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The Conversion of Hepatic Cytochrome P-450 to P-420 in Normal and Phenobarbital- and 3-Methylcholanthrene-Treated Animals

MICHAEL R. FRANKLIN[1]

Department of Biochemistry, The University of Texas Southwestern Medical School at Dallas, Dallas Texas 75235

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SUMMARY

The conversion of rat and rabbit hepatic microsomal cytochrome P-450 to P-420 by mercurials does not proceed to completion. The percentage of cytochrome P-450 resistant to mercurial attack was greater in rats than in rabbits. It was increased by prior treatment with phenobarbital and decreased when the animals had been treated with 3-methylcholanthrene. Correlative studies using ethyl isocyanide as a ligand for reduced cytochrome P-450 instead of carbon monoxide demonstrated a loss of the 455 nm absorbance maximum in this difference spectrum, closely paralleling the loss of cytochrome P-450. The loss of the 455 nm absorbance maximum, however, did not always result in an increase in the absorbance maximum at 430 nm. The effect of low concentrations of mersalyl on the electron paramagnetic resonance spectrum at −172° of microsomes from 3-methylcholanthrene-treated animals suggests the presence of an undetectable form of cytochrome P-450. The differences in the susceptibility of cytochrome P-450 from different sources to mercurial degradation probably reflect changes in the whole population of cytochrome P-450 present in the microsomes.

INTRODUCTION

The conversion of microsomal cytochrome P-450 to P-420 has been shown to occur with a variety of reagents, including organic solvents, enzymatic digestion, and other protein-denaturing procedures (1–5). The inability to achieve complete conversion of cytochrome P-450 to P-420 by mercurial reagents with microsomes from phenobarbital-treated rats (6) prompted a more intensive investigation of this conversion, utilizing microsomes from untreated and 3-methylcholanthrene-treated rats to determine whether methods commonly employed to induce cytochrome P-450 in liver microsomes alter its susceptibility toward mercurials. Also, since much of the work performed on the characterization of cytochrome P-450 (7–10) and its conversion to P-420 (2–5) has employed rabbit rather than rat hepatic microsomes, it was necessary to determine whether species variations apparent in oxidative drug metabolism (11) might also occur with regard to the susceptibility of the cytochrome P-450 to mercurial conversion to P-420. A preliminary account of this work has been reported (12).

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[1] Present address, Department of Biopharmaceutical Sciences, College of Pharmacy, University of Utah, Salt Lake City, Utah 84112.

MATERIALS AND METHODS

Animals either were left untreated or were treated with four daily intraperitoneal injections of phenobarbital (40 mg/kg) or

3-methylcholanthrene (12 mg/kg in corn oil). Hepatic microsomes were prepared as described previously (13). All optical and electron paramagnetic resonance spectra were determined as described in the preceding paper (6). Cytochrome P-450 and P-420 concentrations were determined from the optical spectra, using extinction coefficients of 91 and 110 mM^{-1} cm^{-1}, respectively, with a negative extinction coefficient of -41 mM^{-1} cm^{-1} for the contribution of cytochrome P-450 to the P-420 absorbance maximum (14). (It is recognized that these extinction coefficients may not be correct for the pigments of microsomes from 3-methylcholanthrene-treated animals. The reason for using them will be outlined under DISCUSSION.) In some experiments ethyl isocyanide at a concentration of 1.5 mM was added to the contents of the sample cuvette instead of CO. For convenience, the absorbance maxima of the cytochrome P-450–CO (448 nm) and cytochrome P-450–ethyl isocyanide (453 nm) spectra observed with microsomes from 3-methylcholanthrene-treated animals are referred to as A_{450} and A_{455}, respectively.

Mersalyl acid (*o*-[(3-hydroxymercuri-2-methoxypropyl)carbarmoyl]phenoxyacetic acid), ethyl isocyanide, and 3-methylcholanthrene were obtained from Sigma Chemical Company. Sodium phenobarbital was obtained from Merck & Company.

