# Induction of Drug Metabolism

## II. Qualitative Differences in the Microsomal *N*-Demethylating Systems Stimulated by Polycyclic Hydrocarbons and by Phenobarbital*

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SUMMARY

Hepatic microsomes *N*-demethylate ethylmorphine and 3-methyl-4-methylaminoazobenzene (3-MMAB). The administration of phenobarbital to male rats stimulates the *N*-demethylation of both compounds; 3-methylcholanthrene and 3,4-benzpyrene stimulate the *N*-demethylation of 3-MMAB only. Studies designed to explain this difference in the inductive properties of phenobarbital and the polycyclic hydrocarbons led to the conclusion that polycyclic hydrocarbons cause the synthesis of a new *N*-demethylating system. This conclusion was arrived at through three experimental approaches: (a) by observing a differential inhibitory effect of SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCl), (b) by studying the two-substrate kinetics of *N*-demethylation reactions, and (c) by noting changes in the spectral properties of the reduced microsomal hemoprotein in the presence of ethyl isocyanide. In the untreated rat, one enzyme system is responsible for the *N*-demethylation of both ethylmorphine and 3-MMAB. Phenobarbital causes an increase in this enzyme system. 3-Methylcholanthrene causes the appearance of a second enzyme system which is capable of *N*-demethylating 3-MMAB, but not ethylmorphine. The difference in these two enzyme systems can be explained by the appearance of a second microsomal hemoprotein when polycyclic hydrocarbons are administered. This new hemoprotein (or hemoprotein-lipid complex) has been designated cytochrome $P_1$-450 to distinguish it from cytochrome P-450. Cytochrome $P_1$-450 appears to be present in addition to cytochrome P-450 rather than in place of it in microsomes from rats treated with polycyclic hydrocarbons. When reduced and reacted with ethyl isocyanide, each of these cytochromes exists in two pH-dependent, interconvertible forms. The existence of very small amounts of cytochrome $P_1$-450 in microsomes from untreated and phenobarbital-treated rats has not been excluded.

### INTRODUCTION

A great variety of structurally unrelated drugs and other foreign substances are oxidized by hepatic microsomes. This could mean, at one extreme, that a remarkable number of specific oxidases exist in the

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endoplasmic reticulum, a concept that is difficult to accept on teleological grounds, or, at the other extreme, that an oxidase of remarkable nonspecificity exists. The second possibility becomes more acceptable when these oxidations are visualized as variations of hydroxylation, followed in certain cases by rearrangement (1–3). Rubin and coworkers (4) reasoned that if a single enzyme system is responsible for the metabolism of many drugs, then each of these drugs should serve as an alternative substrate for the enzyme system. They showed that the oxidative demethylation of ethylmorphine was inhibited competitively by hexobarbital, chlorpromazine, zoxazolamine, phenylbutazone, and acetanilide, compounds that are not similar structurally. Moreover, ethylmorphine, hexobarbital, and chlorpromazine were mutually inhibitory, each inhibiting competitively the metabolism of the other. As these authors pointed out, however, whereas acetanilide and zoxazolamine inhibit the metabolism of ethylmorphine competitively, it is not likely that a single enzyme system is responsible for the oxidation of all three compounds, because the administration of certain polycyclic hydrocarbons to animals stimulated the microsomal metabolism of acetanilide and zoxazolamine (5), but not that of the narcotic analgesics (6).

The current study was initiated in an attempt to explain in terms of enzyme specificity the observation that whereas phenobarbital stimulates the microsomal *N*-demethylation of both 3-methyl-4-methylaminoazobenzene and ethylmorphine, the polycyclic hydrocarbons stimulate the *N*-demethylation of only the former compound (7). It has usually been assumed that where there is such differential induction, in this case in the *N*-demethylation of ethylmorphine and 3-MMAB,[2] two *N*-demethylases must exist in the hepatic microsomes of untreated animals, and that phenobarbital causes increases in both *N*-demethylases whereas the polycyclic hydrocarbons cause an increase in only one. The studies to be presented suggest that this is not the case; rather, both substrates are *N*-demethylated by a single *N*-demethylase in microsomes from untreated and phenobarbital-treated rats and another *N*-demethylating system is responsible for the *N*-demethylation of 3-MMAB when 3-methylcholanthrene is administered. In the preceding study (7), a lack of parallelism was observed between the changes in *N*-demethylating activities and cytochrome P-450 contents of microsomes that occurred after animals were treated with polycyclic hydrocarbons. This suggested that the polycyclic hydrocarbons might cause the synthesis of an altered cytochrome P-450. Evidence will be presented to show that this is the case and that this new cytochrome is related to the change in microsomal *N*-demethylase activity that occurs when the polycyclic hydrocarbons are administered.

The first suspicion that the microsomal drug-metabolizing system might be altered qualitatively as well as quantitatively when 3-methylcholanthrene is administered to rats resulted from experiments with SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCl), a potent inhibitor of drug metabolism. SKF 525-A inhibited the *N*-demethylation of 3-MMAB when microsomes from untreated and phenobarbital-treated rats were used, but not when microsomes from 3-methylcholanthrene-treated rats were employed (8). This observation was of interest, for in certain cases SKF 525 A is thought to inhibit drug metabolism by acting as an alternative substrate (9). Thus, the failure of SKF 525-A to inhibit *N*-demethylase for 3-MMAB in microsomes from 3-methylcholanthrene-treated rats could be explained if SKF 525-A cannot serve as a substrate for the *N*-demethylase from these rats.

