Studies on Hepatic Microsomal *N*- and *O*-Dealkylation of Morphine Analogues

W. J. GEORGE AND T. R. TEPHLY

Department of Pharmacology, The University of Michigan Medical School, The University of Michigan, Ann Arbor, Michigan 48104

(Received March 12, 1968)

SUMMARY

N-Dealkylation of ethylmorphine and *O*-dealkylation of norcodeine were studied in hepatic microsomes from male rats. Both reactions appeared to be equally sensitive to carbon monoxide, and the inhibitions produced by CO were light-reversible. Chronic phenobarbital administration stimulated the *N*-dealkylation of ethylmorphine but had no effect on the *O*-dealkylation of norcodeine in hepatic microsomes. Hexobarbital produced a competitive inhibition of ethylmorphine oxidation but effected an inhibition of norcodeine oxidation that was not competitive. Ethylmorphine *N*-dealkylation was more labile to heat treatment than was norcodeine *O*-dealkylation. Fasting-adapted rats that received food for 12 hr out of each 48-hr period for 28 days yielded hepatic microsomes which displayed an increased ethylmorphine oxidation with no change in norcodeine oxidation and no change in hepatic cytochrome P-450 content. These studies indicate different rate-controlling events in the oxidation of ethylmorphine and norcodeine, although each reaction depends upon the participation of cytochrome P-450.

INTRODUCTION

An organized sequence of electron transfer has been shown to function in the 11β-hydroxylation of steroids by adrenal mitochondria (1) and has been proposed to function in the oxidation of drugs by hepatic microsomes (2). In this chain of electron transport a unique hemoprotein, cytochrome P-450, has been postulated to represent an oxygen-activating factor within the hepatic microsomes and to function as the terminal oxidase for mixed function oxidations of drugs, steroids, and carcinogens (3). This might explain why certain drugs and steroids of diverse chemical structure produce mutually competitive inhibition of their metabolism (4) and why agents such as phenobarbital exert an induction of the metabolism of many drugs. Phenobarbital is known to effect an increase in the activity of hepatic microsomal P-450 (5) and NADPH-cytochrome *c* reductase, which may function as a P-450 reductase (2).

However, correlation of all drug oxidation with P-450 content has not always been possible. The administration of 3-methylcholanthrene increases the level of cytochrome P-450 in microsomes, whereas the metabolism of certain substrates is not affected (6). Phenobarbital pretreatment increases the *N*-demethylation of meperidine and methylbarbital, but decreases or has no effect on the demethylation of codeine or methoxyacetanilide (7). In certain rabbit strains, phenobarbital does not stimulate the dealkylation of codeine or the hydroxylation of benzpyrene (8). In addition, there have been numerous reports of species (9) and sex differences (10) which support the view that different mechanisms or enzymes may be involved in the metabolism of various drugs.

Recently, microsomal *N*- and *O*-dealkylation reactions have been studied in this laboratory using analogues of morphine as substrates. Although certain similarities exist for these systems, experiments have

Mol. Pharmacol. 4, 502–509 (1968)

been performed which permit a differentiation between the *N*- and *O*-dealkylation of morphine analogues. These studies form the basis of this report. Certain results have appeared in preliminary form (11).

MATERIALS AND METHODS

Animals. Holtzman rats were used throughout these studies. In certain experiments male rats (200–210 g) were given food for the first 12 hr and fasted for the remaining 36 hr out of every 48-hr period for 28 days. Water was administered ad libitum during this period, and the animals were killed 36 hr after the last feeding period. Livers were quickly excised for the preparation of microsomes.

Preparation of microsomes. Hepatic homogenates were prepared using ice-cold 0.25 M sucrose solutions containing 1 mM disodium EDTA. The homogenate was centrifuged at 12,500 *g* for 20 min at 0–4°. The supernatant fraction was then centrifuged at 104,000 *g* for 90 min in a Spinco model L ultracentrifuge. The microsomal pellets were resuspended with sucrose–EDTA to a volume such that each milliliter contained 5 mg of protein or microsomes from 250 mg of liver. The protein concentration was determined by the biuret method (12).

