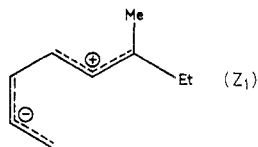


(5) Our own view is that the polarized intermediate would best be stabilized by negative countercharges near atoms 7, 9, and 11.³⁹ A sharp drop in energy, down to ground state, similar to that advocated by Lewis, would then occur, and the first visual intermediate would be formed.

Conclusion

The definitive experimental verification of the sudden polarization effect remains to be done. An extremely strong indication would be given by disrotatory cyclopropane ring closure in hexatrienes substituted by donor groups on the s-trans moiety. The ensuing



polarization should indeed be opposite to that en-

(39) Such charges would also, at least partially, account for the famous shift occurring when chromophore is linked to protein.

countered usually.⁴⁰ A more direct test would be observation of strong intensification of resonance Raman lines of the lowest excited state (as monitored by picosecond spectroscopy) in the hexatriene molecule as it twists into its highly polarizable—and highly polarized—geometry. These two experiments are now being carried out at Berkeley⁴¹ and Harvard,⁴² respectively. Another possibility would be to observe laser-excited fluorescence from twisted olefinic excited states, as presently attempted at Livermore.⁴³

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Tobacco-Specific Nitrosamines: Occurrence, Formation, Carcinogenicity, and Metabolism

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It is now widely accepted that cigarette smoking is causally associated with lung cancer.^{1,2} It is less widely known that smoking is also correlated with an increased incidence of cancer of the oral cavity, esophagus, pancreas, and bladder.²⁻⁶ Tobacco chewing can also cause oral cavity and esophageal cancer.^{3,4,7} In fact, cancer of the mouth is a major cancer among men in India, where the habit of chewing the betel quid containing tobacco is widespread.⁸ Cigarette smoke is known to contain tumor initiators, such as the polynuclear aromatic hydrocarbons, and tumor promoters and cocarcinogens, such as catechol.⁹ These agents can explain many of the observed effects of cigarette smoke condensates in experimental animals and most likely are involved in some of the human cancers associated

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The investigators are interested in the isolation, identification, and reduction of carcinogens in materials which have been associated with human cancer, as well as in the metabolic activation of polynuclear aromatic hydrocarbons, *N*-nitrosamines, other carcinogens, and the reaction of the active species of carcinogens with cellular macromolecules. This paper is no. 17 in the series "A Study of Tobacco Carcinogenesis".

with smoking. However, nitrosamines may also be causative factors in the tobacco-related cancers, especially in those organs which are remote from direct contact with tobacco or tobacco smoke. Thus it is known that nitrosamines can cause esophageal, pancreas, and bladder cancer in experimental animals as well as affect the lung and oral cavity.¹⁰⁻¹²

Since the first report on the carcinogenicity of dimethylnitrosamine,¹³ a wide variety of nitrosamines have been tested in various experimental animals.^{10,14}

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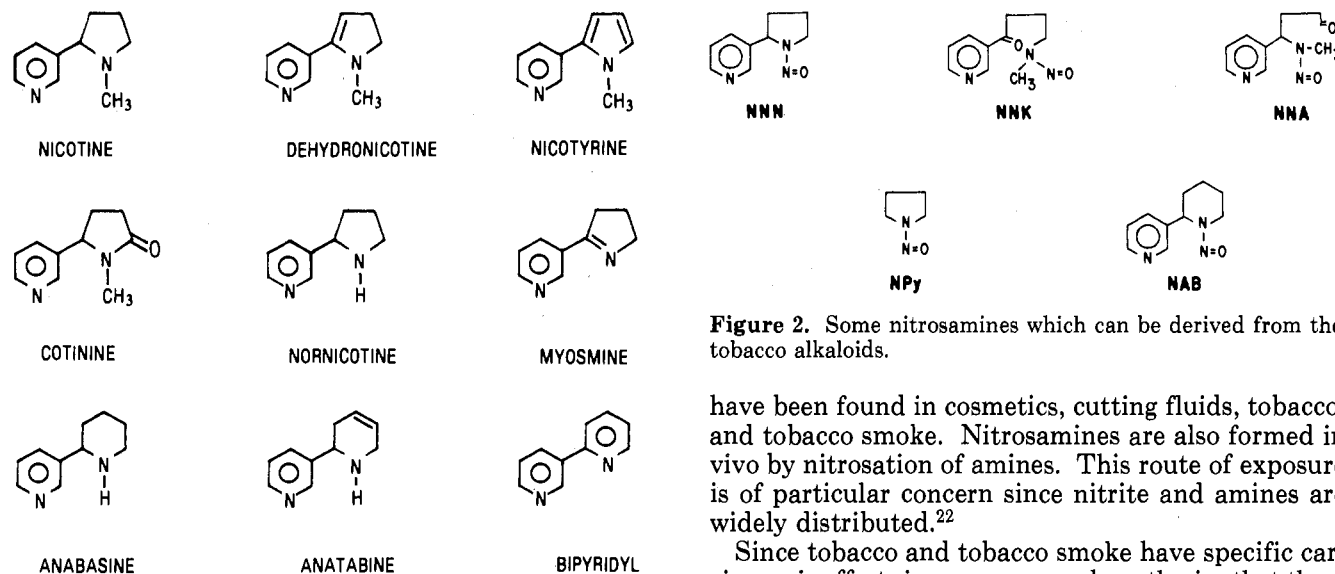