RESULTS

The conversion of cytochrome P-450 to P-420 by various concentrations of mersalyl in liver microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats is shown in Fig. 1. The time course of conversion, especially at higher mersalyl concentrations, was slightly different in 3-methylcholanthrene-treated rats; the rate of conversion between 6 and 10 min was greater than the rate observed (6) using microsomes from phenobarbital-treated rats. However, the greater part of conversion at a specific mersalyl concentration occurred during the first 5 min after the addition of mercurial to the microsomes. Evident in Fig. 1 is the difference in susceptibility of the microsomal cytochrome P-450 of control and 3-methylcholanthrene- and phenobarbital-treated rats toward the mercurial. In 3-methylcholanthrene-treated animals the degradation of cytochrome P-450 was

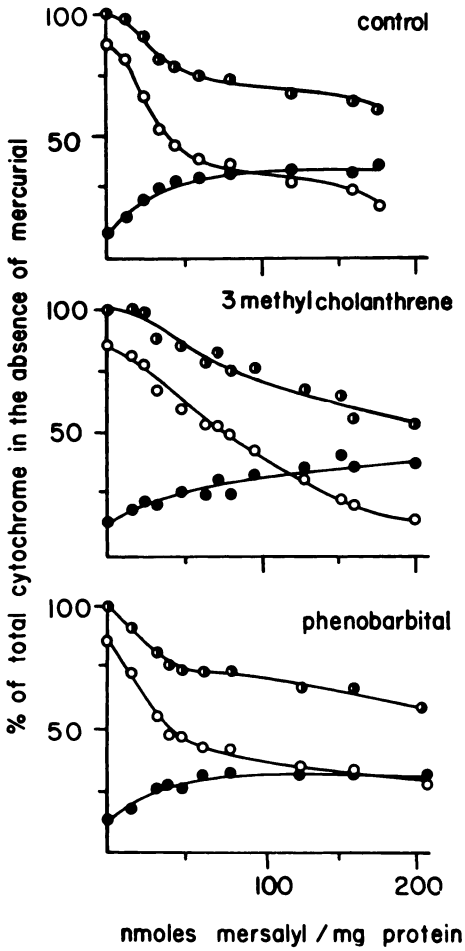

FIG. 1. *Conversion of cytochrome P-450 to P-420 in hepatic microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats*

Using the standard assay described under MATERIALS AND METHODS, the effect of various mersalyl concentrations on cytochrome P-450 was investigated with hepatic microsomes prepared from control and 3-methylcholanthrene- and phenobarbital-treated rats. The cytochrome P-450 concentrations initially present in these three types were 1.00, 1.76, and 2.77/nmoles mg of protein (the means of three, seven, and eight preparations of microsomes, respectively) .●, P-420; ○, cytochrome P-450; ◑, the sum of these two, all expressed as a percentage of the total cytochrome (P-450 + P-420) determined in the absence of mercurial.

almost linear with increasing mersalyl concentrations. This is different from its conversion in control and phenobarbital-treated rats, for which there was a distinct inflection point around 50 nmoles of mersalyl per milligram of protein. The amount of cytochrome P-450 converted at high mercurial concentrations was distinctly less in microsomes prepared from phenobarbital-treated animals than in microsomes from control and 3-methylcholanthrene-treated animals, although, at low mersalyl concentrations, the cytochrome P-450 from 3-methylcholanthrene-treated animals appears less susceptible. The cross-over points, where the concentration of cytochrome P-450 equals the concentration of P-420, occurred at increasing mersalyl concentrations for control, 3-methylcholanthrene-treated and phenobarbital-treated rats, respectively. The amounts of cytochrome present at these points, expressed as a percentage of the cytochrome P-450 and P-420 initially present, are remarkably similar: about 35% for each treatment.

