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Liver Microsomal Metabolism of *N*-Methylcarbazole: Structural Identification of the Four Major Metabolites of *N*-Methylcarbazole using ^{1}H Fourier Transform NMR Spectroscopy

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SUMMARY

NOVAK, R. F., D. R. KOOP AND P. F. HOLLENBERG: Liver microsomal metabolism of *N*-methylcarbazole: Structural identification of the four major metabolites of *N*-methylcarbazole using ^{1}H Fourier transform NMR spectroscopy. *Mol. Pharmacol.* **17:** 128–136 (1980).

^{1}H Fourier transform NMR spectroscopy, in conjunction with mass spectrometry, was employed for the identification of the four major metabolites formed from *N*-methylcarbazole, a cocarcinogen present in tobacco smoke, after incubation *in vitro* with rabbit liver microsomes. The four metabolites were separated by high pressure liquid chromatography and characterized by ultraviolet spectroscopy, mass spectrometry, and ^{1}H Fourier transform NMR spectroscopy. Mass spectrometry established that each of the metabolites contained an oxygen atom and ^{1}H Fourier transform NMR spectroscopy was used to identify the positional isomers of the hydroxylated *N*-methylcarbazole. It was established from comparison of the ^{1}H Fourier transform NMR spectra of the isolated metabolites of *N*-methylcarbazole with those of the corresponding spectra for known synthetic compounds that the four major metabolites formed *in vitro* were: 1-hydroxy-*N*-methylcarbazole, 2-hydroxy-*N*-methylcarbazole, 3-hydroxy-*N*-methylcarbazole, and *N*-hydroxymethylcarbazole.

INTRODUCTION

The particulate matter of tobacco smoke has been shown to be carcinogenic and sarcogenic in a variety of animals and tissues and is capable of acting as a complete carcinogen, effectively inducing squamous cell carcinoma in laboratory animals (2). Tobacco smoke contains significant amounts of the polycyclic aromatic hydrocarbons (PAH)[3] as well as significant quantities of heterocyclic compounds including the *N*-alkylcarbazoles and the alkylindoles (3, 4). Numerous attempts have been made to relate the carcinogenic activity of cigarette smoke to its chemical composition. Large-scale fractionation studies of the tobacco smoke condensate followed by bioassays have shown that a neutral fraction (B) rich in PAH was the most carcinogenic fraction of the tobacco smoke condensate for mouse skin (5, 6). However, the major portion of the carcinogenic activity of this fraction or that of whole smoke could not be explained by the levels of the carcinogenic PAH present (5). Solvent subfractionation of the neutral fraction resulted in a subfraction (BIh) that was highly enriched in PAH (6). Column chromatography of BIh gave 80 subfractions with fractions BIh 56-66, BIh 66-67, and BIh 72-78 exhibiting significant activity as tumor initiators on mouse skin (6). The *N*-alkylcarbazoles, which were found in the BIh 56-66 subfraction, were shown to be active as tumor accelerators or cocarcinogens (5). The *N*-alkylindoles were also shown to act as tumor accelerators (5). The *N*-alkylcarbazoles and *N*-alkylindoles are present in the

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[1] Predoctoral Trainee of the United States Public Health Service, Grant GM-07263. The data in this paper are taken from a thesis submitted by Dennis R. Koop in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of Northwestern University. Present address: Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109.

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[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; HPLC, high pressure liquid chromatography; NMC, *N*-methylcarbazole; NHMC, *N*-hydroxymethylcarbazole; 1-hydroxy-NMC, 1-hydroxy-*N*-methylcarbazole; 2-hydroxy-NMC, 2-hydroxy-*N*-methylcarbazole; 3-hydroxy-NMC, 3-hydroxy-*N*-methylcarbazole; R_t, retention time; and ^{1}H FT NMR, ^{1}H Fourier transform NMR.

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smoke to the extent of 140 and 961 ng per cigarette, respectively (3, 4).

Since only limited information is available on the metabolism of such tumor accelerators, a comprehensive study of the microsomal metabolism of *N*-methylcarbazole was initiated. In order to fully characterize the metabolism of *N*-methylcarbazole by the rabbit liver microsomal mixed function oxidases and to establish the role of *N*-methylcarbazole or its metabolites in the mechanism of tumor acceleration, it was necessary to isolate and identify the major products formed by the liver microsomal preparations.

The results presented here indicate that *N*-methylcarbazole undergoes aromatic hydroxylation at a number of different positions on the aromatic ring as well as aliphatic hydroxylation of the *N*-methyl group and suggest that *N*-methylcarbazole may be a useful substrate for monitoring the levels of activity of different forms of cytochrome *P*-450 in liver. The utility of this substrate for monitoring the levels of the cytochrome *P*-450-dependent mixed function oxidases has been demonstrated subsequently (7).

MATERIALS AND METHODS

Chemicals. The carbazole, acetonitrile, and deuteroacetone (99.9+ atom% D) were obtained from the Aldrich Chemical Company. The NADP$^+$, NADPH, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (Baker's Yeast), bovine serum albumin (Fraction V), sucrose, and Folin and Ciocalteu's phenol reagent were purchased from the Sigma Chemical Company. The *p*-nitroanisole, dimethylsulfate and the 5% palladium on charcoal were supplied by the Eastman Chemical Company. The hexanes (HPLC grade) were purchased from Fisher Scientific and the di[methyl-^{14}C]methyl sulfate was obtained from New England Nuclear. The 2-methyoxycarbazole and 3-methoxycarbazole were a generous gift of Dr. Peter A. S. Smith, University of Michigan. Dr. George Smith, University of Manchester, England, kindly provided the 1,2,3,4-tetrahydro-1-oxocarbazole. All other chemicals were of analytical reagent grade and were obtained from commercial suppliers.

