggesting that modification of DNA by Compound 7 may be more efficient than by Compound 8 (73).

Taken together, the studies on the metabolic activation of NNK and NNN have provided insights into the mechanisms by which they modify DNA and initiate cancer. α-Hydroxylation is one important metabolic step involved in DNA modification by these carcinogens. This metabolic process is likely to be controlled by specific cytochrome P-450 isozymes, the levels of which will probably vary among various tissues and cell types in humans. Indeed, studies of the metabolism of NNK and NNN in human tissues have shown large variations in extents of α-hydroxylation (68). The susceptibility of tissues to the initiation of the carcinogenic process by NNK and NNN may therefore depend partially on the levels of specific cytochrome P-450 isozymes, or perhaps other xenobiotic metabolizing enzymes, in those tissues. Levels of the repair enzyme, O^6-methylguanine-DNA methyltransferase, in various tissues will also have an effect on the initiation of carcinogenesis by NNK, as will rates of cell replication. Mechanistic studies addressing these and related issues pertinent to the induction of cancer by tobacco-specific nitrosamines will have to be approached using laboratory animals. These model studies should provide further insights into mechanisms of nitrosamine carcinogenesis. An advantage of using NNK and NNN for such investigations is the presence of a sizable human population exposed to significant amounts of these compounds. This should allow meaningful comparisons of animal and human data, and thus contribute to a better understanding of mechanisms of human carcinogenesis.

Approaches to human dosimetry of tobacco-specific nitrosamines

There are a number of uncertainties involved in determining the doses of tobacco-specific nitrosamines in humans. Their levels in cigarette smoke are typically measured on smoking machines under a set of defined conditions. Depending on the type of cigarettes, cigars or pipe tobacco, the smoking habits of individuals will differ significantly from these standard conditions. Smokers will adjust their behavior to fulfill their dependence on nicotine. Smokers are also exposed to their own sidestream smoke. Tobacco chewers or snuff-dippers will presumably extract differing amounts of tobacco-specific nitrosamines from tobacco, depending on such factors as salivary flow and on where the tobacco is placed in the mouth. Some amounts of tobacco-specific nitrosamines will also be formed endogenously in smokers and chewers. Previous studies have convincingly demonstrated that urinary *N*-nitrosoproline, a marker for endogenous formation of nitrosamines, is elevated in smokers (74–76). This is due to the high levels of nitrogen oxides in cigarette smoke and to catalysis of their formation by thiocyanate, a metabolite of HCN which occurs in smoke in concentrations up to 500 μg/cigarette (29). Exposure to tobacco-specific nitrosamines in sidestream smoke is even more difficult to estimate because of the many factors which may affect the distribution of these compounds in a given indoor environment. Beyond the question of exposure is the problem of an individual's capacity to metabolically activate or detoxify tobacco-specific nitrosamines. Inter-individual differences of 100-fold have been observed in the extents of metabolic activation of tobacco-specific nitrosamines in cultured human tissues (68). These differences may result from environmental or genetic factors which influence the spectrum of cytochrome P450 isozymes, or other drug metabolizing enzymes. Such polymorphisms are well known, and may correlate with susceptibility to cancer in certain cases (77).

These uncertainties have encouraged the development of methods to quantify dose and extent of metabolic activation of tobacco-specific nitrosamines in humans. Several measures of an individual's exposure to tobacco smoke are already available, including nicotine, cotinine, thiocyanate and carboxyhemoglobin (78). Methods to assess carcinogen exposure via 4-aminobiphenyl–hemoglobin adducts have also been developed and have been applied to smokers and non-smokers (79–81). The results of studies in which these methods have been applied have already provided new insights about uptake and activation of tobacco smoke constituents. At the present time, it is not known whether any of these parameters could serve as a surrogate to assess uptake and activation of other tobacco smoke carcinogens. Hemoglobin adducts or DNA adducts of tobacco-specific nitrosamines might be appropriate dosimeters, and both of these approaches have been investigated.

Figure 2 illustrates the formation of a globin adduct, presumed to result from reaction of hemoglobin with 4-(3-pyridyl)-4-oxobutyl diazohydroxide generated during the metabolism of NNK or NNN (72). Mild base hydrolysis of the globin releases the keto alcohol, Compound 9, which can be detected up to 6 weeks

after a single administration of NNK, and accumulates during chronic dosing of NNK. These observations are in line with the 60-day lifetime of the erythrocyte in rats, and are consistent with models developed by Ehrenberg and co-workers for alkylation of hemoglobin (82). Compound 9, as released upon base treatment of globin from smokers, would thus appear to be a potentially useful dosimeter for exposure to, and metabolic activation of, NNK and NNN. We are presently developing methods for the detection of Compound 9 by gas chromatography–mass spectrometry. In order to be applicable to human samples, sensitivity in the low femtomol range will probably be necessary. Negative ion chemical ionization mass spectrometry with specific ion monitoring appears to be the most promising method at present.