Figure 1. Common tobacco alkaloids in tobacco and/or tobacco smoke.

Most nitrosamines with available hydrogens on the carbons α to the nitrosamine nitrogen are carcinogenic. Nitrosamines generally affect specific organs in a given experimental animal, and the effect is often independent of the route of administration. In animals generally used for bioassay the target organs are frequently the esophagus and liver in the rat, the respiratory tract in the Syrian golden hamster, and the lungs and liver in the mouse.^{10,14} However, other organs such as pancreas and bladder are also affected by nitrosamines with particular structural characteristics.^{10,12,15}

Because of the structural diversity of nitrosamines which can cause cancer in experimental animals and the potency of many of these agents, their occurrence and formation in human environments have been studied intensively.¹⁰ Analytical methods are now available for the routine determination of trace amounts of nitrosamines. Important among these is the methodology involving the "thermal energy analyzer" (TEA), a sensitive and specific detector for nitrosamines, which can be coupled to a gas or liquid chromatograph.¹⁶ By use of this technique and conventional analytical methods, nitrosamines have been identified most commonly in processed meats, cheese, air pollution, unburned tobacco, mainstream and sidestream tobacco smoke, cosmetics, and industrial cutting fluids.¹⁷⁻²¹ Concentrations are often in the parts per billion range, although much higher levels of certain nitrosamines

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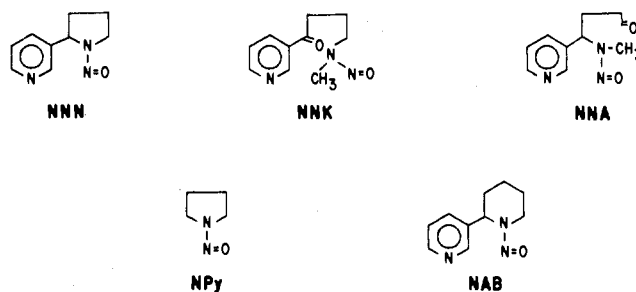


Figure 2. Some nitrosamines which can be derived from the tobacco alkaloids.

have been found in cosmetics, cutting fluids, tobacco, and tobacco smoke. Nitrosamines are also formed in vivo by nitrosation of amines. This route of exposure is of particular concern since nitrite and amines are widely distributed.²²

Since tobacco and tobacco smoke have specific carcinogenic effects in man, one can hypothesize that there may be unique carcinogenic agents in tobacco and tobacco smoke. The tobacco-specific nitrosamines are such a group. These nitrosamines are derived from the tobacco alkaloids (see Figure 1). The most prevalent alkaloid is nicotine, which occurs in general in concentrations of 1-2% in commercial tobacco products.

Both nicotine and nornicotine could give rise to the prototype of tobacco-specific nitrosamines, *N'*-nitrosornicotine (NNN). Nicotine could also be nitrosated to form 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or 4-(*N*-methyl-*N*-nitrosamino)-4-(3-pyridyl)butanal (NNA). In addition, *N*-nitrosopyrrolidine (NPy), which is formed during smoking, could be derived from nicotine and nornicotine.¹⁹ Nitrosation of anabasine would give nitrosoanabasine (NAB). The structures of the nitrosamines which will be considered in this Account are shown in Figure 2. Of course, inspection of Figure 1 reveals other interesting possibilities for nitrosation of the tobacco alkaloids; some of these possibilities are the subjects of ongoing studies.

