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Liver Microsomal Metabolism of *N*-Methylcarbazole: A Probe for Induction, Inhibition, and Species Differences

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SUMMARY

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N-Methylcarbazole is converted to four major metabolites by rabbit liver microsomal preparations: *N*-hydroxymethylcarbazole, 1-hydroxy-*N*-methylcarbazole, 2-hydroxy-*N*-methylcarbazole, and 3-hydroxy-*N*-methylcarbazole. Small amounts of carbazole, presumably formed by the decomposition of *N*-hydroxymethylcarbazole, were also formed. An assay procedure using high pressure liquid chromatography to separate and quantitate the metabolites was developed. The formation of the four major hydroxylated products requires NADPH or NADH. Formation of the hydroxylated products is inhibited by carbon monoxide, SKF-525A or 5,6-benzoflavone to similar extents. Metyrapone inhibited the formation of the 2-hydroxy-*N*-methylcarbazole to a greater extent than any of the others. 7,8-Benzoflavone, which had little effect on the formation of *N*-hydroxymethylcarbazole, inhibited the formation of 3-hydroxy-*N*-methylcarbazole by 30% and the formation of 2-hydroxy-*N*-methylcarbazole by 60% while stimulating the formation of 1-hydroxy-*N*-methylcarbazole. The liver microsomal metabolism of *N*-methylcarbazole by rabbits was compared with that of rats and mice. The major metabolite formed by rabbit liver microsomes is *N*-hydroxymethylcarbazole with slightly smaller amounts of the 3-hydroxy-*N*-methylcarbazole being formed. In rats, the major metabolite is the 1-hydroxy-*N*-methylcarbazole with *N*-hydroxymethylcarbazole being formed in slightly lesser amounts. Metabolism of *N*-methylcarbazole by mice results in the formation of the 3-hydroxy- and *N*-hydroxymethylcarbazole as the major metabolites in essentially equal amounts. Relatively small amounts of 2-hydroxy-*N*-methylcarbazole are formed by microsomes from rabbits, rats, or mice. Phenobarbital treatment of rabbits preferentially induces the formation of 2-hydroxy-*N*-methylcarbazole by 20-fold with lesser induction of the rates of formation of the 1-hydroxy-, 3-hydroxy-, and *N*-hydroxy- metabolites of *N*-methylcarbazole (2-, 4-, and 4-fold, respectively). Although treatment of the rabbits with 3-methylcholanthrene did not alter the rates of formation of 2-hydroxy- or 3-hydroxy-*N*-methylcarbazole, the rates of formation of 1-hydroxy- and *N*-hydroxymethylcarbazole were decreased significantly. In the rat, phenobarbital induction caused a 2-fold increase in the formation of 1-hydroxy- and *N*-hydroxymethylcarbazole without altering the rates of formation of the 2-hydroxy- and 3-hydroxy- metabolites. Induction of the rats with 3-methylcholanthrene caused a 2-fold increase in the rate of formation of 3-hydroxy-*N*-methylcarbazole and a 1.5-fold increase in the rate of formation of *N*-hydroxymethylcarbazole. Steady-state kinetic studies on the metabolism of *N*-methylcarbazole by liver microsomes from rabbits treated with phenobarbital, 3-methylcholanthrene, or 5,6-benzoflavone provided kinetic evidence for the involvement of different forms of cytochrome *P*-450 in the formation of each of the four major metabolites. These results suggest that *N*-methylcarbazole may be an excellent substrate for investigating the multiple forms of cytochrome *P*-450 in crude microsomal preparations as well as for investigating the substrate specificities and kinetic properties of the purified forms of cytochrome *P*-450.

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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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INTRODUCTION

The liver microsomal cytochrome *P*-450-dependent mixed function oxidase system catalyzes the metabolism of a wide variety of substrates, including drugs, steroids, and many xenobiotics (2–4). This system is responsible for the metabolic activation of many procarcinogens, such as benzo[*a*]pyrene, to highly reactive electrophilic metabolites that are thought to account for the mutagenic and carcinogenic character of the chemically unreactive procarcinogen (4). In addition to benzo[*a*]pyrene, which is formed during the pyrolysis of tobacco, tobacco smoke condensate contains a significant quantity of heterocyclic compounds including alkylindoles and *N*-alkylcarbazoles. The maximal carcinogenic character of tobacco smoke condensate is associated with a neutral fraction containing polycyclic aromatic hydrocarbons, heterocyclic compounds, and a weakly acidic fraction (5). The *N*-alkylcarbazoles, which are found in this fraction, were shown to be active as cocarcinogens (5). Structurally similar compounds such as harman and norharman enhance the mutagenicity of benzo[*a*]pyrene in the *Salmonella typhimurium* TA 98 assay system when the S-9 fraction from rat livers is used (6). However, both harman and norharman inhibit the mutagenicity of benzo[*a*]pyrene in *S. typhimurium* TA 98 when mouse liver S-9 fractions are used (7). Although NMC[3] is weakly mutagenic to several strains of *S. typhimurium* in the presence of the rat liver S-9 fraction and cofactors, one of its metabolites, NHMC, is a very potent mutagen exhibiting a mutagenicity comparable to that of benzo[*a*]pyrene.[4] In view of these findings, the metabolism of NMC by the microsomal mixed function oxidases is of interest.

Carbazole is metabolized *in vivo* by the rat and rabbit to 3-hydroxycarbazole (8). The metabolism of NMC *in vitro* by rabbits results in the formation of NHMC (9) and three other monohydroxylated metabolites (1, 10). We have developed a simple procedure utilizing HPLC for the separation and quantitation of the metabolites of NMC. In the present study we have investigated the hydroxylation of NMC by liver microsomes from rabbits, rats, and mice using the HPLC assay. The effects of inducers and inhibitors of the cytochrome *P*-450-dependent mixed function oxidases on the hydroxylation reactions were investigated and kinetic analyses of the reactions were used to probe the multiplicities and regioselectivities of the microsomal enzymes. In addition, the applicability of the NMC assay for monitoring multiple forms of cytochrome *P*-450 in microsomal fractions is discussed.

MATERIALS AND METHODS

Chemicals. The 5,6-benzoflavone and metyrapone were obtained from the Aldrich Chemical Company. The 7,8-benzoflavone was purchased from the Sigma Chemical Company. The SKF-525A was a gift from Dr. Chiadao Chen. The sources and preparation of all other materials are given in the preceding paper (10).