In addition to the SKF 525-A studies, two other approaches were employed to reveal qualitative differences in the *N*-demethylating systems stimulated by polycyclic hydrocarbons and phenobarbital. Two-substrate kinetic analysis of the *N*-demethylation of ethylmorphine and 3-MMAB by microsomes from untreated rats

[2] The abbreviation used is: 3-MMAB, 3-methyl-4-methylaminoazobenzene.

and from animals treated with phenobarbital or 3-methylcholanthrene were made, and the spectral characteristics of the cytochrome P-450 in these microsomes were examined. Because thioacetamide blocks the induction produced by phenobarbital, but not that by 3-methylcholanthrene (7), a study of its effect on these microsomal constituents was included. A study of the effect that morphine administration might have on these measurements was made because it is known that morphine, when administered to male rats, causes a decrease in the microsomal enzyme system responsible for the *N*-demethylation of ethylmorphine and other narcotic drugs (6, 10). Sex and species differences were also studied.

## METHODS

Male Holtzman rats (90–120 g) were employed routinely, but in certain experiments females of the same strain and weight were used. In other studies, male and female Swiss albino mice (20–30 g) were employed. Phenobarbital sodium (in 0.9% NaCl), 3-methylcholanthrene (in corn oil), 3,4-benzpyrene (in corn oil), thioacetamide (in 0.9% NaCl), and morphine sulfate (in 0.9% NaCl) were injected intraperitoneally. Livers were removed 20 hr after the last injection.

Tissue preparations and determinations of microsomal ethylmorphine and 3-MMAB *N*-demethylase activities have been described previously (7).

The carbon monoxide difference spectra of reduced microsomal hemoprotein were determined as described previously (7). The ethyl isocyanide difference spectra were determined in the same way, except that ethyl isocyanide[3] (final concentration, 3.45 mM) was used as the ligand instead of carbon monoxide. The differences between absorption at 430 mμ and 500 mμ and between 455 mμ and 500 mμ were used as measurements of the 430 mμ and 455 mμ peaks.

In certain of the two-substrate studies, it was desired to determine how much of the total formaldehyde formed was contributed by each substrate. This was accomplished by employing gas chromatography to determine the amount of 3-methyl-4-aminoazobenzene formed through the *N*-demethylation of 3-MMAB.[4] After the reaction had been stopped by adding 1 ml of a 2% solution of potassium hydroxide to the incubation medium, 2 ml of chloroform were added and the mixture was shaken for 10 min and then centrifuged at 1500 rpm for 5 min. Twenty-five microliters of the chloroform extract were injected into the chromatograph. Comparison of the peak area with peak areas obtained with authentic 3-methyl-4-aminoazobenzene[4] was used for quantitation. The recovery of 3-methyl-4-aminoazobenzene was 100%. A Barber-Colman model 10 gas chromatograph equipped with a flame ionization detector was employed. The column (liquid phase, 2% SE-30; solid support, Gas-Chrom S) was prepared as described by Anders and associates (11) and conditioned overnight at 275° with the carrier gas (argon) flowing. Column temperature was programmed over a 30-min period, starting at 125° and stopping at 225°. The injection was made when the column temperature was 150°. The flash heater and the detector were maintained at 265°. Inlet pressure was 20 psig, and outlet pressure was atmospheric. The flame ionization detector was operated at a relative gain of $10^{-9}$ amp, and hydrogen and air pressures were 20 and 40 psig, respectively.

Data-processing and statistical analyses were performed as described by Anders and Mannering (9).

## RESULTS

*Inhibition of N-demethylation by SKF 525-A.* When employed in a concentration of $4 \times 10^{-5}$ M, SKF 525-A[5] inhibited the *N*-demethylation of ethylmorphine whether

[3] This compound was prepared by Mr. Don Shoeman.

[4] 3-Methyl-4-methylaminoazobenzene and 3-methyl-4-aminoazobenzene were kindly supplied by Dr. J. A. Miller, McArdle Laboratory for Cancer Research, University of Wisconsin.

[5] Supplied by Smith Kline & French Laboratories, Philadelphia.

TABLE 1

*Effect of SKF 525-A on rat hepatic microsomal N-demethylation*

Treated rats received either phenobarbital sodium, 40 mg/kg, or 3-methylcholanthrene, 20 mg/kg, daily for 4 days. $I_{50}$ is defined as the concentration of SKF 525-A which inhibited *N*-demethylation by 50%. Substrate concentrations were: ethylmorphine, $2 \times 10^{-3}$ M; 3-MMAB, $2 \times 10^{-4}$ M. SKF 525-A concentrations ranged from $2 \times 10^{-5}$ M to $2 \times 10^{-3}$ M. Each value represents the mean and standard error of at least four animals. Values in parentheses are the mean rates of *N*-demethylation, in micromoles per gram of liver per hour, when SKF 525-A was absent.