Occurrence and Formation of Tobacco-Specific Nitrosamines

The prototype of the tobacco-specific nitrosamines, NNN, has been detected in both tobacco smoke and unburned tobacco. Various analytical methods have been used, including gas chromatography (GLC),²³⁻²⁶ combined GLC-mass spectrometry,²⁷ thin-layer chromatography,²⁸ high-pressure liquid chromatography (LC),^{29,30} and combined LC-thermal energy analysis.³¹

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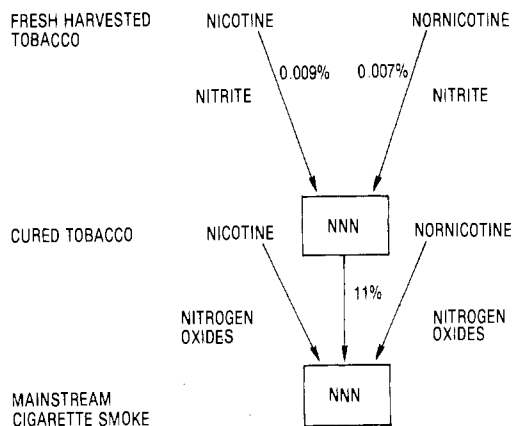


Figure 3. Origins of NNN in tobacco and tobacco smoke (tobacco is known to contain nitrate and nitrite²⁵).

NNN levels in the smoke of a typical American 85-mm nonfilter cigarette range from 140 to 240 ng/cigarette. Surprisingly high levels of NNN were found in unburned tobacco (0.3–9.0 ppm in cigarette tobacco, 3.0–45.3 ppm in cigar tobacco, 3.5–90.6 ppm in chewing tobacco, and 12.1–29.1 ppm in snuff). These levels are among the highest for an environmental nitrosamine in terms of occurrence and human exposure.³² Thus, rather detailed studies were carried out to determine the origins of NNN in tobacco and tobacco smoke.

To study the formation of NNN in tobacco, plants were analyzed at various stages of growth and curing.³³ NNN was not detected prior to harvest or in freshly harvested Burley tobacco but only during and after air curing (0.5–1.1 ppm), which is the process normally employed before use of Burley tobacco in market products. Since either nicotine or nornicotine could have been a precursor to NNN in tobacco, tobacco leaves were fed nicotine-2'-¹⁴C or nornicotine-2'-¹⁴C.³⁴ The yield of NNN from nicotine was 0.009% and from nornicotine, 0.007%. These results showed that both nicotine and nornicotine could be precursors to NNN in tobacco. However, the greater abundance of nicotine in tobacco leaf (20–100 times the concentration of nornicotine) favored nicotine as the major precursor of NNN in tobacco.

The transfer of NNN from cigarette tobacco to mainstream smoke was also studied.³⁰ For this purpose, NNN-2'-¹⁴C was added to cigarettes and the smoke was analyzed. The transfer rate was found to be 11.3%. Since, in this experiment, the tobacco column smoked contained 974 ng of NNN, 110 ng was transferred to the mainstream smoke. Analysis of the mainstream smoke revealed 238 ng of NNN; thus the remaining 128 ng was formed during smoking. Therefore, about 50% of the NNN in mainstream smoke originated by transfer from tobacco while the remainder was formed during smoking.

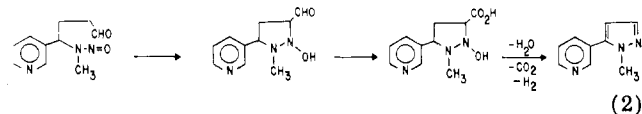
Either nicotine or nornicotine could be a precursor to NNN formed during smoking. To examine this question, nicotine or nornicotine was added to cigarettes and the smoke was analyzed for NNN.²³ In each case, NNN concentration in smoke increased, indicating that

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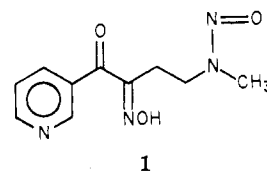
Scheme I Formation of 1-Methyl-5-(3-pyridyl)pyrazole (2) from NNA



both alkaloids are precursors to NNN formed during smoking. However, nicotine is considered the more important precursor due to its higher concentration in tobacco. The results of these studies on the formation of NNN during curing, its transfer to smoke, and its formation during smoking are summarized in Figure 3.

In tobacco samples examined so far, the levels of NAB are significantly less than those of NNN. In fact, NAB has not yet been detected with certainty in unburned tobacco.²⁵ These findings are in line with the major role of nicotine rather than nornicotine as a precursor to NNN since kinetic studies showed that nornicotine and anabasine were nitrosated at similar rates.³⁵ The fact that these rates are relatively high suggests that the formation of NNN and NAB could be favored *in vivo*. When chewing tobacco was incubated with human saliva for 3 h at 37 °C and the mixture analyzed for NNN, the concentration of NNN increased by 44% over that in the chewing tobacco, presumably as a result of further nitrosation.²⁵ Thus, *in vivo* formation of NNN and NAB could constitute an additional exposure of smokers or chewers to these tobacco-specific nitrosamines.