In rabbits the same pattern of conversion of hepatic cytochrome P-450 to P-420 was observed for control and phenobarbital- and 3-methylcholanthrene-treated animals (Fig. 2). The difference in convertibility of the microsomal cytochrome P-450 between control and 3-methylcholanthrene-treated animals and those previously treated with phenobarbital is much more clearly indicated than in rats. The cross-over points (where cytochrome P-450 equals P-420) occur at lower mersalyl concentrations than in the corresponding rat liver microsomes, suggesting that the rabbit microsomal cytochrome P-450 is generally more susceptible to mercurial attack than rat liver microsomal cytochrome P-450. Again, the cytochrome concentrations at these cross-over points, despite the differences in the concentration of mersalyl required to reach these points, are very similar: 38%, 36%, and 32% of the total cytochrome P-450 and P-420 originally present for control, 3-methylcholanthrene-treated, and phenobarbital-treated animals, respectively. Microsomal cytochrome P-450 from both 3-methylcholanthrene-treated rats and rabbits was the most susceptible to conversion. For both species the percentage of cytochrome P-450 remaining at the highest mersalyl concentration employed was observed in the order

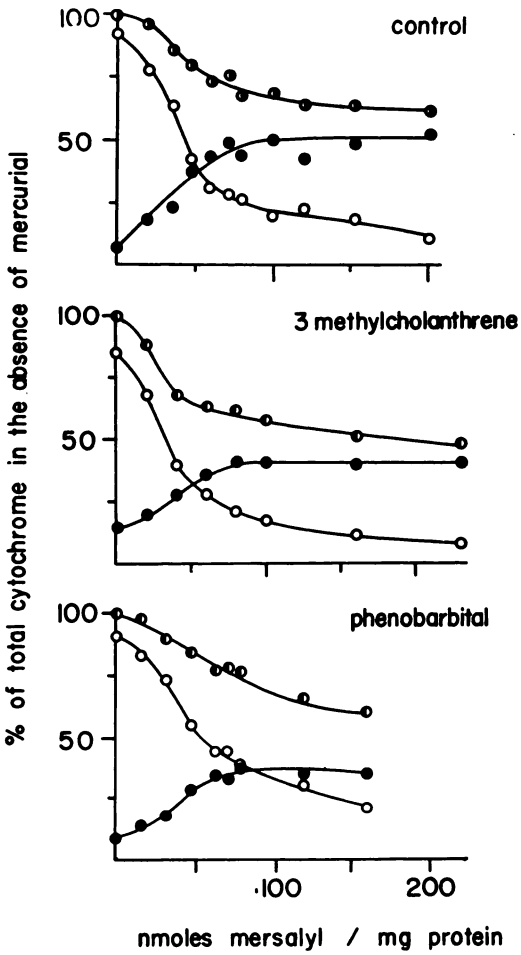

FIG. 2. *Conversion of cytochrome P-450 to P-420 in hepatic microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rabbits*

Using the standard assay described under MATERIALS AND METHODS, the effect of various mersalyl concentrations on cytochrome P-450 was investigated with hepatic microsomes prepared from control and 3-methylcholanthrene- and phenobarbital-treated rabbits. The cytochrome P-450 concentrations initially present in these three types were 1.67, 1.89, and 2.89 nmoles/mg of protein (the means of four, three, and three preparations of microsomes, respectively). ●, P-420; ○, cytochrome P-450; ◑ and ◐, the sum of these two, all expressed as a percentage of the total cytochrome (P-450 + P-420) measured in the absence of mercurial.

