Metabolism of *N*′-Nitrosonornicotine Enantiomers by Cultured Rat Esophagus and in Vivo in Rats

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People who use tobacco products are exposed to considerable amounts of *N*′-nitrosonornicotine (NNN), a well-established esophageal carcinogen in rats. NNN is believed to play a significant role as a cause of esophageal and oral cavity cancer in smokers and snuff dippers. The carcinogenicity of NNN is dependent on its metabolic activation. However, virtually all studies carried out to date on NNN metabolism have used racemic material. In this study, we examined the metabolism of [5-^{3}H]-(*S*)-NNN and [5-^{3}H]-(*R*)-NNN in cultured rat esophagus and in vivo in rats. Cultured rat esophagus metabolized (*S*)-NNN (1 μM) predominantly to products of 2′-hydroxylation, 4-oxo-4-(3-pyridyl)butanoic acid (keto acid) and 4-hydroxy-1-(3-pyridyl)-1-butanone (keto alcohol). In contrast, the major metabolite of (*R*)-NNN under these conditions was 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid), a product of NNN 5′-hydroxylation. The 2′-hydroxylation:5′-hydroxylation metabolite ratio ranged from 6.22 to 8.06 at various time intervals in the incubations with (*S*)-NNN, while the corresponding ratios were 1.12−1.33 in the experiments with (*R*)-NNN. These differences were statistically significant ($P < 0.001$). Since 2′-hydroxylation is believed to be the major metabolic activation pathway of NNN in the rat esophagus, the results demonstrate that (*S*)-NNN is metabolically activated more extensively than (*R*)-NNN in this tissue, and therefore may be more carcinogenic. Rats were treated with 0.3 mg/kg of [5-^{3}H]-(*R*)-NNN, [5-^{3}H]-(*S*)-NNN, or racemic [5-^{3}H]NNN by gavage, and the urinary metabolites were analyzed. The major metabolites were hydroxy acid and keto acid. As in the in vitro studies, products of 2′-hydroxylation predominated in the urine of the rats treated with (*S*)-NNN while products of 5′-hydroxylation were more prevalent in the rats treated with (*R*)-NNN. 2′-Hydroxylation:5′-hydroxylation metabolite ratios ranged from 1.66 to 2.04 in the urine at various times after treatment with (*S*)-NNN, while the ratios were 0.398−0.450 for the rats treated with (*R*)-NNN ($P < 0.001$). The results of this study provide new insights into NNN metabolism in rats and suggest that the carcinogenicity of (*S*)-NNN, the predominant enantiomer in tobacco products, may be greater than that of (*R*)-NNN or racemic NNN.

Introduction

The tobacco-specific nitrosamine *N*′-nitrosonornicotine (NNN)[1] is present in substantial quantities in unburned tobacco as well as cigarette smoke. Levels of NNN in the five leading brands of moist snuff sold in the United States vary from 3.04 to 8.73 μg/g of dry weight of tobacco (*1*). NNN levels in cigarette tobacco range from 45 to 121 454 ng/cigarette and in mainstream smoke from 5 to 1353 ng/cigarette based on studies of products from 12 different countries (*2*, *3*). These extensive international data clearly demonstrate that people who use tobacco products are exposed to significant amounts of NNN.

Numerous studies show that NNN causes tumors of the esophagus and nasal mucosa in rats (*4*). When administered in drinking water, NNN produces mainly esophageal tumors, while nasal tumors are more common in rats treated with NNN by injection (*4*). Treatment of rats with 5 ppm NNN in the drinking water results in an esophageal tumor incidence of 71% (*5*). When NNN is swabbed in the oral cavity of rats together with the related tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), tumors of the oral cavity develop (*6*). NNN causes respiratory tract tumors in mice and hamsters and tumors of the nasal mucosa in mink (*4*). These data, together with the exposure levels discussed above, strongly support the involvement of NNN as a causative factor for esophageal cancer in smokers and cancer of the oral cavity in people who use smokeless tobacco products (*3*, *4*).

The metabolism of NNN is summarized in Scheme 1 (*4*). Pyridine N-oxidation produces *N*′-nitrosonornicotine 1-*N*-oxide (NNN-*N*-oxide), while denitrosation and oxidation yield norcotinine; both pathways result in detoxification. All positions of the pyrrolidine ring are hydroxylated. The major hydroxylation reactions occur α to the *N*-nitroso group, yielding 2′-hydroxy-NNN and 5′-hydroxy-NNN. Hydroxylation at the β-carbons occurs only to a limited extent. 2′-Hydroxy-NNN undergoes spontaneous ring opening, producing a pyridyloxobutyl diazohydroxide. This intermediate reacts with H_2O producing 4-hydroxy-1-(3-pyridyl)-1-butanone (keto alcohol), which in turn is oxidized to 4-oxo-4-(3-pyridyl)butanoic acid

[1] Abbreviations: diol, 4-hydroxy-1-(3-pyridyl)-1-butanol; hydroxy acid, 4-hydroxy-4-(3-pyridyl)butanoic acid; keto acid, 4-oxo-4-(3-pyridyl)butanoic acid; keto alcohol, 4-hydroxy-1-(3-pyridyl)-1-butanone; lactol, 5-(3-pyridyl)-2-hydroxytetrahydrofuran; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NNN-*N*-oxide, *N*′-nitrosonornicotine 1-*N*-oxide.