Since nicotine is the major precursor to NNN in tobacco and tobacco smoke, the reaction of nicotine with sodium nitrite was studied to provide information on formation of other tobacco-specific nitrosamines, especially NNK and NNA, which could arise by oxidative cleavage of the 1'-2' bond or 1'-5' bond of nicotine followed by nitrosation.³⁶ The reaction was investigated under a variety of conditions. All three nitrosamines were formed when the reaction was done under relatively mild conditions (17 h, 20 °C). The yields (0.1–2.8%) were typical of those for formation of nitrosamines from tertiary amines.³⁷ At 90 °C, with a fivefold excess of nitrite, only NNN and NNK were detected. Under these conditions, both NNK and NNA gave secondary products. NNK was nitrosated α to the carbonyl to yield 4-(*N*-methyl-*N*-nitrosamino)-2-oximino-1-(3-pyridyl)-1-butanone (1), while NNA under-



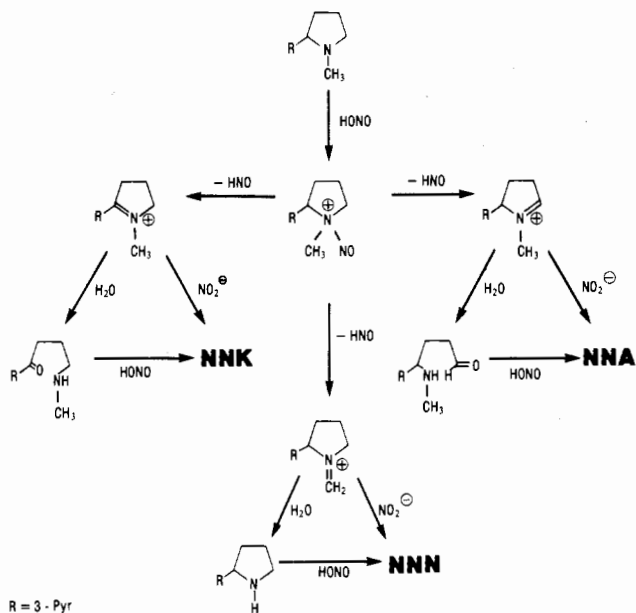
went cyclization followed by oxidation, decarboxylation, and dehydration to give 1-methyl-5-(3-pyridyl)pyrazole (2) as shown in Scheme I. Extensive fragmentation and oxidation of the pyrrolidine ring were also observed under these conditions. The major products resulting from fragmentation of the pyrrolidine ring were *cis*- and

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Scheme II
Formation of Tobacco-Specific
Nitrosamines from Nicotine



trans-3-(3-pyridyl)acrylonitrile and *N*-methylnicotinamide.

The formation of NNN, NNK, and NNA from nicotine probably involves the intermediacy of cyclic iminium salts, as shown in Scheme II.³⁸ These salts can undergo hydrolysis to the free amines which are nitrosated, or, at near neutral pH, can be directly nitrosated to give nitrosamines. The formation of nitrosamines from iminium salts under neutral conditions has been demonstrated in at least two studies and is of interest because iminium salts are known to be intermediates in the mammalian metabolism of nicotine.^{36,39-41} The possibility that tobacco bacteria could nitrosate nicotine via this pathway is currently under investigation.

The formation of NNK and NNA from nicotine in these model studies encouraged us to search for these nitrosamines in tobacco and tobacco smoke. In studies undertaken so far, NNK, but not NNA, has been detected. NNK was most readily analyzed by combined LC-TEA, although conventional LC methods have also been used.^{31,34} Levels of NNN and NNK in tobacco and mainstream cigarette smoke are summarized in Table I. These data are significant because carcinogenicity studies indicated activity for both NNK and NNN. The analytical studies discussed in this section were all done using NNN-2¹⁴C as internal standard. Tobacco was extracted with aqueous ascorbic acid and smoke was collected in traps containing ascorbic acid to prevent artefactual formation of nitrosamines.

