EVIDENCE FOR A NEW P-450 HEMOPROTEIN IN HEPATIC MICROSOMES FROM METHYLCHOLANTHRENE TREATED RATS

N. E. Sladek and G. J. Mannering

Department of Pharmacology, University of Minnesota

Minneapolis, Minnesota 55455

Received July 20, 1966

Previous work from this laboratory (Sladek and Mannering, 1966) suggested that the de novo 3-methyl-4-monomethylaminoazobenzene (3-CH_3-MAB) N-demethylase that results when methylcholanthrene (MC) is administered to rats may be different from that found in normal rats and in rats whose N-demethylase has been increased as a result of treatment with sodium phenobarbital (PB). This conclusion was based on the finding that 2-diethylaminoethyl 2,2-diphenylvalerate (SKF 525-A) in low concentration (4×10^{-5} M) inhibited the N-demethylation of 3-CH_3-MAB by the enzyme system from normal and PB treated rats and the N-demethylation of ethylmorphine (EM) from normal, PB and MC treated rats, but not the N-demethylation of 3-CH_3-MAB by the enzyme system from MC treated rats. Two-substrate kinetic studies employing EM and 3-CH_3-MAB supported this conclusion.

Considerable evidence has been presented for the participation of the hemoprotein, P-450, in the NADPH dependent microsomal systems responsible for the metabolism of many drugs (Cooper et al., 1965; Ernster and Orrenius, 1965; Remmer and Merker, 1965). Recently, Imai and Sato (1966a) suggested the existance of two forms of P-450. Using ethyl isocyanide rather than carbon monoxide as the ligand for reduced hemoprotein, they observed Soret peaks at 430 and 455 mu. The relative size of the two peaks was pH dependent and the authors concluded that "microsomal P-450, at least in the reduced state, exists in two interconvertible forms which are in a pH-dependent equilibrium."

If this is indeed the case, then a change in the protein moiety of P-450 could result in a change in the pH dependent equilibrium of the interconvertible forms of P-450. Specifically, if MC causes such a change, a shift in the relative heights of the 430 and 455 Soret peaks at a given pH might result. The current studies show that such a shift does occur, suggesting that MC treatment causes the formation of a new hemoprotein (P_1-450). Both P-450 and P_1-450, when in reduced form, appear to exist in two interconvertible forms which are in a pH-dependent equilibrium. These studies also strengthen the view that P-450 plays an important role in the metabolism of drugs.

METHODS. Male, Holtzman rats weighing 90 to 100 g were divided into five groups of four animals each, which received daily intraperitoneal injections of the following materials for four days (mg/kg): 1) saline; 2) PB, 40; 3) MC, 20; 4) PB, 40 + MC, 20; 5) morphine sulfate, 20. Morphine was included in the series because it causes a decrease in the enzymes responsible for the N-demethylation of EM and other narcotic drugs (Axelrod, 1956; Mannering and Takemori, 1958). Livers were removed 20 hrs after the last injection, perfused to remove hemoglobin and processed as described previously (Rubin et al., 1964) to obtain microsomal fractions (100,000 x g) for the spectral measurements and microsomal plus soluble fractions (9,000 x g) for the determination of EM N-demethylase and 3-CH_3-MAB N-demethylase activities. The composition of the incubation mixture has been described previously (Rubin et al., 1964). Incubation times and enzyme concentrations were varied to preserve linearity of reaction rates. EM and 3-CH_3-MAB were employed in substrate concentrations of 2×10^{-3} M and 2×10^{-4} M, respectively. When 3-CH_3-MAB was the substrate, reaction rates were determined by measuring the amount of HCHO formed using the chromotropic acid procedure described previously (Takemori and Mannering, 1958). When EM was the substrate, HCHO was measured by a modified procedure of Nash (Anders and Mannering, 1966). The carbon monoxide difference spectrum of the various microsomal preparations was determined using a recording Beckman Model DB dual beam spectrophotometer. Each cuvette (1 cm light path)

contained microsomal preparation equivalent to 250 mg of liver, 0.2 M phosphate buffer, pH 7.4, and a few milligrams of dithionite in a final volume of 3 ml (final pH, 7.0). Carbon monoxide was bubbled into one of the cuvettes for 30 sec. The difference in absorption, 450 - 500 mu, was used as the estimate of P-450. The ethyl isocyanide difference spectra was determined in a similar manner except that ethyl isocyanide (final concentration, 3.45 mM) was used as the ligand instead of CO. The differences between absorption at 430 mu and 500 mu and between 455 mu and 500 mu were used as estimates of the 430 and 455 peaks, respectively.