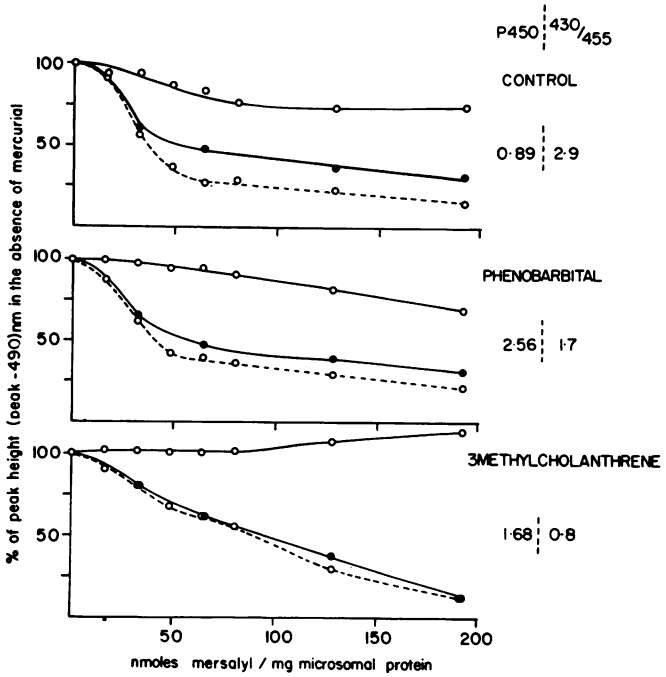

FIG. 3. *Effect of mersalyl on reduced cytochrome P-450–ethyl isocyanide difference spectrum in hepatic microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats*

Using the standard assay described under MATERIALS AND METHODS, and utilizing ethyl isocyanide as well as carbon monoxide as a ligand for reduced cytochrome P-450, the effect of increasing mersalyl concentrations on the two absorbance maxima in the Soret region (455 and 430 nm) of the reduced cytochrome P-450–ethyl isocyanide difference spectrum were compared with the destruction of cytochrome P-450. The values are the means of four preparations each of microsomes from control and 3-methylcholanthrene-treated rats, and five from phenobarbital-treated rats. ●, the concentrations of cytochrome P-450; ○, intensities of the absorbance maxima of the reduced cytochrome P-450–ethyl isocyanide difference spectrum (- - -, at 455 nm; ——, at 430 nm). All values are expressed as a percentage of those obtained in the absence of mercurial. The cytochrome P-450 values given are expressed as nanomoles per milligram of microsomal protein.

3-methylcholanthrene-treated < control < phenobarbital-treated. Because of the differences in convertibility among the cytochromes P-450 of control and phenobarbital- and 3-methylcholanthrene-treated animals and the ability to demonstrate differences between the forms of cytochrome P-450 present in these animals using ethyl isocyanide as a heme ligand (15), the effect of mersalyl on both the 430 nm and 455 nm absorbance maxima in the difference spectrum of the reduced cytochrome P-450–ethyl isocyanide complex was investigated in micrsomes under all three conditions. These changes also were compared with changes in the concentration of cytochrome P-450. In both rats (Fig. 3) and rabbits (Fig. 4) the loss of the 455 nm absorbance maximum of the reduced cytochrome P-450–ethyl isocyanide complex closely paralleled the loss in cytochrome P-450, despite large differences in the A_{430}:A_{455} ratios. The correlations were especially close in 3-methylcholanthrene-treated rats and phenobarbital-treated rabbits. With other treatments the loss of the 455 nm absorbance maximum was greater than the loss of cytochrome P-450 itself, suggesting that in these cases mersalyl preferentially attacked either the functional groups in cytochrome P-450, or those cytochrome P-450 molecules responsible for the 455 nm absorbance maximum of the reduced