Figure 2 illustrates that Compound 9 is also released from DNA of NNK- or NNN-treated rats upon neutral thermal or acid hydrolysis. The levels of modification of hepatic DNA appear to be comparable to those detected in globin, according to presently available data. A sensitive method for detection of Compound 9 could in principle also be applied to human DNA. However, the practicality of this approach for human dosimetry is unclear because of the relatively small amounts of DNA which can routinely be obtained from peripheral blood cells or exfoliated cells.

DNA methylation by NNK exceeds 4-(3-pyridyl)-4-oxobutylation, and the measurement of methylated DNA bases might thus provide another approach to human dosimetry for activation of tobacco-specific nitrosamines. Immunoassays for O^6-methyldeoxyguanosine and 7-methyldeoxyguanosine have been developed (83–85). We have recently analyzed DNA from placenta of smokers and non-smokers for O^6-methyldeoxyguanosine and have detected it in 6/20 samples. However, no apparent relationship to smoking history was observed. The presence of the repair enzyme for O^6-methyldeoxyguanosine complicates its applicability as a dosimeter because its levels are likely to be transient, rather than cumulative. This limitation does not apply to 7-methyldeoxyguanosine. However, the general applicability of DNA methylation assays as dosimeters for specific exposure to NNK is limited because of the presence of other methylating agents such as NDMA in tobacco smoke and the general environment.

Assuming that suitably sensitive methods for the quantitation of tobacco-specific nitrosamine–globin adducts can be developed, this would appear to be an ideal approach for assessing exposure to, and activation of, carcinogens which are presumably associated only with tobacco or other nicotine-containing products. This is in contrast to adducts of carcinogens such as polynuclear aromatic hydrocarbons and 4-aminobiphenyl, which are also detected in non-smokers due to various environmental exposures. These methods would thus seem to have potential applicability to epidemiologic studies as objective markers of exposure to tobacco-specific carcinogens. This would be particularly useful for studies involving relatively weak associations, such as passive smoking and lung cancer.

The use of such measurements to assess risk, as opposed to individual uptake and metabolic activation of carcinogens, requires further research on the relationship of the measured adducts to the probability of tumor development. These studies will have to be carried out in experimental animals under realistic conditions of chronic exposure. Some experiments with NNK, as described above, have already indicated a non-linear relationship between dose and O^6-methyldeoxyguanosine levels in DNA from rat lung (67). Further research is necessary to relate these observations to the dose–response curves for lung tumor development and to those for measurable parameters in man such as tobacco-specific nitrosamine–globin adducts. Such studies will presumably provide important information for assessing individual risk for cancer development upon exposure to tobacco-specific nitrosamines.

Tobacco-specific nitrosamines as possible causative agents for human cancer

The strongest evidence that tobacco-specific nitrosamines cause human cancer comes from epidemiologic studies on the association of snuff-dipping with oral cancer. A Working Group of the International Agency for Research on Cancer, an Advisory Committee to the Surgeon General of the US Public Health Service and a National Insitutes of Health Consensus Conference all reviewed the available data and all came to essentially the same conclusion: snuff-dipping causes oral cancer (2,86,87). The most convincing epidemiologic study implicating snuff-dipping as a cause of oral cancer was carried out by Winn and co-workers who demonstrated that the relative risk associated with snuff-dipping among white non-smoking women was 4.2, and that among chronic users the risk approached 50-fold for cancers of the gum and buccal mucosa (88). Analytical studies summarized in Table I have shown that the total levels of tobacco-specific nitrosamines in dry snuff products are in the range of 30–110 p.p.m. Levels of other known carcinogens in dry snuff are as follows: benzo[*a*]pyrene, 0.1–91 p.p.b.; ^{210}Po, 0.2–0.6 pCi/g; formaldehyde, 2–7 p.p.m.; acetaldehyde, 1.4–3.9 p.p.m.; crotonaldehyde, 0.2–0.6 p.p.m. Thus, tobacco-specific nitrosamines are quantitatively the most abundant known carcinogens in snuff, and the estimated doses of NNK and NNN to a long-term snuff-dipper, as calculated above, are close to the doses of NNK and NNN which induce tumors of the rat oral cavity when applied locally.

Some related observations on the relationship of snuff-dipping and oral cancer are not inconsistent with the hypothesis that tobacco-specific nitrosamines are causative agents. First, a protective effect was found for a diet high in fruits and vegetables (89). This observation is consistent with results of studies which show that a number of isothiocyanates and related compounds, which occur in vegetables, inhibit the metabolic activation of NNK and NNN. Other constituents of fruits and vegetables such as vitamins or β-carotene might also be protective against NNK and NNN but this has not yet been tested. Second, an association has been found between snuff-dipping and cancer of the nasal cavity (3). NNK and NNN both induce nasal tumors in rats, when administered by s.c. injection, and NNN gives nasal tumors when given in the drinking water (41,45–47). However, nasal tumors were not observed when a mixture of NNK and NNN was applied to the oral cavity of rats (42). In every bioassay of NNK, lung tumors have been observed, regardless of the route of administration (Table II). On this basis, it seems possible that snuff-dippers may have an excess risk for lung cancer. This should be tested in epidemiologic studies.

