Changes in Michaelis and Spectral Constants for Aniline in Hepatic Microsomes from Phenobarbital-Treated Rats

A. M. GUARINO,[1,2] T. E. GRAM,[1] P. L. GIGON,[3] F. E. GREENE,[4] AND J. R. GILLETTE

Laboratories of Chemical Pharmacology, National Cancer and Heart Institutes, National Institutes of Health, Bethesda, Maryland 20014

(Received September 27, 1968)

SUMMARY

Recent work has suggested that the rate of hepatic microsomal drug metabolism may be correlated with either the cytochrome P-450 content or the magnitude of the spectral change caused by the addition of substrates to microsomal suspensions. Accordingly, the relationships between these factors were investigated in 0.9% NaCl- and phenobarbital-treated intact, sham-operated, and partially hepatectomized rats. In intact animals phenobarbital produced a 3–4-fold increase in both microsomal aniline hydroxylase activity and cytochrome P-450 content, whereas in sham-operated or partially hepatectomized rats it produced a 4-fold increase in cytochrome P-450 content but only doubled aniline hydroxylase activity. Although phenobarbital treatment doubled the binding constants (K_s) for aniline in all groups, it increased the maximum absorbance change (A_{max}) produced by the additione of aniline to microsomes about 7-fold in unoperated animals but only about 4-fold in the operated groups.

It was found that the Michaelis constant (K_m) and binding constant (K_s) for aniline differ by a factor of 10 in microsomal preparations from intact animals. Phenobarbital treatment was found to elicit significant increases in both K_s and K_m for aniline. These changes in K_m and K_s imply that induction by phenobarbital may be associated with qualitative as well as quantitative changes in the hepatic microsomal aniline hydroxylase.

INTRODUCTION

Gram *et al.* (1) have shown that the magnitude of the stimulatory effect of phenobarbital on hepatic microsomal enzymes in partially hepatectomized rats varies with the substrate employed. For example, this inducer caused a tripling in the maximal rate of *p*-nitroanisole demethylation but only a doubling in the maximal rate of aniline hydroxylation. Associated with these increases in enzyme activity, there was a tripling in the content of cytochrome P-450, which is considered to be involved in drug metabolism (2). Thus, the increase in cytochrome P-450 content paralleled the increase in *p*-nitroanisole demethylase, but not that of aniline hydroxylase. Although these findings indicated that there was no fixed quantitative relationship between the rate of

[1] Research Associates in the Pharmacology-Toxicology Program, National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Maryland 20014.

[2] Present address: Laboratory of Chemical Pharmacology, National Cancer Institute, Bethesda, Maryland 20014.

[3] Sponsored by the Swiss Academy of Medical Sciences.

[4] Supported by Special Fellowship 1-F3-ES-37751-01, Division of Environmental Health Sciences, National Institutes of Health.

aniline hydroxylation and cytochrome P-450 content, they did not preclude the possibility that the rate was more directly related to the maximal spectral changes (A_{max}) caused by the addition of substrates to liver microsomes.

In the present investigation the spectral changes produced by aniline were studied in microsomes from 0.9% NaCl- or phenobarbital-treated rats that had been either unoperated, sham-operated, or partially hepatectomized. These studies revealed that phenobarbital treatment significantly increased A_{max} but that there was no consistent relationship between the maximal velocity of aniline hydroxylation (V_{max}) and A_{max}. They further showed that phenobarbital treatment increases both the apparent Michaelis constant (K_m) for aniline hydroxylation, as well as the binding constant (K_s); this finding was unexpected, because no such changes after phenobarbital treatment have been reported for any other substrate studied to date (3, 4).