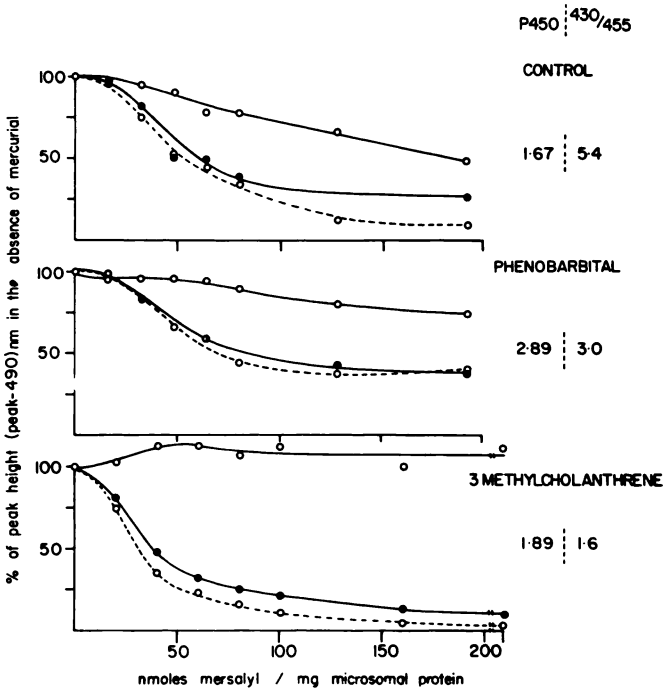

FIG. 4. *Effect of mersalyl on reduced cytochrome P-450–ethyl isocyanide difference spectrum in control and phenobarbital- and 3-methylcholanthrene-treated rabbits*

Using the standard assay described under MATERIALS AND METHODS, and utilizing ethyl isocyanide as well as carbon monoxide as a ligand for the reduced cytochrome P-450, the effect of increasing mersalyl concentrations on the two absorbance bands (455 and 430 nm) of the reduced cytochrome P-450–ethyl isocyanide difference spectrum were compared with the destruction of cytochrome P-450. The values are the means of four preparations of microsomes from control and three each from phenobarbital- and 3-methylcholanthrene-treated rabbits. ●, the concentrations of cytochrome P-450; ○, intensities of the absorbance maxima of the reduced cytochrome P-450–ethyl isocyanide difference spectrum (– – –, at 455 nm; ——, at 430 nm). All values are expressed as a percentage of those obtained in the absence of mercurial. The cytochrome P-450 values given are expressed as nanomoles per milligram of microsomal protein.

cytochrome P-450-ethyl isocyanide spectrum.

The changes in the 430 nm absorbance maximum of the reduced cytochrome P-450–ethyl isocyanide complex shown in Figs. 3 and 4 are difficult to interpret. P-420 in the reduced state shows a 430 nm absorbance maximum with ethyl isocyanide, as do hepatic microsomes, in which little P-420 is detectable with the carbon monoxide ligand. Thus the changes are a combination of the loss of the 430 nm absorbance maximum, associated with cytochrome P-450, and the gain in the 430 nm absorbance maximum, associated with P-420. The different effects that can be observed are shown in Fig. 5, where microsomes from phenobarbital-treated rats, when assayed for the reduced cytochrome P-450–ethyl isocyanide complex, gave an $A_{430}:A_{455}$ absorbance maximum ratio of 1.9 at pH 7.4. The microsomes were also treated with mercurial at pH 8.2, where the ratio of the absorbance maximum at 430 nm relative to 455 nm was 0.8. The absorbance ratios at these two pH values are similar to those seen in microsomes from phenobarbital- and 3-methylcholanthrene-treated rats (1.7 and 0.8, respectively). The changes in the 430 nm absorbance maximum with increasing mersalyl concentrations also compare favorably with those seen in microsomes

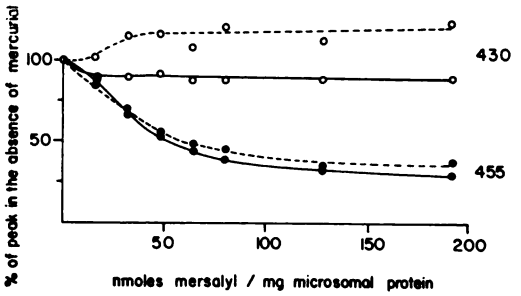

FIG. 5. *Effect of mersalyl on absorbance maxima of reduced cytochrome P-450–ethyl isocyanide difference spectra at pH 7.4 and pH 8.2*