There is sufficient evidence that betel quid chewing is carcinogenic to humans, when the quid contains tobacco. The evidence for cancer causation by betel quid chewing without tobacco is not conclusive (90). Cancer of the oral cavity resulting from this practice is a leading cause of cancer in India (91). As illustrated in Table I, tobacco-specific nitrosaminesa are present in the tobacco used in betel quids. They have also been detected in the saliva of betel quid chewers (92–94). The only other car-

cinogens reported to occur in betel quids and the saliva of betel quid chewers are nitrosamines derived from arecoline, the major alkaloid of areca nut (92–95). These results provide support for the hypothesis that tobacco-specific nitrosamines are involved in oral cancer induction in betel quid chewers.

The possible role of tobacco-specific nitrosamines as causative factors in cancer induction by tobacco smoke is difficult to evaluate because of its complexity. Polynuclear aromatic hydrocarbons, co-carcinogens such as catechol and tumor promoters are all present in tobacco smoke in significant quantities and are likely to play a role in lung cancer. Other carcinogens such as formaldehyde, crotonaldehyde and acetaldehyde, and toxic agents such as acrolein may also be important in lung cancer induction by tobacco smoke. However, the organospecificity of NNK for the lung provides strong support for its potential role in the induction of lung cancer by tobacco smoke. Smokers also have an elevated risk for cancer of the esophagus, nasal cavity and pancreas (1,3). Tobacco-specific nitrosamines, which are organospecific for these tissues in rats, may play a role in the induction of these cancers. The elevated risks of smokers for cancer of the pancreas, kidney and bladder cannot, at this time, be attributed to known tobacco-specific nitrosamines because they have never been shown to induce tumors in these tissues. Indeed, present evidence supports the role of 4-aminobiphenyl and perhaps other aromatic amines as one group of etiologic factors in smoking-induced bladder cancer (79).

Summary and conclusions

Tobacco-specific nitrosamines are a group of carcinogens that are present in tobacco and tobacco smoke. They are formed from nicotine and related tobacco alkaloids. Two of the nicotine-derived nitrosamines, NNK and NNN, are strong carcinogens in laboratory animals. They can induce tumors both locally and systemically. The induction of oral cavity tumors by a mixture of NNK and NNN, and the organospecificity of NNK for the lung are particularly noteworthy. The amounts of NNK and NNN in tobacco and tobacco smoke are high enough that their total estimated doses to long-term snuff-dippers or smokers are similar in magnitude to the total doses required to produce cancer in laboratory animals. These exposures thus represent an unacceptable risk to tobacco consumers, and possibly to non-smokers exposed for years to environmental tobacco smoke. The permission of such high levels of carcinogens in consumer products used by millions of people represents a major legislative failure. Indeed, the levels of tobacco-specific nitrosamines in tobacco are thousands of times higher than the amounts of other nitrosamines in consumer products that are regulated by government authorities.

Although the role of tobacco-specific nitrosamines as causative factors in tobacco-related human cancers cannot be assessed with certainty because of the complexity of tobacco and tobacco smoke, several lines of evidence strongly indicate that they have a major role, especially in the causation of oral cancer in snuff-dippers. Epidemiologic studies have demonstrated that snuff-dipping causes oral cancer. NNK and NNN are quantitatively the most prevalent known carcinogens in snuff, and they induce oral tumors when applied to the rat oral cavity. A role for NNK in the induction of lung cancer by tobacco smoke is likely because of its organospecificity for the lung. Tobacco-specific nitrosamines may also be involved in the etiology of tobacco-related cancers of the esophagus, nasal cavity, and pancreas.

Because they are derived from nicotine, and therefore should be associated only with tobacco, tobacco smoke and other nicotine-containing products, tobacco-specific nitrosamines as well as their metabolites and macromolecular adducts should be ideal markers for assessing human exposure to, and metabolic activation of, tobacco smoke carcinogens. Ongoing research has demonstrated the formation of globin and DNA adducts of NNK and NNN in experimental animals. Sensitive methods for the detection and quantitation of these adducts in humans would provide an approach to assessing individual risk for tobacco-related cancers.

Aside from their suspected role in human cancer, tobacco-specific nitrosamines are useful model compounds for investigating mechanisms of cancer induction in laboratory animals. NNK and NNN induce tumors of the lung, esophagus or nasal cavity. The induction of lung tumors by NNK may be related to the formation and persistence of the promutagenic lesion O^6-methylguanine in DNA of Clara cells. This adduct, as well as other methylated bases and those formed by 4-(3-pyridyl)-4-oxobutylation of DNA by NNK and NNN, result at least partially from metabolic α-hydroxylation of NNK and NNN, a process which seems to be controlled by cytochrome P-450 isozymes. The distribution of these enzymes may be important in the organospecificity of NNK and NNN. The development of assays for quantifying the metabolic activation of NNK and NNN has led to the discovery of compounds which inhibit this process, most notably isothiocyanates. Further studies on the metabolic activation of NNK and NNN, as well as its inhibition, will lead to insights on mechanisms of nitrosamine carcinogenesis in general. The significance of using NNK and NNN for these studies derives from the fact that there are large human populations exposed to these carcinogens. Although it must be the ultimate goal of society to eliminate the use of all tobacco products, it is unlikely that a tobacco-free society will be achieved in the near future. Those populations which continue to smoke or chew tobacco are high risk groups suitable for testing the efficacy of potential chemopreventive agents, and for comparing mechanisms of cancer induction in experimental animals and man.