| | Ethylmorphine *N*-demethylation | | 3-MMAB *N*-demethylation | |
|---|---|---|---|---|
| Treatment | Inhibition by SKF 525-A ($4 \times 10^{-5}$ M) | $I_{50}$ | Inhibition by SKF 525-A ($4 \times 10^{-6}$ M) | $I_{50}$ |
| | % | M $\times 10^5$ | % | M $\times 10^4$ |
| Control | 37 ± 2 ( 8.1) | 9.3 ± 1.1 | 30 ± 1 ( 2.9) | 2.2 ± 0.3 |
| Phenobarbital | 47 ± 2 (28.8) | 5.2 ± 0.6 | 38 ± 3 ( 8.6) | 1.1 ± 0.1 |
| Methylcholanthrene | 35 ± 2 ( 7.9) | 11.2 ± 1.1 | 0 (12.9) | 17.1 ± 0.7 |

the source of microsomes was untreated, phenobarbital-treated, or 3-methylcholanthrene-treated rats (Table 1), and the degrees of inhibition were similar (35–47%). Although this concentration of SKF 525-A inhibited the *N*-demethylation of 3-MMAB when microsomes from untreated and phenobarbital-treated animals were used, it did not inhibit the *N*-demethylation of the aminoazo dye when microsomes from 3-methylcholanthrene-treated rats were employed. As can be seen from the $I_{50}$ values given in the same table, when higher concentrations of SKF 525-A were employed, *N*-demethylation of 3-MMAB by microsomes from 3-methylcholanthrene-treated rats was inhibited, but about 10 times as much SKF 525-A was required to achieve 50% inhibition of 3-MMAB *N*-demethylation when microsomes from rats treated with 3-methylcholanthrene were employed. The $I_{50}$ values for ethylmorphine *N*-demethylation were similar regardless of the source of microsomes.

The finding that SKF 525-A inhibits the *N*-demethylation of 3-MMAB in the untreated rat was unexpected in view of the earlier observation of Takemori and Mannering (6) that the *N*-demethylation of this aminoazo dye was not inhibited by SKF 525-A in low concentration when microsomes from untreated mice were employed. This apparent species difference was reinvestigated and extended to include mice that had been treated with phenobarbital or 3-methylcholanthrene. In Table 2 it can be seen that SKF 525-A in a concentration of $2 \times 10^{-5}$ M inhibited the *N*-demethylation of ethylmorphine regardless of the source of microsomes, but in no case was the *N*-demethylation of 3-MMAB inhibited.

*Two-substrate kinetic studies.* The studies employing SKF 525-A suggested that a single enzyme system is responsible

TABLE 2

*Effect of SKF 525-A on mouse hepatic microsomal N-demethylation*

Treated mice received either phenobarbital sodium, 40 mg/kg, or 3-methylcholanthrene, 20 mg/kg, daily for 4 days. Substrate concentrations were the same as given in Table 1. Each value represents the mean and standard error of three animals. Values in parentheses are the mean rates of *N*-demethylation, in micromoles per gram of liver per hour, when SKF 525-A was absent.

| | Inhibition by SKF 525-A ($2 \times 10^{-5}$ M) | |
|---|---|---|
| Treatment | Ethylmorphine *N*-demethylation | 3-MMAB *N*-demethylation |
| | % | % |
| Control | 53 ± 4 ( 5.9) | 0 ( 5.5) |
| Phenobarbital | 63 ± 6 (11.5) | 0 ( 9.8) |
| Methylcholanthrene | 48 ± 5 ( 6.0) | 0 (10.9) |

for the $N$-demethylation of both ethylmorphine and 3-MMAB by microsomes from untreated and phenobarbital-treated rats, but that a different $N$-demethylating system is introduced after the administration of 3-methylcholanthrene. Kinetic studies described previously by Ariëns *et al.* (13) for interactions of drugs with receptor sites, and by Reiner (14) and Cha (15) for enzymes, were performed to explore this possibility further.

If substrates $A$ and $B$ are incubated together with one enzyme, the concentration of substrate $B$ being held constant while that of $A$ is varied, and both produce a common product that is used to measure the combined velocities ($v$) of the two reactions, a hyperbolic curve will be obtained when $1/v$ is plotted against $1/[A]$ and it will intersect a similarly plotted linear curve obtained when varying amounts of substrate $A$ are incubated under the same conditions without substrate $B$. The intercept can be predicted using the equation $S = V'K/(V - V')$, where $S$ is the concentration of substrate $A$ at the intercept of the two curves, $K$ is the Michaelis constant of substrate $A$, and $V$ and $V'$ are the maximum velocities of the metabolism of substrates $A$ and $B$, respectively. If the curves are to intersect, $V$ must be larger than $V'$. If the maximum velocities are identical, the two curves will merge. If two enzymes are involved, one for the metabolism of substrate $A$ and the other for the metabolism of substrate $B$, the curves will not intersect or merge at any concentration of substrate $A$; that is, the total product formation will always be greater when substrate $B$ is also present. The kinetics is more complex when one of two enzymes present can react with both substrates. In this case, the two curves may intersect, although they need not, but the point of intersection will always be at a greater concentration of substrate $A$ than that predicted by the equation.

Figures 1, 2, and 3 support the view that ethylmorphine and 3-MMAB are $N$-demethylated by the same enzyme system in microsomes from untreated and phenobarbital-treated rats, but by two systems when microsomes from 3-methylcholanthrene-treated animals are employed. A comparison of calculated and experimental intercepts is summarized in Table 3. A note of caution should be added regarding this conclusion: if two multienzyme systems share a single rate-limiting component, these two-substrate kinetic analyses would give the same result as that obtained with a single-enzyme system.