Determination of drug metabolism. The *N*-dealkylation of ethylmorphine and the *O*-dealkylation of norcodeine were performed in incubation mixtures described by Rubin *et al.* (4), except that nicotinamide was omitted from reaction mixtures and formaldehyde was measured by a modification of the method of Nash (13). The *O*-dealkylation of ethylmorphine and of phenacetin was determined by measurement of acetaldehyde production by a modification of the method of Stotz (14).

When experiments were conducted in the presence of carbon monoxide, 25-ml Erlenmeyer flasks equipped with ground glass tops and side arms were used. Flasks were placed on Warburg manometers, and the side arms had rubber tubing attached to them which could easily be clamped. Mixtures containing all reactants except substrate were first incubated at 25° for 3 min in vessels attached to the Warburg manometers. Mixtures of gases were obtained by using a Heidbrink Kinet-O-Meter anesthesia machine equipped with a reservoir bag in which gases were mixed prior to flow to the reaction vessels. A constant concentration of oxygen (20%, v/v) was used throughout, and the concentrations of nitrogen and carbon monoxide were varied. The concentration of carbon monoxide was determined by infrared analysis of the gas mixture (15, 16). At the end of the preliminary incubation period, reactions were started by the injection of substrate through the rubber tubing on a side arm and drug oxidations were determined as described previously with reactions carried out at 37° in a large glass water bath. These reactions were carried out in the dark or in the presence of a strong light source supplied from the lamp (1000-W) of a slide projector. Filtered light was obtained by placing a Corning narrow band interference filter which had a maximum transmission band at 450 mμ in front of the light source. The half-bandwidth of this filter was ±10 mμ.

Kinetic analysis. When data were plotted by the Lineweaver and Burk method (17), lines were drawn with the aid of statistical analyses provided by Wilkinson (18).

Determination of cytochrome P-450. Microsomal suspensions were diluted in 0.1 M phosphate buffer (pH 7.4) to contain 1–2 mg of protein per milliliter. Sodium dithionite (1–2 mg) was added to the diluted preparation, and 1.0-ml aliquots were added to each of two 1-ml cuvettes. Carbon monoxide was bubbled through one of the samples for 30 sec, the cuvettes were capped, and optical densities were determined at 450 and 490 mμ. P-450 content was expressed as $OD_{450-490}$/mg of protein per milliliter.

RESULTS

Effect of carbon monoxide on the N- and O-dealkylation of morphine analogues. Carbon monoxide has been shown to inhibit drug oxidations in hepatic microsomes by combination with cytochrome P-450 (3), and this interaction of P-450

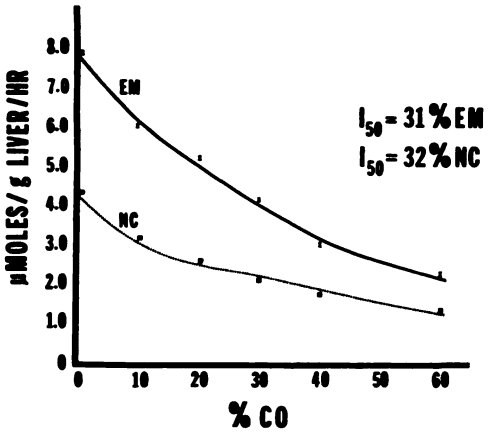

FIG. 1. *Effect of carbon monoxide on norcodeine (NC) and ethylmorphine (EM) oxidation*

Norcodeine oxidation (dashed line) and ethylmorphine oxidation (solid line) were carried out under an atmosphere of 20% oxygen with various concentrations of carbon monoxide and nitrogen. Rates are expressed as micromoles of formaldehyde produced per gram of liver per hour.