Using the standard assay described under MATERIALS AND METHODS and ethyl isocyanide as a ligand for reduced cytochrome P-450, the effect of increasing mersalyl concentrations on the difference spectrum was investigated. ○, absorbance maximum at 430 nm; ●, 455 nm absorbance maximum, using the absorbance at 490 nm as a reference point for both. ——, experiments performed at pH 7.4, where the mean A_{430}:A_{455} ratio was 1.9; – – –, experiments performed at pH 8.2, where the mean A_{430}:A_{455} ratio was 0.8.

from rats treated with these compounds. However, there was no change in the percentage loss in the 455 nm absorbance maximum at the two pH values, suggesting that the differences in mersalyl destruction of this 455 nm absorbance maximum in microsomes from 3-methylcholanthrene-treated as compared to phenobarbital-treated rats were due to a real difference in susceptibility of the cytochrome toward the mercurial, and not to the relative size of the 455 nm absorbance maximum as compared with that at 430 nm.

Thus, in both rats and rabbits, phenobarbital treatment increases the proportion of the 455 nm absorbance maximum in the reduced cytochrome P-450–ethyl isocyanide difference spectrum, but only reduces the susceptibility of cytochrome P-450 to mercurial attack in rabbits. 3-Methylcholanthrene treatment increases the proportion of the 455 nm absorbance maximum in the reduced cytochrome P-450–ethyl isocyanide difference spectrum to a greater extent than phenobarbital. With this treatment the susceptibility of the hepatic microsomal cytochrome P-450 to mercurial attack is increased in both rats and rabbits. Thus, both within and between species and treatments, there is no direct correlation between the A_{430}:A_{455} absorbance maximum ratio, as altered by cytochrome P-450-inducing agents, and susceptibility to mercurial attack. The induction of liver cytochrome P-450 by phenobarbital reduces the A_{430}:A_{455} ratio and decreases the susceptibility to mercurial attack, whereas induction by 3-methylcholanthrene, while also reducing the A_{430}:A_{455} ratio even further than phenobarbital, increases the susceptibility of cytochrome P-450 to mercurial attack.

EPR studies with hepatic microsomes from phenobarbital-treated animals (6) have shown that the conversion of low-spin hemoprotein to the high-spin form by mersalyl produces less high-spin signal ($g = 6.1$) for a given decrease in low-spin signal ($g = 2.25$) than does lowering the pH. It was therefore of interest to determine whether the induction by 3-methylcholanthrene of a hepatic cytochrome P-450, which is more susceptible to mercurial attack than that induced by phenobarbital, would have any effect on the EPR observations. The conversion of low-spin to high-spin signal ($g = 6.1$) in microsomes from both 3-methylcholanthrene-treated rabbits (Fig. 6A) and rats (Fig. 6B) followed the same pattern as in microsomes from phenobarbital-treated animals: the conversion with mersalyl produced less high-spin signal than conversion by lowering the pH. Also, as anticipated from the optical data, the loss (about 75%) of low-spin signal by mersalyl attack was greater in microsomes from 3-methylcholanthrene-treated animals than the loss observed (about 50%) by EPR spectroscopy for similar concentrations of mersalyl with microsomes from phenobarbital-treated animals. It must be emphasized, however, that the presence of a high-spin form of cytochrome P-450 in microsomes from 3-methylcholanthrene-treated animals renders direct comparison of EPR data rather difficult, since the low-spin EPR signal does not represent as large a proportion of the optically detectable cytochrome P-450 (CO complex) as it does in phenobarbital-treated animals. Also, the possible formation of low-spin P-420 from