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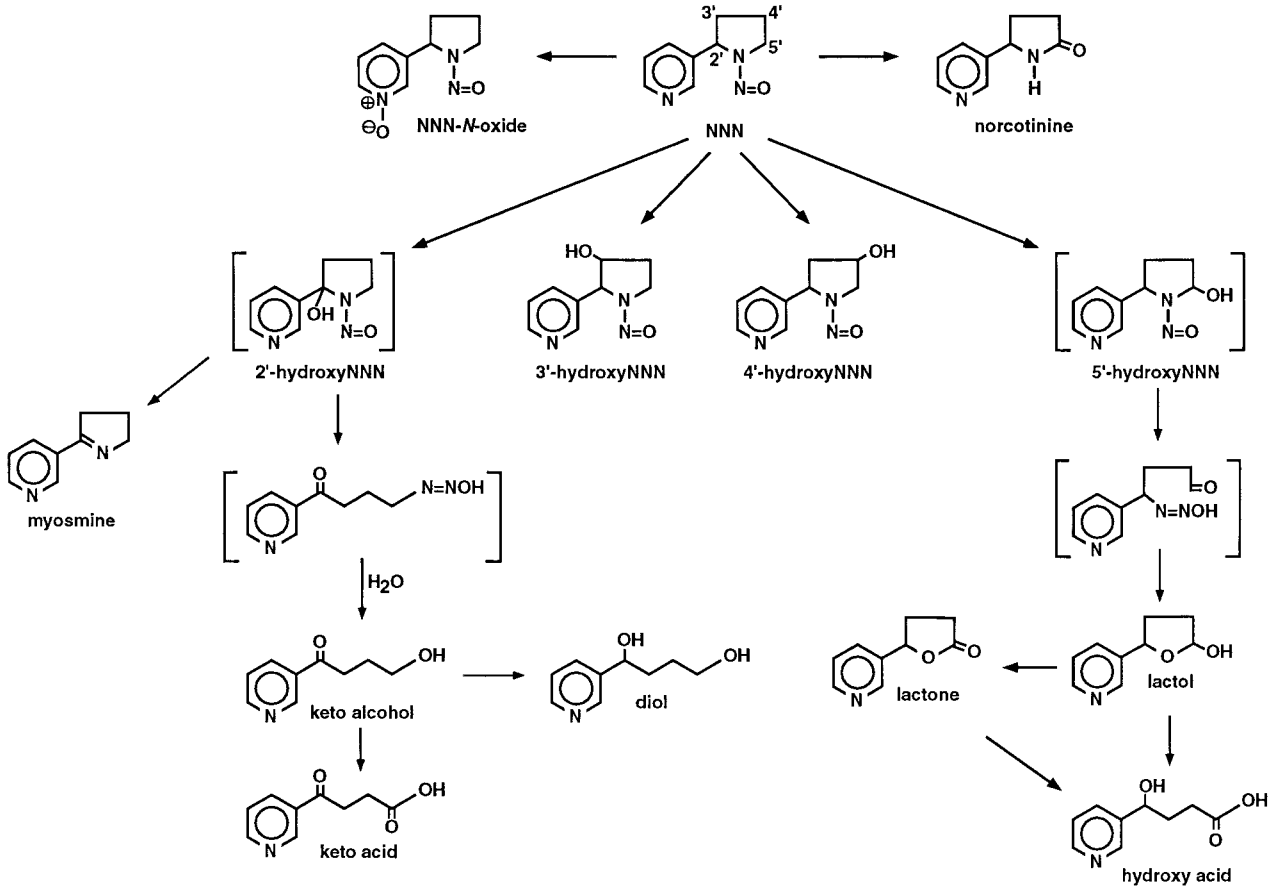
Scheme 1. Metabolism of NNN Based on Studies in Laboratory Animals[a]



[a] See ref *4* for further details.

(keto acid) or reduced to 4-hydroxy-1-(3-pyridyl)-1-butanol (diol). The pyridyloxobutyl diazohydroxide formed by 2′-hydroxylation of NNN also alkylates DNA, yielding adducts which release keto alcohol on hydrolysis. 5′-Hydroxylation of NNN gives 5′-hydroxy-NNN which ring opens to a diazohydroxide that ultimately produces mainly 5-(3-pyridyl)-2-hydroxytetrahydrofuran (lactol) and 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid). DNA adduct formation by this pathway has not been demonstrated, but is likely based on studies of the related nitrosamine *N*-nitrosopyrrolidine (*7*). Studies to date favor the view that 2′-hydroxylation of NNN is the major metabolic activation pathway (*4*). This pathway predominates in rat target tissues such as esophagus and nasal mucosa, and adducts formed by 2′-hydroxylation have been detected in these tissues (*8*, *9*).

NNN has a chiral center at the 2′-position. Recently, we demonstrated that the enantiomeric composition of NNN in cigarette tobacco, moist snuff, and chewing tobacco ranges from 63 to 95% (*S*)-NNN (*10*). Only one previous study examined the metabolism of (*S*)-NNN in rats (*11*). At a dose of 100 mg/kg, urinary metabolites of (*S*)-NNN were similar to those of racemic NNN. In the study presented here, we synthesized the NNN enantiomers labeled with tritium in the pyridine ring and investigated their metabolism by cultured rat esophagus and in vivo in rats.

Experimental Procedures

Caution: *NNN is carcinogenic and mutagenic and therefore should be handled with extreme care, using appropriate protective clothing and ventilation at all times.*

Apparatus. HPLC analyses were carried out with a Waters Associates (Milford, MA) system consisting of two model 510 pumps, a model 440 UV absorbance detector, an IN/US Systems model 2 β-ram radioflow detector (IN/US Systems, Inc., Fairfield, NJ), and either a Rheodyne model 7725 manual injector (Rheodyne Inc., Cotati, CA) or a WISP model 710 Waters Intelligent Sample Processor. Mass spectra were obtained on a Finnigan TSQ 7000 instrument (Finnigan MAT/Themoquest, San Jose, CA). NMR spectra were obtained on a Varian Inova 300 MHz instrument (Varian, Inc., Palo Alto, CA). Optical rotations were determined with a Perkin-Elmer 241 MC polarimeter. Confirmation of stereochemistry was achieved via GC and GC with nitrosamine selective detection (Thermedics Detection, Inc., Chelmsford, MA) using a 30 m × 0.25 mm i.d., 0.25 μm film thickness, Cyclosil-B chiral column (J & W Scientific, Folsom, CA) as previously described (*10*).