Isolation of microsomes. New Zealand white male rabbits weighing between 1.5 and 2.0 kg were used for these studies. Sodium phenobarbital, dissolved in water, was administered intraperitoneally at a dose of 80 mg/kg once a day for 5 days prior to sacrifice. The rabbits were fasted for 18 hr prior to sacrifice. The rabbit livers were perfused *in situ* with ice-cold 0.9% NaCl until blanched, excised, and placed in ice-cold 50 mM Tris-chloride buffer, pH 7.4, containing 1.15% KCl. The microsomes were isolated using the methods of Matsubara *et al.* (8) and Powis and Boobis (9). The isolation procedures were carried out at 0-4° and all pH measurements were made at 23°. The livers were minced with a razor blade and homogenized in 3 vol (w/v) of 50 mM Tris-chloride buffer, pH 7.4, containing 1.15% KCl using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 600*g* for 10 min. The 600*g* pellet was rehomogenized in 2 vol of the same buffer and centrifuged again at 600*g* for 10 min. This step increased the yield of microsomal protein significantly (about 150%) without affecting the specific activity. The supernatant fractions from the 600*g* spins were combined and centrifuged at 10,000*g* for 20 min to remove the mitochondrial fraction. The microsomal pellet obtained by centrifugation of the 10,000*g* supernatant for 1 hr at 100,000*g* was resuspended in 2 vol (w/v of original liver weight) of 50 mM Tris-chloride buffer, pH 7.4, containing 250 mM sucrose and 1 mM EDTA and recentrifuged at 100,000*g* for 1 hr to remove any residual hemoglobin (9). The washed pellet was resuspended in 100 mM potassium phosphate buffer, pH 7.70, containing 250 mM sucrose, at a protein concentration of 15 to 30 mg/ml. The suspension was divided into aliquots, purged with N_2, frozen in acetone-dry ice, and stored at −90°. The microsomes were used within 4 weeks of isolation and, under these storage conditions, there was no significant loss of cytochrome *P*-450 or hydroxylase activity. Protein was determined by the method of Lowry *et al.* (10) using bovine serum albumin as the standard. Cytochrome *P*-450 content was determined by the method of Omura and Sato (11) using an extinction coefficient of 91 $mM^{-1}cm^{-1}$. The specific activity of the preparation from phenobarbital-treated rabbits was approximately 2.9 nmol of cytochrome *P*-450/mg of protein.

Incubations. The microsomal incubations were carried out at 25° in the presence of atmospheric oxygen using 25-ml Erlenmeyer flasks in a Dubnoff shaking incubator. A standard reaction mixture contained the following components: 200 mM phosphate buffer, pH 7.4; 1.5 mM *N*-methylcarbazole, (added in 50 μl of ethanol); 3.0 mM glucose-6-phosphate; 6.0 mM $MgCl_2$; 6 units of glucose-6-phosphate dehydrogenase; 0.60 mM NADP$^+$, and 3 to 6 mg of microsomal protein in a final volume of 3.0 ml. The reaction mixtures were preequilibrated in the water bath at 25° for 3 min prior to the initiation of the reaction by the addition of NADP$^+$. After 15 min, the reaction was terminated by the addition of 3.0 ml of ethyl acetate. The products and unmetabolized substrate were recovered from the reaction mixture by three successive extractions with 3 ml of ethyl acetate. The organic phases were pooled and evaporated to dryness under reduced pressure. The residue was redissolved in 0.20 ml ethyl acetate, layered on a 0.5 × 5-cm silica gel column (30-70 mesh, E.M. Laboratories), and eluted with 9.0 ml of hexanes: isopropanol (100:4 by volume). This latter step was necessary to remove very polar components which bound to the HPLC analytical column and resulted in a rapid loss of resolution. Inclusion of this step had no qualitative or quantitative effect on the elution profiles observed.[4] The eluate from the silica gel column was evaporated to dryness, redissolved in 50 μl of ethyl acetate and analyzed by HPLC.

The separation of the metabolites was accomplished on a Glenco HPLC System I equipped with a Whatman Guard Column (2.0 × 9 mm) packed with H.C. Pellosil (Whatman) and connected to a Whatman Partisil 10 silica column (4.6 mm × 25 cm). The reaction products were eluted under isocratic conditions with a mobile phase of hexanes:isopropanol (100:2.2) at a flow rate of

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[4] Koop, D. R. and Hollenberg, P. F., unpublished observations.

1.05 ml/min. The products were detected in the eluate by measurement of the uv absorbance at 254 nm (Fig. 1).

The major metabolites of *N*-methylcarbazole were collected for structural analysis upon elution from the HPLC using a Gilson Micro Fraction Collector. In order to obtain enough sample for mass spectral and NMR studies of each of the metabolites, the products from five reaction mixtures were collected in this manner. The eluates were pooled and the solvent was removed by evaporation under reduced pressure. The isolated metabolites were stored under nitrogen at −20° until they were used for mass spectral or NMR analysis. The metabolites isolated in this manner were shown to be free of contamination by rechromatography on the HPLC using the conditions described. They were also analyzed for purity by thin-layer chromatography (TLC) on precoated silica gel 60 G-254 glass TLC plates (Merck, 0.25 mM thickness, 5 × 10 cm or 5 × 20 cm). The plates were developed with a mobile phase of benzene:dioxane:diethylamine:H_2O (70:17.5:7.5:0.5 v/v). The metabolites were visualized under uv light or by spraying the plate with 0.2% *p*-dimethylaminobenzaldehyde in 20% H_2SO_4 followed by gentle heating. The metabolites and substrate had the following R_f values: NMC—0.74, carbazole—0.58, NHMC—0.44, 1-hydroxy-NMC—0.41, 3-hydroxy-NMC—0.34, and 2-hydroxy-NMC—0.23.

Ultraviolet spectra. The ultraviolet spectra of the isolated metabolites were recorded on an Aminco DW-2 spectrophotometer operated in the split beam mode with a 2-nm spectral bandpass. Matched quartz cuvettes with a 1.0-cm pathlength were used. The samples were dissolved in hexanes:isopropanol (100:2.2) and the spectra were recorded with hexanes:isopropanol (100:2.2) in the reference cuvette.

Mass spectral studies. Mass spectra were obtained with Finnigan mass spectrometers: Model 3200 E was used for chemical ionization (C.I.) with methane as the reagent gas at 0.7 Torr and Model 3000 was used for electron impact ionization (E.I.) and was operated at an ionizing energy of 70 eV. The purified metabolites were dissolved in ethyl ether and transferred to a solid probe sample tube. The ethyl ether was removed by evaporation and the samples were introduced directly into the ion source via a variable temperature solid sample insertion probe. All spectra were obtained with the source at a temperature of about 90°. Both instruments were interfaced to a System Industries Model System/150 data acquisition system. The spectra were printed using a Houston Instruments Model DP-1 digital plotter.