Animals. White male New Zealand rabbits (1.5–2.0 kg) were used for these studies. Some animals were given phenobarbital (80 mg/kg in water, intraperitoneally) for 5 days, 3-methylcholanthrene (25 mg/kg in corn oil, intraperitoneally) for 2 days, or a single intraperitoneal injection of 5,6-benzoflavone (80 mg/kg in corn oil) 24 hr prior to sacrifice as described by Fouts (11). For some experiments, male Charles River rats (100–160 g) were used. Some animals were treated with phenobarbital or 3-methylcholanthrene as described by Alvares and Kappas (12). Phenobarbital (80 mg/kg) dissolved in water, or 3-methylcholanthrene (25 mg/kg) dissolved in corn oil, were administered intraperitoneally once a day for 4 days prior to sacrifice. The wild-type (Cs^a strain) laboratory colony mice were obtained from Dr. Janardan K. Reddy (Department of Pathology, Northwestern University Medical School).

Isolation of microsomes. The isolation of liver microsomes from the rabbits, rats, and mice was carried out according to the methods described in the accompanying paper (10). Protein was determined by the method of Lowry *et al.* (13), using bovine serum albumin as the standard. The cytochrome *P*-450 content was determined from the reduced CO difference spectrum as described by Omura and Sato (14), using an extinction coefficient of 91 mM^{-1} cm^{-1}. The specific contents of the microsomal preparations used in this study were 1.2, 1.7, 1.9, and 2.9 nmol of cytochrome *P*-450/mg of protein for the control and 5,6-benzoflavone-, 3-methylcholanthrene-, and phenobarbital-pretreated rabbits, respectively. The rat preparations had specific contents of 0.98, 2.07, and 1.65 nmol of cytochrome *P*-450/mg of protein for control and phenobarbital-, and 3-methylcholanthrene-pretreated animals, respectively, and the mouse preparation had a specific content of 1.10 nmol/mg.

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 as previously described (10). A standard reaction mixture contained the following components: 0.2 M phosphate buffer (pH 7.4), 6.0 mM $MgCl_2$, the NADPH regenerating system, 1.5 mM *N*-methylcarbazole in 50 μl of ethanol, and 3 to 6 mg of microsomal protein in a final volume of 3.0 ml. The NADPH-generating system consisted of 0.50 mM $NADP^+$, 3.0 mM glucose-6-phosphate, and 6 units of glucose-6-phosphate dehydrogenase (Bakers Yeast, 150 units/mg). 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 $NADP^+$. All incubations were conducted in duplicate. Reaction mixtures without $NADP^+$ were used as controls.

Analysis. The incubations were terminated by the addition of 3.0 ml of ethyl acetate and *p*-nitroanisole (125 nmol) was added as the internal standard. The reaction mixtures were extracted three times with 3.0-ml aliquots of ethyl acetate and the extracts were analyzed by HPLC with a Glenco HPLC System I liquid chromatograph using a Whatman Partisil 10 silica column (4.6 mm i.d. × 0.25 m) with monitoring at 254 nm as previously

[3] The abbreviations used are: 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; CO, carbon monoxide; and SKF-525A, *N*,*N*-diethylaminoethyl diphenylpropylacetate.

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

described (10). The column was eluted at a flow rate of 1.05 ml/min with hexanes:isopropanol (100:2.2). When *N*-[*methyl*-^{14}C]carbazole (0.045 mCi/mmol) was the substrate, 1-ml fractions of the eluate were collected and transferred to scintillation vials for counting. The samples were counted in 10 ml of RPI 3a70B liquid scintillation fluid in an Isocap 300 refrigerated liquid scintillation counter. The counts per minute were converted to disintegrations per minute by means of a quench curve generated by using ^{14}C-quenched standards (Amersham/Searle). The counting efficiency was approximately 80%.

RESULTS

A typical HPLC separation of the metabolites formed upon incubation of *N*-[*methyl*-^{14}C]carbazole with liver microsomes from phenobarbital-treated rabbits is shown in Fig. 1A. The unmetabolized substrate (NMC) elutes first (R_t = 3.7 min), followed by the internal standard, *p*-nitroanisole (R_t = 6.1 min). Carbazole, formed by the demethylation of the substrate, is the first metabolite to elute (R_t = 7.7 min), followed by 1-hydroxy-NMC (R_t = 9.6 min), 3-hydroxy-NMC (R_t = 13.9 min), 2-hydroxy-NMC (R_t = 17.2 min), and NHMC (R_t = 19.8 min). The retention times were calculated from the time of injection of the sample. Under the isocratic conditions used for this separation, all of the major metabolites are completely separated and eluted within 20 min. As seen in Fig. 1B, each of the major metabolites retained the radioactivity associated with the *N*-[*methyl*-^{14}C] group. This radioactivity was then used to quantitate the amount of product formed from the metabolism of [^{14}C]NMC by microsomes and to generate standard curves that could be used to calculate the amounts of the products formed from unlabeled NMC.

In order to generate standard curves for the major metabolites, the *N*-[*methyl*-^{14}C]carbazole was incubated in microsomal reaction mixtures for various lengths of time, the reactions were terminated, and the reaction mixtures were worked up as described in MATERIALS AND METHODS and analyzed by HPLC. The peak area ratios (peak area of metabolite/peak area of *p*-nitroanisole) were calculated and the amounts of each metabolite (in nmol) determined from the radioactivity measurements. The total nanomoles of each metabolite was obtained after correction for the percentage injection, either by the percentage of total counts or by the area of the *p*-nitroanisole peak. Both methods agreed within 5%. The total nanomoles versus the calculated peak area ratio was plotted for the four hydroxylated metabolites as shown in Figs. 2A and B and the best line determined by linear regression analysis. The correlation coefficient of each line was close to unity and the intercepts were essentially at the origin. The difference in the slopes of these lines compared favorably with the difference in molar extinction coefficients at 254 nm calculated for each metabolite (10). Standard curves for carbazole and NHMC were generated by adding various quantities of the respective compounds to standard reaction mixtures and the samples were extracted and the extract analyzed by HPLC as described. As can be seen in Fig. 2B, the lines generated with synthetic NHMC and with the ^{14}C metabolite are identical. The slopes of the standard