The following HPLC systems were used: (A) a 4.6 mm × 250 mm Luna 5 μm C-18 column (Phenomenex, Torrance, CA), with a guard column, eluted with a linear gradient from 0 to 30% CH_3OH in 20 mM NaH_2PO_4 (pH 5.0) for 60 min at a flow rate of 1 mL/min; (B) the same column used for system A eluted with a linear gradient from 0 to 8% CH_3OH/H_2O (95:5) in 20 mM NaH_2PO_4 (pH 7.0) for 15 min, held for 15 min, then eluted with a linear gradient from 8 to 23% CH_3OH/H_2O (95:5) in 20 mM NaH_2PO_4 (pH 7.0) for 30 min, then held for 20 min, and then eluted with a linear gradient from 23 to 35% CH_3OH/H_2O (95:5) in 20 mM NaH_2PO_4 (pH 7.0) for 15 min; and (C) a 3.9 mm × 300 mm Waters μBondapak C-18 column eluted with a linear gradient from 0 to 25% CH_3OH in 25 mM $CH_3CO_2NH_4$ (pH 4.5) for 60 min.

Chemicals. NNN and its metabolites were synthesized by procedures described in the literature: NNN (*12*), NNN-*N*-oxide (*13*), keto alcohol (*14*), diol (*15*), lactol (*16*), and keto acid and hydroxy acid (*17*). Norcotinine was purchased from Toronto Research Chemicals Inc. (North York, ON). Enantiomers of

[5-^{3}H]NNN were synthesized from the appropriate 5-bromonornicotine precursor by Moravek Biochemicals Inc. (Brea, CA) as described previously (*18*).

(2′*R*,2′*S*)-*N*′-[(1*R*,2*S*,5*R*)-Menthoxycarbonyl]-5-bromonornicotine (3 and 4). To a solution of racemic 5-bromonornicotine (2 g, 8.81 mmol) dissolved in 75 mL of ether and triethylamine (1.84 mL, 13.2 mmol) at 0 °C under an N_2 atmosphere was added (1*R*,2*S*,5*R*)-menthyl chloroformate (**2**, Aldrich Chemical Co., Milwaukee, WI; 2.46 mL, 11.3 mmol) via neat syringe over the course of 8 min with vigorous stirring. Precipitate formed as the chloroformate was added. The mixture was stirred at 0 °C for 10 min and at room temperature for 1.5 h. Following suction filtration, the ethereal solution was washed with 10% NaOH (2 × 35 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated to give a dark orange-yellow oil. Purification by silica gel (70−230 mesh) flash column chromatography (1.25 in. × 16 in.) with elution by $CHCl_3$ (500 mL) and then with $CHCl_3$/CH_3-OH (98:2) afforded 2.2 g (61%) of the title compound as a viscous oil: ^{1}H NMR ($CDCl_3$) δ 8.53 (1H, s, pyr-H2), 8.38 (1H, s, pyr-H6), 7.64 (1H, s, pyr-H4), 4.9−4.8 (1H, m, CHO), 4.4−4.1 (1H, m, H2′), 3.68 (2H, m, H5′), 2.37 (1H, m, OCHC*H*), 2.1−1.8 (4H, m, H3′, H4′), 1.62−1.39 (2H, m, OCHCH_2), 1.36−0.75 [15H, m containing singlets of varying intensity, $(CH_3)_2$CH, CH_3CHCH_2-CH_2]; ESI MS *m*/*z* [M + H]$^+$ 408.8 and 410.8.

Semipreparative HPLC Resolution of *N*′-(Menthoxycarbonyl)-5-bromonornicotine. A semipreparative Whatman Partisil 10 μm column was employed with a hexane/ethyl acetate/$CHCl_3$ (73:23:4) mixture as the eluent. All separations were carried out at a flow rate of 5 mL/min and monitored at 280 nm. A typical sample size consisted of 12.5 mg of the diastereomeric carbamate mixture **3** and **4** dissolved in 50 μL of the eluent. Diastereomer **3** eluted at approximately 17 min and was nearly baseline separated from **4**. The collected fractions were combined on the basis of purities determined by reinjection on the HPLC system. The combined solutions of each pure diastereomer obtained from 40 separations were concentrated to dryness in vacuo, giving 320 mg of the first diastereomer, (2′*R*)-*N*′-[(1*R*,2*S*,5*R*)-menthoxycarbonyl]-5-bromonornicotine (**3**) [$[\alpha]_D^{20}$ 48.47° (*c* 1.77, CH_2Cl_2)], and 250 mg of (2′*S*)-*N*′-[(1*R*,2*S*,5*R*)-menthoxycarbonyl]-5-bromonornicotine (**4**) [$[\alpha]_D^{20}$ −117.56° (*c* 1.885, CH_2Cl_2)].

(2′*R*)-5-Bromonornicotine [(*R*)-1]. A solution of 300 mg of **3** in 3 mL of 10% HCl was heated in a 102 mm Teflon-lined pressure tube at 110 °C for 60 h. The reaction vessel was cooled in dry ice and opened without evidence of excess pressure having developed. The reaction mixture was then washed with ether (3 × 5 mL). The aqueous layer was basified with 10 N NaOH and extracted with $CHCl_3$ (4 × 5 mL). The $CHCl_3$ extracts were combined, dried (Na_2SO_4), filtered, and condensed. This gave 100 mg (60%) of **(*R*)-1** as a golden oil: $[\alpha]_D^{20}$ 52.85° (*c* 0.685, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 8.49 (1H, s, pyr-H2), 8.44 (1H, s, pyr-H6), 7.86 (1H, s, pyr-H4), 4.14 (1H, m, H2′), 3.17−3.11 (1H, m, H5′), 3.09−2.98 (1H, m, H5′), 2.24−2.14 (2H, m, H3′ with broad s for NH), 1.96−1.80 (2H, m, H4′), 1.77−1.57 (1H, m, H3′); GC t_R = 51.57 min; ESI MS *m*/*z* [M + H]$^+$ 226.6 and 228.6.