RESULTS. In Table 1 it can be seen that when rats were treated with PB, percentage increases in all six categories of measurement were approximately equal. Thus, not only did the increases in EM N-demethylase and 3-CH_3-MAB N-demethylase activities parallel the increase in P-450, but the increases in

TABLE 1. Changes in microsomal hemoprotein levels and N-demethylase activities in response to phenobarbital, methylcholanthrene and morphine treatments

Measurement \ Treatment	Phenobarbital	Methylcholanthrene	Methylcholanthrene + Phenobarbital	Morphine
430 peak[1] (% of control)	336 (±17)	140 (±14)	378 (±71)	65 (±14)
455 peak[2] (% of control)	386 (±29)	400 (±61)	723 (±135)	68 (±13)
430 peak + 455 peak[3] (% of control)	353 (±20)	226 (±28)	488 (±98)	66 (±14)
P-450[4] (% of control)	353 (±15)	251 (±42)	547 (±108)	72 (±14)
3-CH_3-MAB N-demethylase activity[5] (% of control)	309 (±21)	441 (±32)	784 (±38)	62 (±10)
EM N-demethylase activity[6] (% of control)	325 (±10)	107 (±4)	377 (±41)	59 (±4)

[1] $\Delta OD_{430-500}$; Control: 0.075. [2] $\Delta OD_{455-500}$; Control: 0.037. [3] ΔOD as in [1] and [2]; Control: 0.112. [4] $\Delta OD_{450-500}$; Control: 0.085. [5] umol HCHO formed/g of liver/hr; Control: 2.90. [6] umol HCHO formed/g of liver/hr; Control: 8.10. Figures in parentheses represent S.E.

both 430 and 455 peaks were also of equivalent proportions. The same proportionalities between the N-demethylase activities and spectral changes were maintained when enzyme activity and hemoprotein were depressed as a result of morphine treatment. On the other hand, when rats were treated with MC, the parallelisms observed in PB and morphine treated rats were not seen. Essentially no increases in EM N-demethylase or the 430 peak were observed, whereas 3-CH_3-MAB N-demethylase and the 455 peak were increased to a similar degree. Thus the ratios of the 455:430 peaks were very similar in normal, PB treated and morphine treated groups (0.49, 0.56 and 0.51, respectively) whereas in the group that received MC the ratio was much greater (1.37). Unlike in the studies where PB and morphine were employed, neither the increase in the 430 peak nor the increase in the 455 peak bears a quantitative relationship to the increase in P-450 that results from MC treatment. However, the increases in P-450 are about equal to the sums of the increases in the 430 and 455 peaks, regardless of treatment, whether it be PB, morphine or MC. This observation strengthens the view that P-450 and the pigments represented by the 430 and 455 peaks are derived from the same precursor. It also supports the suggestion of Imai and Sato (1966a) that the 430 and 455 peaks represent two rather than one form of the hemoprotein. The results obtained when rats were treated with both PB and MC were what would be predicted from results obtained from animals treated singly with these compounds.

Imai and Sato (1966a), in developing their concept of a pH dependent interconvertible hemoprotein, plotted the heights of the 430 and 455 peaks at different pH's. The curves intercepted at about pH 7.4. This intercept should be a function of the pH dependent equilibrium constant. When similar curves were constructed (Fig. 1) for the 430 and 455 peaks obtained with microsomes from normal, PB and MC treated rats, the intercepts derived from microsomes from normal and PB treated rats were the same as that observed by Imai and Sato, who used microsomes from untreated rabbits. The microsomes from MC treated rats gave an intercept at pH 6.9, which reflects a pH

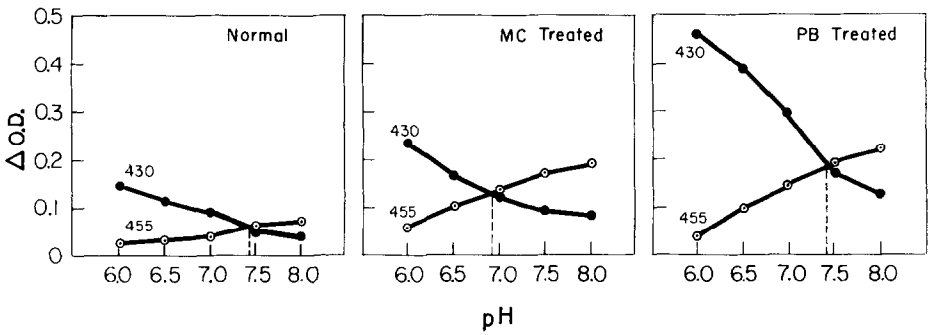

Fig. 1. Dependence of isocyanide difference spectra on pH using microsomes from normal rats and rats treated with MC and PB. Methodology was the same as that described in the text for the data given in Table 1 except that 1.0 M rather than 0.2 M phosphate buffer was employed.

dependent equilibrium constant quite different from that seen with normal microsomes. This is interpreted as strong evidence for a new hemoprotein. The intercept observed with microsomes from MC treated rats must result from a mixture of the original and the new hemoprotein. If the interconvertibility of the new hemoprotein could be measured without comtamination by the original hemoprotein, the intercept would occur at a pH lower than 6.9.