Carcinogenicity of Tobacco-Specific Nitrosamines

The first studies on the carcinogenicity of NAB and NNN were done by Boyland and co-workers, who demonstrated that NAB caused esophageal tumors in

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Table I
NNN and NNK in Tobacco and Tobacco Smoke

product	mainstream, μg/cig		sidestream, μg/cig		tobacco, ppm	
	NNN	NNK	NNN	NNK	NNN	NNK
Burley, NF	3.7	0.32	6.1	0.66	7.0	-
Bright, NF	0.62	0.42	1.7	0.50	0.22	0.37
commercial, NF	0.24	0.11	1.7	0.41	1.7	0.74
commercial, F	0.31	0.19	nd ^a	nd	1.4	0.70
Kentucky, 1R1, NF	0.39	0.16	nd	nd	0.63	0.13
little cigar, F	5.5	4.2	0.88	0.81	45.3	35.4
large cigar, F	3.6	2.4	nd	nd	5.0	2.2
Columbia cigar	3.2	1.9	16.6	15.7	nd	nd

^a nd = not determined.

rats and that NNN induced lung adenomas in mice.^{42,43} In our own studies, the carcinogenicity of NNN and NAB was first compared in Fischer rats.⁴⁴ In the NNN group, 14 of 20 animals developed esophageal tumors after a total dose of 3.6 mmol/rat given in the drinking water. By contrast, NAB at this dose gave only 2 of 20 tumor-bearing animals. NNN also induced tumors of the olfactory epithelium in Sprague-Dawley rats, as demonstrated by Singer and Taylor.⁴⁵

The tumorigenic activities of NNN and NAB were then compared in Syrian Golden hamsters.⁴⁶ In this experiment, NNN and NAB were each given by subcutaneous injection, the total dose being 2 mmol/hamster. Within 83 weeks, 12 of 19 hamsters given NNN developed tumors of the trachea, which is a typical target organ for nitrosamines in this species. No tumors were observed in the animals treated with NAB.

To compare NNN, NNK, and NNA, bioassays were done in strain A mice, which are unusually susceptible to lung adenomas.³⁴ The number of these tumors observed in treated vs. control groups can be used as an indicator of carcinogenic activity. Each compound was given in a total dose of 0.1 mmol/mouse. As judged by multiplicity of lung tumors, both NNN and NNK showed significant activity ($P < 0.05$) compared to controls, and NNK was significantly more active ($P < 0.05$) than NNN. NNA did not show significant tumorigenic activity. The greater tumorigenicity of NNK and NNN in this strain of mice suggests potentially higher carcinogenicity in other rodent species; these bioassays are currently in progress.

Metabolic Studies on NPy and NNN

Nitrosamines, like many other classes of chemical carcinogens, must undergo metabolic transformation to be converted into reactive electrophilic species which can alkylate nucleophilic cellular macromolecules. This process of metabolic activation has been studied extensively by the Millers, who were pioneers in developing these concepts. According to their scheme, an inactive procarcinogen is metabolically transformed to a proximate carcinogen and finally to an ultimate carcinogen; the latter is a reactive electrophile such as

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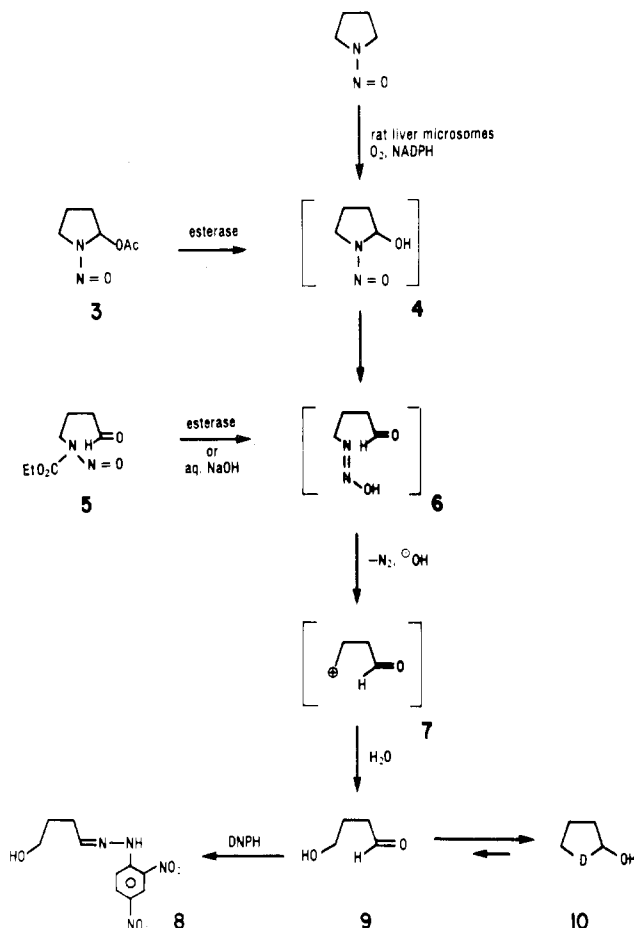
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a carbonium ion.⁴⁷ Such a scheme can be applied to dialkyl or cyclic nitrosamines in several ways, and various critical initial steps have been suggested, including α -hydroxylation, β -hydroxylation, and δ -oxidation.^{10,14,48,49} Since the intermediates generated metabolically may be unstable, indirect means have been used to gain evidence supporting the various pathways. Most studies to date on both cyclic and acyclic nitrosamines support the hypothesis that an initial α -hydroxylation is a critical step in carcinogenesis by nitrosamines. For cyclic nitrosamines, substitution at the α positions often results in decreased carcinogenicity, as demonstrated in studies by Lijinsky, Keefer, and Taylor. For example, 2,5-Me₂NPY was significantly less carcinogenic in the rat than an equimolar dose of NPY.⁵⁰ Similar results were obtained with nitrosopiperidine.⁵¹ Substitution of deuterium atoms α to the nitrosamine function of nitrosomorpholine decreases activity. Thus, 3,3,5,5-tetradeuterionitrosomorpholine was less carcinogenic than nitrosomorpholine.⁵² This reduction in activity was consistent with the slower rate of C-D bond breaking in α -hydroxylation of the deuterated compound.

