Induction of Drug Metabolism

III. Further Evidence for the Formation of a New P-450 Hemoprotein after Treatment of Rats with 3-Methylcholanthrene

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SUMMARY

Drugs have been classified into two groups depending upon whether they form type I or type II difference spectra when they combine with microsomal hemoprotein. Using hexobarbital and aniline as representatives of type I and type II drugs, respectively, the effect that the administration of phenobarbital or 3-methylcholanthrene might have on the binding of microsomal hemoprotein to these drugs was studied. Phenobarbital produced marked increases in type I and type II binding, but 3-methylcholanthrene caused an increase in type II binding only. These changes in the binding properties were reflected in the oxidation of hexobarbital and aniline; as the administration of 3-methylcholanthrene to rats was continued for 5 days, the microsomes from these animals increased their ability to oxidize aniline, but gradually lost their ability to oxidize hexobarbital. The type II binding site was much more stable to digestion with steapsin and to storage in the cold. Thus steapsin or cold storage produced much the same effect on the binding properties of microsomal hemoprotein as did the administration of 3-methylcholanthrene. These studies support the previous conclusion that the administration of 3-methylcholanthrene causes the formation of a new microsomal hemoprotein (cytochrome P_1-450).

INTRODUCTION

In previous studies, Sladek and Mannering (1–3) concluded that the administration of 3-methylcholanthrene to rats causes the appearance in the hepatic microsomes of a cytochrome P-450 (P_1-450) which is different from that found in untreated animals or in those in which hemoprotein has been increased as a result of phenobarbital administration. This conclusion was based largely on the spectral differences seen when ethyl isocyanide was employed as the ligand for the reduced hemoproteins. The sizes of the Soret peaks at 430 and 455 mμ, which appear after the addition of ethyl isocyanide, are dependent upon pH (4). At pH 7.4 the 430 mμ and 455 mμ peaks were of equal size when hepatic microsomes from untreated and phenobarbital-treated rats were employed, but when microsomes from 3-methylcholanthrene-treated rats were used, the peaks were of equal size at pH 6.9. Cytochrome P_1-450 also differs from that found in untreated animals by having a maximum absorbance of its reduced CO complex at slightly less than 450 mμ (5, 6).

The current study provides further evidence for the existence of cytochrome P_1-450, based on its drug-binding properties and on its relative stability after treatment with steapsin or after storage in the cold. Remmer and co-workers (7) and Imai and Sato (8) showed that drugs can be classified into two groups with respect to the difference spectra they produce when they

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combine with hepatic microsomal protein. The spectral change seen with one group of compounds (type I) is characterized by a trough at 419–425 mμ and an absorption peak at 385–390 mμ, and that of the second group (type II), by an absorption peak at 426–435 mμ and a trough at 390–405 mμ. Using hexobarbital and aniline as prototypes of drugs producing type I and type II difference spectra, respectively, the effects that the administration of phenobarbital and 3-methylcholanthrene to rats might have on the binding of these drugs to microsomal hemoprotein were studied. Lipases contained in steapsin (9) or in heated snake venom (10) convert cytochrome P-450 to an inactive form of the hemoprotein, the reduced CO complex of which has maximum absorbance at 420 mμ. Therefore the relative stabilities of cytochromes P-450 and P_1-450 to steapsin treatment were studied. Because it has been noted in this laboratory on numerous occasions that microsomes lose some of their ability to metabolize certain drugs when stored in the cold, the effect of storage on the stabilities of cytochromes P-450 and P_1-450 was also examined.

METHODS

Male Holtzman rats weighing 80–90 or 180–200 g were employed. Phenobarbital sodium (40 mg/kg daily in 0.5 or 1.0 ml of 0.9% NaCl for 4 days) and 3-methylcholanthrene (20 mg/kg daily in 0.5 or 1.0 ml of corn oil for 4 or 5 days) were injected intraperitoneally. "Untreated" rats received either 0.9% NaCl or corn oil. The preparation of hepatic microsomes has been described previously (1).