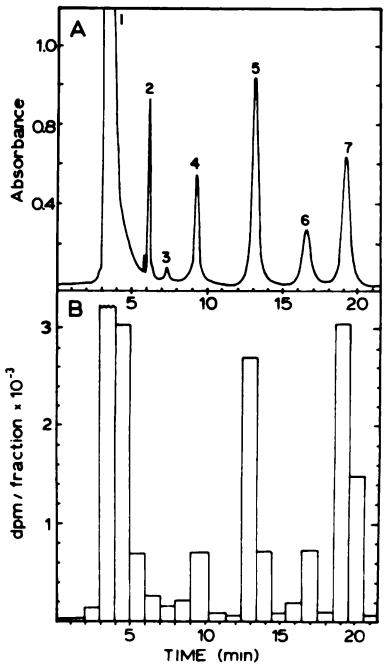

FIG. 1. *HPLC profile of the microsomal metabolites of NMC*
N-[*methyl*-^{14}C]Carbazole (1.6 mM) was incubated with microsomes from phenobarbital-treated rabbits for 15 min at 25°, and the metabolites formed were extracted into ethyl acetate and subjected to HPLC analysis as described in MATERIALS AND METHODS. (A) the absorbance profile at 254 nm. The peak identities are: **1**, NMC; **2**, *p*-nitroanisole; **3**, carbazole; **4**, 1-hydroxy-NMC; **5**, 3-hydroxy-NMC; **6**, 2-hydroxy-NMC; and **7**, NHMC. (B) The radioactivity profile of the same sample obtained by collecting 1.0-ml fractions of the eluate and counting the radioactivity in each fraction as described in MATERIALS AND METHODS.

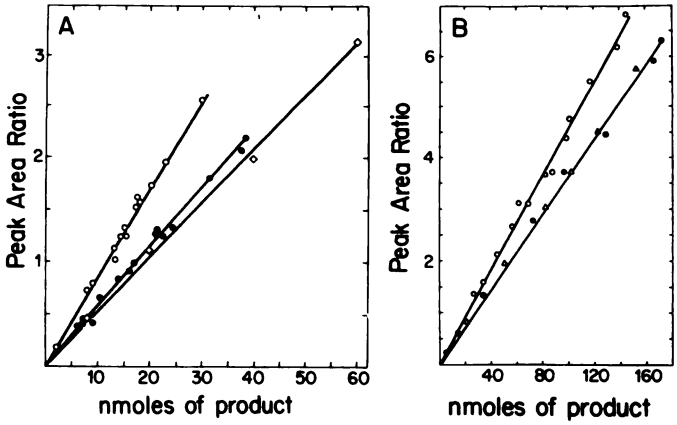

FIG. 2. *Standard curves for the metabolites of NMC*
Microsomes from rabbits induced with phenobarbital were incubated with *N*-[*methyl*-^{14}C]carbazole for various times and the peak area ratios for the metabolites determined as described in MATERIALS AND METHODS. The data include results of two experiments run at different times. The lines were determined by linear regression analyses. (A) Standard curves for carbazole (◇), 1-hydroxy-NMC (○), and 2-hydroxy-NMC (●). (B) Standard curves for 3-hydroxy-NMC (○), NHMC (●), and the curve generated with synthetic NHMC (△).

curves were then used to calculate the nanomoles of the individual metabolites formed when unlabeled NMC was used as the substrate.

When the rabbit liver microsomal metabolites of *N*-[*methyl*-^{14}C]carbazole were separated from the unmetab-

olized substrate by thin-layer chromatography as previously described (10), greater than 95% of the counts associated with the products could be accounted for by the four major metabolites when up to 5% of the substrate was metabolized.[5] The remaining 5% of the radioactivity associated with the products was present as more polar compounds. The more polar metabolites observed on thin-layer chromatography of the reaction mixture were not detected by HPLC. This could be due to the fact that the quantity of these metabolites is very small and, with the peak broadening that is observed with long retention times, they might not be detectable. The more polar products may be diols and/or dihydroxylated products formed from NMC. Experiments are in progress to identify these minor components.

Characterization of the metabolism of N-methylcarbazole. As shown in Fig. 3, the rates of formation of the four major metabolites were linear with the concentration of microsomal protein up to approximately 6.0 mg/3 ml. The metabolite ratios also remained relatively constant up to a protein concentration of 6 mg/3 ml. Carbazole, which results from demethylation of NMC, is formed at a very low rate and becomes detectable at approximately 6.0 mg of microsomal protein. The rate of formation of carbazole increases as the concentration of protein is increased. Since NHMC is thought to be an intermediate in the demethylation reaction, the formation of carbazole at higher protein concentrations may be due to the presence of higher levels of NHMC in the reaction mixture when higher protein concentrations are used. Since most incubations were conducted with less than 6 mg of microsomal protein, the formation of carbazole was not observed.

Figure 4 shows the time course for the NADPH-supported formation of the four major metabolites of NMC by liver microsomes isolated from untreated rabbits. The formation of all four metabolites is linear for at least 10 min. The metabolite ratios are relatively constant with time for the first 20 min. Similar time courses were observed when microsomes isolated from rabbits treated with phenobarbital, 3-methylcholanthrene, or 5,6-benzoflavone were used in the reaction mixture. All subsequent experiments were conducted with a 10-min incubation time to insure that the reaction was a measure of the initial rates of formation of the metabolites.

Cofactor requirement for the metabolism of N-methylcarbazole. As can be seen in Table 1, the formation of all four hydroxylated metabolites shows an absolute requirement for NADPH. Although NADPH is the preferred electron donor for cytochrome *P*-450-mediated mixed function oxidation reactions, NADH supports the formation of all four metabolites of *N*-methylcarbazole to different extents. The rate of formation of 1-hydroxy-NMC and 2-hydroxy-NMC is about 15–20% of that obtained with NADPH while the rates of formation of NHMC and 3-hydroxy-NMC are about 30 and 40% of the NADPH-supported rates, respectively.

When both reduced pyridine nucleotides were included in the reaction mixture at a concentration of 1 mM, the rates of formation of the 2-hydroxy-NMC and NHMC were equal to the sum of the rates obtained with each of the pyridine nucleotides separately, while the rate of formation of 3-hydroxy-NMC was less than the sum of the two rates, but greater than that observed with NADPH alone. The addition of NADH did not increase the rate of 1-hydroxy-NMC formation over that observed with NADPH alone. The stimulation of many NADPH-supported mixed function oxidation reactions by NADH has been reported (15).