Further information on the role of α -hydroxynitrosamines as active intermediates in nitrosamine carcinogenesis has been obtained in recent years by the use of α -acetoxy nitrosamines as model compounds.^{53,54} Numerous α -acetoxy nitrosamines have been synthesized, and most, including 2-(AcO)NPY, were mutagenic in *Salmonella typhimurium* without enzymatic activation.⁵³⁻⁵⁹ In addition, methyl(acetoxymethyl)nitrosamine and 1-acetoxypyrrolpropyl nitrosamine both showed primarily local carcinogenic effects in experimental animals.⁶⁰⁻⁶² These results are consistent with the role of α -hydroxylation in activation of dialkyl nitrosamines, since in the presence of esterase, the α -acetoxy compounds are readily hydrolyzed to the corresponding α -hydroxynitrosamines.

Despite the apparent importance of α -hydroxylation as an activation step for cyclic nitrosamines, limited information was available on metabolism of these compounds by this process.⁶³⁻⁶⁵ This was due, in part,

Scheme III
Intermediates and Products Resulting from
 α -Hydroxylation of NPY^a



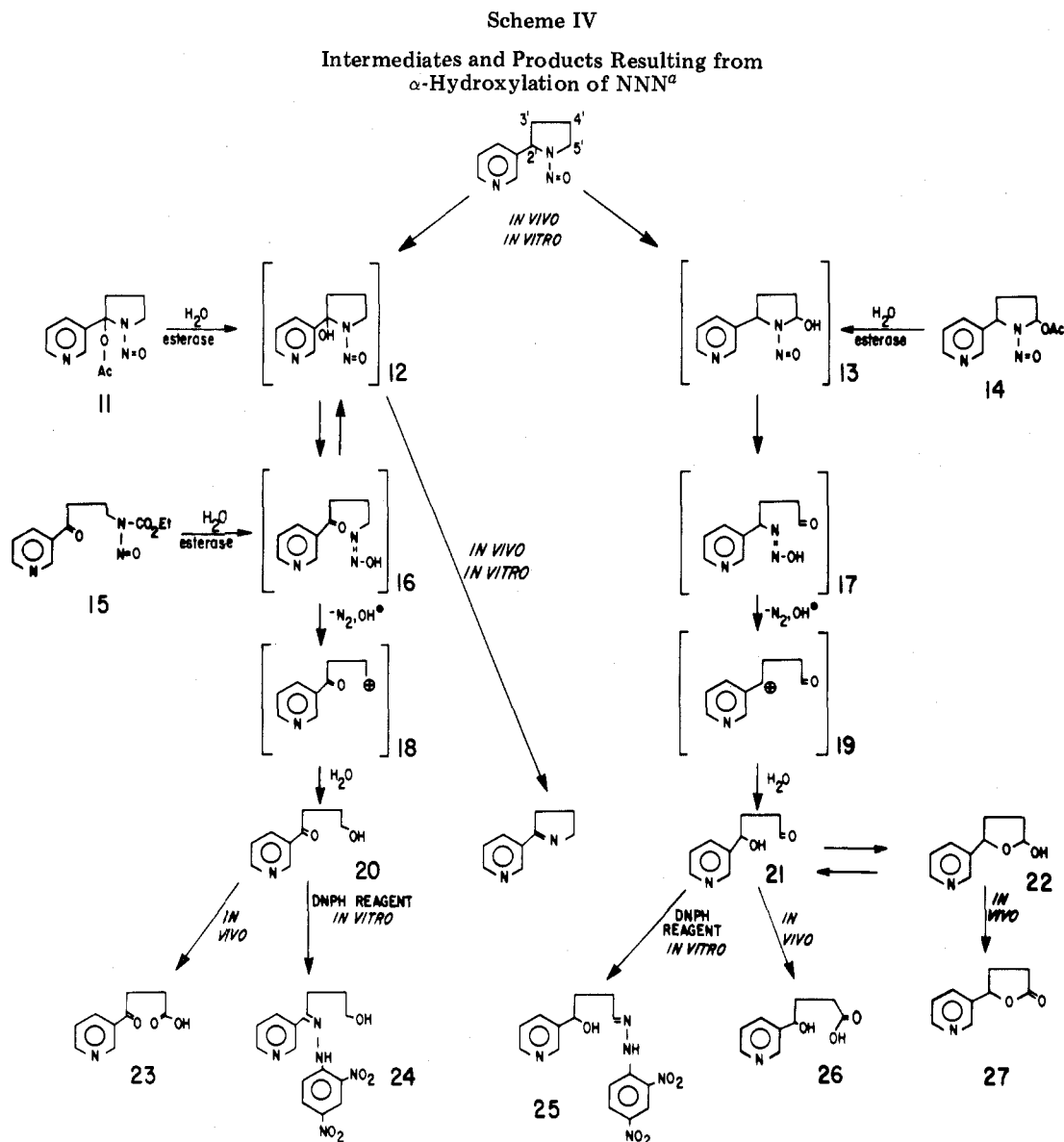
^a Reproduced with permission from *Cancer Research*.⁶⁶