The protein content of microsomal suspensions was determined by the method of Lowry *et al.* (11). Difference spectra produced by binding of aniline or hexobarbital to hemoprotein were obtained by the method of Remmer and co-workers (7), using a Shimadzu model MPS 50 spectrophotometer. Aniline and hexobarbital concentrations were 3.3 mM; higher concentrations did not alter the binding spectra. Protein concentrations were 1.05–1.12 mg/ml.

Microsomal aniline hydroxylase activity was determined by the modification by Sasame and Gillette of the method of Brodie and Axelrod (12) as given by Kato and Gillette (13), with slight changes in the incubation medium. Microsomes (100,000 × *g* pellet) from 500 mg of liver were incubated for 15 min at 37° with 1 mM aniline. Microsomal hexobarbital oxidase activity was determined as described previously (14), with minor modifications. Microsomes (9000 × *g* supernatant frac-

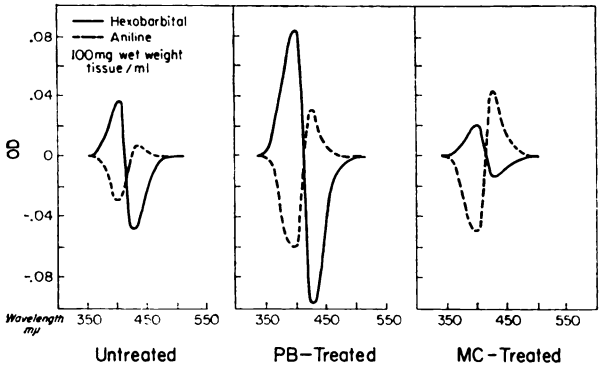

FIG. 1. *Effects of 3-methylcholanthrene (MC) and phenobarbital (PB) treatment of rats on drug binding to microsomal hemoprotein*

Microsome concentration was 100 mg of liver (wet weight) per milliliter. Male rats (180–200 g) were given daily intraperitoneal injections with 40 mg of phenobarbital sodium or 20 mg of 3-methylcholanthrene per kilogram of body weight for 4 days. The concentrations of aniline and hexobarbital were each 3.3 mM. The concentrations of protein in the preparations from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats were 1.1, 1.4, and 1.3 mg/ml, respectively.

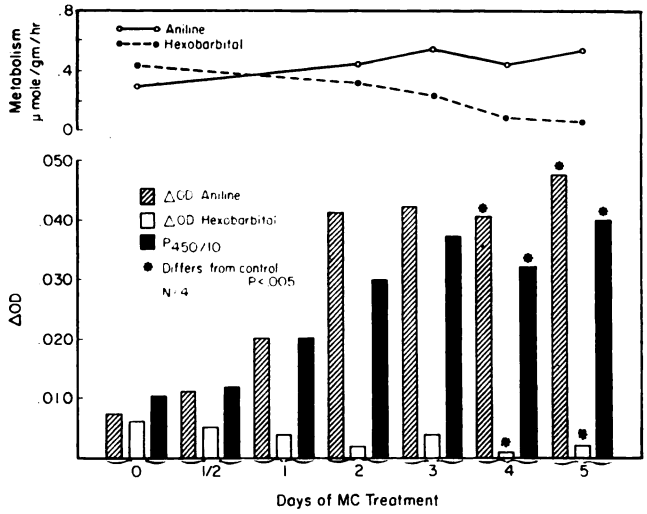

FIG. 2. *Drug binding and metabolism by hepatic microsomes from rats*

Male rats (80–90 g) were given daily intraperitoneal injections with 20 mg of 3-methylcholanthrene (MC) per kilogram of body weight for 5 days. ΔOD aniline = $OD_{430-500}$; ΔOD hexobarbital = $OD_{500-420}$; ΔOD P-450 = $OD_{450-500}$. The concentrations of aniline and hexobarbital were each 3.3 mM; the concentrations of protein were 1.05–1.12 mg/ml.

tion) from 500 mg of liver were incubated for 30 min at 37° with 0.4 mM hexobarbital.