Effect of inhibitors of cytochrome P-450 on the metabolism of N-methylcarbazole. The catalytic activity of the cytochrome *P*-450-dependent mixed function oxidase system is inhibited by carbon monoxide (CO). When *N*-methylcarbazole was incubated in the presence of NADPH and microsomes from rabbits induced with phe-

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

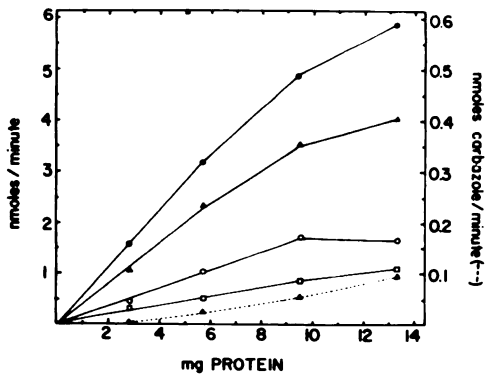

FIG. 3. *Metabolism of NMC as a function of the microsomal protein concentration*

The reaction mixtures were the same as described in MATERIALS AND METHODS, except that the concentration of NMC was 1.0 mM. The microsomal protein was from untreated rabbits and the amount of protein added was varied as indicated. The reaction mixtures were incubated at 25° for 15 min and the reactions were then terminated, extracted with ethyl acetate, and analyzed by HPLC as described in MATERIALS AND METHODS. The points represent the average of duplicates from a single experiment. The metabolites are carbazole (▲), 1-hydroxy-NMC (○), 2-hydroxy-NMC (□), 3-hydroxy-NMC (△), and NHMC (●).

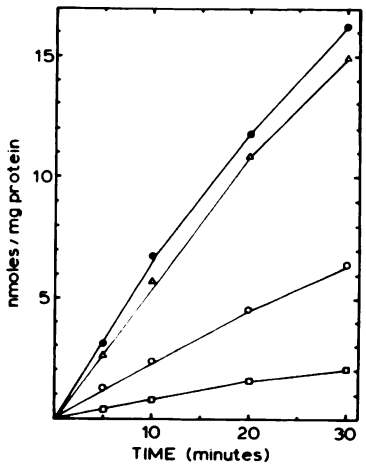

FIG. 4. *Metabolism of NMC as a function of time*

The experimental details were similar to those described in the legend to Fig. 3, except that the amount of microsomal protein was constant (4.1 mg) and the incubation times were varied as indicated. The metabolites are: 1-hydroxy-NMC (○), 2-hydroxy-NMC (□), 3-hydroxy-NMC (△), and NHMC (●).

TABLE 1

Cofactor requirement for the metabolism of N-methylcarbazole

The reaction mixtures contained potassium phosphate buffer, pH 7.4 (0.2 M), *N*-methylcarbazole (1.0 mM), liver microsomal protein from phenobarbital-treated rabbits (4 mg), and NADPH (1.0 mM), NADH (1.0 mM), or both, in a total volume of 3.0 ml. The reactions were initiated by the addition of the reduced pyridine nucleotide, and the reaction mixtures were incubated for 10 min at 25°. The metabolites formed were extracted and quantitated for HPLC as described in MATERIALS AND METHODS. The results are averages of duplicates from a single experiment.

Cofactor added	Activity			
	1-Hydroxy-NMC	2-Hydroxy-NMC	3-Hydroxy-NMC	NHMC
	(nmol products formed/min/mg protein)			
NADPH	0.61	1.34	2.57	3.01
NADH	0.09	0.25	1.11	0.80
NADPH + NADH	0.61	1.71	3.18	3.95
None	0[a]	0	0	0

[a] The minimum amount detectable is approximately 0.10 nmol. This corresponds to a rate of about 0.003 nmol/min/mg protein.

nobarbital under an atmosphere containing 80% CO and 20% O_2, the formation of all four metabolites was inhibited by at least 60% (Table 2). These results indicate that the hydroxylation of NMC is catalyzed by cytochrome *P*-450. This conclusion is also supported by the results obtained when other inhibitors of the mixed function oxidase system were included in the reaction mixtures (Table 2). SKF-525A (50 μM) inhibited the rates of formation of 1-hydroxy-, 2-hydroxy-, and 3-hydroxy-NMC by 40 to 50%, whereas NHMC formation was inhibited only 25% by the same concentration of SKF-525A. Metyrapone, an inhibitor of many cytochrome *P*-450-catalyzed hydroxylations (16), exhibits marked differences in its ability to inhibit the formation of the four metabolites. At a concentration of 10 μM, metyrapone inhibits the formation of 1-hydroxy-NMC, 3-hydroxy-NMC, and NHMC by 40 to 50% while inhibiting the formation of 2-hydroxy-NMC by greater than 70%. A similar pattern was observed in the presence of 100 μM metyrapone. The addition of 7,8-benzoflavone (α-naphthoflavone) to the incubation mixture has a differential effect on the rate of formation of each of the metabolites. Although 7,8-benzoflavone has little effect on the rate of formation of NHMC, it inhibits the formation of 2-hydroxy-NMC and 3-hydroxy-NMC by 60 and 30%, respectively, and stimulates the formation of 1-hydroxy-NMC to 150% of the control rate at a concentration of 10 μM.

The effect of various concentrations of 7,8-benzoflavone on the rates of formation of the four metabolites is shown in Fig. 5. The four activities are clearly differentiated by the effect of 7,8-benzoflavone. NHMC formation is slightly inhibited by 20 μM 7,8-benzoflavone, while the inhibition of the 2- and 3-hydroxylase activities is much greater, and they parallel each other, reaching maximum inhibition at about 200 μM 7,8-benzoflavone. The 1-hydroxylase activity is stimulated throughout the concentration range studied. These results indicate that in liver microsomes from phenobarbital-induced rabbits there are at least three different hydroxylation activities

TABLE 2

Effects of microsomal monooxygenase inhibitors on N-methylcarbazole hydroxylation

The reaction mixtures were the same as described in MATERIALS AND METHODS except that the concentration of NMC was 1.0 mM. The microsomal protein (3 to 5 mg) was from phenobarbital-treated rabbits. The reactions were initiated by the addition of $NADP^+$ and incubated for 10 min at 25°. The metabolites were extracted and quantitated by HPLC as described in MATERIALS AND METHODS. The velocities are expressed relative to those obtained without inhibitor. Metyrapone, 5,6-benzoflavone, and 7,8-benzoflavone were added in acetonitrile (10 μl). Acetonitrile was added to the appropriate control incubations.