METHODS

Animals. In previous investigations (5, 6), microsomal enzyme activity was reduced to a minimum level 4–7 days after partial hepatectomy, and hence unoperated controls were compared with sham and partially hepatectomized rats 6 days after operation. Male Sprague-Dawley rats (NIH) weighing 80–90 g at the time of surgery were used in these studies. Animals had access to Purina laboratory chow and water at all times. All surgical manipulations were carried out under ether by the same procedures as described previously (1, 7), and the day of surgery was designated day 0. After laparotomy, the liver of the rat was exteriorized; a heavy cotton suture was applied to the hilus to isolate the medial and left lateral lobes, and these lobes were excised. It was confirmed that removal of these lobes resulted in removal of about 75% of the liver. In the sham operations the rats were anesthetized and the livers were exteriorized and manipulated as with the partially hepatectomized animals. The duration of laparotomy was the same in both groups (3–5 min). The abdominal incision was closed in two stages; first, the muscle layers were closed with chromic 000 gut suture, and then the skin was closed with several 11-mm Michel wound clips. Starting on day 2 the control animals received intraperitoneal injections of 0.5 ml of 0.9% NaCl per 100 g of body weight, and a second group of rats received sodium phenobarbital, 80 mg/kg (0.5 ml/100 g of body weight), once daily for 4 days. The last dose was administered 24 hr before the animals were killed.

Animals were decapitated, and their livers were homogenized with 2 volumes of 1.15% KCl containing 0.02 M Tris-HCl, pH 7.4, in a Potter homogenizer fitted with a motor-driven Teflon pestle. All tissue manipulations were performed at 0–4°. The homogenate was centrifuged at $9000 \times g$ for 20 min. The supernatant fraction was carefully decanted and centrifuged at $105{,}000 \times g$ for 60 min in a Spinco model L ultracentrifuge. The microsomal pellet was resuspended in 1.15% KCl–Tris-HCl buffer by gentle manual homogenization.

Analytical procedures. Microsomal preparations were assayed for protein by the method of Lowry *et al.* (8) prior to the incubation or spectral studies to assure that all samples would contain the same concentration of microsomal protein. Assays of aniline hydroxylase activity were conducted essentially as described previously (1). To each 20-ml serum bottle were added Tris-HCl buffer (2.0 ml, 0.3 M, pH 7.4), aniline hydrochloride (20 μmoles), and a cofactor mixture (1 ml) containing sodium isocitrate (35 μmoles), isocitrate dehydrogenase (2.5 units), NADP (2 μmoles), and $MgCl_2$ (20 μmoles). To duplicate vessels were added 10 mg of microsomal protein (1 ml) and 1.15% KCl (containing 0.02 M Tris-HCl) to give a total volume of 5.0 ml. Incubations were carried out with shaking in a Dubnoff apparatus for 10 min at 37° under air. For the experiments in which Michaelis constants were determined, the incubation time was 20 min, and the aniline HCl concentration was varied from 0.01 to 1.5 mM. Enzyme activity was linear with time and protein concentration under these conditions.

After the samples had been incubated for the appropriate time, the reaction was stopped by the addition of 2.0 ml of a saturated aqueous solution of NaCl. A 5-ml aliquot was transferred to a 45-ml glass-stoppered centrifuge tube containing about 1 g of solid NaCl and 30 ml of diethyl ether which contained 1.5% (v/v) isoamyl alcohol. The tubes were stoppered and mechanically shaken for 20 min. After the phases had been allowed to separate, a 20-ml aliquot of the ether phase was transferred to a clean 45-ml centrifuge tube which contained 4.0 ml of 0.5 M K_3PO_4 containing 1% (w/v) aqueous phenol. The tubes were stoppered, shaken for 5 min, and centrifuged. Thirty minutes after the addition of the alkaline phenol solution, the optical density of the aqueous phase was measured at 620 mμ in a Gilford model 2000 spectrophotometer. Standards (*p*-aminophenol) and tissue blanks were carried through the entire procedure.

