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Assay for Microsomal α-Hydroxylation of *N'*-Nitrosonornicotine and Determination of the Deuterium Isotope Effect for α-Hydroxylation[1]

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ABSTRACT

A high-pressure liquid chromatographic assay was developed for microsomal α-hydroxylation (2'-hydroxylation and 5'-hydroxylation) of *N'*-nitrosonornicotine. *N'*-Nitrosonornicotine was incubated with rat liver microsomes and a reduced nicotinamide adenine dinucleotide phosphate-generating system at 37°. After addition of 2,4-dinitrophenylhydrazine reagent, the mixtures were analyzed by reverse-phase high-pressure liquid chromatography. The 2,4-dinitrophenylhydrazones of 4-hydroxy-1-(3-pyridyl)-1-butanone and 4-hydroxy-1-(3-pyridyl)-butanal, which are the products of 2'-hydroxylation and 5'-hydroxylation, were quantified by ultraviolet light detection at 254 nm. K_m's for 2'-hydroxylation and 5'-hydroxylation of *N'*-nitrosonornicotine by liver microsomes from Aroclor-treated male F-344 rats were 1.81 and 1.96 mM, while V_{max}'s were 0.53 and 1.05 nmol/min/mg protein, respectively. Aroclor pretreatment of rats resulted in a 20-fold induction of 2'-hydroxylation, but only a 1.9-fold induction of 5'-hydroxylation. The deuterium isotope effect for α-hydroxylation of *N'*-nitrosonornicotine was determined by comparing the rates of 2'-hydroxylation and 5'-hydroxylation of *N'*-nitrosonornicotine, *N'*-[2',5',5'-D]nitrosonornicotine, *N'*-[2'-D]nitrosonornicotine, and N'-[5',5'-D]nitrosonornicotine. The deuterium isotope effect ($V_{max\ H}/V_{max\ D}$) was 2.4 to 2.7 for 5'-hydroxylation and 1.2 for 2'-hydroxylation.

INTRODUCTION

NNN[4] has been detected in relatively high concentrations in unburned tobacco and mainstream and sidestream cigarette smoke. Exposure of smokers to NNN is at least 10 times as great as to benzo(*a*)pyrene. Tobacco chewers are also exposed to high levels of this tobacco-specific carcinogen (12, 14). NNN causes nasal cavity and esophageal tumors in rats, tracheal tumors in hamsters, and lung adenomas in mice (3, 11, 13, 15, 24). Thus, NNN and the other tobacco-specific nitrosamines could be causative agents in tobacco-related cancers (26).

The metabolic activation of NNN and other cyclic nitrosamines may proceed by α-hydroxylation. Certain lines of evidence support this hypothesis. Model compounds for 2'-hydroxy-NNN (Compound 2) and 5'-hydroxyNNN (Compound 3), which are the products of α-hydroxylation of NNN (Compound 1), were mutagenic toward *Salmonella typhimurium* TA 100 without activation (see Chart 1) (5). Similar results have been obtained with α-acetoxy derivatives of the related cyclic nitrosamine, NPYR, as well as of acyclic nitrosamines (1, 2, 4, 23, 25, 27, 28). In structure-activity studies on NPYR and other cyclic nitrosamines, substitution of methyl groups or deuterium atoms in the α positions decreased carcinogenicity (17-19). Despite the apparent importance of α-hydroxylation in cyclic nitrosamine activation, little is known about the inducibility or inhibition of the enzymes which catalyze this process. Until recently, when an assay for α-hydroxylation of NPYR was developed, no methods were available to study α-hydroxylation of cyclic nitrosamines (6). In this paper, we describe a high-pressure liquid chromatographic assay for microsomal α-hydroxylation of NNN. This assay has been used to measure the deuterium isotope effects for 2'-hydroxylation and 5'-hydroxylation of α-deuterated NNN derivatives.