Inhibitor	Concentration	Activity			
		1-Hydroxy-NMC	2-Hydroxy-NMC	3-Hydroxy-NMC	NHMC
	(μM)	*(% control)*			
CO	80% CO:20% O_2	38	30	40	41
SKF-525A	50	60	52	62	75
	500	19	11	22	21
Metyrapone	10	49	27	60	55
	100	27	6	38	41
5,6-Benzoflavone	100	—[a]	42	51	60
7,8-Benzoflavone	10	146	42	70	93
	100	132	33	58	88

[a] 5,6-Benzoflavone has the same retention time as the 1-hydroxy-NMC and prevents the determination of this metabolite.

having different sensitivities to inhibition by 7,8-benzoflavone. The ability of 7,8-benzoflavone to differentially stimulate and inhibit the formation of different metabolites by the liver microsomal mixed function oxidases has previously been reported. For example, Billings and McMahon (17) reported that in microsomes from untreated mice, 100 μM 7,8-benzoflavone stimulated the 2-

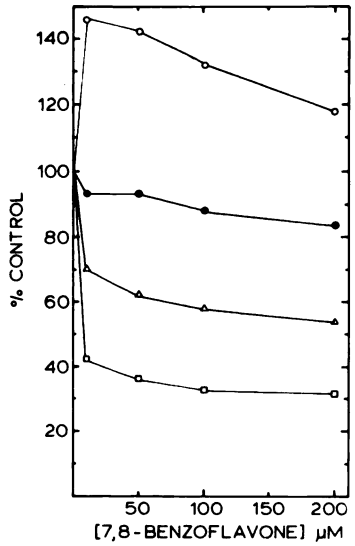

FIG. 5. *Effect of 7,8-benzoflavone on the metabolism of NMC*

The experimental details were similar to those described in the legend to Table 2 except that the concentration of 7,8-benzoflavone was varied as indicated. The results are expressed relative to the rate obtained in the absence of the inhibitor. The points represent the average of duplicates from a single experiment. The metabolites are: 1-hydroxy-NMC (○), 2-hydroxy-NMC (□), 3-hydroxy-NMC (△), and NHMC (●).

hydroxylation of biphenyl and inhibited the 3- and 4-hydroxylase activities.

Species differences in the metabolism of N-methylcarbazole. The results of studies on the metabolism of NMC by liver microsomes from untreated rabbits, rats, and mice are shown in Fig. 6. The microsomes isolated from rabbits and mice form 3-hydroxy-NMC and NHMC at the greatest rates followed by lower rates of formation of the 1-hydroxy- and 2-hydroxy-NMC. In contrast, the liver microsomes from the rat exhibit a very different product profile with 1-hydroxy-NMC formed at the greatest rate followed by lower rates of formation of NHMC and 3-hydroxy-NMC. The 2-hydroxylase activity is low in microsomes from all three species.

Effect of inducers on the metabolism of N-methylcarbazole. Liver microsomes from rabbits or rats induced with phenobarbital, 3-methylcholanthrene, or 5,6-benzoflavone exhibit different catalytic activities for various substrates (2–4) as well as differences in regioselectivity for a single substrate (18–20). The variable activities and specificities are attributable to different forms of cytochrome *P*-450 induced by these compounds in the livers of rats and rabbits. As shown in Table 3, liver microsomes from rabbits and rats induced with phenobarbital or 3-methylcholanthrene exhibit marked differences in their regiospecificity toward NMC. The metabolism of benzo[*a*]pyrene by the microsomes was determined by the fluorescent assay method of Nebert and Gelboin (21), and is included in the table for comparative purposes.

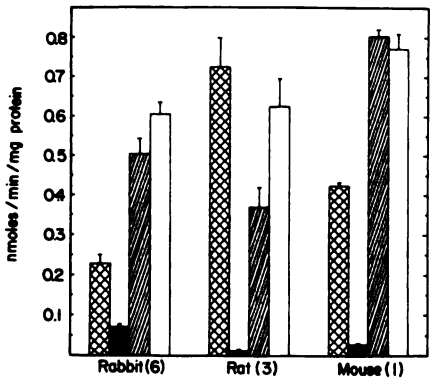

FIG. 6. *Species differences in the metabolism of NMC*

The experimental details were the same as described in the legend to Fig. 3, except that liver microsomes were isolated from untreated rabbits, rats, or mice and used at a protein concentration of approximately 5 mg per assay. The double-hatched bars indicate the amount of 1-hydroxy-NMC formed, the solid bars indicate the amount of 2-hydroxy-NMC formed, the hatched bars represent the 3-hydroxy-NMC formed, and the open bars indicate the NHMC formed. The number of experiments is shown in parentheses and the standard errors are shown.

The major metabolite formed by liver microsomes from untreated rabbits is NHMC. 3-Hydroxy-NMC is formed at a slightly slower rate and the ratio of the two activities (3-hydroxy-NMC/NHMC) for microsomes from untreated rabbits is 0.84. Liver microsomes from phenobarbital-induced rabbits show a 2-fold increase in the rate of formation of 1-hydroxy-NMC and a 4-fold increase in the rates of formation of 3-hydroxy-NMC and NHMC. However, the ratio of these two activities (3-hydroxy-NMC/NHMC) is the same as that obtained with control microsomes, i.e., 0.84. The most notable result of induction with phenobarbital is the almost 20-fold enhancement in the rate of formation of the 2-hydroxy isomer. Treatment of rabbits with 3-methylcholanthrene induces cytochrome *P*-448 as opposed to cytochrome *P*-450, but, as seen in Table 3, the increased aryl hydrocarbon hydroxylase activity associated with this form of cytochrome in the mouse and the rat (22) is not observed. This is in agreement with the studies on the induction of the hydroxylase activity in rabbits reported by Atlas *et al.* (23). Microsomes isolated from 3-methylcholanthrene-induced rabbits show decreased rates of formation for 1-hydroxy-NMC and NHMC, whereas the other two activities are essentially unchanged from those of control microsomes. This results in a shift in the product profile from that of control microsomes, with 3-hydroxy-NMC becoming the major metabolite formed. With the 3-methylcholanthrene-induced microsomes, the ratio of 3-hydroxy-NMC to NHMC is 1.47, indicative of this shift in the product profile.