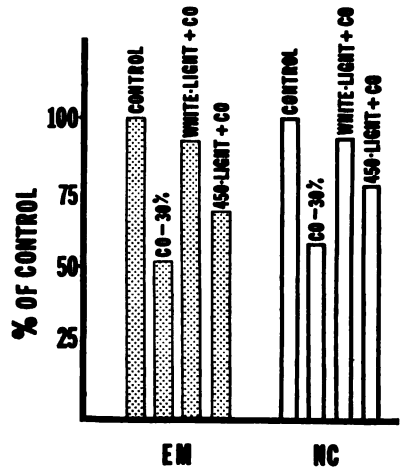

FIG. 2. *Effect of light on carbon monoxide inhibition of ethylmorphine (EM) and norcodeine (NC) oxidation*

Incubation atmospheres consisted of nitrogen, 50%; oxygen, 20%; and carbon monoxide, 30%. Control reactions were performed in the dark, and white light was provided by a 1000-W lamp from a slide projection system. A narrow band interference filter was used to obtain light at 450 mμ.

with carbon monoxide was competitive with oxygen. Figure 1 shows the effect of carbon monoxide on the *N*-dealkylation of ethylmorphine and on the *O*-dealkylation of norcodeine in rat hepatic microsomes. Both reactions were inhibited by carbon monoxide, and the 50% inhibitory (I_{50}) values obtained when ethylmorphine was used as substrate were in good agreement with those obtained when norcodeine was the substrate. This would indicate that both reactions utilize cytochrome P-450 and that the same hemoprotein participates in both reactions.

Figure 2 shows that light reverses the carbon monoxide inhibition of both ethylmorphine and norcodeine oxidation. When white light was supplied, almost complete restoration of drug oxidations was observed. Light at 450 mμ was less effective in restoring the drug oxidations. It is possible that insufficient light intensity was obtained after filtering. Both restorations were statistically significant ($p < .05$).

Effect of phenobarbital on the oxidation of ethylmorphine and norcodeine in hepatic microsomes. Phenobarbital is known to stimulate the metabolism of many drugs by endoplasmic reticulum of liver when it is administered chronically (19–21). It can be seen in Fig. 3 that microsomes derived from rats treated with phenobarbital for 4 days prior to death had a greater capacity to metabolize ethylmorphine than microsomes from untreated animals. Livers from these animals also showed a 3.5-fold increase in their content of cytochrome

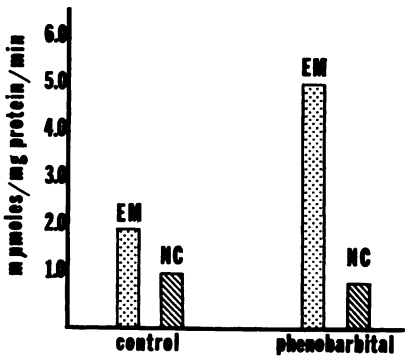

FIG. 3. *Effect of phenobarbital on ethylmorphine (EM) and norcodeine (NC) oxidation*

Rats received single daily intraperitoneal injections of saline or phenobarbital sodium (40 mg/kg) for 4 successive days and were killed 24 hr after the last injection.

TABLE 1
Cytochrome P-450 content in microsomes from saline- and phenobarbital-treated rats

Conditions for these experiments were as described for Fig. 3. Each value represents the mean of seven experiments ± the standard error.

Treatment	P-450 ($OD_{450-490}$/mg protein/ml)
Saline	0.056 ± 0.011
Phenobarbital	0.194 ± 0.052

P-450 (Table 1). However, no increase in norcodeine dealkylation occurred. The oxidation of ethylmorphine and norcodeine in hepatic microsomes from phenobarbital-treated rats displayed the same sensitivity to carbon monoxide that was observed for hepatic microsomes of untreated rats (Fig. 1).