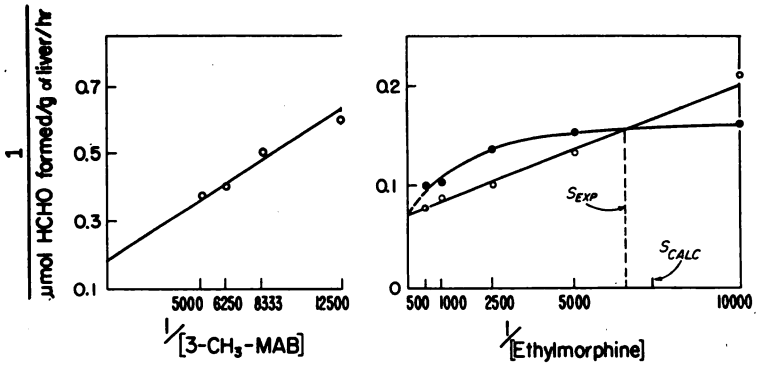


FIG. 1. *Two-substrate kinetic studies employing hepatic microsomes from untreated rats*

$S_{calc} = V'K/(V - V')$, where $V'$ is the maximum velocity of 3-methyl-4-methylaminoazobenzene (3-$CH_3$-MAB) $N$-demethylation (5.3 µmoles/g of liver per hour), $V$ is the maximum velocity of ethylmorphine $N$-demethylation (13.6 µmoles/g of liver per hour), and $K$ is the Michaelis constant for ethylmorphine $N$-demethylation ($2.1 \times 10^{-4}$ M). When both substrates were employed, 3-methyl-4-methylaminoazobenzene was present at a fixed concentration of $2 \times 10^{-4}$ M (●——●). Statistical analysis (9) was used to draw the curves when single substrates were employed, but not when two substrates were used.

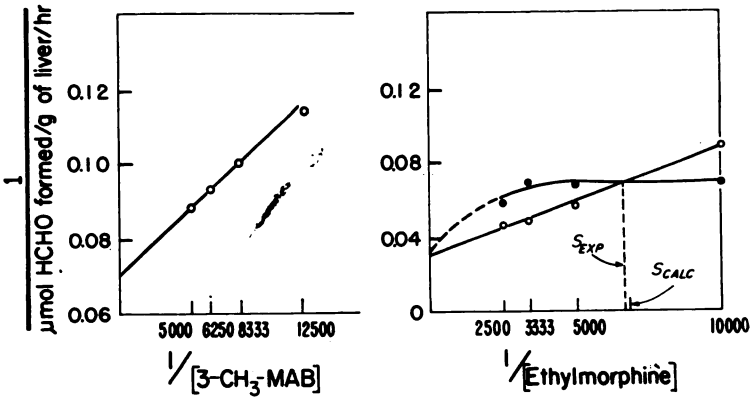


FIG. 2. *Two-substrate kinetic studies employing hepatic microsomes from phenobarbital-treated rats*

Rats received phenobarbital sodium, 40 mg/kg/day, for 4 days. $S_{calc} = V'K/(V - V')$, where $V'$ is the maximum velocity of 3-methyl-4-methylaminoazobenzene (3-$CH_3$-MAB) *N*-demethylation (14.3 µmoles/g of liver per hour), $V$ is the maximum velocity of ethylmorphine *N*-demethylation (33.3 µmoles/g of liver per hour), and $K$ is the Michaelis constant for ethylmorphine *N*-demethylation ($2.0 \times 10^{-4}$ M). When both substrates were employed, 3-methyl-4-methylaminoazobenzene was present at a fixed concentration of $2 \times 10^{-4}$ M (●——●). Statistical analysis (9) was used to draw the curves when single substrates were employed, but not when two substrates were used.

The degree of inhibition of 3-MMAB metabolism by ethylmorphine and that of ethylmorphine by 3-MMAB was determined using saturating concentrations of both substrates. 3-Methyl-4-aminoazobenzene was measured by gas chromatography as described previously. Formaldehyde production was also measured, and it was thus possible to calculate the amount of norethylmorphine formed from ethylmorphine. The results (Table 4) show that *N*-demethylation of ethylmorphine was markedly inhibited by 3-MMAB in all of the preparations. The reason for the greater inhibition of ethylmorphine *N*-demethylation when the enzyme was obtained

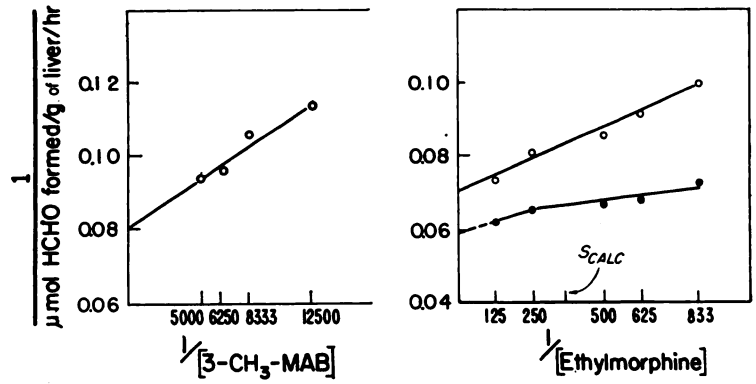


FIG. 3. *Two-substrate kinetic studies employing hepatic microsomes from 3-methylcholanthrene-treated rats*

Rats received 3-methylcholanthrene, 20 mg/kg/day, for 4 days. $S_{calc} = V'K/(V - V')$, where $V'$ is the maximum velocity of 3-methyl-4-methylaminoazobenzene (3-$CH_3$-MAB) *N*-demethylation (12.5 µmoles/g of liver per hour), $V$ is the maximum velocity of ethylmorphine *N*-demethylation (14.3 µmoles/g of liver per hour), and $K$ is the Michaelis constant for ethylmorphine *N*-demethylation ($4.0 \times 10^{-4}$ M). When both substrates were employed, 3-methyl-4-methylaminoazobenzene was present at a fixed concentration of $2 \times 10^{-4}$ M (●——●). Statistical analysis (9) was used to draw the curves when single substrates were employed, but not when two substrates were used.