A different effect on metabolite formation from NMC

TABLE 3

Effect of cytochrome P-450 induction on N-methylcarbazole metabolism

The experimental details were identical to those described in the legend to Table 2 except for the source of the microsomes. The animals were treated as described in MATERIALS AND METHODS. The number of experiments is shown in parentheses. Results are reported as averages ± standard errors. The amount of 3-hydroxybenzo[*a*]pyrene (3-HO-BP) formed from benzo[*a*]pyrene was determined by the method of Nebert and Gelboin (21). Results are expressed as nmol 3- HO-BP formed/mg protein/30 min and are averages of duplicates from a single experiment.

Treatment	Activity				
	1-Hydroxy-NMC	2-Hydroxy-NMC	3-Hydroxy-NMC	NHMC	3-HO-BP
	(nmol products formed/min/mg protein)				
Rabbit					
None (6)	0.27 ± 0.024	0.07 ± 0.018	0.50 ± 0.042	0.61 ± 0.030	2.20
Phenobarbital (6)	0.47 ± 0.039	1.38 ± 0.123	2.21 ± 0.107	2.65 ± 0.127	3.66
3-Methylcholanthrene (4)	0.12 ± 0.017	0.07 ± 0.016	0.46 ± 0.039	0.31 ± 0.033	2.19
Rat					
None (3)	0.72 ± 0.079	0.01 ± 0.003	0.37 ± 0.052	0.62 ± 0.069	1.76
Phenobarbital (3)	1.31 ± 0.088	0.02 ± 0.001	0.49 ± 0.029	1.08 ± 0.072	2.52
3-Methylcholanthrene (3)	0.87 ± 0.099	0.01 ± 0.001	0.78 ± 0.051	0.85 ± 0.065	6.74

is observed with microsomes from untreated or phenobarbital- or 3-methylcholanthrene-induced rats. Phenobarbital treatment results in a 2-fold increase in 1-hydroxy-NMC and NHMC formation with little effect on the 2- or 3-hydroxylase activities. The 20-fold enhancement of 2-hydroxylation observed in the rabbit is not seen in the rat. Induction with 3-methylcholanthrene results in a 6-fold stimulation of benzo[*a*]pyrene metabolism. This inducer also causes a 2-fold increase in the 3-hydroxylation of NMC and a smaller (1.5 fold) increase in the rate of formation of NHMC.

Effect of N-methylcarbazole concentration on the rates of product formation. The effect of varying the concentration of NMC on the rate of formation of each of the major metabolites was investigated using liver microsomes from untreated and phenobarbital-, 3-methylcholanthrene-, and 5,6-benzoflavone-induced rabbits. The concentration of NMC in the reaction mixture was varied from 0.025 to 0.8 mM and the rates of metabolite formation determined as described in MATERIALS AND METHODS. As shown in Fig. 7A, the double reciprocal plot for the formation of NHMC is linear when microsomes from all four sources are used in the reaction mixture. The apparent Michaelis constant for NMC in the formation of the *N*-methylol is about the same with the four microsomal preparations (0.24, 0.11, 0.16, and 0.14 mM for untreated and 3-methylcholanthrene-, phenobarbital-, and 5,6-benzoflavone-induced microsomes, respectively). The increase in the rate of formation of NHMC with phenobarbital induction is evident from the increase in the V_{max}, while treatment with 3-methylcholanthrene or 5,6-benzoflavone shows a decreased V_{max}.

The double reciprocal plots for the formation of 2-hydroxy-NMC are shown in Fig. 7B. Linear double reciprocal plots are observed with microsomes from untreated and phenobarbital- and 5,6-benzoflavone-treated rabbits. With microsomes from 3-methylcholanthrene-induced rabbits, the 2-hydroxylase activity is inhibited by high concentrations of the substrate, as evidenced by the upward curvature in the double reciprocal plot. Although the results in Table 3 indicated that induction with 3-methylcholanthrene had no effect on the specific activity of the microsomes for 2-hydroxylation, it is interesting that an enhancement in this activity is observed with 3-methylcholanthrene-induced microsomes at lower substrate concentrations. The large enhancement of 2-hydroxylase activity observed with the phenobarbital-induced microsomes is evident at all concentrations of NMC. There is no significant change in the apparent K_m for NMC in the formation of 2-hydroxy-NMC by the microsomes from untreated or phenobarbital- or 5,6-benzoflavone-treated animals, suggesting that the differences in the ability of the three microsomal preparations to hydroxylate at the 2 position are most likely due to a change in the specific content of one form of cytochrome *P*-450 that is present in all three microsomal preparations. Extrapolation of the linear portion of the reciprocal plot with microsomes from 3-methylcholanthrene-treated animals also yields an apparent K_m that is not significantly different from the other values, but this activity is inhibited at high concentrations of NMC.

In contrast to the linear double reciprocal plots obtained for the formation of NHMC and 2-hydroxy-NMC, the double reciprocal plots for the 1- and 3-hydroxylase activities are nonlinear (Figs. 7C and D, respectively) suggesting that two or more enzymes (or forms of the same enzyme) are catalyzing the formation of each of the metabolites (24). The nonlinearity of the double reciprocal plots could also be explained by the presence in the microsomes of a form (or forms) of enzyme that exhibits substrate activation. The data presented here do not permit us to differentiate between these two possibilities.