The *O*-dealkylation of ethylmorphine and the *O*-dealkylation of phenacetin were also studied in hepatic microsomes from phenobarbital-treated rats. When the *O*-dealkylation of ethylmorphine was measured, no increase in oxidation rates in microsomes obtained from phenobarbital-treated rats was observed (Table 2). Therefore, it appears that the *O*-dealkylation of norcodeine and the *O*-dealkylation of ethylmorphine proceed through similar enzymic mechanisms. However, the *O*-dealkylation of phenacetin was increased to about the same extent as that observed for the *N*-dealkylation of ethylmorphine. This suggests that there are two enzymic systems in hepatic microsomes concerned with *O*-dealkylation.

TABLE 2
Effect of phenobarbital on the O-dealkylation of ethylmorphine and phenacetin

Conditions were as described for Fig. 3. Each value represents the mean of four experiments ± the standard error.

Substrate	Activity (μmoles/g liver/hr)	
	Saline	Phenobarbital
Ethylmorphine	4.63 ± 0.32	4.71 ± 0.15
Phenacetin	3.18 ± 0.78	7.87 ± 1.81

Inhibition of ethylmorphine and norcodeine oxidation by hexobarbital. It has been shown that hexobarbital competitively inhibits the oxidation of ethylmorphine in rat hepatic microsomes (4). If norcodeine and ethylmorphine utilized the same rate-limiting step in their oxidative metabolism, one would expect hexobarbital to inhibit the *O*-dealkylation of norcodeine competitively. Figure 4 shows that whereas hexobarbital did produce a competitive inhibition of ethylmorphine oxidation, a different inhibitory pattern was obtained when norcodeine was the substrate. This would indicate that different rate-limiting events control the oxidation of these substrates or that an alternative mechanism operates in the *O*-dealkylation of norcodeine.

Differential lability of microsomal oxidative systems. In the course of these experiments it was noted that upon storage of microsomes at 0–5° the system concerned with norcodeine oxidation was less labile than that involved with ethylmorphine oxidation. This differential lability was also observed if microsomal preparations were incubated at various temperatures prior to assay for oxidation of ethylmorphine or norcodeine. Results of such experiments are presented in Fig. 5. When microsomal preparations were incubated at 45° prior to study, ethylmorphine oxidation was more rapidly diminished than was norcodeine oxidation. Indeed, there is a suggestion that, after 5 min preliminary incubation, norcodeine oxidation may even be stimulated. These results further indicate that norcodeine and ethylmorphine oxidations have different rate-limiting steps in their oxidative sequence, or that a different system is involved. There was no correlation between the loss of drug-oxidizing activity and decreases in cytochrome P-450. Cytochrome P-450 measurements remained virtually unchanged for 15 min, with a maximal decrease of about 48% seen after 60 min preliminary incubation at 45° (Table 3).

Oxidation of ethylmorphine and norcodeine in fasting-adapted rats. Certain feeding schedules have been shown to affect