(2′*S*)-5-Bromonornicotine [(*S*)-1]. A solution of 230 mg of **4** in 3 mL of 10% HCl was heated in a 102 mm Teflon-lined pressure tube at 110 °C for 60 h. The workup described above produced 100 mg (79%) of **(*S*)-1** as a golden oil: $[\alpha]_D^{20}$ −46.42° (*c* 0.53, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 8.50 (1H, s, pyr-H2), 8.46 (1H, pyr-H6), 7.87 (1H, s, pyr-H4), 4.14 (1H, m, H2′), 3.18−3.13 (1H, m, H5′), 3.10−2.99 (1H, m, H5′), 2.24−2.14 (2H, m, H3′ with broad s for NH), 1.95−1.80 (2H, m, H4′), 1.75−1.55 (1H, m, H3′); GC t_R = 52.28 min; ESI MS *m*/*z* [M + H]$^+$ 226.6 and 228.6.

Metabolism by Cultured Rat Esophagus. Five male F-344 rats (240−260 g) were sacrificed by CO_2 asphyxiation and the esophagi removed. Each esophagus was split longitudinally into two equal sections, each measuring ∼40 mm × 6 mm. The fragments were placed with the adventitia side down in separate 60 mm × 15 mm scored culture dishes (Fisher Scientific, Itasca, IL). Tissues were covered with 6 mL of Williams medium supplemented with glutamine (2 mM), gentamycin (100 μg/mL), insulin (1 milliunit/mL), and dexamethasone (0.4 μg/mL). To each dish was added 60 μL of 0.9% saline containing either 100 μM [5-^{3}H]-(*R*)-NNN (4.45 Ci/mmol) or [5-^{3}H]-(*S*)-NNN (4.59 Ci/mmol) for a final concentration of 1 μM. The dishes were placed in a 37 °C controlled atmosphere incubator atop a rocking apparatus. The dishes were rocked at a rate of 20 cpm, alternately exposing the sections to the atmosphere (95% O_2/5% CO_2) and to the culture medium. Aliquots of 450 μL of medium were removed at 1, 2, 4, 6, 8, 10, and 23 h and placed in 1.5 mL Eppendorf tubes. Tubes were then placed on dry ice and stored at −20 °C until the contents were assayed.

Analysis of Metabolites. Medium samples were allowed to thaw at room temperature for 40 min. The samples were then vortexed, and a 0.2 mL aliquot was removed and filtered through a 0.45 μm nylon Acrodisc filter. One hundred microliters of each sample was then subjected to HPLC analysis using system A. Metabolites were detected and quantified using radioflow detection. Metabolites were identified by co-injection with reference metabolites using HPLC system A. Selected samples were reinjected on HPLC systems B and C to confirm the identity of the metabolites. Metabolite concentrations were expressed as picomoles per milliliter of medium.

Metabolism in Vivo. Male F-344 rats (Charles River, Kingston, NY) were maintained on NIH-07 pellet diet (Dyets, Inc., Bethlehem, PA) and were housed individually in glass metabolism cages. The rats (240−260 g) were divided into three groups of three rats each. Each rat received a single intragastric gavage dose (0.3 mg/kg of body weight) of either racemic [5-^{3}H]-NNN, [5-^{3}H]-(*R*)-NNN, or [5-^{3}H]-(*S*)-NNN in 0.5 mL of saline. The amount of radioactivity and the specific activity of each compound were as follows: racemic [5-^{3}H]NNN, 55.2 μCi and 108.9 μCi/μmol; [5-^{3}H]-(*R*)-NNN, 49.6 μCi and 97.9 μCi/μmol; and [5-^{3}H]-(*S*)-NNN, 52.2 μCi and 103 μCi/μmol. Urine was collected after 6, 12, 18, 24, 36, and 48 h in vessels cooled to 0−4 °C. Urine samples were frozen on dry ice and stored at −20 °C until they were assayed.

Analysis of Urine. Urine samples were allowed to thaw at room temperature for 40 min. The samples were vortexed, and a 0.3 mL aliquot was removed and filtered through a 0.45 μm nylon Acrodisc filter. One hundred microliters of each sample was then subjected to HPLC analysis using system A. Metabolites were detected and quantified using radioflow detection. Metabolite concentrations were expressed as picomolar and were adjusted for urine volume. Identification of metabolites was initially determined by comparison of retention times of injected reference metabolites using HPLC system A. Selected samples were reinjected on HPLC systems B and C to confirm the identity of the metabolites.

Statistical Analysis. Statistical comparison of 2′-hydroxylated and 5′-hydroxylated metabolites was carried out using SigmaStat (version 2.0). The ratio of 2′-hydroxylated to 5′-hydroxylated metabolites was determined for each sample that was analyzed. The groups were then compared using an unpaired Student's *t* test.

Results

The synthesis of [5-^{3}H]-(*S*)- and -(*R*)-NNN is summarized in Scheme 2. The method is based on that described by Seeman et al. for resolution of nornicotine (*19*). Derivatization of racemic 5-bromonornicotine (**1**) with (1*R*,2*S*,5*R*)-menthyl chloroformate (**2**) gave the diastereomeric carbamates **3** and **4**, which were separated by normal phase HPLC and hydrolyzed individually to (*S*)- and (*R*)-5-bromonornicotine. These were converted to [5-^{3}H]-(*S*)- and [5-^{3}H]-(*R*)-NNN by catalytic tritium exchange and nitrosation as previously described (*18*). The products were analyzed by chiral column GC with nitrosamine selective detection (*10*). The tritium-labeled NNN enantiomers each coeluted with the corre-

