STUDIES ON THE INDUCTION OF CO-BINDING PIGMENTS IN LIVER MICROSOMES BY PHENOBARBITAL AND 3-METHYLCHOLANTHRENE *

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Treatment of rats with phenobarbital causes parallel increases in drug metabolizing activity and the concentration of the hemoprotein present in liver microsomes (Remmer and Merker, 1965; Ernster and Orrenius, 1965). However, when 3-methylcholanthrene (3-MC) is given, the rate of metabolism of certain drugs does not increase with the increase in hemoprotein level (Sladek and Mannering, 1966). This microsomal hemoprotein, in the reduced form, binds with CO to give a characteristic spectral peak at 450 $m\mu$ and has been called cytochrome P-450 or the CO-binding pigment (Omura and Sato, 1964). When ethyl isocyanide is used as the ligand for reduced hemoprotein in liver microsomes, spectral peaks at 430 $m\mu$ and 455 $m\mu$ are observed (Imai and Sato, 1966). After a single 3-MC injection, Alvares et al. (1967) observed that increases in 3-methyl-4-monomethylaminoazobenzene N-demethylase paralleled temporally the increase in ratio of the 455:430 peaks, but did not parallel the increase in cytochrome P-450. Recently, Bresnick et al. (1967) investigating the distribution of labelled 3-MC within the hepatocyte, observed some labelling of the microsomal fraction and the labelled material appeared to be protein-bound. If 3-MC or its metabolite is bound to the microsomal hemoprotein, the complex could possess different spectral properties than the native cytochrome and this binding could account for the change in the ratio of the 455:430 peaks observed in 3-MC treated rats. Evidence presented in this paper shows that ethionine, an inhibitor of protein synthesis, which prevents the inductive

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effects of 3-MC (Conney et al. 1956), was able to prevent, the induction of 3, 4-benzpyrene hydroxylase activity, the increase in the CO-binding pigment and the change in ratio of the 455:430 peaks observed when rats were treated with 3-MC. Evidence is also presented which shows that in 3-MC treated rats, the CO-binding pigment has a maximum absorption at 448 mμ instead of 450 mμ. These studies suggest that 3-MC causes the synthesis of a hemoprotein with spectral properties different from cytochrome P-450 and suggests the presence of more than one CO-binding pigment in rat liver microsomes.

METHODS: Male Sprague-Dawley rats weighing 130-150 g were used. 3-MC or phenobarbital was administered by a single i.p. injection in a dose of 20 mg/kg in corn oil or 40 mg/kg in saline, respectively. Animals received saline or DL-ethionine (500 mg/kg, dissolved in saline) 60 and 30 minutes prior to the injection of 3-MC or corn oil. Controls received saline and corn oil injected i.p. The animals were sacrificed 24 hours after the last injection. Livers were perfused with a cold solution of isotonic (1.15%) KCl to remove hemoglobin and were homogenized in 0.25 M sucrose in the cold with a Dounce homogenizer. For the determination of 3,4-benzpyrene hydroxylase activity, 50 μg of the substrate was dissolved in 0.1 ml of acetone and incubated for 15 minutes at 37° in a final volume of 3.1 ml with whole liver homogenate equivalent to 2 mg of wet weight liver. The composition of the incubation mixture and the assay for the hydroxylated metabolites of 3,4-benzpyrene was similar to that described previously (Kuntzman et al., 1966) except that the incubation mixture contained 2 Kornberg units of glucose 6-phosphate dehydrogenase. Liver homogenates were centrifuged at 9000 xg for 20 minutes. Microsomes were prepared by centrifugation of the 9000 xg supernatant at 100,000 xg for 60 minutes. The microsomal pellet was suspended in cold isotonic KCl solution so that each milliliter contained the equivalent of 250 mg of liver and this suspension was used for spectral measurements. The carbon monoxide and the ethyl isocyanide difference spectra were determined using a recording Cary, Model 15, dual beam spectrophotometer, as described previously (Sladek and Mannering, 1966), with

the following modifications: 1 M, instead of 0.1 M, phosphate buffer pH 7.4 and ethyl isocyanide in a final concentration of 4.5 mM were used. Each cuvette contained 1 ml of a dithionite-reduced microsomal preparation and 2 ml of 1.0 M phosphate buffer, pH 7.4.

RESULTS: The CO difference spectra of liver microsomes from untreated rats and rats treated with a single injection of phenobarbital or 3-MC is shown in figure 1. In confirmation of previous work (Garfinkel, 1958; Klingenberg, 1958)

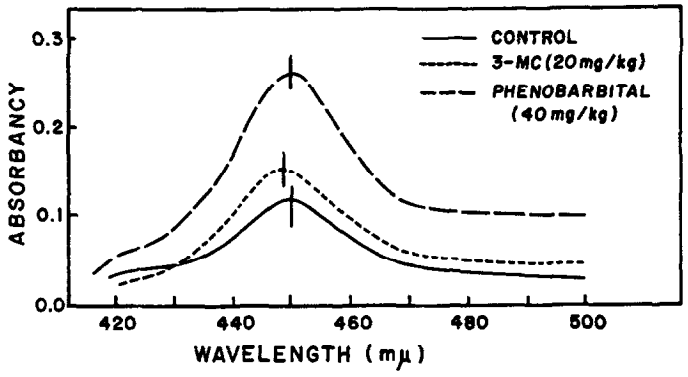

Fig. 1. CO difference spectra of reduced microsomes. Treated rats received a single injection of the drug administered i.p. Rats were sacrificed 24 hours following injection of vehicle or drug.