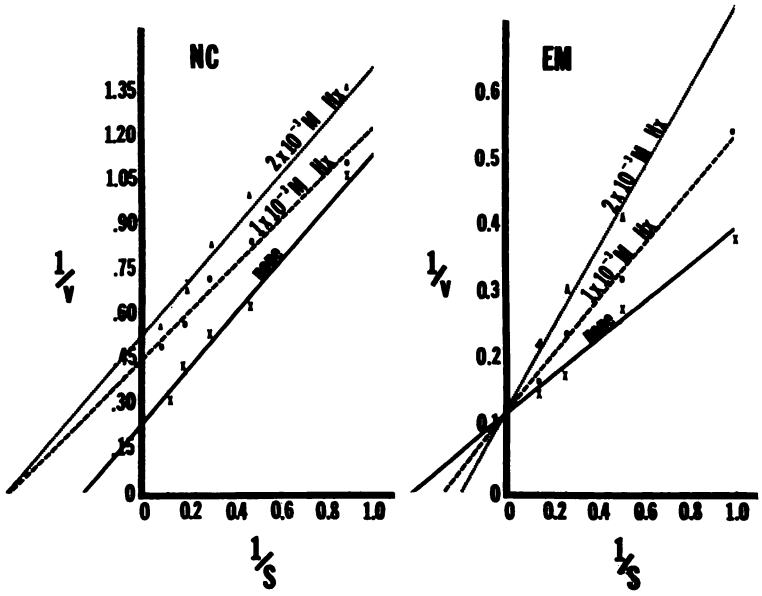

FIG. 4. *Effect of hexobarbital on ethylmorphine (EM) and norcodeine (NC) oxidation*

S = molarity $\times 10^{-4}$; v = velocity at given substrate concentration in micromoles of formaldehyde formed per gram of liver per hour. Sodium hexobarbital (*Hx*) concentrations were 1×10^{-3} M and 2×10^{-3} M.

enzyme synthesis. Potter *et al.* (22) have described the effects of adaptation in rats that were fed for 12 and fasted for 36 hr out of every 48-hr period. Figure 6 shows results from hepatic microsomes derived from rats which had been on such a feeding schedule for 28 days. It can be seen that ethylmorphine oxidation was greatly stimulated in microsomal preparations from fasting-adapted rats whereas norcodeine oxidation was not increased significantly. It is interesting that cytochrome P-450 values were not significantly different in these preparations when compared to those from normal rats. These results indicate that dietary manipulation can differentially

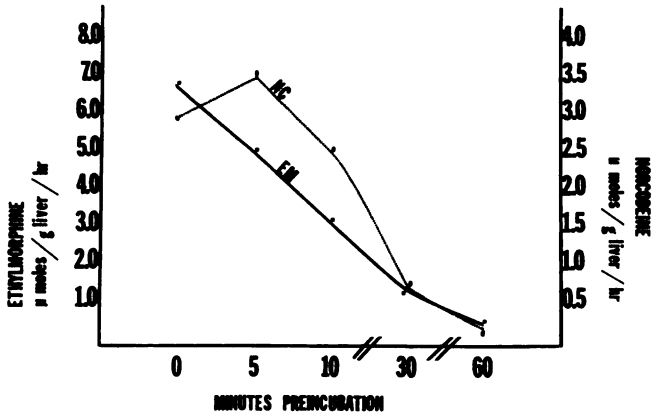

FIG. 5. *Effect of preliminary incubation at 45° on ethylmorphine (EM) and norcodeine (NC) oxidation*

The ordinate on the left side of the figure refers to ethylmorphine oxidation, and the ordinate on the right side refers to norcodeine oxidation. The abscissa refers to the duration of preliminary incubation in minutes prior to removal of microsomes for assay.

TABLE 3
Effect of preliminary incubation at 45° on cytochrome P-450

Each value represents the mean of four experiments ± the standard error.

Preliminary incubation (min)	$OD_{450-490}$ (% of control)
5	91.83 ± 8.35
10	84.55 ± 8.30
15	87.85 ± 5.49
20	77.03 ± 2.54
30	70.38 ± 8.57
60	52.73 ± 6.41