Scheme 2. Synthesis of [5-^{3}H]-(*R*)-NNN and [5-^{3}H]-(*S*)-NNN

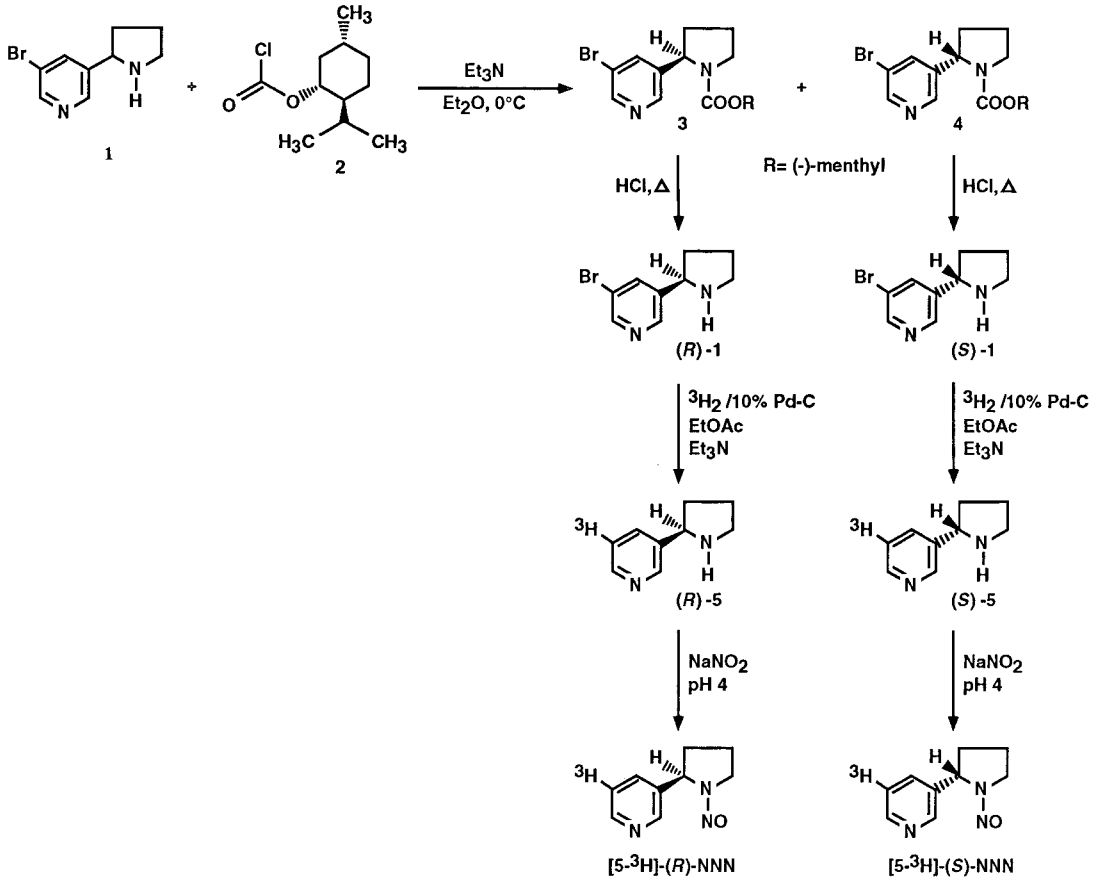


Table 1. 2′-Hydroxylation:5′-Hydroxylation Ratios in the Metabolism of (*S*)-NNN and (*R*)-NNN by Cultured Rat Esophagus[a]

	2′-hydroxylation:5′-hydroxylation ratio						
	1 h	2 h	4 h	6 h	8 h	10 h	23 h
(*S*)-NNN	8.06 ± 1.17	7.11 ± 0.764	6.89 ± 0.987	7.00 ± 1.03	6.79 ± 0.984	6.56 ± 0.926	6.22 ± 0.614
(*R*)-NNN	1.33 ± 0.134	1.15 ± 0.123	1.16 ± 0.0644	1.18 ± 0.0935	1.15 ± 0.0409	1.13 ± 0.0704	1.12 ± 0.0783
P[b]	<0.001	<0.001	<0.001	0.008	<0.001	<0.001	0.008
(*S*)- + (*R*)-NNN[c]	3.43 ± 1.79	2.74 ± 1.38	2.67 ± 1.15	2.63 ± 1.08	2.58 ± 1.02	2.62 ± 1.14	2.22 ± 0.794

[a] [5-^{3}H]-(*S*)-NNN or [5-^{3}H]-(*R*)-NNN was added to cultures of rat esophagus, and metabolites were analyzed at various times. 2′-Hydroxylation is the sum of keto acid, keto alcohol, and diol, while 5′-hydroxylation is the sum of hydroxy acid and lactol. Keto acid and hydroxy acid are not interconverted in cultured rat esophagus (*20*). Values are means ± SD. [b] Comparisons of ratios from (*S*)-NNN to (*R*)-NNN. [c] Calculated values.

sponding unlabeled standards, which have been synthesized previously (*10*). The GC retention times of (*S*)- and (*R*)-NNN were 41.7 and 44.9 min, respectively.

We added [5-^{3}H]-(*S*)- or -(*R*)-NNN to cultures of rat esophagi. HPLC analysis of metabolites showed peaks corresponding in retention time to hydroxy acid, keto acid, keto alcohol, diol, lactol, and norcotinine (Scheme 1). With the exception of lactol and norcotinine, the identities of these metabolites in rat esophagus cultures have been previously confirmed (*8*, *20*). Lactol has been established as a metabolite of NNN in rat esophageal microsomes (*21*). In the study presented here, we confirmed the identities of lactol and norcotinine by HPLC analysis in systems B and C. We also detected a small peak corresponding in retention time to NNN-*N*-oxide, but did not investigate it further.

We observed substantial differences between the metabolism of (*S*)-NNN (Figure 1A) and (*R*)-NNN (Figure 1B). Keto acid and keto alcohol, products of 2′-hydroxylation, were the major metabolites at every time point in rat esophagus cultured with (*S*)-NNN. In contrast, hydroxy acid, a product of 5′-hydroxylation, was the major metabolite of (*R*)-NNN. Figure 1C shows the average of the metabolites of (*S*)- and (*R*)-NNN; these are remarkably similar to that seen previously in the metabolism of racemic NNN under the same conditions (*8*). Using the data in panels A and B of Figure 1, we calculate that, in a racemic mixture, 71% of the 2′-hydroxylation products would be formed from (*S*)-NNN and 69% of the 5′-hydroxylation products from (*R*)-NNN.