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Refererences

1. International Agency for Research on Cancer (1986) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 38. Tobacco Smoking*. IARC, Lyon, France, pp. 309–314.
2. International Agency for Research on Cancer (1985) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 37. Tobacco Habits Other Than Smoking: Betel-Quid and Areca Nut Chewing; and Some Related Nitrosamines*. IARC, Lyon, France, pp. 113–116.
3. Brinton,L.A., Blot,W.J., Becker,J.A., Winn,D.M., Browder,J.P., Farmer,J.C.,Jr and Fraumeni,J.F,Jr (1984) A case-control study of cancers of the nasal cavity and paranasal sinuses. *Am. J. Epidemiol.*, **119**, 896–906.
4. Doll,R. and Peto,R. (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.*, **66**, 1191–1308.
5. Wynder,E.L. and Gori,G.B. (1977) Contribution of the environment to cancer: an epidemiologic exercise. *J. Natl. Cancer Inst.*, **58**, 825–832.
6. National Research Council (1986) *Environmental Tobacco Smoke. Measuring Exposures and Assessing Health Effects*. National Academy Press, Washington, DC, p. 17.
7. United States Department of Health and Human Services (1983) *Why People Smoke Cigarettes*. USPHS Publication No. (PHS) 83-50195. US Government Printing Office, Washington, DC.