MATERIALS AND METHODS

Apparatus. High-pressure liquid chromatography was carried out with a Waters Associates Model ALC/GPC-204 high-speed liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model 660 solvent programmer, a Model U6K septumless injector, a Model 440 UV-visible detector, and a 3.9-mm x 30-cm C_{18}-μBondapak column (Waters Associates, Milford, Mass.). Liver homogenization was performed with a polytron homogenizer (Willems type; Kinematic GmbH, Lucerne, Switzerland). Centrifugation was done with a Sorvall RC2-B centrifuge and a Spinco Model L ultracentrifuge. NMR spectra were determined with a Hitachi-Perkin Elmer Model R-24 spectrometer in $CDCl_3$ solution and are reported as ppm downfield from tetramethylsilane as internal reference. Mass spectra were run with a Hewlett-Packard Model 5982A dual source instrument. TLC was done with Silica Gel 60 F glass plates (EM Laboratories, Elmsford, N. Y.). Alumina (basic, activity II to III) was obtained from ICN Nutritional Biochemicals, Cleveland, Ohio.

Chemicals. NNN was prepared as previously described, as were the 2,4-dinitrophenylhydrazone Compounds 12 and 13 of 4-hydroxy-1-(3-pyridyl)-1-butanone (Compound 8) and 4-hydroxy-1-(3-pyridyl)butanal (Compound 10) (5, 16). NADH, NADPH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, Mo. Aroclor 1254 was obtained from Analabs, Inc., Hamden, Conn. 2,4-Dinitrophenylhydrazine was obtained from

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[4] The abbreviations used are: NNN, *N'*-nitrosonornicotine; 2'-hydroxy-NNN, 2'-hydroxy-*N'*-nitrosonornicotine; 5'-hydroxy-NNN, 5'-hydroxy-N'-nitrosonornicotine; NPYR, *N*-nitrosopyrrolidine; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; [2'-D]NNN, *N'*-[2'-D]nitrosonornicotine; [5',5'-D]NNN, *N'*-[5',5'-D]nitrosonornicotine; [2',5',5'-D]NNN, N'-[2',5',5'-D]nitrosonornicotine; m, multiplet.

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Eastman Kodak Co., Rochester, N. Y.

[2′,5′,5′-D]NNN (Compound 14). A 40% sodium deuteroxide solution (Stohler Isotope Chemicals, Waltham, Mass.; 3 ml, 30 mmol) was added in one batch to NNN (5.2 g; 30 mmol) in 14 ml of D_2O, and the mixture was refluxed for 100 min. After cooling, 3 g of anhydrous K_2CO_3 were added, and the mixture was extracted 3 times with CH_2Cl_2. The CH_2Cl_2 extracts were combined and dried over K_2CO_3. After filtration and concentration, the residue was purified by column chromatography (basic alumina, Activity II to III), eluting with benzene and benzene:ether. The resulting product (4.7 g; 90%) was pure according to TLC ($CHCl_3$:methanol, 15:1) and had R_F identical to that of NNN. NMR spectrum: 8.60 to 8.20 ppm and 7.60 to 7.00 ppm, 4H, m (pyridyl H); 2.80 to 1.80 ppm, 4H (3′-CH_2 + 4′-CH_2); mass spectrum, *m/e* (relative intensity): 180 (M^+, 42.0); 106 (100); 105 (52.9); 78 (70.7); 51 (45.8).

