DIFFERENCES IN THE KINETICS OF BENZPYRENE HYDROXYLATION BY HEPATIC DRUG-METABOLIZING ENZYMES FROM PHENOBARBITAL AND 3-METHYLCHOLANTHRENE-TREATED RATS

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Inducers of hepatic drug-metabolizing enzymes in liver microsomes can readily be categorized into two groups (1) inducers that stimulate the metabolism of many drugs and (2) inducers that stimulate the metabolism of only a few drugs. Phenobarbital (PB) is a prototype of the first class of inducers and 3-methylcholanthrene (3-MC) is an example of the second class. Evidence for more than one mechanism for the stimulation of drug metabolism by these two classes of inducers has recently been comprehensively reviewed by Conney (1967) and Mannering (1967). Studies on the effect of PB and 3-MC on testosterone hydroxylation revealed that the administration of PB to adult male rats increased the 7α-, 16α- and 6β-hydroxylation of testosterone. In contrast, 3-MC treatment stimulated the formation of 7α-hydroxytestosterone, but markedly inhibited the rate of 16α-hydroxylation and had little or no effect on the formation of 6β-hydroxytestosterone (Kuntzman et al., 1967). SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate), an inhibitor of many drug metabolizing enzymes, inhibits the N-demethylation of 3-methyl-4-methylaminoazobenzene by hepatic microsomes from non-treated rats, but has little effect on the metabolism of the aminoazo dye by microsomes from rats that have been treated with 3-MC (Sladek and Mannering, 1966). Furthermore, 3-MC induces the formation of a microsomal cytochrome with spectral characteristics different from those observed in liver microsomes obtained from control or PB-

treated rats (Alvares et al., 1967; Kuntzman et al., 1967). Studies on the intramicrosomal distribution of drug-metabolizing enzymes in rabbits show that the increase in enzyme activity caused by PB treatment occurs in both the rough-surfaced and smooth-surfaced microsomal subfractions while the increase in enzyme activity caused by treatment with 3-MC occurs in one microsomal subfraction or the other, but not in both fractions (Gram et al., 1967).

PB and 3-MC treatment are known to stimulate the activity of benzpyrene hydroxylase in liver microsomes (Conney et al., 1957, 1960) but the kinetic properties of the induced enzyme have not been measured. The present investigation was undertaken in an attempt to assess whether pretreatment of rats with PB or 3-MC causes a quantitative and/or qualitative change in the enzyme system involved in the hydroxylation of 3,4-benzpyrene by rat liver. The kinetics of 3,4-benzpyrene hydroxylation were compared in untreated, PB- and 3-MC-treated rats.

METHODS: Male Sprague-Dawley rats weighing 130-150 g were used. 3-MC (20 mg/kg) dissolved in corn oil was administered by a single i.p. injection. Sodium phenobarbital dissolved in saline was administered i.p. at a dose of 40 mg/kg once daily for 4 days. All animals were sacrificed 24 hours after the last injection. Livers were removed and homogenized in 0.25 M sucrose in the cold with a Dounce homogenizer. Liver homogenates were centrifuged at 9000 x g for 20 minutes. The homogenates or 9000 x g supernatant fractions were diluted with 0.25 M sucrose such that each ml contained an equivalent of 20 mg or 40 mg, respectively, of wet weight liver. Varying concentrations of 3,4-benzpyrene were used for the determination of 3,4-benzpyrene hydroxylase activity. The composition of the incubation mixture and the assay for the hydroxylated metabolites of 3,4-benzpyrene have been described previously (Kuntzman et al., 1966; Alvares et al., 1967). Each incubation flask contained homogenate equivalent to 2 mg of liver, wet weight, or 9000 x g supernatant equivalent to 4 mg of liver, wet weight, and were incubated at 37° for 5 minutes. The data were plotted according to the method of Lineweaver

and Burk (1934) and were analyzed statistically by use of the Student's "t" test (Snedecor, 1956).

RESULTS: Liver homogenates obtained from untreated rats or from rats to which either PB or 3-MC was administered, were incubated with 2,4,6,8 and 10 μg of 3,4-benzpyrene and a NADPH-generating system as previously described and the rate of formation of 8-hydroxy-3,4-benzpyrene was determined. Figure 1 shows the data, which were obtained in these studies, plotted

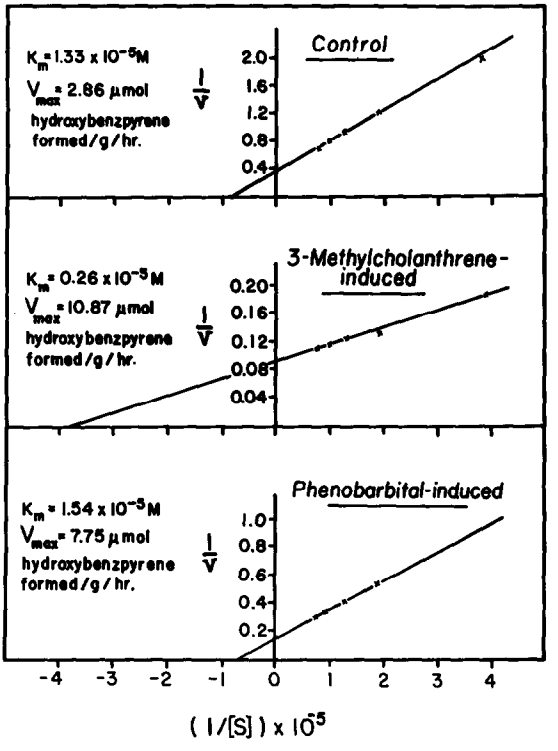

Fig. 1. 1/v versus 1/S plot for the hydroxylation of 3,4-benzpyrene by rat liver. Velocities are given as μmol hydroxybenzpyrene formed/g liver/hr; substrate concentrations are in moles/liter. Each point represents the mean value of 5 different experiments. Each experiment was done with a different rat.