TABLE 3
*Two-substrate kinetic studies: comparison of calculated and experimental intercepts*

Phenobarbital sodium, 40 mg/kg, was injected once daily for 4 days. Experimental and calculated intercepts were obtained as described in the text and in Figs. 1 and 2. Each value represents the mean and standard error of four animals. Statistical comparisons of calculated and experimental intercepts are given in the footnotes below.

| Treatment | Calculated intercept | Experimental intercept |
|---|---|---|
| | M × $10^4$ | M × $10^4$ |
| None | 1.75 ± 0.10 | 1.89 ± 0.18[a] |
| Phenobarbital | 1.72 ± 0.14 | 1.74 ± 0.16[b] |

[a] $p > 0.40$.
[b] $p > 0.80$.

from 3-methylcholanthrene-treated rats is not apparent.

Inhibition of the *N*-demethylation of 3-MMAB by ethylmorphine was about the same when enzyme from untreated or phenobarbital-treated rats was used, but no inhibition occurred when the enzyme was obtained from rats that had been treated with 3-methylcholanthrene, although the concentration of the inhibitor was approximately 10 times that of the substrate. Even though 3-methylcholanthrene causes the formation of a new *N*-demethylating system with properties quite different from those of the enzyme originally found in the microsomes, the level of the original enzyme system is maintained during treatment with 3-methylcholanthrene. This component of the total enzyme system should be susceptible to inhibition by ethylmorphine. 3-Methylcholanthrene causes about a 4-fold increase in 3-MMAB *N*-demethylation. Only 25% of the total *N*-demethylation is due to the original enzyme, and, since ethylmorphine inhibits this enzyme by about 30%, it would inhibit total *N*-demethylation by only about 7.5%, an amount that would not be readily detected by the procedures employed.

TABLE 4
*Effect of ethylmorphine on 3-MMAB N-demethylation, and of 3-MMAB on ethylmorphine N-demethylation*

Phenobarbital sodium, 40 mg/kg, and 3-methylcholanthrene, 20 mg/kg, were injected once daily for 4 days. Ethylmorphine and 3-MMAB were employed at concentrations of $3 \times 10^{-3}$ M and $4 \times 10^{-4}$ M, respectively. Each value represents the mean and standard error of at least four animals. Values in parentheses are the mean rates of *N*-demethylation, in micromoles per gram of liver per hour, when inhibitor was absent.

| Treatment | Inhibition | |
|---|---|---|
| | Substrate = ethylmorphine; inhibitor = 3-MMAB | Substrate = 3-MMAB; inhibitor = ethylmorphine |
| | % | % |
| Control | 43 ± 2 ( 8.7) | 30 ± 1 ( 3.1) |
| Phenobarbital | 53 ± 4 (26.1) | 33 ± 3 ( 9.4) |
| Methylcholanthrene | 74 ± 2 ( 8.3) | 0 (12.3) |

*Effects of phenobarbital, 3-methylcholanthrene, and thioacetamide on microsomal hemoprotein content and N-demethylase activities.* Table 5 summarizes the changes that occurred in microsomal hemoprotein content and *N*-demethylase activities after treatment with phenobarbital, 3-methylcholanthrene, 3,4-benzpyrene, thioacetamide, or various combinations of these compounds. When maximum stimulatory doses of phenobarbital and 3-methylcholanthrene were administered concurrently, additive stimulation was observed not only in 3-MMAB *N*-demethylase activity as previously reported (7), but also in cytochrome P-450 levels. On the other hand, concurrent administration of maximum stimulatory doses of 3,4-benzpyrene and 3-methylcholanthrene did not increase 3-MMAB *N*-demethylase activity or cytochrome P-450 levels above the levels observed when either was administered singly.

*Use of ethyl isocyanide to reveal a change in hemoprotein that occurs when 3-methylcholanthrene is administered.* Imai and Sato (16) suggested the existence of two forms of cytochrome P-450. Using ethyl isocyanide rather than carbon monoxide as the ligand for reduced microsomal hemoprotein, they observed Soret peaks at

TABLE 5

*Effects of phenobarbital sodium, 3-methylcholanthrene, 3,4-benzpyrene, and thioacetamide administration on microsomal N-demethylase activities and cytochrome P-450 levels*

Control values for ethylmorphine and 3-MMAB *N*-demethylase activities and for cytochrome P-450 levels are given in Table 7. Each value represents the mean of at least three male rats.

| Treatment | Percent of control | | |
|---|---|---|---|
| | Ethylmorphine *N*-demethylase | 3-MMAB *N*-demethylase | Cytochrome P-450 |
| Thioacetamide[a] | 20 | 13 | 20 |
| Phenobarbital[b] | 337 | 344 | 366 |
| Phenobarbital[b] and thioacetamide[a] | 90 | 107 | 150 |
| Benzpyrene[c] | 113 | 338 | 300 |
| Benzpyrene[c] and thioacetamide[a] | 34 | 207 | 140 |
| Methylcholanthrene[c] | 100 | 445 | 275 |
| Methylcholanthrene[c] and thioacetamide[a] | 27 | 310 | 165 |
| Phenobarbital[b] and methylcholanthrene[c] | 380 | 799 | 676 |
| Benzpyrene[c] and methylcholanthrene[c] | 89 | 480 | 281 |
| Methylcholanthrene[d] | 62 | 332 | 187 |
| Phenobarbital[e] | 458 | 429 | 459 |
| Phenobarbital[e] and methylcholanthrene[f] | 389 | 868 | 695 |

[a] 50 mg/kg/day for 4 days.
[b] 40 mg/kg/day for 4 days.
[c] 20 mg/kg/day for 4 days.
[d] 20 mg/kg/day for 21 days.
[e] 50 mg/kg twice daily for 5 days.
[f] 20 mg/kg/day for 5 days.