[2′-D]NNN (Compound 16). A solution of myosmine (Compound 9) (16) (1.55 g, 10.5 mmol) and sodium borodeuteride (0.77 g, 18.4 mmol; Stohler Isotope Chemicals) in 120 ml of ethanol:H_2O (1:3) was stirred at room temperature for 4 days (22). It was then extracted with $CHCl_3$ 4 times, dried (K_2CO_3), and concentrated. The residue (1.5 g) was greater than 95% pure [2′-D]nornicotine (Compound 15) according to TLC (silica gel, $CHCl_3$:CH_3OH:NH_4OH, 85:15:2). The product thus obtained was used without further purification for the synthesis of Compound 16 according to the procedure described below for the nitrosation of [5′,5′-D]nornicotine (Compound 18). [2′-D]-NNN (1.7 g, 95%) was purified by preparative TLC (silica gel, $CHCl_3$:CH_3OH, 15:1). NMR spectrum: 8.75 to 8.25 ppm and 7.25 to 7.00 ppm, 4H, m (pyridyl H); 4.35 to 3.50 ppm, 2H, m (5′-CH_2); 2.80 to 1.65 ppm, 4H, m (3′-CH_2 + 4′-CH_2). MS, *m/e* (relative intensity): 178 (M^+, 100); 148 (49.8); 106 (82.4); 105 (68.4).

[5′,5′-D]NNN (Compound 19). A solution of norcotinine (Compound 17) (21) (200 mg, 1.2 mmol) in 10 ml of dry tetrahydrofuran was added dropwise to a suspension of lithium aluminum deuteride (Stohler Isotope Chemicals; 4.8 mmol) in 15 ml of dry tetrahydrofuran. After refluxing for 24 hr, the mixture was cooled in an ice bath, and 0.2 ml of H_2O was added slowly, followed by 0.4 ml of 2 N NaOH and finally by 1 ml of H_2O. The solution was filtered and the solid was washed several times with $CHCl_3$. The combined filtrate and $CHCl_3$ washings were then dried (K_2CO_3) and concentrated. The residue thus obtained was purified by preparative TLC (silica gel, $CHCl_3$:CH_3OH:NH_4OH, 85:15:2) to give [5′,5′-D]nornicotine (Compound 18) (130 mg, 0.87 mmol) which was nitrosated in D_2O with $NaNO_2$ (152 mg, 2.2 mmol) at pH 3 to 4. The reaction was carried out at room temperature for 4 hr. After extraction (3 times with $CHCl_3$), drying (K_2CO_3), and concentration, the residue was purified by preparative TLC (silica gel, $CHCl_3$:CH_3OH, 15:1) to give [5′,5′-D]NNN (Compound 19) (55 mg, 36%) with R_F identical to that of NNN. NMR spectrum: 8.70 to 8.00 ppm, 1.5H, m (pyridyl H) and 7.60 to 6.90 ppm, 1.5H, m (pyridyl H); 5.80 to 4.80 ppm, 1H, m (2′-CH); 2.80 to 1.70 ppm, 4H, m (3′-CH_2 + 4′-CH_2); mass spectrum, *m/e* (relative intensity) 180 (64.5); 179 (M^+, 34.5); 106 (100); 105 (78.0); 79 (51.3).

Assay for Microsomal α-Hydroxylation of NNN. Liver microsomes were prepared either from male F-344 rats treated with 500 mg Aroclor per kg 4 days prior to sacrifice or from untreated rats. Preparation of microsomes followed a previously described procedure (10). Protein was determined by the method of Lowry *et al.* (20).

A standard assay was carried out in the following manner. A mixture of 20 μmol of NNN, 0.6 μmol of NADPH, 0.4 ml of microsomal suspension (9.0 mg of protein/ml), 10 units of glucose-6-phosphate dehydrogenase, 10 μmol of glucose 6-phosphate, and 10 μmol of $MgCl_2$ was brought to a total volume of 2.0 ml with 0.185 mmol Tris-HCl buffer (pH 7.5), and incubated at 37° for 60 min. This incubation mixture was then quenched with 2 ml of ethanol. After removal of protein by centrifugation, 3 ml of the supernatant were pipetted and treated with 0.3 ml of 0.15 M 2,4-dinitrophenylhydrazine reagent. After standing overnight, a 0.5-ml aliquot was neutralized with 0.02 ml of 10 N NaOH and a 0.04-ml aliquot of this sample was analyzed by high-pressure liquid chromatography, using a reverse-phase C_{18}-μBondapak column and UV detection (254 nm). A linear 1-hr 40-min gradient from Solvent A to Solvent B with a flow rate of 2 ml/min was used. Solvent A was 40% CH_3OH:H_2O (70:30):60% 0.02% KH_2PO_4 buffer (pH 5.5). Solvent B was CH_3OH:H_2O (70:30). The formation of dinitrophenylhydrazone Compounds 12 and 13 was determined quantitatively by comparison of their peak heights with those of standards.