Keto acid, keto alcohol, and diol are all products of 2′-hydroxylation, while hydroxy acid and lactol are products of 5′-hydroxylation. We calculated the 2′-hydroxylation:5′-hydroxylation ratio for each enantiomer. These data, which are summarized in Table 1, demonstrate that the 2′-hydroxylation:5′-hydroxylation ratio was significantly greater in the metabolism of (*S*)-NNN than in that of (*R*)-NNN at each time point. The 2′-hydroxylation:5′-hydroxylation ratios were also calculated at each time point for the average of the metabolites produced from (*S*)-

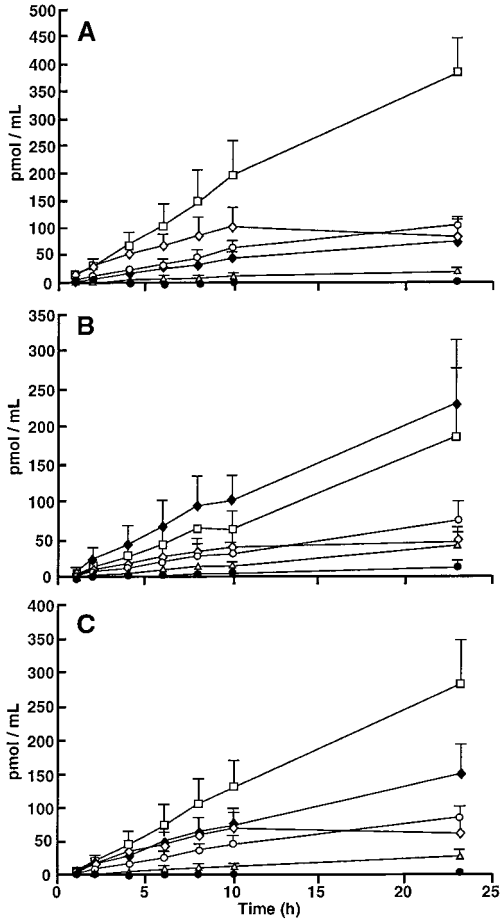


Figure 1. Formation of metabolites in rat esophagus cultured with (A) [5-^{3}H]-(*S*)-NNN and (B) [5-^{3}H]-(*R*)-NNN. Panel C is the calculated average of the data depicted in panels A and B: (□) keto acid, (◇) keto alcohol, (◆) hydroxy acid, (○) norcotinine, (△) diol, and (●) lactol.

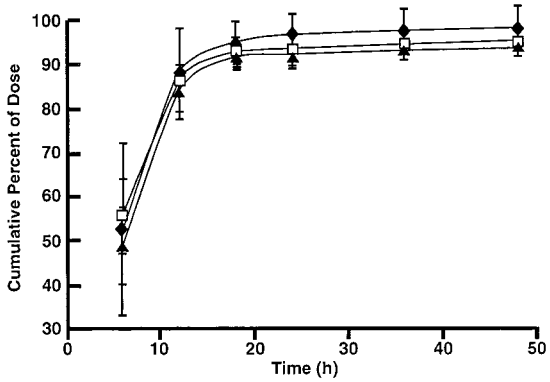


Figure 2. Excretion of radioactivity in the urine of rats treated with (◆) [5-^{3}H]-(*R*)-NNN, (□) [5-^{3}H]-(*S*)-NNN, and (▲) racemic [5-^{3}H]NNN.

NNN and (*R*)-NNN (Table 1). The ratios ranged from 2.22 to 3.43 which is consistent with previous results (*8*, *20*).

For the in vivo studies, we chose a dose of 0.3 mg/kg of body weight, administered by gavage. Rats treated chronically with 5 ppm NNN in their drinking water, or approximately 0.3 mg/kg of body weight per day, develop tumors of the esophagus (*5*). Therefore, this dose is similar to the daily dose in the carcinogenicity study. As shown in Figure 2, more than 90% of the radioactivity was excreted in the urine, consistent with previous studies (*22*, *23*). There was no difference in excretion among the individual enantiomers and racemic mixture.

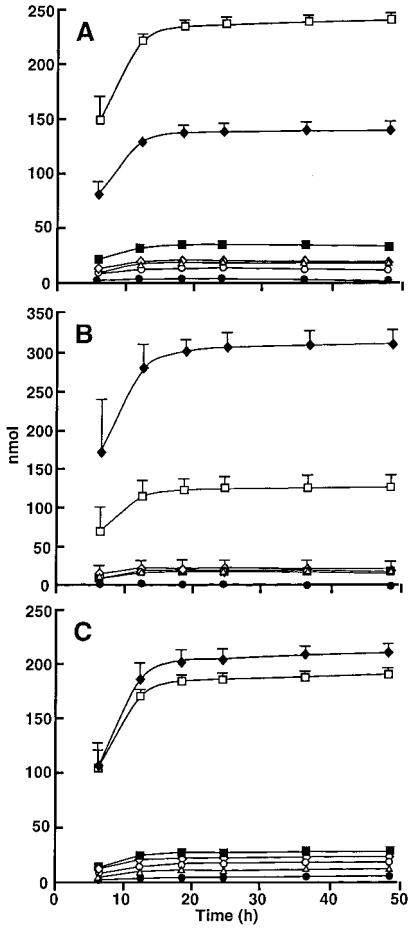


Figure 3. Excretion of NNN metabolites in the urine of rats treated with (A) [5-^{3}H]-(*S*)-NNN, (B) [5-^{3}H]-(*R*)-NNN, and (C) racemic [5-^{3}H]NNN: (□) keto acid, (◆) hydroxy acid, (■) NNN-*N*-oxide, (◇) NNN, (△) diol, (○) norcotinine, and (●) lactol.