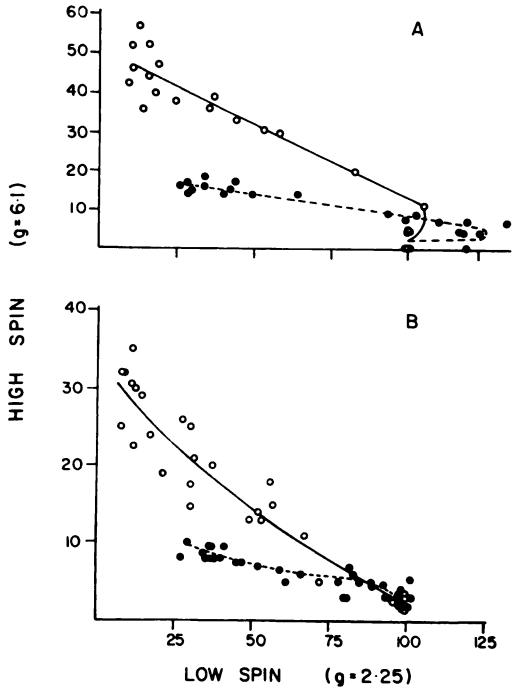

FIG. 6. *Effects of pH changes and mersalyl on electron paramagnetic resonance spectra of hepatic microsomes from 3-methylcholanthrene-treated rats and rabbits*

A. Hepatic microsomes from four preparations of 3-methylcholanthrene-treated rabbits were used at concentrations of 10–15 mg of protein per milliliter. Changes in the high- and low-spin signals were obtained with changes in pH from 7.4 to 4.0 (○) and with mersalyl concentrations (●) from 0 to 240 nmoles/mg of protein. The low-spin signal obtained at pH 7.4 in each preparation was normalized to 100, and the high-spin signal was adjusted to the same extent.

B. Hepatic microsomes from five preparations of 3-methylcholanthrene-treated rats were used at protein concentrations of 12.5–15 mg/ml. The pH changes were the same as above, and mersalyl concentrations of 0–200 nmoles/mg of protein were used. The signals were normalized as before.

this high-spin cytochrome P-450 would mask decreases in the low-spin signal because of conversion of low-spin cytochrome P-450 to high-spin P-420.

Of particular interest, from the EPR data, are the effects of low concentrations of mersalyl upon the spin signals of microsomes from 3-methylcholanthrene-treated animals. In rabbits (Fig. 6A), contrary to what would be expected, there was an increase (up to 25%) in the low-spin signal, with a slight increase in the high-spin signal, with low concentrations (0–30 nmoles/mg of protein) of mersalyl. In rats (Fig. 6B) only the increase in high-spin signal, without a reduction of "original" low-spin signal, could be demonstrated. Observations by Peisach and Blumberg (16) have shown that microsomes from 3-methylcholanthrene-treated animals contain a high-spin signal (g = 8.1) which is not detectable at liquid nitrogen temperatures but is measurable at the temperature of liquid helium. The results we observed may represent the conversion of this high-spin form (undetectable under our conditions) to a detectable low-spin form of the hemoprotein, as well as its conversion to a high-spin form of P-420.

DISCUSSION

The results presented here suggest that treatment of animals with compounds which cause induction of hepatic microsomal cytochrome P-450 can alter the susceptibility of this cytochrome with respect to its conversion to P-420 by mercurials. The concentrations of mercurial causing conversion are much higher than would be required for stoichiometric titration of the sulfhydryl groups acting as ligands to the heme moiety. Thus changes in susceptibility toward conversion do not necessarily imply an alteration in the immediate vicinity of the heme, and do not exclude the synthesis of a new hemoprotein as a result of induction. Alternatively, the induction may either have resulted in the synthesis of new or different proteins associated with the endoplasmic reticulum or have caused an alteration in conformation or structure of proteins already existing in the membrane. Imai and Siekevitz (17) have attributed the greater resistance of cytochrome P-450 from 3-methylcholanthrene-treated rats toward KSCN as an increase in the hydrophobicity of the environment of the heme. Both changes in environment and new hemoprotein synthesis could result in changes in the number of exposed thiol groups available for interaction with the mercurial and thus

could alter the concentration of mercurial needed for conversion of cytochrome P-450 to P-420.