8. Advisory Committee to the Surgeon General (1986) *The Health Consequences of Using Smokeless Tobacco*. US Department of Health and Human Services, NIH Publication No. 86–2874, Bethesda, MD, pp. 141–183.
9. International Agency for Research on Cancer (1986) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 38. Tobacco Smoking*. IARC, Lyon, France, pp. 60–65.
10. International Agency for Research on Cancer (1986) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 38. Tobacco Smoking*. IARC, Lyon, France, pp. 164–167.
11. Schmeltz,I. and Hoffmann,D. (1977) Nitrogen-containing compounds in tobacco and tobacco smoke. *Chem. Rev.*, **77**, 295–311.
12. Preussmann,R. and Stewart,B.W. (1984) *N*-Nitroso carcinogens. In Searle,C.E. (ed.), *Chemical Carcinogens*. American Chemical Society, Washington, DC, Vol. 2, pp. 643–828.
13. Hecht,S.S., Chen,C.B., Ornaf,R.M., Jacobs,E., Adams,J.D. and Hoffmann,D. (1978) Reaction of nicotine and sodium nitrite: formation of nitrosamines and fragmentation of the pyrrolidine ring. *J. Org. Chem.*, **43**, 72–76.
14. Hoffmann,D., Rathkamp,G. and Liu,Y.Y. (1974) On the isolation and identification of volatile and non-volatile *N*-nitrosamines and hydrazines in cigarette smoke. In Bogovski,P. and Walker,E.A. (eds), *N-Nitroso Compounds in the Environment. IARC Scientific Publications No. 9*. International Agency for Research on Cancer, Lyon, France, pp. 159–165.
15. Hoffmann,D., Hecht,S.S., Ornaf,R.M. and Wynder,E.L. (1974) *N'*-Nitrosonornicotine in tobacco. *Science*, **186**, 265–267.
16. Hecht,S.S., Chen,C.B., Hirota,N., Ornaf,R.M., Tso,T.C. and Hoffmann,D. (1978) Tobacco-specific nitrosamines: formation by nitrosation of nicotine during curing of tobacco and carcinogenicity in strain A mice. *J. Natl. Cancer Inst.*, **60**, 819–824.
17. Hoffmann,D. and Hecht,S.S. (1985) Nicotine-derived *N*-nitrosamines and tobacco related cancer: current status and future directions. *Cancer Res.*, **45**, 935–944.
18. Brunnemann,K.D., Genoble,L. and Hoffmann,D. (1987) Identification and analysis of a new tobacco-specific *N*-nitrosamine, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol. *Carcinogenesis*, **8**, 465–469.
19. Adams,J.D., Brunnemann,K.D. and Hoffmann,D. (1983) Rapid method for the analysis of tobacco-specific *N*-nitrosamines by gas liquid chromatography with a Thermal Energy Analyzer. *J. Chromatogr.*, **256**, 347–351.
20. International Agency for Research on Cancer (1985) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 37. Tobacco Habits Other Than Smoking: Betel-Quid and Areca Nut Chewing, and Some Related Nitrosamines*. IARC, Lyon, France, pp. 242–246.
21. International Agency for Research on Cancer (1985) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 37. Tobacco Habits Other Than Smoking: Betel-Quid and Areca Nut Chewing, and Some Related Nitrosamines*. IARC, Lyon, France, pp. 210–213.
22. Andersen,R.A. and Kemp,T.R. (1985) Accumulation of 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone in alkaloid genotypes of Burley tobacco during postharvest processing: comparisons with *N'*-nitrosonornicotine and probable nitrosamine precursors. *Cancer Res.*, **45**, 5287–5293.
23. MacKown,C.T., Eivazi,F., Sims,J.L. and Bush,L.P. (1984) Tobacco-specific *N*-nitrosamines: effect of burley alkaloid isolines and nitrogen facility management. *J. Agric. Food Chem.*, **32**, 1269–1272.
24. Brunnemann,K.D., Genoble,L. and Hoffmann,D. (1985) *N*-Nitrosamines in chewing tobacco: an international comparison. *J. Agric. Food Chem.*, **33**, 1178–1181.
25. Adams,J.D., O'Mara-Adams,K.J. and Hoffmann,D. (1987) Toxic and carcinogenic agents in undiluted mainstream smoke and sidestream smoke of different types of cigarettes. *Carcinogenesis*, **8**, 729–731.
26. Hoffmann,D., Adams,J.D., Lisk,D., Fisenne,I. and Brunnemann,K.D. (1987) Toxic and carcinogenic agents in dry and moist snuff. *J. Natl. Cancer Inst.*, **79**, 1281–1286.
27. US Department of Agriculture, Food Safety and Quality Service (1978) Nitrates, nitrites and ascorbates (or isoascorbates) in bacon. *Fed. Reg.*, **43**, 20992–20995.
28. US Food and Drug Administration (1980) Dimethyl nitrosamine in malt beverages: availability of guide. *Fed. Reg.*, **45**, 39341–39342.
29. International Agency for Research on Cancer (1986) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 38. Tobacco Smoking*. IARC, Lyon, France, pp. 86–87.
30. Hecht,S.S., Chen,C.B., Dong,M., Ornaf,R.M., Hoffmann,D. and Tso,T.C. (1977) Studies on non-volatile nitrosamines in tobacco. *Beitr. Tabakforsch.*, **9**, 1–6.
31. Andersen,R.A., Kaperbauer,M.J., Burton,H.R., Hamilton,J.L. and Yoder, E.E. (1982) Changes in chemical composition of homogenized leaf-cured and air cured Burley tobacco stored in controlled environments. *J. Agric. Food Chem.*, **30**, 663–668.
32. Hoffmann,D. and Adams,J.D. (1981) Carcinogenic tobacco-specific *N*-nitrosamines in snuff and saliva of snuff-dippers. *Cancer Res.*, **41**, 4305–4308.