The major metabolites of NNN at this dose were hydroxy acid and keto acid. Smaller amounts of NNN-*N*-oxide, norcotinine, diol, lactol, and unchanged NNN were also observed. Diol and lactol had not been reported previously as urinary metabolites of NNN. Therefore, we confirmed their identities by analysis in HPLC systems B and C, in which they coeluted with standards. Time-dependent excretion of urinary metabolites of (*S*)-NNN, (*R*)-NNN, or racemic NNN is summarized in Figure 3A–C. In the rats treated with (*S*)-NNN, keto acid was the most prevalent urinary metabolite, followed by hydroxy acid. However, in the rats treated with (*R*)-NNN, hydroxy acid was the most prevalent metabolite, followed by keto acid. The amounts of hydroxy acid and keto acid present in the urine of the rats treated with racemic NNN were consistent with these results. Total urinary excretion of each metabolite is summarized in Figure 4.

2′-Hydroxylation:5′-hydroxylation ratios at each time point are presented in Table 2. These results demonstrate that (*S*)-NNN undergoes significantly more 2′-hydroxylation than (*R*)-NNN. In the rats treated with racemic NNN, approximately 66% of the 2′-hydroxylation products are formed from (*S*)-NNN while 74% of the 5′-hydroxylation metabolites are produced from (*R*)-NNN.

Discussion

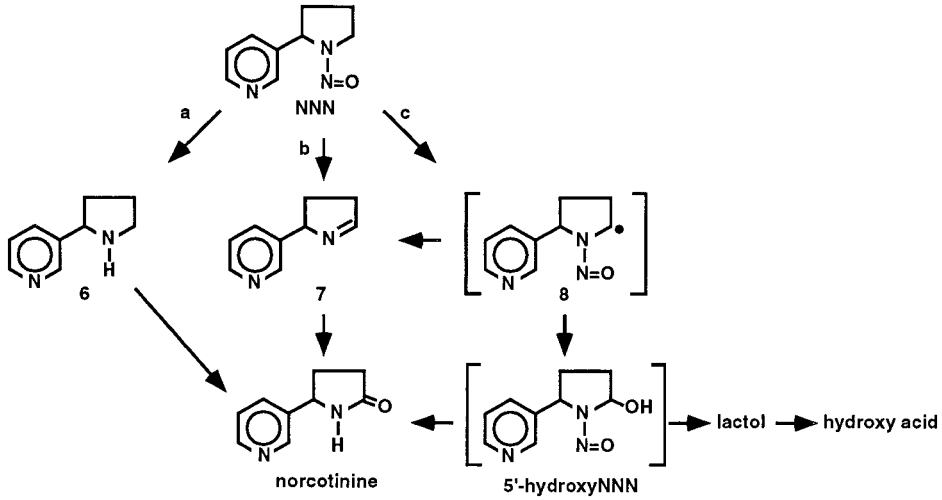
The results of this study demonstrate clear stereoselectivity in rat esophageal metabolism of NNN enanti-

Table 2. 2′-Hydroxylation:5′-Hydroxylation Ratios in the Metabolism of (*S*)-NNN, (*R*)-NNN, and Racemic NNN in Vivo in Rats[a]

	2′-hydroxylation:5′-hydroxylation ratio[b]					
	6 h	12 h	18 h	24 h	36 h	48 h
(*S*)-NNN	1.88 ± 0.043	1.66 ± 0.108	1.74 ± 0.237	1.81 ± 0.025	1.78 ± 0.081	2.04 ± 0.175
(*R*)-NNN	0.398 ± 0.032	0.431 ± 0.047	0.450 ± 0.046	0.449 ± 0.019	0.438 ± 0.072	0.405 ± 0.042
racemic NNN	1.01 ± 0.057	0.880 ± 0.047	0.884 ± 0.020	0.750 ± 0.087	0.766 ± 0.040	0.786 ± 0.038

[a] [5-^{3}H]-(*S*)-NNN, [5-^{3}H]-(*R*)-NNN, or racemic [5-^{3}H]NNN (0.3 mg/kg) was administered to male F-344 rats by gavage, and urine was collected at various times. Values are means ± SD. [b] All differences among the three groups were significant at each time interval ($P < 0.001$) except (*S*)-NNN vs racemic NNN, 18 h ($P = 0.003$); (*R*)-NNN vs racemic NNN, 24 h ($P = 0.004$); and (*R*)-NNN vs racemic NNN, 36 h ($P = 0.002$).

Scheme 3. Some Possible Pathways of NNN Metabolism to Norcotinine



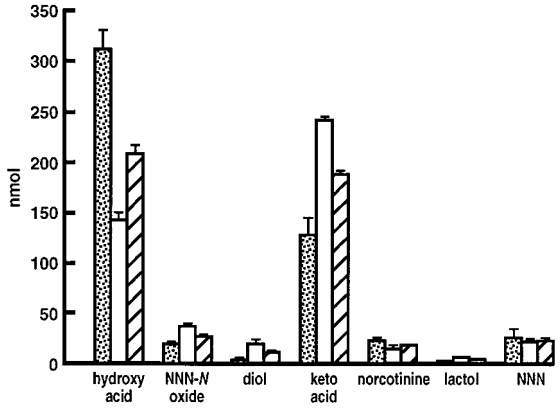


Figure 4. Urinary metabolites in rats treated with [5-^{3}H]-(*R*)-NNN (stippled bars), [5-^{3}H]-(*S*)-NNN (white bars), and racemic [5-^{3}H]NNN (crosshatched bars).