to the inherent instability of the α -hydroxynitrosamines. In our studies on the metabolism of cyclic nitrosamines, we have used model compounds to determine the probable products of metabolic α -hydroxylation and have then searched for these products as metabolites. In this way, metabolic α -hydroxylation of NPY and NNN was demonstrated.⁶⁶⁻⁶⁸

The approach for NPY is outlined in Scheme III. α -Hydroxylation of NPY would give 2-(HO)NPY (4) which is expected to undergo spontaneous ring opening to 3-formyl-1-propanediazohydroxide (6); this intermediate would lose N₂ and hydroxide to give oxocarbenium ion 7. This oxocarbenium ion could react with cellular macromolecules as well as be trapped by water to give 4-hydroxybutanal (9), which exists predominantly as the cyclic hemiacetal, 2-hydroxytetrahydrofuran (10). To validate this scheme, 2-(AcO)NPY⁵⁶ (3) and 4-(N-carbethoxy-N-nitrosamino)butanal (5) were synthesized as precursors to the unstable intermediates 4 and 6. Hydrolysis of both 3 and 5 gave 2-hydroxytetrahydrofuran (10) as the major product, in support of the intermediacy of 4, 6, and 7

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as shown in Scheme III. Both 3 and 5 were mutagenic in *S. typhimurium* strains TA 100 and TA 1535 without enzymatic activation, presumably because of in situ hydrolysis to 4, 6, and 7.

Metabolic α -hydroxylation of NPy was then demonstrated in vitro by isolation of 4-hydroxybutyraldehyde 2,4-dinitrophenylhydrazone (8) after incubation of NPy with liver microsomes. The formation of 10 was also demonstrated in vivo by isolation of 8 from the urine of rats injected with NPy.

Metabolic α -hydroxylation of the tobacco-specific carcinogen NNN was studied using a similar approach. Despite the apparent similarity of NNN and NPy, some differences were apparent in the model and metabolic studies. Scheme IV summarizes the main features of these experiments. Both 2'-(AcO)NNN (11) and 5'-(AcO)NNN (14) were synthesized as model precursors to 2'-(HO)NNN (12) and 5'-(HO)NNN (13). The syntheses were done by procedures reported for 2-(AcO)NPy.⁵⁶ Hydrolysis of the 2'-acetate 11 gave, as major products, myosmine (50–60%) and the keto alcohol 20 (5–10%). The 5'-acetate 14 gave predominantly the lactol 22 (60–70%). Formation of 20 and 22

from the 2'-acetate and the 5'-acetate can be rationalized as shown in Scheme IV. However, the high yield of myosmine in the solvolysis of 11 and the rapid rate of decomposition of 11 in H₂O (half-life at 37 °C \approx 10 min vs. 180 min for 14) indicated that loss of ⁻OAc and NO⁺ was a competing pathway to formation of 12. Therefore, nitrosourethane 15 was also prepared as a model compound for the 2'-hydroxylation pathway. Hydrolysis of 15 gave predominantly 20.