430 mμ and 455 mμ. The relative sizes of the two peaks were pH-dependent, and it was concluded that reduced cytochrome P-450 exists in two interconvertible forms. With the thought that ethyl isocyanide might reveal qualitative differences in microsomal hemoprotein, measurements of hemoprotein reacted with ethyl isocyanide were made.

The effects of phenobarbital, 3-methylcholanthrene, thioacetamide, and combinations of these compounds on microsomal *N*-demethylase activities, cytochrome P-450 levels, and the heights of the 430 mμ and 455 mμ peaks that result when reduced microsomal hemoprotein is reacted with ethyl isocyanide are summarized in Table 6. The ratios of the 455 mμ to 430 mμ peaks were about the same in microsomes from untreated and from thioacetamide-, phenobarbital-, and thioacetamide- plus phenobarbital-treated animals, namely 0.49 (Table 7), 0.49, 0.56, and 0.48, respectively. However, a much different ratio was seen in microsomes from 3-methylcholanthrene-treated animals, specifically, 1.37. The ratio was even higher when 3-methylcholanthrene and thioacetamide were administered simultaneously. When 3-methylcholanthrene and phenobarbital were administered together, the ratio was about halfway between the ratios observed when the two inducing agents were given singly. The marked change in the 455:430 mμ peak ratio that occurred after 3-methylcholanthrene treatment is offered as evidence for a second cytochrome P-450. This newly recognized cytochrome has been referred to in a preliminary study as cytochrome $P_1$-450[7] (17).

[7] Soon after we presented evidence for the existence of cytochrome $P_1$-450 (17), Alvares *et al.* (18) and Hildebrandt *et al.* (19) showed that the maximum absorbance of reduced microsomal hemoprotein (bound to carbon monoxide) seen after the administration of a polycyclic hydrocarbon differed slightly from that observed in the microsomes from untreated animals. Alvares

*Effect of sex, species differences, and morphine on microsomal N-demethylases and cytochrome P-450.* A variety of factors are known to influence the rate of drug metabolism (2, 3, 20). Among these are sex, species differences, and the administration of morphine. An attempt was made to correlate what is already known about the effects of these factors on drug metabolism with differences that might be seen in microsomal hemoprotein. The results are given in Table 7.

The well-known greater ability of the male rat relative to the female to *N*-demethylate drugs is again seen in Table 7, where the difference between the sexes in the rates of ethylmorphine and 3-MMAB *N*-demethylation were about 2.7- and 1.7-fold, respectively. Parallel differences in cytochrome P-450 levels were not seen; the level of cytochrome P-450 in the male rat was only 1.1 times greater than that observed in microsomes from female rats. The cytochrome P-450 in male rats is more efficient with respect to *N*-demethylase activity than it is in the female, which suggests a qualitative difference in the microsomal hemoproteins in the two sexes. This is in agreement with the observations of Schenkman *et al.* (21). It is of interest that this qualitative difference is not reflected in a notable change in the ratio of the 430:455 mμ peak heights. An alternative explanation is that cytochrome P-450 is not the rate-limiting component in the *N*-demethylating system of the female rat.

No such sex differences were seen in the mouse; microsomal *N*-demethylase activities, cytochrome P-450 levels, and the 430 mμ and 455 mμ peaks obtained with ethyl isocyanide varied little between the sexes. The 455:430 mμ peak ratios of 0.33 (male) and 0.35 (female) in mouse microsomes are sufficiently different from the ratios of 0.49 (male) and 0.55 (female) observed in rat microsomes to suggest that there may be a qualitative difference in the microsomal hemoproteins of these species.

In male rats, morphine treatment depressed both microsomal *N*-demethylase activities and the level of cytochrome P-450, but had no such effect in female rats. The 455:430 mμ peak ratio was not changed.

## DISCUSSION

The observation that phenobarbital stimulates the *N*-demethylation of both ethylmorphine and 3-methyl-4-methylaminoazobenzene, whereas 3-methylcholanthrene stimulates the *N*-demethylation of the latter compound only might be explained in several ways. (a) In the untreated rat, one enzyme system *N*-demethylates ethylmorphine and a second enzyme system *N*-demethylates 3-MMAB. Phenobarbital causes an increase in both enzyme activities, but 3-methylcholanthrene causes an increase in the activity of the second enzyme system only. (b) In the untreated rat, one enzyme system is responsible for the *N*-demethylation of both ethylmorphine and 3-MMAB. Phenobarbital stimulates this enzyme system. However, 3-methylcholanthrene causes the formation of a new enzyme system that is capable of *N*-demethylating 3-MMAB, but not ethylmorphine. This hypothesis does not exclude the possibility that the new enzyme system may exist in such small amounts in untreated animals that its activity is hidden by the greater activity

*et al.* observed a maximum absorption at 448 mμ; accordingly, this hemoprotein is referred to as cytochrome P-488. Hildebrandt *et al.* recorded the maximum absorbance at 446 mμ, and this gave rise to cytochrome P-446. We do not feel that these designations reflect the true maximum absorbance of cytochrome $P_1$-450, because the absorption spectra observed by these investigators undoubtedly represent mixtures of cytochromes P-450 and $P_1$-450. If cytochromes P-450 and $P_1$-450 have different absorption maxima, the observed maximum would vary with the relative amounts of the cytochromes in the microsomes. In fact, this could account for the small differences in the absorption maxima observed by Alvares *et al.* and by Hildebrandt *et al.* In view of this consideration we shall continue to use the $P_1$-450 designation because the hemoprotein is a variant P-450, but recognize at the same time that it does not have a maximum absorption at exactly 450 mμ. It is hoped that more acceptable names will be found for both cytochromes P-450 and $P_1$-450.