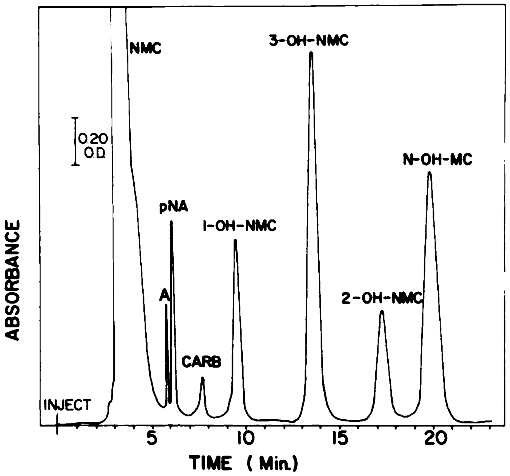

FIG. 1. *HPLC analysis of the metabolites of N-methylcarbazole formed by rabbit liver microsomes*

The incubation conditions and the conditions for the HPLC analysis are described in MATERIALS AND METHODS. The eluate was monitored at 254 nm.

Proton NMR studies. ^1H FT NMR spectra were recorded on a Varian CFT-20 spectrometer operating at 80 MHz and internally locked on the signal from deuteroacetone or deuterochloroform. The metabolites were dissolved in 0.5 ml of either deuteroacetone or deuterochloroform and transferred to a 4.5-mm NMR tube. For samples less than 10 μg, the compound was dissolved in 25 μl of the appropriate solvent and transferred to an NMR microtube (1.7 mm o.d.). Water was removed from the sample solutions by the addition of molecular sieves (4 Å, Fisher Scientific). The spectra were obtained at room temperature. The spectra were recorded over a sweep width of 1000 Hz with 8192 data points. Tetramethylsilane (TMS) was added as the internal standard.

Chemical methods. NMC was synthesized by the method of Stevens and Tucker (12) using carbazole and dimethylsulfate. The product, which was recrystallized from ethanol, had a melting point of 87° (lit. 88°) and was pure as determined by TLC and HPLC. *N*-[*methyl*-^{14}C]Carbazole was synthesized by the same method using di[^{14}C-*methyl*]methyl sulfate.

NHMC was synthesized by refluxing carbazole and formaldehyde in a methanol solution containing potassium carbonate (13). The white needle-shaped crystals that formed on cooling the reaction mixture were recrystallized from benzene and had a melting point of 129° (lit. 129°). The recrystallized product was determined to be pure by TLC and HPLC.

Samples of 2- and 3-methoxycarbazole, kindly provided by Dr. P. A. S. Smith from the University of Michigan, were *N*-methylated by the method of Sawicki (14), using dimethyl sulfate and KOH in acetone. The reaction yielded the starting material and the less polar *N*-methylated derivative of the methoxycarbazoles. The *N*-methyl-2-methoxycarbazole and the *N*-methyl-3-methoxycarbazole derivatives were isolated in pure form by HPLC using the conditions previously described for the isolation of the metabolites.

The 2- and 3-monohydroxylated metabolites of NMC, which had been isolated from five microsomal incubation mixtures as previously described, were methylated to the corresponding methoxy derivatives as described by Smith *et al.* (15). The isolated metabolites were dissolved in 4% NaOH and dimethyl sulfate was added. The reaction was allowed to proceed for 2 hr at ~100° in a steam bath, after which time the mixture was extracted with 10 ml of ethylacetate and dried with $MgSO_4$. The extract contained two components as shown by HPLC and TLC. The less polar 2- and 3-methoxy-*N*-methylcarbazole derivatives were isolated in pure form by HPLC as described previously.

The synthesis of 1-hydroxy-NMC was accomplished by dehydrogenation of 1,2,3,4-tetrahydro-9-methyl-1-oxocarbazole as described by Horning *et al.* (16). This compound was methylated with dimethyl sulfate using the method of Stevens and Tucker (12). The methylated product was recovered from the reaction mixture by extracting with ethyl ether and the ether was removed by distillation after drying with sodium sulfate. The 1,2,3,4-tetrahydro-9-methyl-1-oxocarbazole was dehydrogenated by refluxing in mesitylene for 2 hours in the presence of 5% palladium on a charcoal catalyst (16). The 1-hydroxy-NMC was separated from the starting compound by TLC on a preparative TLC plate (Merck 20 × 20 cm, 2.0 mm thickness). The plate was developed with benzene:dioxane:diethylamine:water (70:17.5:5:0.5). The starting compound had an R_f of 0.69 and the 1-hydroxy-NMC had an R_f of 0.35. The 1-hydroxy-NMC was detected under uv light, scraped off the plate with a spatula, and eluted from the silica support with dichloromethane. The solvent was removed by distillation. The product was further purified by HPLC using hexanes: isopropanol (100:3.9) with a flow rate of 0.60 ml/min, and had a retention time of 11.4 min.

RESULTS

Metabolite separation. A typical HPLC profile obtained for the metabolites of NMC is shown in Fig. 1. The four major metabolites are completely separated under conditions of isocratic solvent flow and elute within 20 min after injection under the conditions used for these studies. The *p*-nitroanisole was added as an internal standard after the reaction was terminated. The retention times (R_t) measured from the time of injection of the sample are: NMC, 3.7 min; p-nitroanisole, 6.1 min; carbazole, 7.7 min; 1-hydroxy-NMC (metabolite 1), 9.6 min; 3-hydroxy-NMC (metabolite 2), 13.9 min; 2-hydroxy-NMC (metabolite 3), 17.2 min; and NHMC (metabolite 4), 19.8 min. The peak that elutes between NMC and *p*-nitroanisole (Fig. 1, peak A) is present in eluates of the ethyl acetate extracts of control microsomal reaction mixtures to which NMC was not added and therefore does not represent a metabolite of NMC, but a microsomal contaminant.