DISCUSSION. These results are subject to several interpretations. One interpretation assumes a single hemoprotein in normal and PB treated rats which is involved in the N-demethylation of both EM and 3-CH_3-MAB. The reaction involving this hemoprotein is inhibited by SKF 525-A. The hemoprotein may or may not be interconvertible to two forms in its original state, but after reacting with ethyl isocyanide, it is distinguishable in two forms by peaks at 430 mu and 455 mu. MC treatment results in the formation of a new hemoprotein which contributes to 3-CH_3-MAB N-demethylase activity, but which does not function or functions poorly in the N-demethylation of EM. The reaction involving this new hemoprotein is not inhibited by SKF 525-A.

A second interpretation insists not only on the formation of a new hemoprotein as the result of MC treatment, but also on the association of each of the two interconvertible forms with a given type of drug metabolism. Thus

the form associated with the 430 peak is involved in a type of drug metabolism represented by the N-demethylation of EM, whereas the form associated with the 455 peak is involved in a second type of drug metabolism, in this case, the N-demethylation of 3-CH_3-MAB. This concept conforms with the observations of Remmer et al., (1966) and Imai and Sato (1966b) who found that drugs can be divided into two groups depending upon the spectral changes that occur when these drugs combine with microsomal hemoprotein.

A third interpretation would deny the interconvertability of the hemoprotein in its natural state. Accordingly, the 430 and 455 peaks would represent different hemoproteins, each involved in a different type of drug metabolism, or at least one being more involved with one type of drug metabolism than the other.

Because the N-demethylation of EM was not increased after MC treatment while levels of P-450 were increased, it was concluded in a previous study (Sladek and Mannering, 1966) that P-450 is not rate limiting in the overall reaction involving the N-demethylation of EM in normal microsomes. The current findings provide a basis for other interpretations. In Table 1 it can be seen that whereas MC produced an increase in P-450, it caused little increase in the hemoprotein represented by the 430 peak. Thus, if the hemoprotein associated with the 430 peak is involved in the N-demethylation of EM and the hemoprotein associated with the 455 peak is not, then in effect, MC has not increased the amount of hemoprotein that is functional with respect to EM N-demethylation. This can also be interpreted to mean that the increase in P-450 seen after MC treatment is due to an increase in P_1-450, which functions in the N-demethylation of 3-CH_3-MAB, but not in the N-demethylation of EM.

ACKNOWLEDGMENTS: This research was supported by USPHS grant No. GM-12543. The authors gratefully acknowledge the able technical assistance of Mrs. Sheila Ham, Miss Janice Holen and Miss Margaret Kuhfeld. We are grateful to Mr. Don Shoeman for the synthesis of ethyl isocyanide.

References

Anders, M. W. and Mannering, G. J., Mol. Pharmacol., in press, July, 1966.
Axelrod, J., Science, 124, 263, 1956.
Cooper, D. Y., Levin, S., Narasimhulu, S. and Rosenthal, O., Sciences, 147, 400 (1965).
Ernster, L. and Orrenius, S., Federation Proc., 24, 1190 (1965).
Imai, Y. and Sato, R., Biochem. Biophys. Res. Commun., 23, 5 (1966a).
Imai, Y. and Sato, R., Biochem. Biophys. Res. Commun., 22, 620 (1966b).
Mannering, G. J. and Takemori, A. E., J. Pharmacol. Exptl. Therap., 127, 187 (1959).
Remmer, H. and Merker, H. J., Ann. N. Y. Acad. Sci., 123, 79 (1965).
Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillette, J., Narasimhulu, S., Cooper, D. Y. and Rosenthal, O., Mol. Pharmacol., 2, 187, (1966).
Rubin, A., Tephly, T. R. and Mannering, G. J., Biochem. Pharmacol., 13, 1007 (1964).
Sladek, N. E. and Mannering, G. J., Federation Proc., 25, 418 (1966).
Takemori, A. E. and Mannering, G. J., J. Pharmacol. Exptl. Therap., 123, 171 (1958).