It is not altogether surprising that 3-methylcholanthrene treatment of animals altered the susceptibility of hepatic cytochrome P-450 toward mercurials, since much evidence (10, 15, 18, 19) has been produced to demonstrate the synthesis of a different form of cytochrome P-450 after the administration of polycyclic hydrocarbons to an animal. One could conclude from our results that this new form of cytochrome P-450 is more easily converted to P-420 and results in the more complete conversion seen in 3-methylcholanthrene-treated animals. Such a simple assumption, however, may be complicated by the suggestion (10) that the hepatic cytochrome P-450 induced by 3-methylcholanthrene has an extinction coefficient over twice that of the total cytochrome P-450 population of phenobarbital-induced and control animals. Thus a loss of 1 cytochrome P-450 molecule (upon conversion to P-420) would initially show up as a loss of 2 assuming an extinction coefficient of 91 mM^{-1} cm^{-1}. Assuming that this cytochrome is preferentially attacked by mersalyl, one would have to use a "sliding scale" for the extinction coefficient as the percentage of this new cytochrome in the remaining population decreased. Further complications also arise, since we are using a negative extinction coefficient for the contribution of cytochrome P-450 to the absorbance maximum of the P-420, and this is derived from the cytochrome P-450 by assuming the original extinction coefficient of 91 mM^{-1} cm^{-1}. Recently it has been reported (20) that the P-420 derived from the cytochrome P-450 induced by 3-methylcholanthrene has a higher extinction coefficient than P-420 derived from untreated animals. Thus the conversions of cytochrome P-450 to P-420 shown for 3-methylcholanthrene-treated animals have only been interpreted within the conventional framework, as used for phenobarbital-treated and normal animals.

There is a close correlation between the loss of the 455 nm absorbance maximum of the reduced cytochrome P-450–ethyl isocyanide complex and the loss of cytochrome P-450, despite the wide variation in intensity of this absorbance maximum in relation to that at 430 nm. This suggests that the 455 lnm absorbance maximum is a property of al or most of the population of cytochrome P-450. Alternatively, if the 455 nm absorbance maximum of the reduced cytochrome P-450–ethyl isocyanide complex is due to a small population existing in a different "state," this small population does not differ in its range of mersalyl susceptibility from that of the whole population.

Thus, either 3-methylcholanthrene treatment of animals, which is well known for its effect of increasing the 455 absorbance maximum of the reduced cytochrome P-450–ethyl isocyanide complex, alters the whole population of cytochrome P-450 or the cytochrome synthesized in response to 3-methylcholanthrene treatment, and responsible for the increase in the 455 nm absorbance maximum, has the same susceptibility to mersalyl conversion to P-420 as the original population, which still comprises about 50% of the total liver cytochrome P-450 (21). Since after 3-methylcholanthrene treatment almost all the cytochrome P-450 is convertible by mersalyl, the evidence suggests that induction alters the total population of cytochrome P-450, at least with regard to its mercurial sensitivity. The electron paramagnetic resonance data, showing differences in the effects of the mercurial on microsomes from 3-methylcholanthrene-treated animals compared with those previously obtained for phenobarbital-treated animals (6), are not incompatible with this suggestion, since spin changes may occur, such as those at low mersalyl concentrations, without a change in optical properties of the reduced cytochrome P-450–carbon monoxide complex.

In summary, differences exist in the susceptibility of hepatic cytochrome P-450 toward mercurial conversion to P-420, both between and within species, depending upon the prior treatment of the animals. These changes probably reflect differences in the whole population of cytochrome P-450 in the liver microsomes, not changes in the proportions of various types of cytochrome P-450.

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