Ultraviolet absorption spectra. The ultraviolet absorption spectrum for NMC and for each of the metabolites was recorded after separation of the compounds on the HPLC. The spectra of the eluates containing the separated metabolites were recorded in methanol and in the HPLC solvent (hexanes:isopropanol, 100:2.2) and no major differences were observed between the spectra in the two solvents. The spectra for the four major metabolites in the HPLC solvent are shown in Fig. 2. The spectrum for each metabolite is unique. The spectrum of NHMC is nearly identical to that observed for NMC. Compared with NHMC, the three phenolic metabolites show a bathochromic shift of a portion of the spectrum that is consistent with hydroxylation of an aromatic compound; the absorption maximum at about 291 nm for NHMC is shifted by approximately 10 nm to the red in the 2- and 3-hydroxy- metabolites, while the maximum at 235 nm in NHMC is shifted about 10 nm to the red in the 1-hydroxy- metabolite.

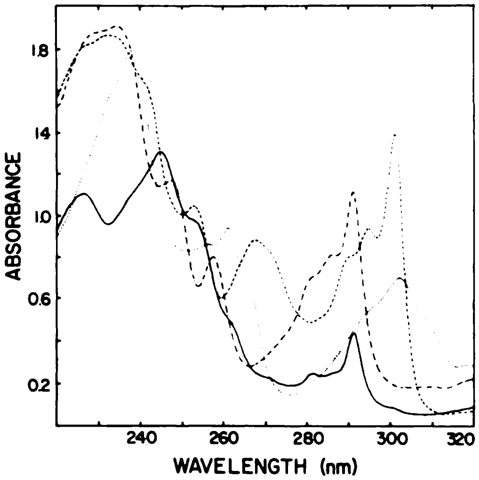

FIG. 2. *The ultraviolet spectrum of the isolated metabolites of NMC*

The metabolites isolated by HPLC were dissolved in hexanes: isopropanol (100:2.2) and the spectra were recorded on an Aminco DW-2 spectrophotometer with a 2.0-nm spectral bandpass in matched quartz cuvettes (1-cm pathlength). The concentrations were: NHMC (—·—), 55.5 μM; 1-hydroxy-NMC (——), 41.2 μM; 2-hydroxy-NMC (····), 34.5 μM; and 3-hydroxy-NMC (- - -), 70.8 μM.

The molar extinction coefficient at 254 nm for each metabolite was determined by using *N*-[*methyl*-^{14}C]carbazole as the substrate for the hydroxylations. The radiolabeled products were isolated as they eluted from the HPLC column and diluted to 10 ml in a volumetric flask with the HPLC solvent (hexanes:isopropanol, 100:2.2). The concentration of the isolated metabolite in this solution was determined from the radioactivity and the absorbance of the same solution was measured at 254 nm. The extinction coefficients at 254 nm for the metabolites (calculated by substituting the concentration and absorbance into Beers Law) are: NMC, 12.2 $\text{mM}^{-1}\text{cm}^{-1}$; 1-hydroxy-NMC, 22.9 $\text{mM}^{-1}\text{cm}^{-1}$; 2-hydroxy-NMC, 19.4 $\text{mM}^{-1}\text{cm}^{-1}$; 3-hydroxy-NMC, 14.4 $\text{mM}^{-1}\text{cm}^{-1}$; and NHMC, 12.0 $\text{mM}^{-1}\text{cm}^{-1}$.

Mass spectral studies. The chemical ionization mass spectra of all four major metabolites of NMC are shown in Fig. 3. All four spectra exhibit marked similarities being characterized by a quasi-molecular ion at *m/e* of 198 $(MH)^+$. Recombination ions of $(M+29)^+$ and $(M+41)^+$ corresponding to $(M+C_2H_5)^+$ and $(M+C_3H_5)^+$ are observed at *m/e* of 226 and 238, respectively, providing additional support for assignment of the molecular mass of the compounds at 197. This molecular mass is consistent with the incorporation of one atom of oxygen into the substrate molecule suggesting monohydroxylation of the substrate. The spectrum for NHMC also has a major fragment at *m/e* of 180 corresponding to the loss of water and a small ion at 168 corresponding to carbazole, a contaminant of the sample resulting from the decomposition of the NHMC.

The electron impact mass spectra of all four major metabolites are shown in Fig. 4. The molecular ion at *m/e* of 197 is predominant in the spectra of the three phenolic metabolites confirming the molecular mass of 197 obtained from the chemical ionization spectra. The molecular ion at *m/e* 197 is small for NHMC with a large ion at *m/e* 167 owing to the relative instability of the hydroxymethyl group. The small degree of fragmentation