according to the method of Lineweaver and Burk (1934). There was a marked difference in the kinetic constants found for the 3,4-benzpyrene hydroxylase system in liver homogenates from normal and PB-induced rats when compared with those constants obtained from 3-MC-induced rats. The Michaelis constant (Km) for the hydroxylation of 3,4-benzpyrene by liver from untreated rats

(1.33×10^{-5}M) was not significantly different ($P > 0.70$) from that obtained with liver from rats treated with PB (1.54×10^{-5}M); however, it was significantly different ($P < 0.01$) from the Km value (0.26×10^{-5}M) obtained for the hydroxylation of 3,4-benzpyrene by liver from 3-MC-treated rats. The maximal velocity (V_{max}) was increased 4-fold after a single injection of 3-MC and 2.7-fold in rats treated with PB for four days.

Similar differences in kinetic constants were obtained using the 9000 xg supernatant which contains the microsomal plus soluble fractions (Table 1).

Table 1

Effect of 3-MC pretreatment on the kinetics of 3,4-benzpyrene hydroxylation by rat liver 9000 xg supernatant fraction

Treatment	Km $\times 10^{+5}$M	V_{max} μmol hydroxybenzpyrene formed/g/hr
Control	1.41 ± 0.30[1]	1.88 ± 0.20
3-MC	0.20 ± 0.02[2]	4.10 ± 0.35[2]

3-MC, 20 mg/kg in corn oil was administered by a single i.p. injection. For the analysis of enzyme kinetic data, all calculations were performed with a GE 265 computer using a FORTRAN program written by Cleland (1963).

[1] Each value represents the mean ± S.E. from 4 experiments, each with a different rat.

[2] Values significantly different from control values ($P < 0.01$).

The Km for the hydroxylation of 3,4-benzpyrene by the 9000 xg supernatant from untreated rats (1.41×10^{-5}M) was significantly different ($P < 0.01$) from that obtained with rats treated with 3-MC (0.20×10^{-5}M). The Km values obtained with 9000 xg supernatant were similar to those obtained with whole liver homogenates. It would have been desirable to use a more purified 3,4-benzpyrene hydroxylating system from liver microsomes, but unfortunately, the oxidative enzymes in liver microsomes which metabolize steroids and drugs have resisted all attempts at solubilization and purification. There-

fore, the Km values presented in this paper may be influenced by non-specific binding (Gillette, 1963) and by the transport of the substrate to the enzyme. Under the conditions of the assay, the rate of hydroxylation of 3,4-benzpyrene was linear for 12.5 minutes using homogenates from untreated and PB-treated rats and linear for 7.5 minutes using homogenates from 3-MC-treated rats. After these times, a marked decrease in the rate of hydroxylation was observed.

DISCUSSION: Evidence to support the view that different mechanisms are involved in producing the inductive effects seen when PB and 3-MC are administered is the effect of these inducers on microsomal hemoproteins which have been implicated as a component of the drug-metabolizing enzyme system in liver microsomes. 3-MC induction results in a shift of maximum absorption of the microsomal hemoprotein from 450 $m\mu$ to 448 $m\mu$ when CO is used as the ligand for the reduced hemoprotein (Alvares et al., 1967; Kuntzman et al., 1967). Furthermore, when ethyl isocyanide was used as a ligand instead of CO, 3-MC was shown to induce the hemoprotein associated with an absorption maximum at 455$m\mu$ and not at 430$m\mu$ (Sladek and Mannering, 1966a; Alvares et al., 1967). PB induces both the 455 and 430 peaks and does not cause a shift in the 450$m\mu$ peak (Alvares et al., 1967a). These results indicate that unlike PB, 3-MC causes a qualitative change in the CO-binding hemoprotein of hepatic microsomes. If the hemoprotein is a component of the drug-metabolizing enzyme system, and it is only quantitatively increased by an inducing agent, one should then see only an increase in the V_{max}, but no change in the Km of the hydroxylation of 3,4-benzpyrene. This type of induction is seen in the studies which indicate that PB treatment did not significantly change the Km for the hydroxylation of benzpyrene. Other workers have reported no change in Km after PB-treatment for procaine esterase (Remmer, 1962) for O-demethylation of p-nitroanisole (Netter and Seidel, 1964) for N-demethylation of ethylmorphine, for oxidation of hexobarbital and for sulfoxidation of chlorpromazine (Rubin et al., 1964) though the V_{max} values increased

after induction. Similarly, treatment of rats with 3,4-benzpyrene increased the V_{max} for p-nitroanisole O-demethylation without influencing the Km of the demethylase (Netter and Seidel, 1964). However, if the inducing agent produces a qualitative change in the nature of the hemoprotein that is functional with respect to 3,4-benzpyrene hydroxylation, a change in the Km for the hydroxylation reaction would occur. The present studies show that pretreatment with 3-MC does result in a significant lowering of the Km for the hydroxylation of 3,4-benzpyrene suggesting that 3-MC induced the formation of a hydroxylase with greater affinity for the substrate than the enzyme that was normally present in the liver. These studies demonstrate that differences in the inducing properties of the barbiturates and the polycyclic hydrocarbons are not due solely to the amount of enzyme stimulated, but may also be due to qualitative changes occurring in the new enzyme synthesized.

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