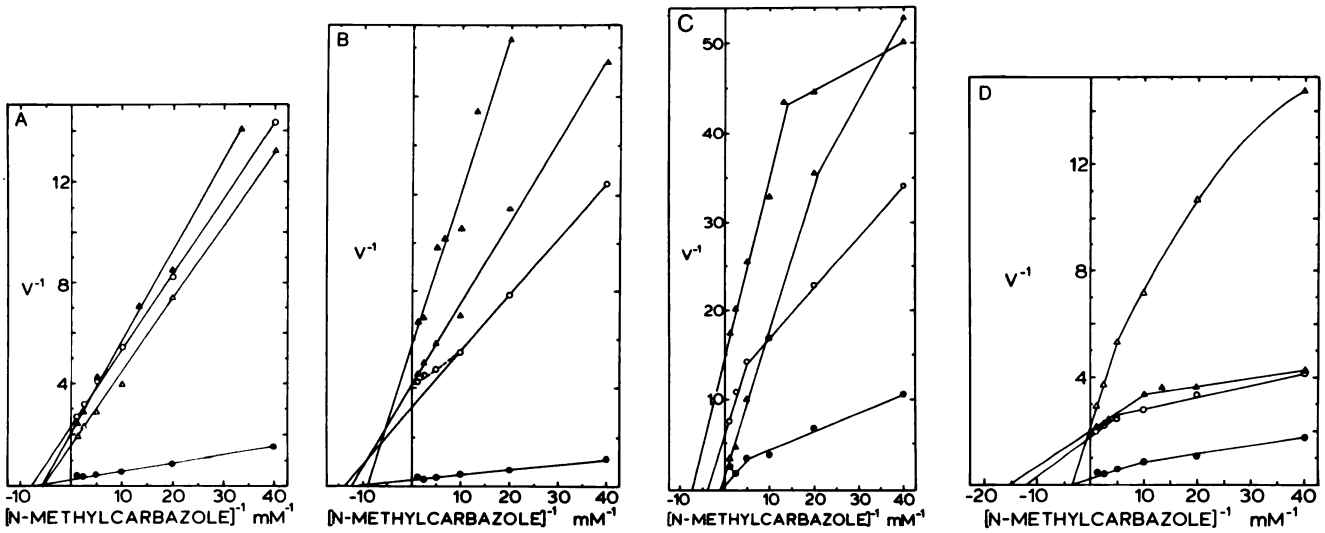

FIG. 7. *Double reciprocal plots of the rates of formation of the major metabolites of NMC as a function of the NMC concentration*

The reactions were run for 10 min at 25° with microsomes from untreated or phenobarbital-, 3-methylcholanthrene-, or 5,6-benzoflavone-treated rabbits. The experimental details were similar to those described in MATERIALS AND METHODS, except that the concentration of NMC was varied from 0.025 to 0.80 mM. The ordinate is the reciprocal of the initial rate expressed in nmol/min/mg protein. The experimental points represent the average of duplicates from a single experiment. (A) NHMC formation, (B) 2-hydroxy-NMC formation, (C) 1-hydroxy-NMC formation, (D) 3-hydroxy-NMC formation. The curves represent the results with microsomes from untreated (△) and phenobarbital- (●), 3-methylcholanthrene- (○), and 5,6-benzoflavone-induced (▲) rabbits.

The marked differences in the double reciprocal plots for the formation of 1-hydroxy-NMC (Fig. 7C) exhibited by the microsomes from rabbits treated with phenobarbital, 3-methylcholanthrene, or 5,6-benzoflavone suggest that the distribution of the enzymic activities for 1-hydroxylation is different for each inducer. Extrapolation of the apparently linear portions of the double reciprocal plots does not provide an accurate estimation of the apparent Michaelis constants for either the high or the low K_m activities (24). Therefore, it is difficult to determine if the induced activities for 1-hydroxylation are kinetically different from those in untreated microsomes.

In contrast to the 1-hydroxylase activity, the double reciprocal plots for the formation of 3-hydroxy-NMC (Fig. 7D) by microsomes from rabbits treated with 3-methylcholanthrene or 5,6-benzoflavone are similar. While the double reciprocal plots are nonlinear, it is apparent that there is a predominance of a low K_m activity for the 3-hydroxylation. As with the 1-hydroxylase activity, it is difficult to determine if induction results in the formation of a kinetically distinct species that was not present in microsomes from untreated animals, or is the result of an increase in the specific content of a cytochrome *P*-450 species that is also present in the untreated microsomes. For both the 1- and 3-hydroxylase activities (Figs. 7C and D), substrate inhibition by high concentrations of NMC was observed with phenobarbital-induced microsomes. However, an increased rate of formation of both metabolites was observed at all concentrations of NMC tested after phenobarbital induction and is consistent with the result depicted in Table 3, obtained with a single substrate concentration.

DISCUSSION

The metabolism of NMC by rabbit liver microsomes results in the formation of four major metabolites that have been identified by mass spectral and ^{1}H-NMR analysis (10). As shown in Fig. 8, these metabolites include three isomeric phenolic metabolites, 1-hydroxy-NMC, 2-hydroxy-NMC, and 3-hydroxy-NMC and the product of an aliphatic hydroxylation, NHMC. The formation of the hydroxylated products required NADPH as a source of reducing equivalents (Table 1). However, NADH was also capable of supporting the reaction and the addition of NADH to the NADPH system resulted in increased formation of some of the metabolites. Carbon monoxide inhibited the formation of all four major products, as did other classical inhibitors of the hepatic cytochrome *P*-450-dependent mixed function oxidases such as SKF-525A, metyrapone, 5,6-benzoflavone, and 7,8-benzoflavone (Table 2). These results, in addition to the increase in the metabolism of NMC seen after pretreatment of the animals with inducers of cytochrome *P*-450 such as phenobarbital and 3-methylcholanthrene (Table 3), demonstrate that the metabolism of NMC is catalyzed by one or more cytochrome *P*-450-dependent monooxygenases.

The lack of linearity of product formation at higher protein concentrations (greater than 2 mg/ml of reaction mixture) may be due to a variety of factors including product inhibition in which the monohydroxylated products serve as dead-end inhibitors, competitive inhibition

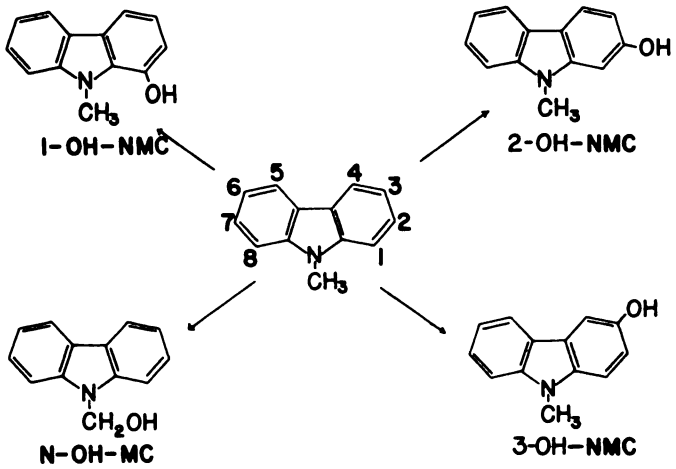

FIG. 8. *Chemical structures of NMC and the four major liver microsomal metabolites*

by the products in which the monohydroxylated metabolites compete with NMC for further hydroxylation, or the presence of an endogenous inhibitor in the microsomes which has an effect on metabolism that is only apparent at higher protein concentrations. The possibility that the monohydroxylated products are competing with NMC for metabolism is supported by the fact that metabolites that are more polar than the monohydroxylated metabolites have been observed on thin-layer chromatography of the reaction mixture.[5] These compounds may represent dihydroxy metabolites that result from the further metabolism of the monohydroxylated NMC. Studies on the effect of incubation conditions in which the NMC concentration is limiting on the production of these metabolites and the identification of these metabolites are in progress.