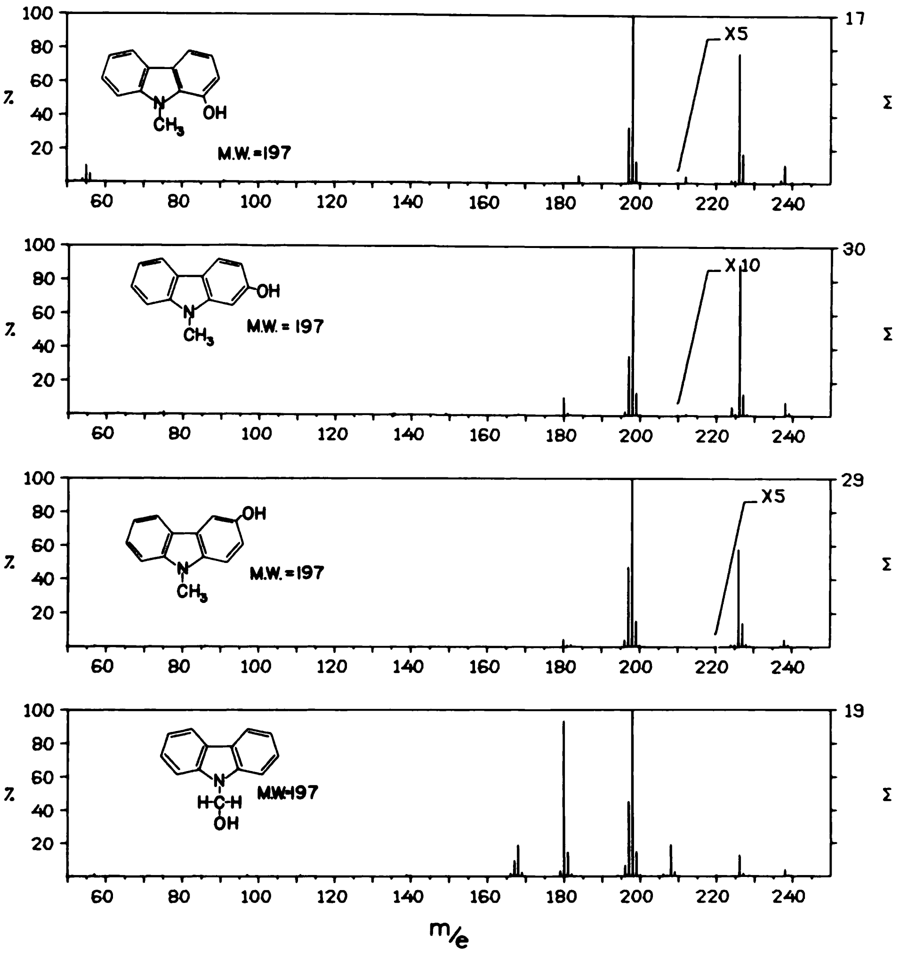

FIG. 3. *Chemical ionization mass spectra of the four major metabolites of NMC*
Sample preparation and analysis conditions are described in MATERIALS AND METHODS.

of these metabolites probably reflects the high stability of the carbazole ring structure (17). The fragmentation pattern is basically the same for the three phenolic metabolites with ions at *m/e* of 182, 168, 154, and 153 corresponding to the losses of CH_3 (M^+—15), CH_3N (M^+—29), CH_3 + CO (M^+—43), and CH_3 + HCO (M^+—44) from the molecular ion, respectively. The major difference between the three phenolic metabolites is the fragment ion at *m/e* 154. For 1-hydroxy-NMC this fragment is approximately 40% of the base peak while it is less than 5% in the 2- and 3-hydroxy- metabolites. The position of the hydroxyl group (*ortho* to the indole nitrogen) in 1-hydroxy-NMC permits the transfer of the proton from the hydroxyl group to the nitrogen, thus facilitating the loss of CO which results in the appearance of the ion at *m/e* 154. This mechanism has been suggested for the structurally similar 8-hydroxyquinoline (18). This proton transfer cannot occur in the 2- and 3-hydroxylated metabolites, and as a result there is relatively little formation of the ion at *m/e* 154. Although this difference in the three phenolic metabolites was observed, positive identification of the 1-hydroxy-NMC could not be made based solely on the mass spectral results.

Nuclear magnetic resonance results. Although mass spectrometry established that an hydroxyl group had been incorporated into each of the metabolites of NMC, these data did not permit the determination of the positions of hydroxylation. In order to unambiguously identify the positional isomers of the ring hydroxylated NMC, ^1H FT NMR spectroscopy was utilized.

The ^1H FT NMR spectrum of NMC in deuteroacetone is shown in Fig. 5. The spectrum consists of a singlet (δ = 3.85 ppm) ascribable to the protons of the *N*-methyl group and multiplet (δ = 7.0 to 8.1 ppm) representative of the aromatic ring protons.

The ^1H FT NMR spectrum of metabolite 4 isolated from the microsomal reaction mixture, NHMC, in deuteroacetone is shown in Fig. 6a. The spectrum consists of an asymmetrical triplet, centered at δ = 5.35 ppm, a doublet (δ = 5.9 ppm), and a multiplet of the aromatic protons (δ = 7.0 to 8.2 ppm). The asymmetrical triplet may be assigned to the hydroxyl proton ($-CH_2\mathit{OH}$) and the doublet to the methylene protons ($-C\mathit{H}_2OH$), respectively. Integration of the spectrum yields area ratios of 8:2:1 (aromatic:methylene:hydroxyl), and ^1H homonuclear decoupling experiments showed that the methylene and hydroxyl protons were coupled to each other. For example, placement of the decoupler frequency on the methylene doublet (δ = 5.9 ppm) resulted in the collapse of the triplet to a singlet. Furthermore, addition of trace amounts of ^2H_2O to the acetone-d_6 solution of metabolite 4 initially produced the collapse of the triplet to a singlet

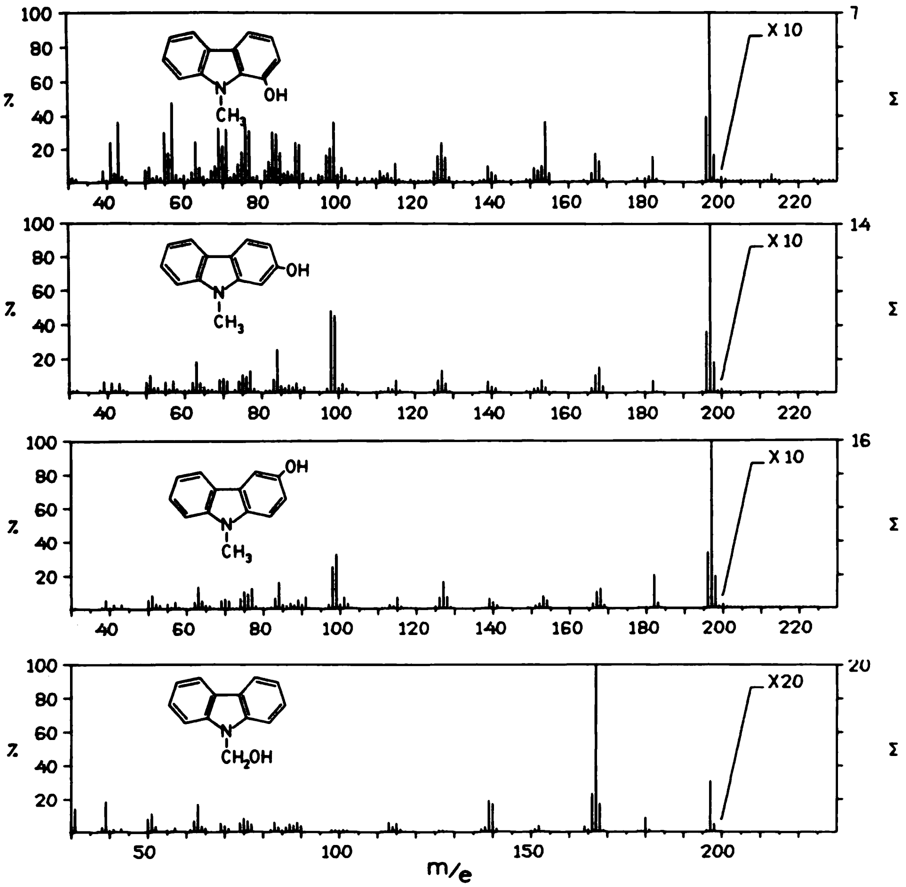

Fig. 4. *Electron impact mass spectra of the four major metabolites of NMC*

Sample preparation and analysis conditions are described in MATERIALS AND METHODS.