RESULTS AND DISCUSSION

The α-hydroxylation of NNN is outlined in Chart 1 (5). Hydroxylation of the 2′- and 5′-carbons of NNN (Compound 1) gives the unstable compounds 2′-hydroxy-NNN (Compound 2) and 5′-hydroxy-NNN (Compound 3). Rapid, nonenzymatic ring opening of Compounds 2 and 3 gives the unstable diazohydroxide Compounds 4 and 5, which spontaneously decompose to carbonium ions (Compounds 6 and 7). These carbonium ions, which may be the ultimate carcinogens derived from NNN, are trapped by water to give 4-hydroxy-1-(3-pyridyl)-1-butanone (Compound 8) (from 2′-hydroxylation), and 4-hydroxy-1-(3-pyridyl)butanal (Compound 10) (from 5′-hydroxylation). 2′-Hydroxy-NNN (Compound 2) also decomposes nonenzymatically to myosmine (Compound 9) (8). The ketone (Compound 8) and the aldehyde [Compound 10, which exists predominantly as lactol (Compound 11)] can be trapped as the corresponding 2,4-dinitrophenylhydrazone Compounds 12 and 13.

A high-pressure liquid chromatographic system was developed to assay for Compounds 12 and 13 in mixtures resulting from incubation of NNN with liver microsomes and a NADPH-generating system. A typical high-pressure liquid chromatogram is shown in Chart 2. The indicated peaks are the 2,4-dinitrophenylhydrazone Compounds 12 and 13, which were collected and identified by comparison of their mass spectra to reference samples. Recovery experiments showed that Compounds 8 and 11 were stable under the incubation conditions and that formation of Compounds 12 and 13 was efficient. The detection limit for 2,4-dinitrophenylhydrazone Compounds 12 and 13 in analyses of typical incubation mixtures was approximately 0.1 nmol. No significant interfering UV absorption was present at the retention volumes of Compounds 12 and 13 in chromatograms of control incubations, but the intensities of the other peaks did not change. The deviation coefficients for measurement of Compounds 12 and 13 were 5.0 and 2.6%, respectively (see Table 1).