Microsomal suspensions were digested with steapsin as described by Omura and Sato (10). After incubation with 0.07% steapsin (crude pancreatic lipase, Sigma type II) for 24 hr at 0° under N_2, the preparation was sedimented at 100,000 × *g* for 1 hr and resuspended in the original volume of 1.15% KCl solution, and the difference spectra and the cytochrome P-450 and protein contents were determined. The preparation was then incubated with 0.2% steapsin at 37° for 1 hr under N_2, and the remainder of the procedure was repeated.

Microsomes were used on the day they were prepared, except in the storage studies, when microsomal suspensions in 1.15% KCl (100,000 × *g* pellet) were stored for 7 days at −5°, thawed, resedimented at 100,000 × *g* for 1 hr, and resuspended in 1.15% KCl solution.

RESULTS

In Fig. 1 it can be seen that phenobarbital treatment produced hepatic microsomes which gave markedly increased difference spectra with both hexobarbital (type I binding) and aniline (type II binding), but that 3-methylcholanthrene-treated rats yielded microsomes in which only the aniline-binding component was increased. In fact, hexobarbital binding appeared to decrease as a result of 3-methylcholanthrene treatment. This observation has also been noted in a recent communication of Schenkman and co-workers (15), which appeared while the current studies were being prepared for publication. The decline of hexobarbital binding is seen more clearly in the time course study, which compared the binding properties of the microsomes with their ability to oxidize hexobarbital and aniline (Fig. 2). Hexobarbital binding decreased over the 5-day period of 3-methylcholanthrene treatment, while aniline binding increased dramatically during the first 2 days and then leveled off. The concomitant changes which occurred in the rates of hexobarbital and aniline oxidation during the period of 3-methylcholanthrene treatment strengthen the view that the difference spectra seen when drugs react with microsomal hemoprotein bear a relationship to the kinetics of the oxidation of those drugs by mixed-function oxidases.

The effects of steapsin digestion on the dif-

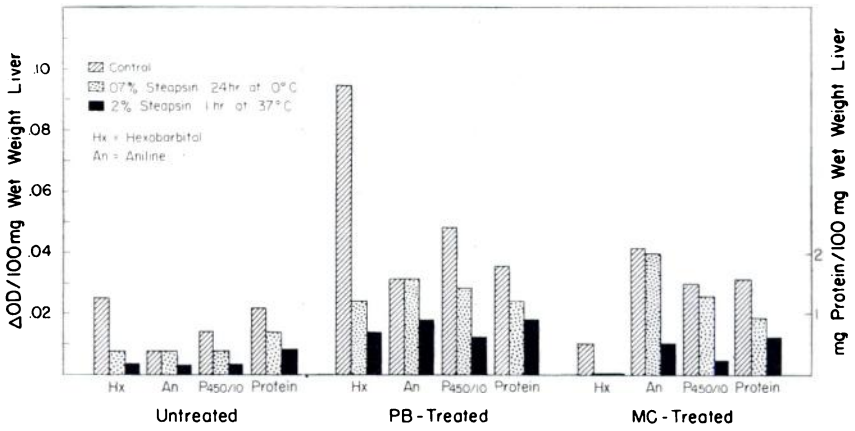

FIG. 3. *Effects of steapsin digestion on drug binding by hepatic microsomes*

Values are expressed on the basis of 100 mg of liver and are the means of five experiments, each of which employed the pooled livers from four rats. Male rats (180–200 g) were given daily intraperitoneal injections with 40 mg of phenobarbital sodium (PB) or 20 mg of 3-methylcholanthrene (MC) per kilogram of body weight for 4 days. ΔOD criteria and drug and protein concentrations are given in the legend to Fig. 2.

ference spectra of hepatic microsomes from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats are shown in Figs. 3 and 4. In Fig. 3, where results were calculated on the basis of wet liver weight, it may be seen that microsomes from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats had a greatly reduced capacity to combine with hexobarbital after they had been incubated with 0.07% steapsin for 24 hr at 0°. On the other hand, regardless of the treatment of the animals, the aniline-binding capacity of the microsomes was not affected by incubation with 0.07% steapsin. It is to be noted that the loss of cytochrome P-450 that occurred as a result of treatment with 0.07% steapsin was disproportionate to the losses of hexobarbital and aniline binding. Thus, while there were losses of 73, 66, and 100% of the hexobarbital-binding component and concomitant decreases in cytochrome P-450 concentrations of 46, 41, and 35% in microsomes from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats, respectively, no loss of the