When tested in *S. typhimurium* TA 100, all three model compounds were mutagenic without activation. The 2'-acetate 11 was weakly mutagenic, the 5'-acetate 14 was moderately mutagenic, and the nitrosourethane 15 was highly mutagenic. These differences in mutagenicity may be partially due to differing rates of hydrolysis of 11, 14, and 15. When NNN was tested at comparable doses in the presence of hepatic supernatants, no significant activity was observed. However, NNN was mutagenic at higher doses.

Evidence for metabolic α -hydroxylation of NNN was obtained in vitro or in vivo. When NNN was incubated with rat liver microsomes, the formation of 20 and 21 was demonstrated by isolation of the 2,4-dinitro-

phenylhydrazones **24** and **25**. When rats were treated with NNN, 73–85% of the dose was excreted in the urine, but **20** and **21** were not detected. However, products of further oxidation of **20** and **21**, the keto acid **23**, hydroxy acid **26**, and lactone **27** were isolated. The formation of **20** and **21** in vitro is most readily explained by initial 2'-hydroxylation or 5'-hydroxylation of NNN, as indicated in Scheme IV. The metabolites **23**, **26**, and **27** were formed, at least partially, by metabolic oxidation of **20** and **21**. Pathways other than an initial α -hydroxylation of NNN could have been involved in the formation of **23**, **26**, and **27**, which are also observed in the metabolism of nicotine.⁶⁹

The results of these in vitro and in vivo experiments demonstrate that both NPy and NNN undergo metabolic α -hydroxylation in the rat. The mutagenicity data discussed above are consistent with the involvement of α -hydroxylation as the critical step in the metabolic activation of NPy and NNN. Further evidence on the role of α -hydroxylation in the activation of these compounds is currently being sought through carcinogenicity studies of α -deuterated NNN derivatives and through studies of the binding of NPy and NNN to DNA and RNA of target tissues.

Prospects

The results described in this review indicate that the tobacco-specific carcinogens, NNN and NNK, may be causative factors in the various cancers associated with tobacco usage. These nitrosamines are derived predominantly from the major tobacco alkaloid, nicotine, by nitrosation during the curing and smoking of tobacco. Other tobacco alkaloids may also be precursors to carcinogenic nitrosamines. Since NNN and NNK form during curing, it is feasible to reduce their concentrations in tobacco by appropriate management of

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the curing and related processes. Formation during smoking can also be inhibited. The reduction of these and related nitrosamines in tobacco and tobacco smoke is one approach to reduce tobacco-related cancers.

A second approach begins with an understanding of the metabolic activation and detoxification of tobacco-specific nitrosamines. The enzymes that mediate these transformations can be induced or inhibited by environmental modifiers. Such modifiers may increase or decrease the carcinogenic effects of these nitrosamines. NNN and NPy, as well as NNK, all undergo metabolic α -hydroxylation which is a likely activation process. Specific induction of α -hydroxylation could lead to greater carcinogenic activity; the modifier causing this would act as a cocarcinogen. Similarly, specific inhibition of α -hydroxylation could have a protective effect against carcinogenesis by these nitrosamines. The identification of these modifiers through metabolic studies and bioassays is important for a more complete characterization of the causative factors in tobacco carcinogenesis and for the prevention of tobacco-related cancer.

Note Added in Proof. Recently *N'*-nitrosoanatabine, another tobacco-specific *N*-nitrosamine, has been identified in tobacco (0.6–13 ppm), cigarette smoke (0.33–4.6 $\mu\text{g}/\text{cig}$), and cigarette sidestream smoke (0.15–1.5 $\mu\text{g}/\text{cig}$).⁷⁰ Data on the carcinogenic activity of this nitrosamine are not yet available.

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Alkylidene Complexes of Niobium and Tantalum

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Complexes containing a heteroatom-substituted carbene ligand, such as $\text{W}(\text{CO})_5[\text{C}(\text{Ph})(\text{OMe})]$, were first prepared by Fischer 15 years ago.¹ Hundreds are now known which contain metals from groups 6–8 (usually in a 0 or +1 formal oxidation state if the carbene is taken to be a neutral two-electron ligand).

By comparison, isolable complexes of carbenes which contain only C and H (primary and secondary alkyl-

idene complexes²) are rare, possibly because alkylidene ligands are not "stabilized" by a heteroatom substituent and the complexes therefore are more reactive. They (especially primary alkylidene complexes) had been postulated for several years as intermediates in the decomposition of transition-metal alkyl complexes³ and

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(2) The alkylidene nomenclature is a useful, less misleading alternative to the carbene nomenclature. An alkylidene is derived from an alkyl ligand by removing an α -hydrogen atom. A primary alkylidene is derived from a primary alkyl ligand, methylene being a unique member of this family. A secondary alkylidene (or disubstituted methylene) is derived from a secondary alkyl ligand.

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