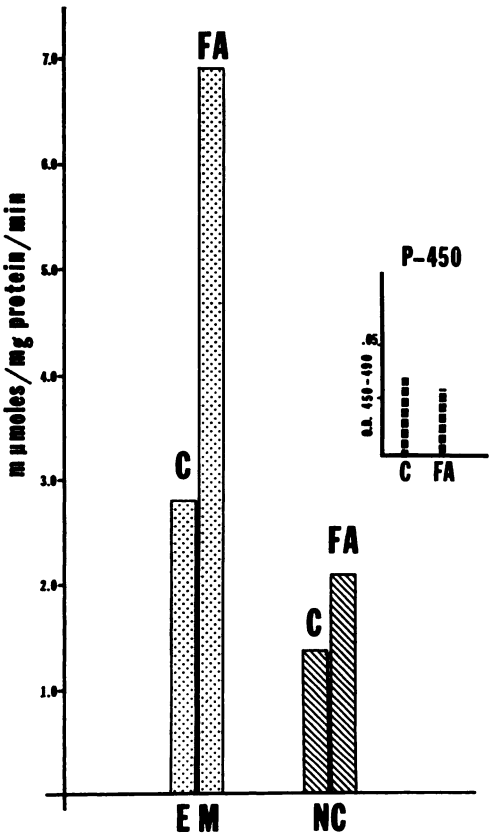

FIG. 6. *Drug oxidation in microsomes from fasting-adapted rats*

FA = fasting-adapted rats obtained by feeding rats for first 12 hr out of each 48-hr time period for 28 days. *C* = control rats, which received a regular diet of laboratory chow ad libitum. Cytochrome P-450 values represent $OD_{450-490}$/mg of protein per milliliter.

affect drug-oxidative reactions. Furthermore, there seems to be no correlation between P-450 content and microsomal drug oxidation in these preparations. These results differ from those obtained from hepatic microsomes of rats fasted for 72 hours. In these experiments no significant differences were observed when rates of ethylmorphine or norcodeine oxidation were compared with rates obtained from microsomes prepared from rats fed ad libitum.

DISCUSSION

These studies indicate that within hepatic microsomes different rate-limiting events exist for the *N*- and *O*-dealkylation of morphine analogues. Although the participation of cytochrome P-450 is involved in both reactions, it was possible to show that the P-450 content did not correlate with either ethylmorphine *N*-dealkylation or norcodeine *O*-dealkylation. Phenobarbital increased the P-450 content in livers of male rats without affecting microsomal *O*-dealkylation of norcodeine. Stimulation of ethylmorphine *N*-dealkylation was observed in microsomes from fasting-adapted rats which had no change in P-450 content. That different rate-limiting steps were operative in these reactions was further indicated when hexobarbital was tested as an inhibitor of ethylmorphine *N*-dealkylation and norcodeine *O*-dealkylation. Hexobarbital competitively inhibited the *N*-dealkylation of ethylmorphine, whereas the inhibition of norcodeine dealkylation was not competitive. Also, preliminary incubation of microsomes at 45° revealed a differential lability in the factors which control the rate of these reactions. Ethylmorphine *N*-dealkylation appeared to be more sensitive to preliminary incubation than was norcodeine *O*-dealkylation. The decrease in activity which was observed with this procedure did not correlate with decreases in P-450. Recent studies have shown that NADPH-cytochrome *c* reductase activity is not affected by preliminary incubation.

These data permit several interpretations concerning the composition and regulation of the hepatic microsomal oxidative sys-

tems. First, there may be two regulatory sites within the same system, and one of these regulatory sites might be rate-limiting for one substrate or group of substrates while the other is rate-limiting for another group. Factors to be considered are substrate-enzyme binding characteristics and differential requirements for electron flux. These factors and possibly others would serve as a basis for the existence of two regulatory sites within the same system. For example, the reduction of cytochrome P-450 by NADPH-cytochrome P-450 reductase may be rate-limiting with respect to ethylmorphine *N*-dealkylation, whereas the binding of the substrate to P-450 may be limiting with respect to norcodeine *O*-dealkylation. This possibility has been considered previously (23). Second, there may exist in the electron chain alternative forms of a given component which differ from one another such that one is functional for certain groups of compounds and the other is operative when other groups of substrates are made available. An example of this would be the existence of several forms of cytochrome P-450. Different hemoproteins which could be measured as P-450 because they contain similar prosthetic groups may function for different classes of substrates. Multiple forms of P-450, each with different binding properties but with qualitatively similar catalytic properties, would explain many of the results obtained in the current study. Different binding spectra obtained for different groups of compounds have been described previously (24, 25), and different forms of P-450 have recently been identified (26). Finally, we must not exclude the possibility that there exist two or more entirely different enzyme systems which may have few or no common transfer intermediates. Although this proposal appears to be the least tenable at this time, it must not be discarded until additional evidence is provided.