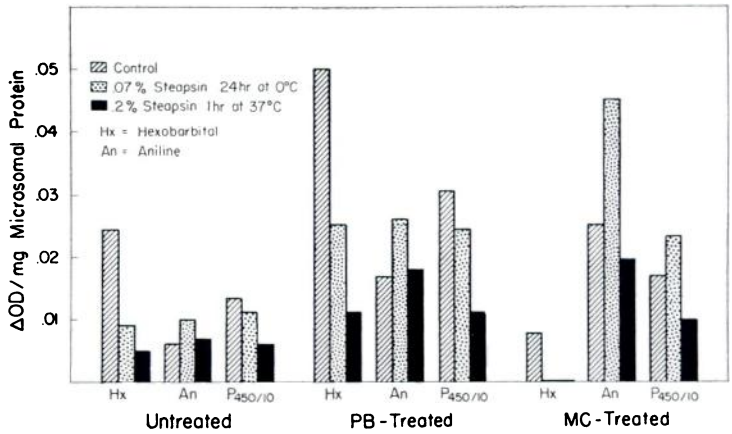

FIG. 4. *Data from Fig. 3 expressed on the basis of milligrams of microsomal protein rather than weight of wet liver*

Symbols and abbreviations are explained in the legends to Figs. 2 and 3.

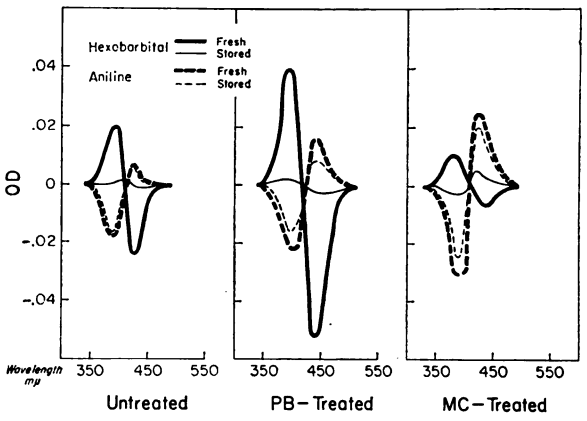

FIG. 5. *Effects of storage at −5° for 7 days on drug binding to microsomal hemoprotein*

Male rats (180–200 g) were given phenobarbital (PB) and 3-methylcholanthrene (MC) as described for Fig. 1. The concentrations of aniline and hexobarbital were each 3.3 mM; the concentrations of protein were 1.05–1.12 mg/ml.

aniline-binding component occurred in any of the microsomes, regardless of source.

Attempts were made to determine the amount of cytochrome P-420 formed from cytochrome P-450 as a result of steapsin treatment; however, cytochrome P-420 is not stable when incubated with steapsin and therefore estimates could not be accurate. After incubation with 0.07% steapsin at 0° for 24 hr, the level of cytochrome P-420 in the 100,000 × *g* pellet was less than 10% of that of cytochrome P-450; after incubation with 0.2% steapsin at 37° for 1 hr, about equal amounts of cytochromes P-420 and P-450 were found in the pellet. The presence of cytochrome P-420 is not thought to affect the measurement of the total drug-binding capacity of the microsomal particles. When snake venom was used to convert all of the cytochrome P-450 to cytochrome P-420 while still in the microsome, no drug binding was seen.[3] Cytochrome P-420 was also found in the supernatant fraction of the incubation mixture, but because of the loss of cytochrome P-420 during incubation, the amount present was not considered meaningful with respect to the inventory of microsomal hemoprotein.