TABLE 6

*Changes in microsomal hemoprotein levels and N-demethylase activities in response to phenobarbital sodium, 3-methylcholanthrene, and thioacetamide administration*

Control values are given in Table 7. Each value represents the mean and standard error of four male rats.

| Treatment | Percent of control | | | | | | |
|---|---|---|---|---|---|---|---|
| | Ethylmorphine *N*-demethylase activity | 3-MMAB *N*-demethylase activity | 430 mμ peak | 455 mμ peak | 430 mμ peak + 455 mμ peak | Cytochrome P-450 | 455:430 mμ peak ratio |
| Thioacetamide[a] | 11 ± 4 | 13 ± 2 | 45 ± 10 | 45 ± 10 | 45 ± 10 | 37 ± 7 | 0.49 ± 0.02 |
| Phenobarbital[b] | 325 ± 10 | 309 ± 21 | 336 ± 17 | 386 ± 29 | 353 ± 20 | 353 ± 15 | 0.56 ± 0.04 |
| Phenobarbital[b] and thioacetamide[a] | 100 ± 12 | 107 ± 4 | 85 ± 18 | 85 ± 20 | 88 ± 20 | 96 ± 24 | 0.48 ± 0.04 |
| Methylcholanthrene[c] | 107 ± 4 | 441 ± 32 | 140 ± 14 | 400 ± 61 | 226 ± 28 | 251 ± 42 | 1.37 ± 0.09 |
| Methylcholanthrene[c] and thioacetamide[a] | 28 ± 9 | 310 ± 16 | 70 ± 9 | 223 ± 21 | 120 ± 11 | 140 ± 16 | 1.61 ± 0.26 |
| Methylcholanthrene[c] and phenobarbital[b] | 377 ± 41 | 784 ± 38 | 378 ± 71 | 723 ± 135 | 488 ± 98 | 547 ± 108 | 0.94 ± 0.01 |

[a] 50 mg/kg/day for 4 days.
[b] 40 mg/kg/day for 4 days.
[c] 20 mg/kg/day for 4 days.

TABLE 7

*Microsomal hemoprotein levels and N-demethylase activities in male and female rats, male and female mice, and morphine-treated male and female rats*

Each value represents the mean and standard error of four animals.

| Source of microsomes | Ethylmorphine *N*-demethylase activity | 3-MMAB *N*-demethylase activity | 430 mμ peak ($\Delta A_{430} - A_{500}$)[a] | 455 mμ peak ($\Delta A_{455} - A_{500}$)[a] | 430 mμ peak + 455 mμ peak | Cytochrome P-450 ($\Delta A_{450} - A_{500}$)[a] | 455:430 mμ peak ratio |
|---|---|---|---|---|---|---|---|
| | *μmoles HCHO formed/g liver/hr* | | | | | | |
| Untreated male rat | 8.10 ± 0.73 | 2.90 ± 0.26 | 0.075 ± 0.006 | 0.037 ± 0.003 | 0.112 ± 0.008 | 0.085 ± 0.007 | 0.49 ± 0.01 |
| Untreated female rat | 3.08 ± 0.18 | 1.67 ± 0.17 | 0.069 ± 0.005 | 0.038 ± 0.003 | 0.106 ± 0.008 | 0.075 ± 0.012 | 0.55 ± 0.02 |
| Morphine[b]-treated male rat | 4.78 ± 0.32 | 1.80 ± 0.29 | 0.049 ± 0.011 | 0.025 ± 0.005 | 0.074 ± 0.016 | 0.061 ± 0.012 | 0.51 ± 0.01 |
| Morphine[b]-treated female rat | 3.49 ± 0.14 | 1.90 ± 0.20 | 0.080 ± 0.010 | 0.043 ± 0.005 | 0.124 ± 0.015 | 0.096 ± 0.016 | 0.53 ± 0.01 |
| Untreated male mouse | 7.63 ± 0.55 | 8.13 ± 0.55 | 0.132 ± 0.013 | 0.045 ± 0.008 | 0.177 ± 0.024 | 0.128 ± 0.010 | 0.33 ± 0.02 |
| Untreated female mouse | 9.95 ± 1.78 | 8.04 ± 1.55 | 0.133 ± 0.017 | 0.047 ± 0.007 | 0.180 ± 0.024 | 0.135 ± 0.008 | 0.35 ± 0.01 |

[a] Per microsomal preparation equivalent to 250 mg of wet liver.

[b] 20 mg/kg/day for 4 days.

of the predominating enzyme system, which is capable of *N*-demethylating both substrates. Phenobarbital causes an increase in the major enzyme system without increasing or disproportionately increasing the activity of the minor enzyme system. The minor, but not the major, enzyme system is stimulated by the administration of 3-methylcholanthrene. It is now found in sufficient quantity with respect to the enzyme system that predominated in untreated animals that it becomes recognizable as a second enzyme system, and in fact now becomes the predominating system for the *N*-demethylation of 3-MMAB. (c) A third hypothesis combines certain features of hypotheses (a) and (b). As in the first hypothesis, two *N*-demethylating enzymes are found in untreated animals, each showing specificity toward one of the substrates. Phenobarbital stimulates the synthesis of both enzyme systems. 3-Methylcholanthrene produces no changes in the activities of either of these enzyme systems, but causes the formation of a third enzyme system which is capable of *N*-demethylating 3-MMAB, but not ethylmorphine.