The 2′-hydroxylation and 5′-hydroxylation of NNN were lin-

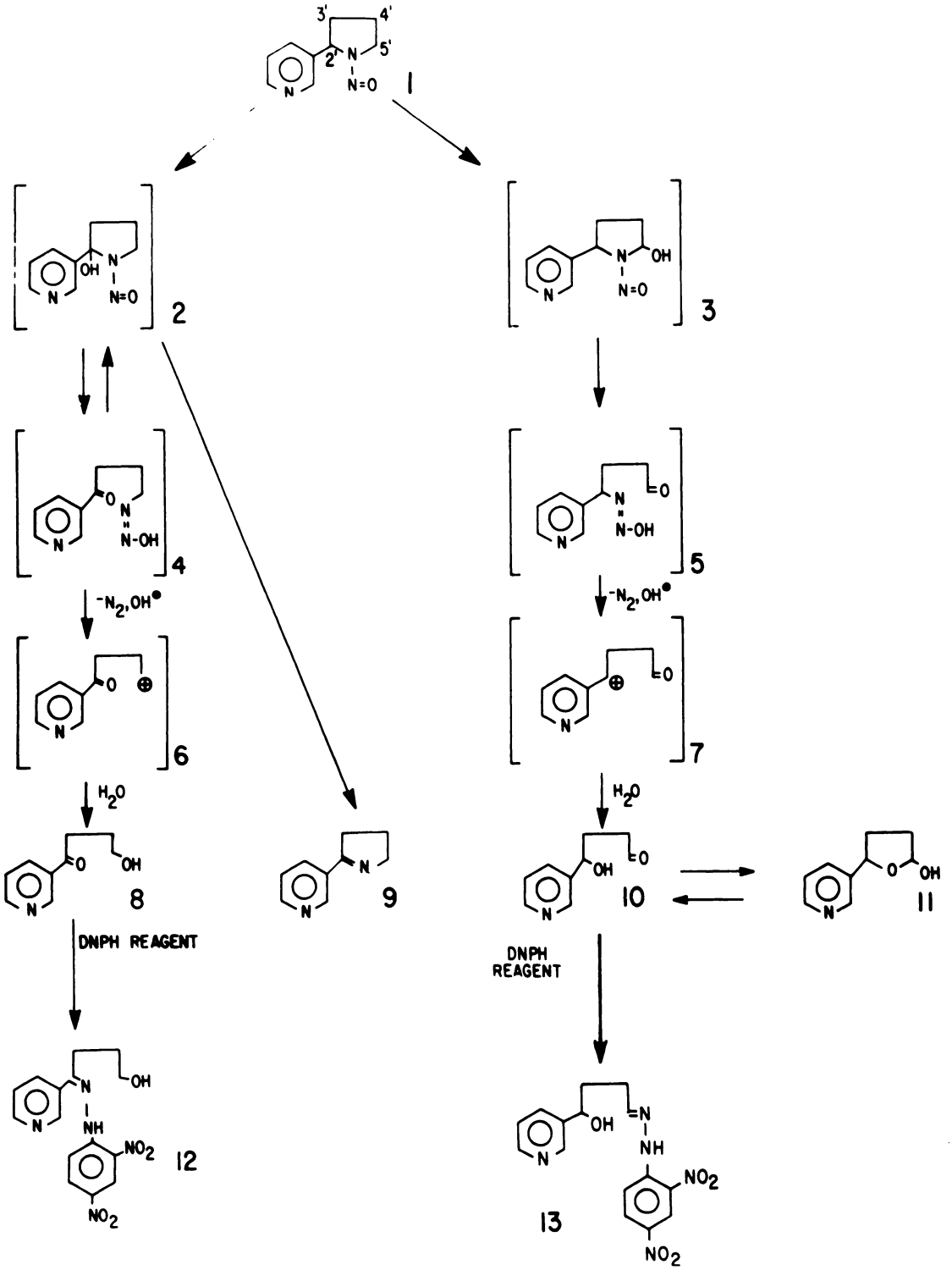

Chart 1. Intermediates and products resulting from microsomal α-hydroxylation of NNN. *DNPH reagent*, 2,4-dinitrophenylhydrazine reagent.

ear for at least 60 min. The effects of variations in protein concentration on the 2′-hydroxylation and 5′-hydroxylation of NNN are shown in Chart 3. These data were obtained using liver microsomes from Aroclor-treated rats. The rates of product formation were linear, with protein concentration from 0.2 to 1.9 mg protein per ml. The effect of variation in substrate concentration is presented in the form of a Lineweaver-Burk plot in Chart 4. The K_m's for 2′-hydroxylation and 5′-hydroxylation were 1.81 and 1.96 mM, respectively, and the V_{max}'s for 2′-hydroxylation and 5′-hydroxylation were 0.53 and 1.05 nmol/min/mg protein. In comparison, K_m for α-hydroxylation of NPYR by liver microsomes from Aroclor-treated rats was 16.5 mM, and V_{max} was 5.6 nmol/min/mg protein (6). The rates of 2′-hydroxylation of NNN determined by this assay may be