In terms of milligrams of microsomal protein, steapsin treatment (0.07%) greatly reduced the binding of hexobarbital but greatly increased aniline binding (Fig. 4). Microsomes from 3-methylcholanthrene-treated rats which had been incubated with 0.07% steapsin contained about 7.5 times more aniline-binding component per milligram of protein than did unincubated microsomes from untreated rats.

Storage in the cold decreased both the hexobarbital- and the aniline-binding components of microsomes, but the loss of the aniline-binding component was much less than that of the hexobarbital-binding component (Fig. 5). It is to be noted that the configuration of the hexobarbital binding spectrum seen with the stored microsomes from 3-methylcholanthrene-treated rats resembles the type II binding spectrum seen with aniline. Similar type II binding has been observed with hexobarbital to microsomes from female rats which had been treated with 3,4-benzpyrene (15), and with phenobarbital to microsomes from untreated male rabbits (8).

DISCUSSION

While it is not known exactly how drugs combine with microsomal hemoprotein to produce type I and type II binding spectra, studies have been performed which permit meaningful speculation. Schenkman and coworkers (16) proposed that compounds

[3] Unpublished results.

producing type II spectral changes interact with the iron at the CO-binding site of the heme, but that the interaction of type I compounds was at a different site. Using ferriheme as a model, it was observed that changes in pH produced a spectrum resembling that seen when type I drugs combine with cytochrome P-450. Schenkman and Sato (17) postulated that type I binding compounds increase the electronegativity or polarity of the sixth ligand of the heme. They suggested that the spectral shift might be due to the displacement of the sixth ligand from one part of the enzyme to a more polar region. They further postulated that ferricytochrome exists in two interconvertible forms. One form, with an intact sixth ligand, reacts with substrates of the mixed-function oxidase before the substrates are oxidized. After interaction with the substrate, the first form is converted to the second form. The second form differs from the first in that it has substrate bound to the apoenzyme and the sixth ligand of the heme is altered. Lacking a type I binding site, but with the type II binding site intact, cytochrome P_1-450 resembles the second of the two hemoprotein forms suggested by Schenkman and Sato. However, it lacks reversibility to the first form, for it will not function in the oxidation of hexobarbital, a type I compound. Cytochrome P_1-450 might be thought of as an aberrant cytochrome P-450 in which the type I binding site (sixth ligand?) is missing or somehow rendered incapable of reacting with type I compounds. This could result from the administration of 3-methylcholanthrene in several ways: (a) synthetic pathways in the liver may have been altered so that a defective hemoprotein is produced; (b) the polycyclic hydrocarbons may cause an alteration of the lipid environment of a "normal" hemoprotein so that type I compounds are no longer "accessible" to the type I binding site, or the nature of the binding site itself may be so influenced by the change in lipid that it no longer combines with type I drugs; or (c) the polycyclic hydrocarbons or their metabolites may combine with the type I binding site so as to render it nonfunctional. In any event, the altered cytochrome must be considered a variant of P-450 hemoprotein.

Schenkman and co-workers (15) recently pointed out the similarity of the spectral characteristics of microsomal hemoprotein from 3,4-benzpyrene-treated rats to those of microsomal hemoprotein bound to a type I compound such as hexobarbital or 3,4-benzpyrene itself. They concluded that cytochrome P-450 exists in only two forms, the native enzyme and the enzyme-substrate complex. While these studies show why certain spectral measurements cannot be employed to reveal differences in cytochromes, they do not nullify all other evidence that has accumulated to show the existence of a new cytochrome P-450 after the administration of polycyclic hydrocarbons. The concept of direct binding of a polycyclic hydrocarbon or its metabolites to normal cytochrome P-450 to produce a polycyclic hydrocarbon–hemoprotein derivative implies that the formation of this derivative is independent of the inductive effect produced by the compound. It would mean that the polycyclic hydrocarbon would induce the increased synthesis of normal cytochrome P-450, much as phenobarbital and many other compounds induce increased production of normal cytochrome P-450, and that the polycyclic hydrocarbon would then combine with this hemoprotein much as it would *in vitro*. This being the case, it would be expected that the polycyclic hydrocarbon would combine with normal cytochrome P-450 whether it was caused to be formed as a result of phenobarbital or of 3-methylcholanthrene administration. When phenobarbital and 3-methylcholanthrene were administered to rats simultaneously, each in amounts known to produce a maximum inductive effect, the amount of cytochrome P_1-450 formed, as judged from the relative heights of the 455 mμ and 430 mμ peaks formed with ethyl isocyanide, was not consistent with the concept of equal combination of 3-methylcholanthrene with new, normal cytochrome P-450 regardless of the manner of induction, whether by phenobarbital or by 3-methylcholanthrene (3). The A_{455}:A_{430} peak height ratio with microsomes from 3-methylcholanthrene-treated rats was 1.37;