33. Brunnemann,K.D., Scott,J.C. and Hoffmann,D. (1983) *N* Nitrosoproline, an indicator for *N*-nitrosation of amines in processed tobacco. *J. Agric. Food Chem.*, **31**, 905–909.
34. Brunnemann,K.D., Masaryk,J. and Hoffmann,D. (1983) Role of tobacco stems in the formation of *N*-nitrosamines in tobacco and cigarette mainstream and sidestream smoke. *J. Agric. Food Chem.*, **31**, 1221–1224.
35. Hoffmann,D., Dong,M. and Hecht,S.S. (1977) Origin in tobacco smoke of *N'*-nitrosonornicotine, a tobacco-specific carcinogen. *J. Natl. Cancer Inst.*, **58**, 1841–1844.
36. Adams,J.D., Lee,S.J., Vinchkoski,N., Castonguay,A. and Hoffmann,D. (1983) On the formation of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone during smoking. *Cancer Lett.*, **17**, 339–346.
37. Adams,J.D., Lee,S.J. and Hoffmann,D. (1984) Carcinogenic agents in cigarette smoke and the influence of nitrate on their formation. *Carcinogenesis*, **5**, 221–223.
38. Castonguay,A., Lin,D., Stoner,G.D., Radok,P., Furuya,K., Hecht,S.S., Schut,H.A.J. and Klaunig,J.E. (1983) Comparative carcinogenicity in A/J mice and metabolism by cultured mouse peripheral lung of *N'*-nitrosonornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and their analogues. *Cancer Res.*, **43**, 1223–1229.
39. Hecht,S.S., Adams,J.D., Numoto,S. and Hoffmann,D. (1983) Induction of respiratory tract tumors in Syrian golden hamsters by a single dose of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and the effect of smoke inhalation. *Carcinogenesis*, **4**, 1287–1290.
40. Hecht,S.S., Trushin,N., Castonguay,A. and Rivenson,A. (1986) Comparative carcinogenicity and DNA methylation in F344 rats by 4-(methylnitrosamino-1-(3-pyridyl)-1-butanone and *N*-nitrosodimethylamine. *Cancer Res.*, **46**, 498–502.
41. Hoffmann,D., Rivenson,A., Amin,S. and Hecht,S.S. (1984) Dose–response study of the carcinogenicity of tobacco-specific nitrosamines in F344 rats. *J. Cancer Res. Clin. Oncol.*, **108**, 81–86.
42. Hecht,S.S., Rivenson,A., Braley,J., DiBello,J., Adams,J.D. and Hoffmann,D. (1986) Induction of oral cavity tumors in F344 rats by tobacco-specific nitrosamines and snuff. *Cancer Res.*, **46**, 4162–4166.
43. Hoffmann,D., Castonguay,A., Rivenson,A. and Hecht,S.S. (1981) Comparative carcinogenicity and metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine in Syrian golden hamsters. *Cancer Res.*, **41**, 2386–2393.
44. LaVoie,E.J., Prokopczyk,G., Rigotty,J., Czech,A., Rivenson,A. and Adams,J.D. (1987) Tumorigenic activity of the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosmino)-4-(3-pyridyl)-1-butanol (iso-NNAL) and N'-nitrosonornicotine (NNN) on topical application to Sencar mice. *Cancer Lett.*, **37**, 277–283.
45. Hoffmann,D., Raineri,R., Hecht,S.S., Maronpot,R.R. and Wynder,E.L. (1975) Effects of *N'*-nitrosonornicotine and *N'*-nitrosoanabasine in rats. *J. Natl. Cancer Inst.*, **55**, 977–981.
46. Hecht,S.S., Young,R. and Maeura,Y. (1983) Comparative carcinogenicity in F344 rats and Syrian golden hamsters of *N'*-nitrosonornicotine and *N'*-nitrosonornicotine-1-*N*-oxide. *Cancer Lett.*, **20**, 333–340.
47. Castonguay,A., Rivenson,A., Trushin,N., Reinhardt,J., Stathopoulos,S., Weiss,C.J., Reiss,B. and Hecht,S.S. (1984) Effects of chronic ethanol consumption on the metabolism and carcinogenicity of *N'*-nitrosonornicotine in F344 rats. *Cancer Res.*, **44**, 2524–2529.
48. Prokopczyk,G., Adams,J.D., LaVoie,E.J. and Hoffmann,D. (1987) Effect of snuff and nicotine on DNA methylation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Carcinogenesis*, **8**, 1395–1397.
49. Hirsch,J.M. and Johansson,S.L. (1983) Effect of long-term application of snuff on the oral mucosa—an experimental study in the rat. *J. Oral Pathol.*, **12**, 187–198.
50. Konstantinidus,A., Smulow,J.B. and Sonnenschein,C. (1982) Tumorigenesis at a predetermined oral site after one intraperitoneal injection of *N*-nitroso-*N*-methylurea. *Science*, **216**, 1235–1237.
51. Park,N.H., Sapp,J.P. and Herbosa,E.G. (1986) Oral cancer induced in hamsters with herpes simplex infection and simulated snuff-dipping. *Oral Surg. Oral Med. Oral Pathol.*, **62**, 164–168.
52. Hirsch,J.M., Svennerholm,B. and Vahlne,A. (1984) Inhibition of herpes simplex virus replication by tobacco extracts. *Cancer Res.*, **44**, 1991–1997.
53. Chung,F.-L., Juchatz,A., Vitarius,J. and Hecht,S.S. (1984) Effects of dietary compounds on target tissue α-hydroxylation of *N*-nitrosopyrrolidine and *N'*-nitrosonornicotine. *Cancer Res.*, **44**, 2924–2928.
54. Chung,F.-L., Wang,M. and Hecht,S.S. (1985) Effects of dietary indoles and isothiocyanates on *N*-dinitrosodimethylamine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone α-hydroxylation and DNA methylation in rat liver. *Carcinogenesis*, **6**, 529–543.
55. International Agency for Research on Cancer (1985) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 37.*