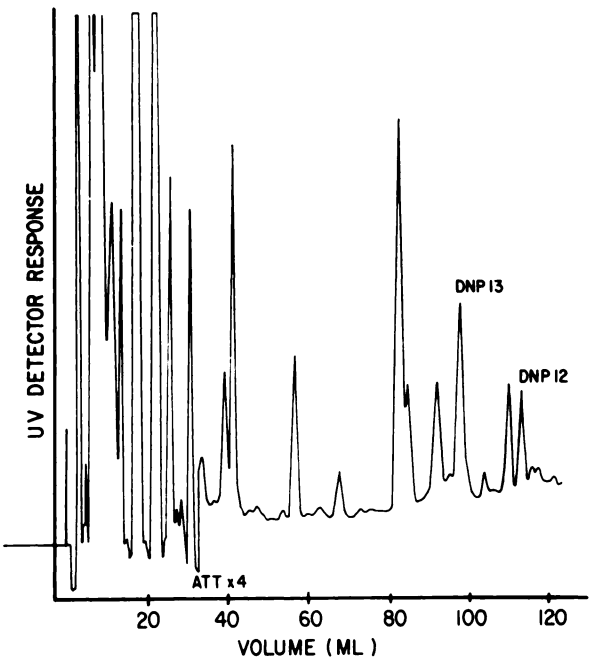

Chart 2. High-pressure liquid chromatogram of a mixture obtained by incubation of NNN with liver microsomes from Aroclor-treated rats, followed by addition of 2,4-dinitrophenylhydrazine reagent. The indicated peaks, *DNP 13* and *DNP 12*, are the dinitrophenylhydrazone Compounds 13 and 12 (see Chart 1) from 5′-hydroxylation and 2′-hydroxylation of NNN, respectively. *ATT × 4*, 4-fold increase in sensitivity.

Table 1

α-Hydroxylation of NNN by rat liver microsomes

Incubation mixtures consisted of NNN, rat liver microsomes, NADPH, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, and $MgCl_2$ in Tris-HCl buffer (pH 7.5) at 37°. Incubations were for 60 min prior to addition of 2,4-dinitrophenylhydrazine reagent.

Conditions	% of complete incubation[a]	
	2′-Hydroxylation	5′-Hydroxylation
Complete incubation[a]	100[b]	100[b]
Incubation time = 0[a, c]	<5	7
−NADPH, +NADH (0.7 μmol)[a]	<5	<5
Complete incubation[d]	5	53

[a] Liver microsomes prepared from Aroclor-treated rats were used.

[b] Dinitrophenylhydrazone (Compound 12) from 2′-hydroxylation per min per mg protein = 0.40 ± 0.02 nmol. Dinitrophenylhydrazone (Compound 13) from 5′-hydroxylation per min per mg protein = 0.76 ± 0.02 nmol; mean ± S.D. for 5 incubations.

[c] Ethanol was added to the incubation mixture before microsomes were added.

[d] Liver microsomes prepared separately from 3 untreated rats were used. Dinitrophenylhydrazone (Compound 12) from 2′-hydroxylation per min per mg protein = 0.02 ± 0.0006 nmol; dinitrophenylhydrazone (Compound 13) from 5′-hydroxylation per min per mg protein = 0.40 ± 0.03 nmol; mean ± S.D. for 3 rats.

artificially low, since 2′-hydroxy-NNN (Compound 2) can also decompose to myosmine (Compound 9), which was not measured.

2′-Hydroxylation and 5′-hydroxylation of NNN were NADPH dependent, as shown in Table 1, which also summarizes data on Aroclor induction. Pretreatment with Aroclor in corn oil resulted in a 20-fold induction of 2′-hydroxylation, but only a 1.9-fold induction of 5′-hydroxylation. These differences are currently under further study.