ACKNOWLEDGMENTS

This research was supported in part by an institutional grant (NSF 89) and in part by United States Public Health Service Research Grant AM-12168.

REFERENCES

1. T. Omura, R. Sato, D. Y. Cooper, O. Rosenthal and R. W. Estabrook, *Federation Proc.* **24**, 1181 (1965).
2. T. Omura, E. Sanders, R. W. Estabrook, D. Y. Cooper and O. Rosenthal, *Arch. Biochem. Biophys.* **117**, 660 (1966).
3. D. Y. Cooper, S. Levin, S. Narasimhulu, O. Rosenthal and R. W. Estabrook, *Science* **147**, 400 (1965).
4. A. Rubin, T. R. Tephly and G. J. Mannering, *Biochem. Pharmacol.* **13**, 1007 (1964).
5. H. Remmer and H. J. Merker, *Ann. N. Y. Acad. Sci.* **123**, 79 (1965).
6. N. E. Sladek and G. J. Mannering, *Federation Proc.* **25**, 418 (1966).
7. J. F. Henderson and P. Mazel, *Biochem. Pharmacol.* **13**, 1471 (1964).
8. R. L. Cram, M. R. Juchau and J. R. Fouts, *Proc. Soc. Exptl. Biol. Med.* **118**, 872 (1965).
9. H. S. Posner, C. Mitoma and S. Udenfriend, *Arch. Biochem. Biophys.* **94**, 269 (1961).
10. R. Kato and J. R. Gillette, *J. Pharmacol. Exptl. Therap.* **150**, 279 (1965).
11. W. J. George and T. R. Tephly, *Pharmacologist* **9**, 203 (1967).
12. E. Layne, *in* "Methods in Enzymology," (S. P. Colowick and N. O. Kaplan, eds.), Vol. 3, p. 450. Academic Press, New York, 1957.
13. T. Nash, *Biochem. J.* **55**, 416 (1953).
14. E. Stotz, *J. Biol. Chem.* **148**, 585 (1943).
15. W. J. Potts, Jr., "Chemical Infrared Spectroscopy," Vol. I, "Techniques," p. 92. Wiley, New York, 1963.
16. L. J. Bellamy, "Infrared Spectra of Complex Molecules," p. 125. Wiley, New York, 1958.
17. H. Lineweaver and D. Burk, *J. Am. Chem. Soc.* **56**, 658 (1934).
18. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
19. H. Remmer, *Arch. Exptl. Pathol. Pharmakol.* **237**, 296 (1959).
20. A. H. Conney, C. Davidson, R. Gastel and J. J. Burns, *J. Pharmacol. Exptl. Therap.* **130**, 1 (1960).
21. M. W. Anders and G. J. Mannering, *Mol. Pharmacol.* **2**, 341 (1966).
22. V. R. Potter, M. Watanabe, J. E. Becker and H. Pitot, *in* "Advances in Enzyme Regulation" (G. Weber, ed.), Vol. 5, p. 303, Pergamon, London, 1966.
23. T. R. Tephly and G. J. Mannering, *Mol. Pharmacol.* **4**, 10 (1968).
24. H. Remmer, J. Schenkman, R. W. Estabrook, H. Sasame, J. Gillette, S. Narasimhulu, D.

Y. Cooper and O. Rosenthal, *Mol. Pharmacol.* **2**, 187 (1966).
25. Y. Imai and R. Sato, *Biochem. Biophys. Res. Commun.* **22**, 620 (1966).
26. A. Hildebrandt, H. Remmer, and R. W. Estabrook, *Biochem. Biophys. Res. Commun.* **30**, 607 (1968).