a difference spectrum with a peak of 450 mμ was observed when CO was bubbled through dithionite-reduced microsomes obtained from untreated rats. A similar peak at 450 mμ was observed with liver microsomes from phenobarbital-treated rats. On the other hand, when rats were treated with 3-MC, a peak shift occurred and the CO-binding pigment now exhibited a maximum absorption at 448 mμ instead of 450 mμ. † These findings suggested that the peak shift may be due to either the binding of the hemoprotein with 3-MC or that the polycyclic hydrocarbon was inducing the synthesis of a hemoprotein, with spectral properties not observed in liver microsomes of untreated rats. In vitro addition

† The ethyl isocyanide difference spectrum of microsomes from rats injected with 3-MC at a dose of 20 mg/kg per day for 3 days, exhibited a spectral shift from 455 mμ to 453 mμ. No such shift occurred with the 430 mμ peak. The shift in the 455 peak was not apparent after a single 3-MC injection.

of 3-MC in a final concentration of 0.30 mM to the microsomal preparation from untreated rats did not cause a spectral shift from 450 mμ to 448 mμ. To determine if 3-MC was indeed causing the induction of a spectrally-different hemoprotein, the effect of ethionine on the induction of 3,4-benzpyrene hydroxylase activity, the levels of the CO-binding pigment and the ethyl isocyanide difference spectral peaks - the 455 and 430 peaks - was studied (Table 1). Administration of 3-MC caused increases in 3,4-benzpyrene hydroxyl-

TABLE 1. Effect of DL-ethionine on 3,4-benzpyrene hydroxylase activity, the CO-binding pigment and the 455 and 430 peaks in methylcholanthrene-treated rats

	Benzpyrene hydroxylase activity [1]	CO-binding pigment [2]	Ethyl isocyanide difference spectra		Ratio of 455:430 peaks
			455 peak [3]	430 peak [4]	
Control	61.6 (± 9.9)	0.067 (±0.010)	0.037 (±0.007)	0.052 (±0.008)	0.71 (±0.03)
Ethionine	70.7 (± 9.6)	0.070 (±0.010)	0.045 (±0.003)	0.065 (±0.007)	0.70 (±0.03)
3-MC	364.3 (±32.3)	0.110 (±0.019)	0.084 (±0.012)	0.057 (±0.011)	1.48 (±0.08)
Ethionine + 3-MC	93.9 (±18.4)	0.068 (±0.004)	0.041 (±0.006)	0.055 (±0.006)	0.74 (±0.08)

3-MC, 20 mg/kg in corn oil, was administered by a single i.p. injection. Animals received saline or DL-ethionine (500 mg/kg) 60 and 30 min prior to the injection of 3-MC or corn oil. Controls received saline and corn oil injected i.p. The animals were killed 24 hours after the administration of 3-MC or corn oil. [1] mμg hydroxybenzpyrene formed/mg/15 minutes; [2] Δ O.D.450-500; [3] Δ O.D.455-500; [4] Δ O.D.430-500. Values represent mean of 4 experiments. Figures in parenthesis represent S.E.

ase activity and in the CO-binding pigment. However, the increase in hydroxylase activity did not bear a quantitative relationship to the increase in the hemoprotein. Administration of ethionine, prior to the injection of 3-MC, prevented the increase in 3,4-benzpyrene hydroxylase activity and also blocked the increase in the CO-binding pigment seen when 3-MC is administered alone. When rats were treated with 3-MC, essentially no increase is seen in the 430 peak, whereas, the 455 peak increased almost three-fold when compared to controls. Thus, the ratio of the 455:430 peaks increased from 0.71 in controls to 1.48 in rats treated with 3-MC. When

ethionine was administered prior to 3-MC, increases in the 455 peak and the ratio of the 455:430 peaks seen in rats treated with 3-MC, were completely blocked.

DISCUSSION: Phenobarbital stimulates the hepatic microsomal metabolism of many drugs; polycyclic hydrocarbons such as 3-MC, selectively stimulate the metabolism of relatively few drugs and do not influence the metabolism of others. These findings suggest that the polycyclic hydrocarbons and drugs, of which phenobarbital is a prototype, may stimulate drug metabolism by different mechanisms. One of the differences between phenobarbital and 3-MC induction is the effect on microsomal hemoprotein. Recent evidence (Alvares et al., 1967) indicates that induction of enzyme activity by 3-MC in microsomal subfractions paralleled increases in the 455 peak when the ethyl isocyanide difference spectra were studied. Furthermore, experiments in our laboratory, the results of which are presented in this paper, show that 3-MC induction results in a shift of maximum absorption from 450 mμ to 448 mμ when CO is used as the ligand. This peak shift can be prevented if ethionine is administered prior to 3-MC. It would, therefore, be a misnomer to abbreviate the CO-binding pigment as P-450 in 3-MC induced rats. The observation that ethionine, an inhibitor of protein synthesis, blocks the effect of 3-MC on the spectra of microsomal cytochromes suggests that 3-MC induces the formation of a microsomal cytochrome with spectral characteristics different from those observed in liver microsomes obtained from control or phenobarbital-treated rats. It might be proposed that the 430 and 455 peaks observed in the presence of ethyl isocyanide may represent two different hemoproteins (Sladek and Mannering, 1966) and that 3-MC induces the hemoprotein associated with the 455 peak.

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