For the spectral studies, all samples were diluted to a final concentration of 3 mg of protein per milliliter with 1.15% KCl–Tris-HCl. The magnitudes of the spectral changes following consecutive additions of aniline HCl were measured with a Shimadzu model MPS-50L recording spectrophotometer, as described by Schenkman *et al.* (9), except that the wavelengths used were 430 mμ and 470 mμ. Two 3-ml aliquots of a microsomal suspension were placed in separate cuvettes, and a baseline was recorded at room temperature. The baseline was subtracted from the change in light absorbance caused by consecutive additions of aniline HCl (0.15–1.5 mM, final concentrations). The addition of aniline HCl at these concentrations caused no significant change in the pH of the microsomal suspension. The amounts of aniline HCl added did not cause volume changes sufficient to alter the optical properties of the suspensions, but above 1.5 mM aniline HCl there were profound changes in absorbance, which seemed to be due to clumping of the microsomes.

The apparent spectral dissociation constants (K_s) and the absorbance maxima (A_{max}) were obtained by a least squares method in which the data were fitted to the Michaelis-Menten model as reported previously (10, 11), except that all values were retained regardless of their standard error. Statistical comparisons were made using Student's *t*-test (12).

RESULTS

Relationships between K_s and V_{max} for aniline in microsomes from 0.9% NaCl- and phenobarbital-treated rats. The addition of aniline to microsomal suspensions results in the appearance of a peak at about 430 mμ (9, 13). As shown in Fig. 1,

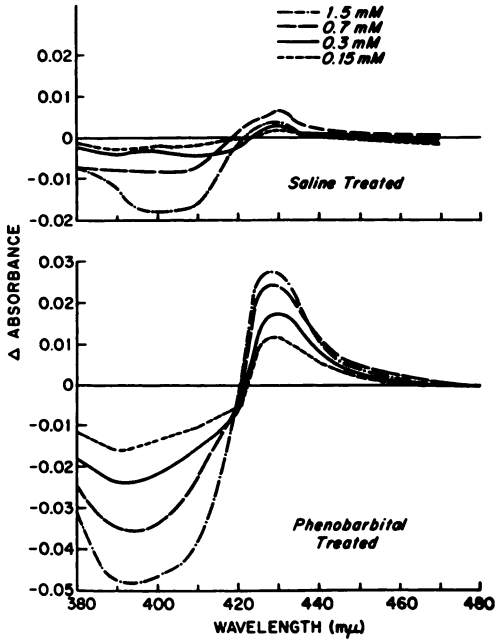

FIG. 1. *The effect of aniline hydrochloride concentration on the magnitude of the spectral changes caused in hepatic microsomal suspensions prepared from 0.9% NaCl- and phenobarbital-treated animals*

The experimental conditions are described in METHODS.

prior treatment with phenobarbital markedly enhances the magnitude of this spectral change. It is interesting that the typical concentration-dependent changes are noted following consecutive additions of aniline HCl to microsomal suspensions from phenobarbital-treated but not from 0.9% NaCl-treated animals. With microsomes from NaCl-treated animals, 1.5 mM

aniline HCl consistently gave a smaller difference in absorbance than did 0.7 mM aniline HCl. Because of the many problems associated with making these measurements, it is difficult to offer an explanation for the consistent lack of proportionality at the higher aniline concentrations in the control animals. However, it should be emphasized that this effect was never observed when microsomes from phenobarbital-treated animals were used.

Hepatic microsomes from rats subjected to a variety of treatments (Table 1) showed inconsistent relationships between aniline metabolism (V_{max}) and cytochrome P-450 content. In the unoperated control rats, phenobarbital administration increased both aniline metabolism and cytochrome P-450 content about 3–4-fold. Thus, phenobarbital treatment did not alter the rate of metabolism per unit of cytochrome P-450 in unoperated rats. In sham-operated animals, however, phenobarbital doubled the rate of aniline hydroxylation but caused a 4-fold increase in cytochrome P-450 content, which decreased the activity in relation to the cytochrome P-450 content from 2.8 to 1.6. A similar decrease was obtained with hepatectomized rats; the ratios of aniline hydroxylase activity to cytochrome P-450 were 1.7 and 0.9 in the 0.9% NaCl- and phenobarbital-treated rats, respectively.