Tobacco Habits Other Than Smoking: Betel-Quid and Areca Nut Chewing: and Some Related Nitrosamines. IARC, Lyon, France, pp. 216–219.
56. International Agency for Research on Cancer (1985) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 37. Tobacco Habits Other Than Smoking: Betel-Quid and Areca Nut Chewing: and Some Related Nitrosamines*. IARC, Lyon, France, pp. 251–255.
57. Hecht,S.S., Castonguay,A., Rivenson,M., Mu,B. and Hoffmann,D. (1983) Tobacco-specific nitrosamines: carcinogenicity, metabolism, and possible role in human cancer. *J. Environ. Health Sci. Cl.* **1**, 1–54.
58. Mochizuki,M., Anjo,T. and Okada,M. (1980) Isolation and characterization of *N*-alkyl-*N*-(hydroxymethyl) nitrosamines from *N*-alkyl-*N*-(hydroperoxymethyl)nitrosamines by deoxygenation. *Tetrahedron Lett.*, **21**, 3693–3696.
59. Loechler,E.L., Green,C.L. and Essigmann,J.M. (1984) *In vivo* mutagenesis by O^6-methylguanine built into a unique site in a viral genome. *Proc. Natl. Acad. Sci. USA*, **81**, 6271–6275.
60. Barbacid,M. (1986) Oncogenes and human cancer: cause or consequence? *Carcinogenesis*, **7**, 1037–1042.
61. Pegg,A.E. (1984) Methylation of the O^6 position of guanine in DNA is the most likely initiating agent in carcinogenesis by methylating agents. *Cancer Invest.*, **2**, 223–231.
62. Singer,B. (1984) Alkylation of the O^6 of guanine is only one of many chemical events that may initiate carcinogenesis. *Cancer Invest.*, **2**, 233–238.
63. Hecht,S.S., Chen,C.B. and Hoffmann,D. (1980) Metabolic 3-hydroxylation and *N*-oxidation of *N'*-nitrosonornicotine. *J. Med. Chem.*, **23**, 1175–1178.
64. Castonguay,A., Tharp,R. and Hecht,S.S. (1984) Kinetics of DNA methylation by the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the F344 rat. In O'Neill,I.K., von Borstel,R.C., Miller,C.T., Long,J. and Bartsch,H. (eds), *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer*. IARC Scientific Publications, Lyon, France, Vol. 37, pp. 805–810.
65. Castonguay,A., Foiles,P.G., Trushin,N. and Hecht,S.S. (1985) A study of DNA methylation by tobacco specific *N*-nitrosamines. *Environ. Health Perspect.*, **62**, 197–202.
66. Belinsky,S.A., White,C.M., Boucheron,J.A., Richardson,F.C., Swenberg,J.A. and Anderson,M. (1986) Accumulation and persistence of DNA adducts in respiratory tissue of rats following multiple doses of the tobacco-specific carcinogen 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Res.*, **46**, 1280–1284.
67. Belinsky,S.A., White,C.M., Devereux,T.R., Swenberg,J.A. and Anderson,M.W. (1987) Cell selective alkylation of DNA in rat lung following low dose exposure to the tobacco-specific carcinogen 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Res.*, **47**, 1143–1148.
68. Castonguay,A., Stoner,G.D., Schut,H.A.J. and Hecht,S.S. (1983) Metabolism of tobacco-specific *N*-nitrosamines by cultured human tissues. *Proc. Natl. Acad. Sci. USA*, **80**, 6694–6697.
69. Belinsky,S.A., Walker,V.E., Maronpot,R.R., Swenberg,J.A. and Anderson,M.W. (1987) Molecular dosimetry of DNA adduct formation and cell toxicity in rat nasal mucosa following exposure to the tobacco specific nitrosamine 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl) 1-butanone and their relationship to induction of neoplasia. *Cancer Res.*, **47**, 6058–6065.
70. Tjälve,H., Castonguay,A. and Rivenson,A. (1985) Microautoradiographic localization of bound metabolites in the nasal cavities of F344 rats treated with the tobacco-specific carcinogen 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone. *Natl. Cancer Inst.*, **74**, 185–190.
71. Hecht,S.S., Spratt,T.E. and Trushin,N. (1988) Evidence for 4-(3-pyridyl)-4-oxobutylation of DNA in F344 rats treated with the tobacco-specific nitrosamines 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine. *Carcinogenesis*, **9**, 161–165.
72. Carmella,S.G. and Hecht,S.S. (1987) Formation of hemoglobin adducts upon treatment of F344 rats with the tobacco-specific nitrosamines 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine. *Cancer Res.*, **47**, 2626–2630.
73. Hecht,S.S. and Lin,D. (1986) Comparative mutagenicity of 4-(carbethoxynitrosamino)-4-(3-pyridyl)-1-butanal, and 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone, model compounds for α-hydroxylation of *N'*-nitrosonornicotine. *Carcinogenesis*, **7**, 611–614.
74. Hoffmann,D. and Brunnemann,K.D. (1983) Endogenous formation of *N*-nitrosoproline in cigarette smokers. *Cancer Res.*, **43**, 5570–5574.
75. Ladd,K.F., Newmark,H.L. and Archer,M.C. (1984) *N*-Nitrosation of proline in smokers and nonsmokers. *J. Natl. Cancer Inst.*, **73**, 83–87.
76. Bartsch,H., Ohshima,H., Muñoz,N., Crespi,M., Cassale,V., Ramazotti,V., Lambert,R., Minaire,Y., Forichon,J. and Walters,C.L. (1984) *In vivo* nitrosation, precancerous lesions and cancers of the gastrointestinal tract. On-going studies and preliminary results. In O'Neill,I.K., Von Borstel,R.C., Miller,C.T., Long,J. and Bartsch,H. (eds), *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer. IARC Scientific Publications No. 57*. International Agency for Research on Cancer, Lyon, France, pp. 957–964.