To determine the kinetic deuterium isotope effects for α-hydroxylation of NNN, [2′,5′,5′-D]NNN (Compound 14), [2′-D]NNN (Compound 16), and [5′,5′-D]NNN (Compound 19) were synthesized. The syntheses are outlined in Chart 5; the position of deuterium was confirmed by NMR spectra. In each case, the appropriate α position was greater than 95% deuterated. In [5′,5′-D]NNN, some deuterium was also incorporated into the pyridine ring, as observed previously in the synthesis of [5′,5′-D]nicotine (22). In each case, Lineweaver-Burk plots were obtained for the deuterated compound and for NNN, using the same liver microsomes from Aroclor-treated rats. The K_m's for NNN and the deuterated compounds were similar, ranging from 1.47 to 1.81 mM for 2′-hydroxylation, and from 1.00 to 1.96 mM for 5′-hydroxylation. The ratios $V_{max\ H}:V_{max\ D}$ for NNN compared to each deuterated compound are summarized in Table 2. The deuterium isotope effect for 5′-hydrox-

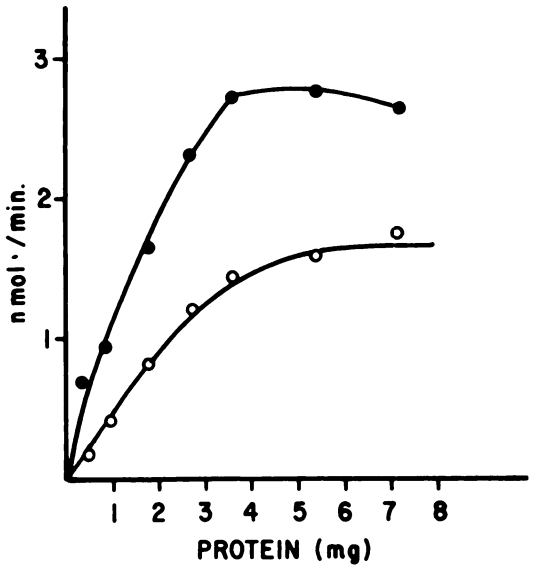

Chart 3. Effect of microsomal protein concentration on the rates of 2′-hydroxylation and 5′-hydroxylation of NNN; ●, dinitrophenylhydrazone Compound 12 from 2′-hydroxylation; ○, dinitrophenylhydrazone Compound 13 from 5′-hydroxylation.

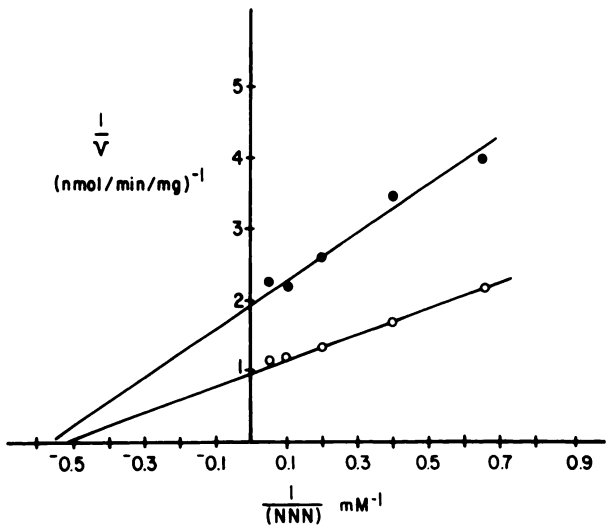

Chart 4. Double reciprocal plot of the effect of variations in substrate concentration on the rates of 2′-hydroxylation and 5′-hydroxylation of NNN; ●, dinitrophenylhydrazone Compound 12 from 2′-hydroxylation; ○, dinitrophenylhydrazone Compound 13 from 5′-hydroxylation.