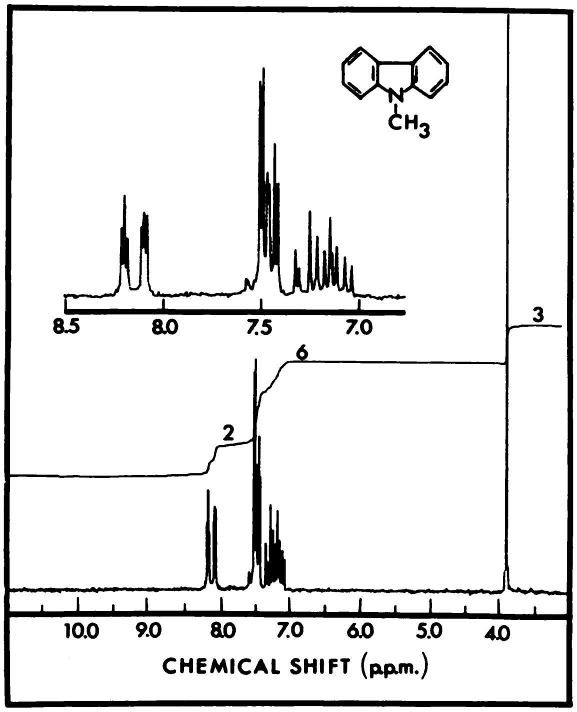

Fig. 5. *¹H FT NMR spectrum of NMC in acetone-d₆*

The instrumental parameters were: sweep width = 1000 Hz, pulse width = 5 µsec, number of transients = 100, data points = 8192, and sensitivity enhancement = −0.05 sec. The inset contains an expanded spectrum of the aromatic protons.

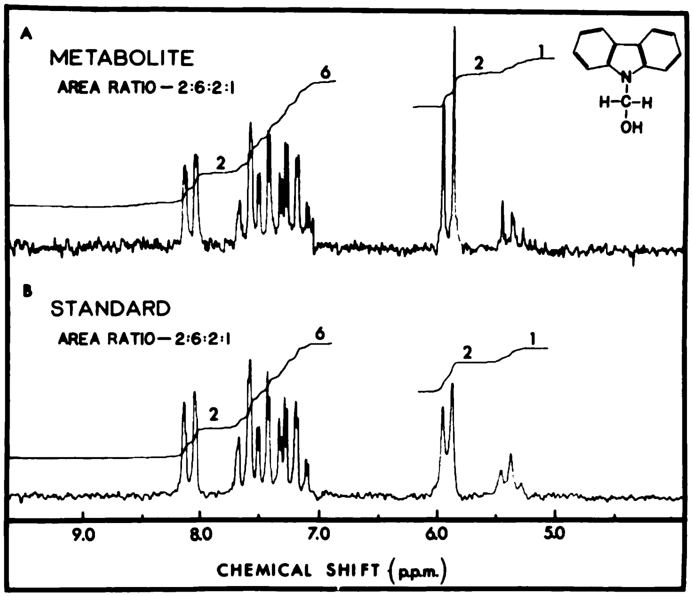

Fig. 6. *¹H FT NMR spectra of NHMC*

(A) Isolated from the microsomal reaction mixture by HPLC. (B) Synthetic NHMC. Both spectra were obtained with the sample dissolved in 0.5 ml of deuteroacetone. The instrumental parameters were: sweep width = 1000 Hz, pulse width = 20 µsec, number of transients = 260, data points = 8192, and sensitivity enhancement = −0.80 sec. The vertical scale is 800. The chemical shifts are reported in parts per million (ppm) relative to the internal reference, TMS, δ = 0.0.

and subsequently resulted in the total disappearance of the signal. The aromatic region (δ = 7.0 to 8.2 ppm) appears to be relatively unaffected in position and multiplicity in comparison to the spectrum of NMC (Fig. 5). These results are consistent with the analysis of metabolite 4 as NHMC. This analysis was further confirmed by synthesis of NHMC and comparison of the ^{1}H FT NMR spectrum of the synthetic product with that isolated from microsomal reaction mixtures. As shown in Fig. 6b, both spectra are virtually identical.

The ^{1}H FT NMR spectrum for metabolite 1 (1-hydroxy-NMC), a ring-hydroxylated metabolite, is shown in Fig. 7a. The spectrum consists of a singlet occurring at δ = 4.4 ppm, associated with the *N*-methyl protons, and a multiplet in the aromatic region δ = 7.2–8.2 ppm, which is significantly altered from that of NMC (see Fig. 5). Compared with NMC and the other metabolites, the methyl peak of the 1-hydroxy metabolite is shifted downfield by 1 ppm. The hydroxyl proton signal is not observed and may be hidden under the aromatic region or may be sufficiently broadened by the presence of a small amount of water to prevent detection. The ^{1}H FT NMR spectrum of 1-hydroxy-NMC synthesized by the dehydrogenation of 1,2,3,4-tetrahydro-9-methyl-1-oxocarbazole as described in MATERIALS AND METHODS is identical to that of metabolite 1 (Fig. 7b).