The major urinary metabolite formed after the administration of carbazole to rats and rabbits was found to be the glucuronide of 3-hydroxycarbazole (8). The major metabolites in rat bile of ellipticine, a plant alkaloid that is structurally related to carbazole, were the sulfate and glucuronide conjugates of 9-hydroxyellipticine, which corresponds to the 3-hydroxy derivative of carbazole (25). Since more than 90% of the dose of ellipticine was excreted within 24 hours after dosing, it was suggested that 9-hydroxy ellipticine and other hydroxylated metabolites are readily conjugated to form water soluble products that are readily excreted from the body and do not accumulate (25).

Multiple forms of cytochrome *P*-450 are present in liver microsomes (26, 27). In the past few years, as many as six different forms of cytochrome *P*-450 have been isolated from liver microsomes of rabbits and have been shown to be distinct proteins based on differences in their spectral, catalytic, and immunological properties (27). Studies on the metabolism of warfarin, chlorobenzene, and biphenyl have led to the suggestion that the multiple products formed by liver microsomes are due to the catalytic activity of different forms of the hemeprotein (17–20). The results presented in this report are consistent with those observations. Treatment of rabbits with phenobarbital induces a form of cytochrome *P*-450 that exhibits a marked specificity for the formation of 2-

hydroxy-NMC compared to control enzymes (Table 3). Preliminary studies using the reconstituted system and purified forms of rabbit liver cytochrome *P*-450 indicate that 2-hydroxy-NMC is a major product formed by rabbit liver LM_2, the major form of cytochrome *P*-450 induced by phenobarbital (27), whereas rabbit liver LM_4, which is the major form induced by 3-methylcholanthrene (27), catalyzes the formation of 3-hydroxy-NMC and NHMC without forming any 1- and 2-hydroxy-NMC (28). In addition, the 2-hydroxylation of NMC by rabbit liver microsomes is very sensitive to inhibition by metyrapone (70% inhibition at 10 μM) and 7,8-benzoflavone (60% at 10 μM) (Table 2), whereas the 1-hydroxylation of NMC is activated by 7,8-benzoflavone. 7,8-Benzoflavone also exhibits different inhibitory effects on the formation of 3-hydroxy-NMC and NHMC. Although 3-hydroxy-NMC formation is inhibited slightly by 7,8-benzoflavone, there is no effect on the formation of NHMC. Treatment of the rabbits with 3-methylcholanthrene further differentiates between these two activities, resulting in a 50% decrease in the specific activity for NHMC formation without significantly affecting the rate of formation of the 3-hydroxy-NMC. The kinetic results with microsomes from untreated rabbits and rabbits treated with 3-methylcholanthrene also indicate that the two products are formed by different enzymes. Treatment of rabbits with 3-methylcholanthrene results in the induction of a low K_m activity toward 3-hydroxylation when compared with control activity (Fig. 7D) while this inducer has no effect on the K_m for NMC in NHMC formation (Fig. 7A). If the same enzyme were catalyzing the formation of the two metabolites, then both activities should have been changed in parallel by induction with 3-methylcholanthrene and a similar effect would have been observed in the saturation kinetic studies.

Species differences in the metabolism of NMC are also readily apparent (Fig. 6). In the rabbit and mouse, NHMC and 3-hydroxy-NMC are the major metabolites produced by microsomes from untreated animals, while in the rat, 1-hydroxy-NMC is formed at the greatest rate. Induction with phenobarbital, which greatly enhances the rate of formation of 2-hydroxy-NMC in the rabbit, has very little effect on 2-hydroxylation in the rat (Table 3). Treatment with 3-methylcholanthrene increases the rates of formation of NHMC and 3-hydroxy-NMC by rat liver microsomes, but has no effect on 3-hydroxylation by rabbit microsomes and causes a decrease in the rate of formation of NHMC (Table 3). Lu and co-workers (26, 29) have demonstrated that rabbit and rat 3-methylcholanthrene-inducible cytochrome *P*-450 preparations are different proteins as judged by immunological and catalytic differences and by differences in their subunit molecular weights. These results suggest that some of the species differences in the metabolism of NMC may reflect the catalytic activity of different forms of cytochrome *P*-450 having different regioselectivities for the substrate molecule.

Changes in the microsomal metabolite profiles that are observed after treatment of animals with inducers such as phenobarbital or 3-methylcholanthrene are generally attributed to changes in the concentrations of the various forms of cytochrome *P*-450 in the microsomes. However, it should be recognized that inducers may also alter the metabolite profiles by mechanisms unrelated to changes in the concentrations of the different forms of cytochrome *P*-450. These may include changes in the interaction between the reductase and cytochrome *P*-450, changes in the phospholipid concentration in the microsomal membrane, changes in the orientation of the cytochrome *P*-450 in the microsomal membrane, or changes in the interaction of the cytochrome b_5 with the cytochrome *P*-450-containing electron transport chain.

The metabolism of NMC by liver microsomes from rabbits produces four major metabolites that can be readily separated and quantitated by HPLC. Product separation is accomplished under isocratic conditions in 20 min and as little as 0.05 nmol of product can be measured. The metabolite profile is markedly influenced by treatment of the animals with different inducers and by species differences. The results indicate that different forms of cytochrome *P*-450 may be responsible for the formation of the various products. Therefore, the metabolism of NMC appears to be a useful assay for use in monitoring the different forms of the cytochrome *P*-450-dependent monooxygenases in liver microsomes and in assessing the catalytic capabilities of postmitochondrial supernatants and microsomal preparations used in toxicology tests such as the Ames mutagenesis assay (30). In addition, the metabolism of NMC may be used to monitor the different forms of cytochrome *P*-450 in various tissues and should prove valuable in the further characterization of the substrate specificity of the purified forms of cytochrome *P*-450.

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