SKF 525-A in low concentration did not inhibit the *N*-demethylation of 3-MMAB by microsomes from 3-methylcholanthrene-treated rats, whereas the *N*-demethylation of ethylmorphine was inhibited markedly. On the other hand, the *N*-demethylation of both substrates was inhibited by SKF 525-A when enzyme from untreated or phenobarbital-treated rats was used. If the effect of 3-methylcholanthrene was only to increase the amount of the pre-existing enzyme system, SKF 525-A should still have inhibited the *N*-demethylation of 3-MMAB after induction. Since it did not, hypothesis (a) is ruled out. The observed effects of SKF 525-A are not inconsistent with either hypothesis (b) or (c).

The two-substrate kinetic studies showed that a single enzyme system was responsible for the *N*-demethylation of both 3-MMAB and ethylmorphine by microsomes from untreated and phenobarbital-treated rats, but that in microsomes from 3-methylcholanthrene-treated rats more than one enzyme system was involved in the *N*-demethylation of these substrates. Thus hypothesis (c) can be discarded and hypothesis (b) is strengthened. The observation that one inducing agent will stimulate the metabolism of two drugs, whereas another will stimulate the metabolism of only one of the same two drugs, had frequently been used to argue that two different enzymes for the metabolism of the two drugs must exist in microsomes from untreated animals. This argument is no longer valid.

The difference between the *N*-demethylating system of animals treated with 3-methylcholanthrene and that of untreated animals can be explained by the presence of a qualitatively different cytochrome P-450. As a result of 3-methylcholanthrene treatment, a new cytochrome is formed (cytochrome $P_1$-450) which differs from that which exists in microsomes from untreated rats in that it no longer functions in the *N*-demethylation of ethylmorphine. Cytochrome $P_1$-450 appears to be synthesized in addition to cytochrome P-450 rather than in place of it. That cytochrome P-450 was being synthesized at its normal rate in 3-methylcholanthrene-treated rats was demonstrated in studies employing thioacetamide. The administration of thioacetamide depressed *N*-demethylation as well as microsomal hemoprotein content. The ratio of the 455 mμ and 430 mμ peak heights remained the same as that in control animals, which suggests that the qualitative nature of the microsomal hemoprotein had not changed. When administered with phenobarbital, thioacetamide prevented the increased *N*-demethylase activity and microsomal hemoprotein levels normally observed when phenobarbital alone was given. Again no change in the ratio of the 455:430 mμ peak heights was observed. When 3-methylcholanthrene was administered to rats, a large increase in the 455 mμ peak was observed, but no increase was seen in the 430 mμ peak. Accordingly, the ratio of the 455 mμ peak to the 430 mμ peak increased dramatically from 0.49 to 1.37. If the ratio of 1.37 reflects the characteristics of a mixture of "old" hemoprotein (cytochrome P-450) and "new" hemoprotein (cytochrome $P_1$-450),

and if thioacetamide inhibits the synthesis of cytochrome P-450, but not that of cytochrome $P_1$-450, it would be expected that simultaneous administration of thioacetamide and 3-methylcholanthrene would result in a ratio greater than 1.37. This was the case; the observed ratio was 1.61. Furthermore, concurrent administration of phenobarbital and 3-methylcholanthrene should give a ratio between 0.49 and 1.37 if both cytochrome P-450 and cytochrome $P_1$-450 were synthesized. The observed ratio was 0.94 in these animals. Thus, it would appear that hepatic microsomes from 3-methylcholanthrene-treated rats contain at least two hemoproteins, cytochrome P-450 and cytochrome $P_1$-450, each of which, when reduced and bound to ethyl isocyanide, exists in two pH-dependent, interconvertible forms.

In this and in a preliminary study (17) ethyl isocyanide was employed as a ligand for reduced hemoprotein to demonstrate the presence of a new hepatic microsomal mixed function oxidase, cytochrome $P_1$-450, in 3-methylcholanthrene-treated rats. Spectral analysis of reduced hemoprotein bound to carbon monoxide led to the same conclusion (18, 19, 22).

Madix and Bresnick (23) recently reported an increased efficacy of liver chromatin as a template for RNA synthesis after administration of 3-methylcholanthrene. Since 3-methylcholanthrene causes the synthesis of a new enzyme, the idea might be entertained that the enhanced activity of this chromatin is due not to an increase in the efficacy of genomes normally transcribed, but rather to the additional transcription of genomes not usually expressed in the liver cells.

The studies that compared the hemoproteins in male and female rats and in mice indicate that qualitative differences in cytochrome P-450 may exist naturally without having to be produced by exogenous agents. Omura and Sato (24) suggested that because the solubilization of cytochrome P-450 to the inactive cytochrome P-420 is effected by agents which attack phospholipids, it is likely that microsomal phospholipids are responsible for the peculiar spectral properties of cytochrome P-450 and that in its normal state this hemoprotein is "embedded" in microsomal lipids. It is quite possible that the species and strain differences seen in cytochrome P-450, and for that matter the differences between cytochrome P-450 and cytochrome $P_1$-450, may be due to differences in the association of the hemoprotein with microsomal lipid, or to differences in the lipid itself, rather than to differences in the hemoprotein. It is also quite possible that many factors known to change the quality of the drug-metabolizing enzymes, such as starvation or treatment with certain hormones, may produce their effects by altering the microenvironment of the hemoprotein.

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