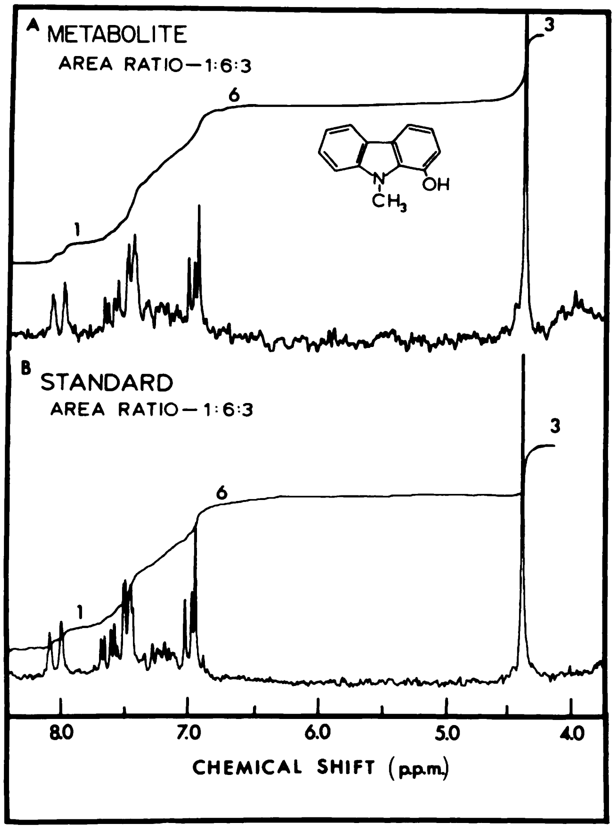

FIG. 7. *^{1}H FT NMR spectra of 1-hydroxy-NMC*

(A) Isolated by HPLC from the microsomal reaction mixture. The isolated metabolite was dissolved in 25 μl of deuteroacetone and the NMR spectrum recorded using the microprobe technique (1.7-mm tube). The instrumental parameters were: sweep width = 1000 Hz, pulse width = 20 μsec, pulse delay = 10 sec, number of transients = 3624, data points = 8192, and sensitivity enhancement = −0.50 sec. The vertical scale is 3000. (B) Synthetic 1-hydroxy-NMC. The sample was dissolved in 0.5 ml of deuteroacetone. The instrumental parameters were: sweep width = 1000 Hz, pulse width = 20 μsec, number of transients = 2095, data points = 8192, and sensitivity enhancement = −0.80 sec. The vertical scale is 8000. The chemical shifts are in ppm relative to TMS, δ = 0.

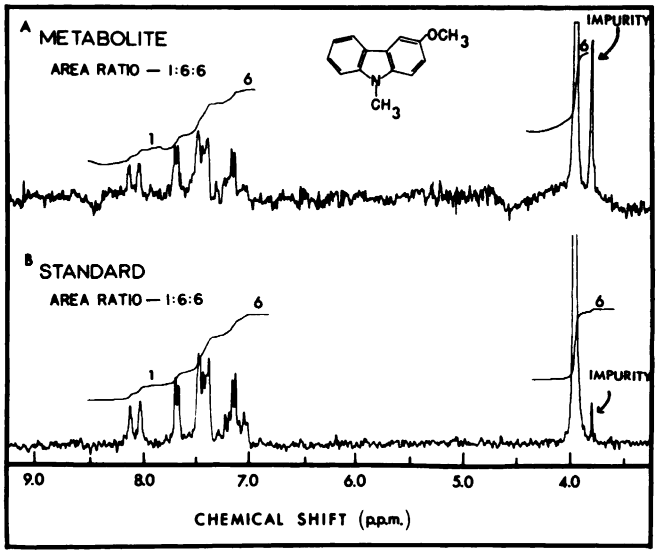

FIG. 8. *^{1}H FT NMR spectra of 3-methoxy-NMC*

(A) The isolated metabolite was methylated as described in MATERIALS AND METHODS and the methylated product was dissolved in 25 μl of deuteroacetone. The NMR spectrum was taken using the microprobe technique (1.7 mm tube). The instrumental parameters were: sweep width = 900 Hz, pulse width = 20 μsec, number of transients = 5678, data points = 8192, and sensitivity enhancement = −0.80 sec. The vertical scale is 1000. (B) Synthetic 3-methoxy-NMC. The sample was dissolved in 0.5 ml of deuteroacetone. The instrumental parameters were: sweep width = 1000 Hz, pulse width = 15 μsec, number of transients = 100, sensitivity enhancement = −0.80 sec, and data points = 8192. The vertical scale is 200. The chemical shifts are in ppm relative to TMS, δ = 0. The signal occurring at δ = 3.9 ppm consists of two singlets: the *N*-methyl- signal, α = 3.95 ppm, and the methoxy- signal, δ = 3.90 ppm.

Figure 8a presents the ^{1}H FT NMR spectrum of the methylated derivative of 3-hydroxy-NMC, metabolite 2. Methylation with dimethyl sulfate was carried out in order to convert the hydroxyl group to a methoxy derivative for comparative purposes. The spectrum consists of two very closely spaced singlets, δ = 3.98 and δ = 4.00 ppm, which are associated with the *N*-methyl and the methoxy-methyl groups, respectively, and the aromatic proton signals occurring in the region 7.0–8.2 ppm. Comparison of the spectrum of the methylated derivative of the isolated metabolite (Fig. 8a) with that of 3-methoxy-NMC (Fig. 8b) established the identity of metabolite 2 as 3-hydroxy-NMC.

Similarly, the ^{1}H FT NMR spectrum of the methylated derivative of metabolite 3 is presented in Fig. 9A. The spectrum may be generally described as consisting of two closely spaced singlets (δ = 3.85 and 3.95 ppm) that result from the N-methyl and methoxy-methyl protons, respectively, and a multiplet associated with the aromatic ring protons. For comparison, the spectrum of the synthetic 2-methoxy-NMC is also shown (Fig. 9B). As can be readily observed, both spectra are virtually identical and thus metabolite 3 is identified as 2-hydroxy-NMC.