77. Ayesh,R., Idle,J.R., Ritchie,J.C., Crothers,M.J. and Hetzel,M.R. (1984) Metabolic oxidation phenotypes as markers for susceptibility to lung cancer. *Nature*, **312**, 169–170.
78. O'Neill,I.B., Brunnemann,K.D., Dodet,B. and Hoffmann,D. (1987) *Environmental Carcinogens: Methods of Analysis and Exposure Measurement. Vol. 9. Passive Smoking*. IARC Publications, Lyon, France, Vol. 81, pp. 293–337.
79. Bryant,M.S., Skipper,P.L., Tannenbaum,S.R. and Maclure,M. (1987) Hemoglobin adducts of 4-aminobiphenyl in smokers and nonsmokers. *Cancer Res.*, **47**, 602–608.
80. Törnqvist,M., Osterman-Golkar,S., Kautiainen,A., Jensen,S., Farmer,P.B. and Ehrenberg,L. (1986) Tissue doses of ethylene oxide in cigarette smokers determined from adduct levels in hemoglobin. *Carcinogenesis*, **7**, 1519–1521.
81. Harris,C.C., Vahakangas,K., Newman,M.J., Trivers,G.E., Shamsuddin,A., Sinopoli,N., Mann,D.L. and Wright,W.E. (1985) Detection of benzo[*a*]pyrene diol-epoxide-DNA adducts in peripheral blood lymphocytes and antibodies to the adducts in serum from coke oven workers. *Proc. Natl. Acad. Sci. USA*, **82**, 6672–6676.
82. Ehrenberg,L. and Osterman-Golkar,S. (1976) Alkylation of macromolecules for detecting mutagenic agents. *Teratogen. Carcinogen. Mutagen.*, **1**, 105–127.
83. Wild,C.P., Smart,G., Saffhill,R. and Boyle,J.M. (1983) Radioimmunoassay of O^6-methyldeoxyguanosine in DNA of cells alkylated *in vitro* and *in vivo*. *Carcinogenesis*, **4**, 1605–1609.
84. Foiles,P., Trushin,N. and Castonguay,A. (1985) Measurement of O^6-methyldeoxyguanosine in DNA methylated by the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone using a biotin-avidin enzyme-linked immunosorbent assay. *Carcinogenesis*, **6**, 989–993.
85. Degan,P., Montesano,R. and Wild,C.P. (1988) Development of antibodies against N^7-methyldeoxyguanosine (N^7-MEDg) to determine exposure to alkylating agents. In Bartsch,H., Hemminki,K. and O'Neill,I.K. (eds), *Methods for Detecting DNA Damaging Agents in Humans: Applications in Cancer Epidemiology and Prevention*. IARC Sci. Publ. No.89. International Agency for Research on Cancer, Lyon, France, in press.
86. Advisory Committee to the Surgeon General (1986) *The Health Consequences of Using Smokeless Tobacco*. US Department of Health and Human Services, NIH Publication, No. 86-2874, Bethesda, MD, pp. 33–47.
87. National Institutes of Health (1986) *Consensus Development Conference Statement: Health Implications of Smokeless Tobacco Use*. National Institutes of Health, Bethesda, MD, pp. 1–8.
88. Winn,D.M., Blot,W.J., Shy,C.M., Pickle,L.W., Toledo,A. and Fraumeni,J.R.,Jr (1981) Snuff-dipping and oral cancer among women in the southern United States. *New Engl. J. Med.*, **304**, 745–749.
89. Winn,D.M., Ziegler,R.G., Pickle,L.W., Gridley,G., Blot,W.J. and Hoover,R.N. (1984) Diet in the etiology of oral and pharyngeal cancer among women from the southern United States. *Cancer Res.*, **44**, 1216–1222.
90. International Agency for Research on Cancer (1985) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 37. Tobacco Habits Other Than Smoking: Betel-Quid and Areca Nut Chewing: and Some Related Nitrosamines*. IARC, Lyon, France, pp. 186–188.
91. International Agency for Research on Cancer (1985) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 37. Tobacco Habits Other Than Smoking: Betel-Quid and Areca Nut Chewing: and Some Related Nitrosamines*. IARC, Lyon, France, pp. 141–202.
92. Sipahimalani,A.T., Chadha,M.S., Bhide,S.V., Pratap,A.I. and Nair,Y. (1984) Detection of *N*-nitrosamines in the saliva of habitual chewers of tobacco. *Food. Chem. Toxicol.*, **22**, 261–264.
93. Wenke,G., Brunnemann,K.D. and Hoffmann,D. (1984) A study of betal quid carcinogenesis. IV. Analysis of the saliva of chewers. A preliminary report. *J. Cancer Res. Clin. Oncol.*, **108**, 110–113.
94. Nair,J., Ohshima,H., Friesen,M., Croisy,A., Bhide,S.V. and Bartsch,H. (1985) Tobacco-specific and betel nut-specific *N*-nitroso compounds: occurrence in saliva and urine of betel quid chewers and formation *in vitro* by nitrosation of betel quid. *Carcinogenesis*, **6**, 295–303.
95. Prokopczyk,B., Rivenson,A., Bertinato,P., Brunnemann,K.D. and Hoffmann,D. (1987) 3-(Methylnitrosamino) propionitrile: occurrence in saliva of betel quid chewers, carcinogenicity, and DNA methylation in F344 rats. *Cancer Res.*, **47**, 467–471.

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A recently completed lifetime bioassay of NNK and NNAL, given in the drinking water to F344 rats, produced significant incidences of pancreatic acinar and duct tumors in addition to lung tumors. NNK and NNAL are the only tobacco carcinogens known to induce cancer of the pancreas (D.Hoffman, A.Rivenson, and S.S.Hecht, Induction of cancer of the pancreas in rats by tobacco-specific N-nitrosamines (Submitted to *Nature*.)