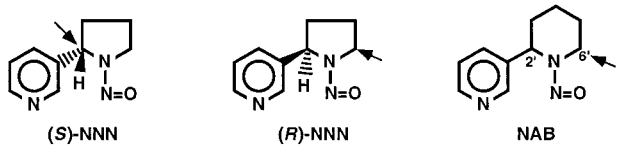


Figure 5. Structures of (*S*)-NNN, (*R*)-NNN, and NAB. Arrows indicate the predominant positions of α-hydroxylation in cultured rat esophagus.

omers. (*S*)-NNN preferentially undergoes 2′-hydroxylation in this tissue, while (*R*)-NNN is preferentially hydroxylated at the 5′-position. Previous studies carried out with 1 μM racemic NNN have shown that 2′-hydroxylation is favored compared to 5′-hydroxylation in cultured rat esophagus, as well as in rat esophageal microsomes. 2′-Hydroxylation:5′-hydroxylation ratios of **3** were reported in these studies (*8*, *20*). The results presented here indicate that 71% of the 2′-hydroxylation products were formed from (*S*)-NNN while 69% of the 5′-hydroxylation products were formed from (*R*)-NNN. 2′-Hydroxylation is believed to be the major pathway of metabolic activation of NNN leading to esophageal tumorigenesis. Evidence in support of this hypothesis includes the detection of DNA adducts resulting from 2′-hydroxylation in rat esophagus cultured with NNN (*8*) and higher 2′-hydroxylation:5′-hydroxylation ratios in esophagus and other target tissues of NNN compared to liver, a nontarget site (*8*, *20*, *21*, *24*, *25*). Moreover, comparative studies of NNN and the weak esophageal carcinogen *N*′-nitrosoanabasine (NAB, Figure 5) demonstrate that 2′-hydroxylation is strongly favored in the metabolism of NNN by rat esophagus compared to that of NAB (*11*). Collectively, these results suggest that (*S*)-NNN will be more carcinogenic to the rat esophagus than (*R*)-NNN and that the carcinogenicity of NNN, based on studies carried out to date exclusively with racemic material, may have been underestimated. Considering that (*S*)-NNN is the predominant enantiomer present in tobacco, the results of a carcinogenicity study of the NNN enantiomers could have considerable implications in risk assessment. We are planning a comparative bioassay of the NNN enantiomers and racemic NNN in rats.

Our results and those previously published indicate that there is considerable stereoselectivity in rat esophageal metabolism of NNN and NAB (Figure 5). 5′-Hydroxylation and 6′-hydroxylation are the predominant pathways of metabolism of (*R*)-NNN and racemic NAB, respectively, in cultured rat esophagus, while 2′-hydroxylation is the predominant pathway of (*S*)-NNN metabolism (*11*). Several studies provide strong evidence for the presence of a high-affinity cytochrome P450 or P450s in the rat esophagus which metabolize NNN, *N*-nitrosoben-

zylmethylamine, and *N*-nitrosomethylamylamine (*21*, *26*−*28*). These P450s have not been fully characterized, but it is known that P450 2A3 is involved to a limited extent (*26*, *27*). The differences in metabolism shown here between the NNN enantiomers, and among the NNN enantiomers and NAB, should prove to be useful in characterizing the enzymes involved in rat esophageal metabolic activation of nitrosamines.

The results of the in vivo studies with racemic NNN are consistent with those of a previous investigation (*23*). As the dose of NNN was decreased from 300 mg/kg to 3 mg/kg in our earlier study, there was a decrease in 5′-hydroxylation and a corresponding increase in 2′-hydroxylation such that the 2′-hydroxylation:5′-hydroxylation ratios were 0.24 at 300 mg/kg and 0.84 at 3 mg/kg (*23*). In the study presented here, with an NNN dose of 0.3 mg/kg, the 2′-hydroxylation:5′-hydroxylation ratio was 0.94. These results are also consistent with those obtained in a study of NNN metabolism in rat liver microsomes (*21*). A 2′-hydroxylation:5′-hydroxylation ratio of 0.23 was observed at an NNN concentration of 200 μM, compared to 0.71 at 1 μM, demonstrating higher 2′-hydroxylation:5′-hydroxylation ratios at lower substrate concentrations (*21*). The liver contains a mixture of NNN α-hydroxylating enzymes, probably P450s (*21*). Among these, there is apparently a high-affinity enzyme which favors 2′-hydroxylation over 5′-hydroxylation at low NNN concentrations (*21*). This may be the same enzyme that is present in the rat esophagus (*21*). In support of this concept, the in vivo studies of the NNN enantiomers demonstrated preferential 2′-hydroxylation of (*S*)-NNN and 5′-hydroxylation of (*R*)-NNN, as seen in the esophagus, although the differences were not as great. This is probably due to the presence of multiple NNN α-hydroxylating enzymes in the liver, some of which have a stronger proclivity for 5′-hydroxylation of (*R*)-NNN than for 2′-hydroxylation of (*S*)-NNN.

Our results may provide some insights concerning the origins of norcotinine in NNN metabolism. Possible mechanisms of norcotinine formation are summarized in Scheme 3. In mechanism a, NNN would undergo direct denitrosation to nornicotine (**6**), followed by oxidation to norcotinine. In mechanism b, NNN would lose HNO yielding isomyosmine (**7**), which would be oxidized to norcotinine. In mechanism c, NNN would be oxidized to 5′-radical **8**. Radical **8** would give both isomyosmine (**7**) and 5′-hydroxy-NNN, both of which could ultimately yield norcotinine. A related radical has been proposed as an intermediate in the α-hydroxylation and denitrosation of *N*-nitrosodimethylamine (*29*). The results do not appear to support mechanism c since radical **8** would be the likely precursor to both norcotinine and hydroxy acid. Therefore, the ratio of these two metabolites would be expected to be constant in the metabolism of (*S*)-NNN and (*R*)-NNN, but this was not observed, either in the esophagus or in vivo. These results suggest that mechanism c is not important in norcotinine formation from NNN in the rat, unless there are different mechanisms for the two NNN enantiomers. The results would favor the view that norcotinine is formed by route a or b, although it should be noted that administration of nornicotine (**6**) to rats did not result in detectable amounts of norcotinine in urine (*23*).

In summary, the results of this study clearly demonstrate distinct differences in the metabolism of (*S*)- and (*R*)-NNN. 2′-Hydroxylation is the predominant pathway of metabolism of (*S*)-NNN by cultured rat esophagus and in rats treated by gavage, while 5′-hydroxylation is the favored pathway of (*R*)-NNN metabolism under these conditions. These results provide new insights into mechanisms of metabolic activation of NNN and suggest that the risk associated with exposure to NNN via tobacco products may have been underestimated.

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