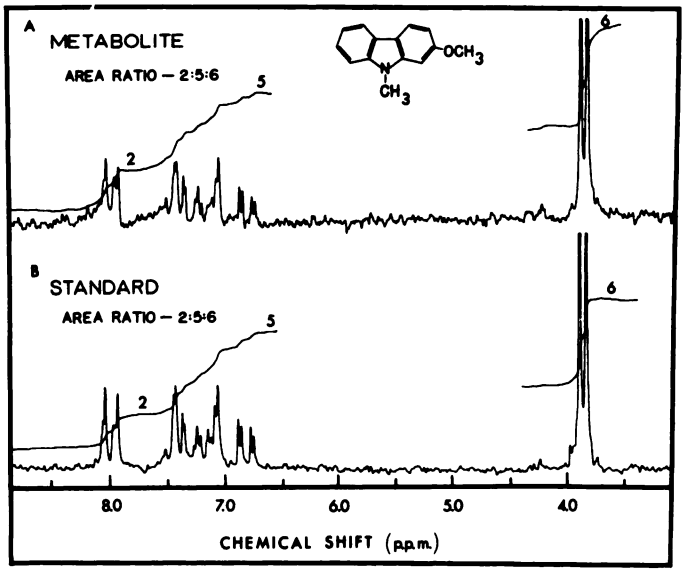

FIG. 9. *^{1}H FT NMR of 2-methoxy-NMC*

(A) The isolated metabolite was methylated as described in MATERIALS AND METHODS and the methylated product was dissolved in 25 μl of deuteroacetone. The NMR spectrum was recorded using the microprobe technique. The instrumental parameters were: sweep width = 800 Hz, pulse width = 20 μsec, data points = 8192, sensitivity enhancement = −0.50 sec, and the number of transients = 5000. The vertical scale is 1000. (B) Synthetic 2-methoxy-NMC. The sample was dissolved in 0.5 ml of deuteroacetone. The instrumental parameters were: sweep width = 800 Hz, pulse width = 20 μsec, pulse delay = 5.0 sec, number of transients = 35, data points = 8192, and the sensitivity enhancement = −0.50 sec. The vertical scale is 500. The chemical shifts are reported in ppm relative to TMS, $\delta = 0$. The apparent doublet occurring at approximately 3.90 ppm is actually composed of two separate singlets: the *N*-methyl-, $\delta = 3.95$ ppm, and the methoxy-, $\delta = 3.85$ ppm.

DISCUSSION

Rabbit liver microsomes in the presence of NADPH are able to metabolize NMC, a component of cigarette smoke that is an active cocarcinogen, to a variety of hydroxylated products that can be readily separated by HPLC. In order to characterize the results of the microsomal metabolism of NMC and investigate the role of NMC and/or its metabolites in the cocarcinogenic activity, it was necessary to identify the metabolites. The results obtained from mass spectrometry unequivocally established that the four major metabolites of NMC contained only one added oxygen atom; however, the sites of hydroxylation on the substrate molecule could not be determined from these data. Therefore ^{1}H Fourier transform NMR spectroscopy was used to identify the positional isomers of the monohydroxylated products of NMC. Since only small quantities of the metabolites 1-hydroxy-NMC and 3-hydroxy-NMC were formed during the microsomal incubations, it was necessary to use the microprobe technique, which for ^{1}H requires only ~50–100 ng of sample to yield spectra suitable for analysis. From the ^{1}H FT NMR results it was determined that the isolated metabolites of *N*-methylcarbazole are NHMC and the 3-ring hydroxylated products: 1-hydroxy-, 2-hydroxy-, and 3-hydroxy-NMC.

Johns and Wright (19) have reported that the major urinary metabolite in rats and rabbits after administration of carbazole is 3-hydroxycarbazole conjugated with glucuronic acid. Since the 3-position is the position of the highest electron density in the carbazole nucleus, it was suggested that the attacking agent is an electron-deficient species such as a hydroxyl cation or a hydroxyl free radical (19). The formation of NHMC as a product of liver microsomal metabolism of NMC has also been reported by Gorrod and Temple (20). It was suggested that NHMC was formed as an intermediate in the dealkylation of NMC and that, due to its inherent instability, it decomposed nonenzymically to give carbazole and formaldehyde (20).

The monohydroxylated products represent greater than 95% of the products formed during the liver microsomal metabolism of NMC and the addition of inhibitors of epoxide hydrase such as cyclohexene oxide and TCPO to the incubation mixtures do not cause a significant increase in the amounts of the monohydroxylated products formed.[4] However, these results are insufficient evidence for precluding the formation of an arene oxide intermediate in the hydroxylation reactions since the arene oxide intermediate may be highly unstable and may undergo a rapid hydrolysis to form the monohydroxylated products.

The metabolism of ellipticine (5,11-dimethyl-(6*H*)-pyrido[4,3-*b*]carbazole), an alkaloid that exhibits significant antitumor activity *in vivo* and is structurally related to carbazole, has been studied (21, 22). When metabolized either *in vivo* (21) or by rat liver microsomal preparations (22), ellipticine is hydroxylated primarily in position 9, which corresponds to the 3-position on the carbazole nucleus. Metabolism studies using 7,9-dideutero-ellipticine demonstrated that the reaction proceeds with the conservation of deuterium at the position of hydroxylation, suggesting that the aromatic hydroxylation reaction at the 9-position does not involve an arene oxide intermediate.

Some carbazole derivatives are biologically active and display significant antibacterial, antifungal, and insecticidal activities (23). Numerous derivatives of ellipticine have been shown to exhibit antitumor activity *in vivo* (24). Some of these derivatives also exhibit mutagenicity in the Ames test (24). Furthermore, some carbazole derivatives have been shown to function as carcinogens (25). However, little information is available on the metabolism of these carbazole derivatives and the role of metabolism in carcinogenesis and/or mutagenesis. Preliminary results in our laboratory demonstrate that, although NMC is a relatively weak mutagen for *Salmonella typhimurium* TA-100 in the presence of rat liver S-9 fractions, NHMC is an extremely potent mutagen exhibiting a mutagenic potency comparable to that of benzo[*a*]pyrene.[5] Since the mutagenic activity of NHMC requires the presence of the rat liver S-9 fraction, NHMC is not the ultimate mutagenic form, but requires additional activation by the S-9 fraction in order to express its mutagenic potential.

The formation of multiple products from a single sub-

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[5] Koop, D. R., Scarpelli, D. G. and Hollenberg, P. F., unpublished observations.

strate by the liver microsomal mixed function oxidases has been reported for several substrates (26–28). The formation of multiple products has been attributed to the presence of multiple forms of cytochrome *P*-450 in the microsomal fraction (29, 30) and has been used to monitor the levels of the different forms of cytochrome *P*-450 present in the liver as well as in other tissues (26–30). The formation of several metabolites of NMC that can be readily separated by HPLC using isocratic elution suggests that the metabolism of NMC might be used to advantage to monitor levels of the microsomal mixed function oxidases in standard microsomal preparations of various tissues, as demonstrated in the accompanying paper (7).

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