that obtained with microsomes from phenobarbital-treated rats was 0.56; and that with microsomes from rats treated with both compounds was 0.94. This implies that the formation of cytochrome P_1-450 is part of the inductive process, not incidental to it. The addition of 3-methylcholanthrene to microsomes from untreated rats (final concentration, 0.1 mM) does not alter the $A_{455}:A_{430}$ peak height ratio seen with ethyl isocyanide.[3]

Treating microsomes with steapsin or storing them in the cold produces much the same effect on the binding properties of microsomal hemoprotein as the administration of 3-methylcholanthrene. The type I binding site diminishes, but the type II binding site remains intact. The loss of the type I binding site after treatment with steapsin suggests that reactivity of the type I binding site is highly dependent upon the association of the hemoprotein to lipid. Steapsin contains proteolytic enzymes as well as lipases, and the possibility of the loss of type I binding as a result of proteolysis should not be disregarded.

The possibility should be considered that microsomes from untreated animals contain two different P-450 hemoproteins, one being more or less predisposed to type I binding, and the other, to type II binding. On the basis of the current binding studies, one could propose that in untreated animals both cytochromes P-450 and P_1-450 exist, that after treatment with phenobarbital, concentrations of both cytochromes are increased, and that after 3-methylcholanthrene treatment, only cytochrome P_1-450 is increased. Previous studies from this laboratory (2, 3), in which microsomal ethylmorphine *N*-demethylase and 3-methyl-4-methylaminoazobenzene *N*-demethylase activities were compared in microsomes from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats, do not support the view that cytochrome P_1-450 exists to any measurable degree in microsomes from untreated rats.

The type I binding spectrum has a maximum at about the minimum of the type II binding spectrum, and vice versa. Thus, in microsomes possessing both binding sites, it is not possible to determine whether a predominantly type I binding compound has any affinity for the type II binding site or whether type II compounds will in fact combine to some degree with the type I binding site. In microsomes from 3-methylcholanthrene-treated rats, the type I binding site appears to be absent or nearly so, and the type II binding site is exaggerated. Storage also depletes the type I binding site. One would expect to find the smallest number of type I binding sites in stored microsomes from 3-methylcholanthrene-treated rats. These microsomes show type II binding with hexobarbital (Fig. 5), a drug which typically gives a type I binding spectrum. Thus it would appear that hexobarbital combines with both type I and type II binding sites but that the type II binding spectrum is obscured when the more reactive type I binding site is also present. The other possibility is that cytochrome P_1-450 combines with hexobarbital so as to give a type II spectrum whereas cytochrome P-450 does not.

Schenkman and co-workers (15) observed type II binding of hexobarbital to microsomes from female rats which had been treated with 3,4-benzpyrene, but not from male rats. These studies with the two sexes were not strictly comparable, because the males were treated with 3,4-benzpyrene for only 1 day and the females were treated for 5 days. In the current studies, type I binding of hexobarbital, although greatly reduced, was still evident in microsomes after male rats had been treated with 3-methylcholanthrene for 5 days (Fig. 2). Apparently there was sufficient type I binding to obscure any type II binding of hexobarbital which might have occurred. Microsomes from female rats are normally much less capable of type I binding than microsomes from males (18); therefore, it might be expected that type I binding would be even lower with microsomes from females treated with polycyclic hydrocarbons than with microsomes from similarly treated males. This could explain the sex difference in hexobarbital binding.

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