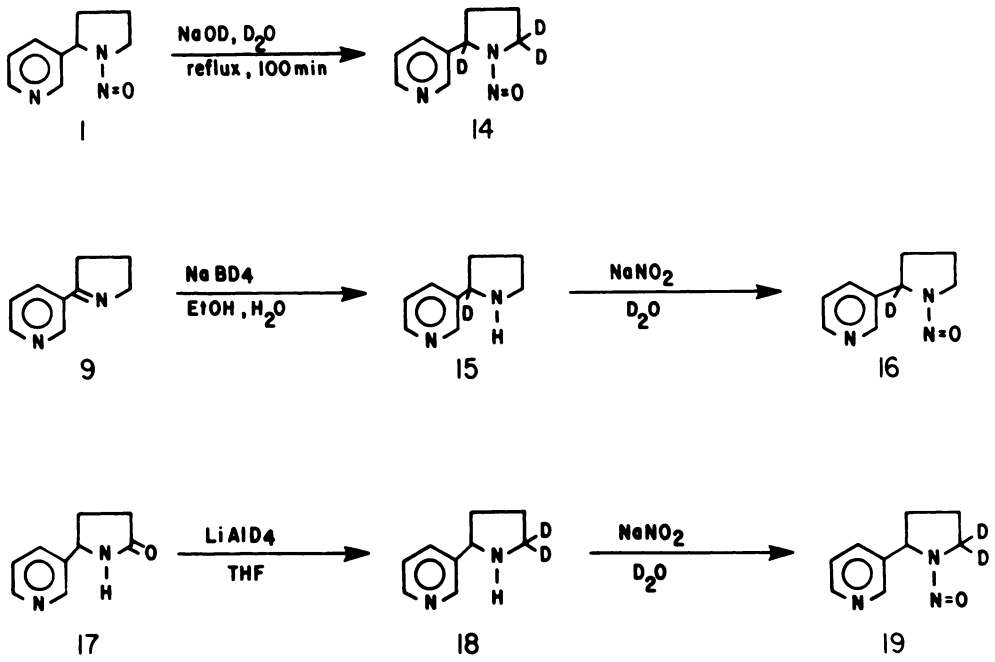

Chart 5. Synthesis of [2′,5′,5′-D]NNN, [2′-D]NNN, and [5′,5′-D]NNN.

Table 2

Deuterium isotope effect for α-hydroxylation of NNN

Each deuterated compound (0.34 to 27.5 mM) was incubated with liver microsomes (3.6 mg protein) from Aroclor-treated rats and K_m and V_{max} for 2′-hydroxylation and 5′-hydroxylation were determined from the resulting Lineweaver-Burk plots. These values were compared to those obtained for NNN using the same microsomal preparation.

	$\frac{V_{max\,H}}{V_{max\,D}}$[a]		$\frac{K_{m\,H}}{K_{m\,D}}$[b]	
Substrate	2′-Hydroxylation	5′-Hydroxylation	2′-Hydroxylation	5′-Hydroxylation
[2′-D]NNN	1.2		1.2	
[5′,5′-D]NNN		2.7		2.0
[2′,5′,5′-D]NNN	1.2	2.4	1.1	1.3

[a] The ratio of V_{max} for hydroxylation of NNN over V_{max} for hydroxylation of deuterated NNN.

[b] The ratio of K_m for hydroxylation of NNN over K_m for hydroxylation of deuterated NNN.

ylation was 2.4 to 2.7, while a value of 1.2 was obtained for 2′-hydroxylation. The values obtained for 5′-hydroxylation are in good agreement with those reported for α-hydroxylation of dimethylnitrosamine (7, 8). The small isotope effect for 2′-hydroxylation may be a reflection of the greater acidity of the 2′-hydrogen, but further studies are necessary to confirm this. Bioassays of these deuterated NNN derivatives are currently in progress.

The assay described here will allow further research on the influence of environmental modifiers or potential chemopreventive agents on the α-hydroxylation of NNN. Since α-hydroxylation is presumed to be the activation step for NNN, such studies should provide leads concerning enhancement or inhibition of carcinogenesis by NNN.

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