Sham operation or partial hepatectomy did not alter K_s, but regardless of the surgical manipulations to which the rats were subjected, phenobarbital treatment doubled the K_s value (Table 1). Phenobarbital treatment also increased A_{max}, causing a 7-fold increase in the microsomes obtained from unoperated controls and a 4-fold increase in those from sham-operated and partially hepatectomized rats. The effects of phenobarbital treatment on A_{max} values of liver microsomes from sham-operated and partially hepatectomized animals thus paralleled its effects on cytochrome P-450.

Effect of phenobarbital treatment on Michaelis and spectral constants. The find-

TABLE 1

Effect of prior treatment with phenobarbital on rate of aniline hydroxylation, cytochrome P-450 content, and magnitude of spectral change in hepatic microsomes from unoperated, sham-operated, and partially hepatectomized rats

Dosage and dosage schedules are described in METHODS. All values are means ± standard deviation for five animals.

	V_{max} (*p*-aminophenol formed)	Cytochrome P-450 concentration	K_s	Maximum spectral change ($A_{430-470}$) per mg protein
	mμmoles/mg/min	*mμmoles/mg microsomal protein*	*mM*	$\times 10^{-3}$
Unoperated control				
0.9% NaCl (A)	0.53 ± 0.24	0.44 ± 0.12	0.147 ± 0.042	1.66 ± 0.82
Phenobarbital (B)	1.95 ± 0.10[a]	1.68 ± 0.09[a]	0.293 ± 0.026[a]	11.80 ± 3.11[a]
B:A	3.68	3.82	1.99	7.11
Sham-operated				
0.9% NaCl (A)	0.81 ± 0.16	0.29 ± 0.06	0.119 ± 0.066	2.38 ± 1.03
Phenobarbital (B)	1.72 ± 0.23[a]	1.15 ± 0.10[a]	0.272 ± 0.058[a]	9.88 ± 0.44[a]
B:A	2.22	3.96	2.29	4.15
Partial hepatectomy				
0.9% NaCl (A)	0.40 ± 0.09	0.23 ± 0.04	0.148 ± 0.051	2.50 ± 0.09
Phenobarbital (B)	0.99 ± 0.26[a]	1.09 ± 0.12[a]	0.262 ± 0.023[a]	9.70 ± 1.45[a]
B:A	2.48	4.74	1.77	3.88

[a] Significantly different ($p < 0.05$) from the corresponding 0.9% NaCl-treated group.

TABLE 2

Effect of prior treatment of rats with phenobarbital on kinetic and spectral constants for aniline HCl in hepatic microsomes

Dosage and dosage schedules are described in METHODS. All values are means ± standard deviation for four animals.

Treatment	K_m	K_s	V_{max} (*p*-aminophenol formed)	Maximum spectral change ($A_{430-470}$) per mg protein
	mM	*mM*	*mμmoles/mg/min*	$\times 10^{-3}$
0.9% NaCl (A)	0.041 ± 0.01	0.329 ± 0.068	0.56 ± 0.02	3.26 ± 0.38
Phenobarbital (B)	0.099 ± 0.016	0.536 ± 0.012	1.25 ± 0.12	16.7 ± 0.83
p value	<0.025	<0.025	<0.005	<0.001
B:A	2.40	1.63	2.24	5.12

ing that phenobarbital treatment increased K_s suggested the possibility that K_m for aniline hydroxylation might also be increased. Unoperated rats (80–90 g) were treated with phenobarbital as described above, and hepatic microsomes were used for the determination of Michaelis and spectral constants. As reported previously (9), K_m and K_s for aniline obtained with hepatic microsomes from control animals differ by a factor of 10 (Table 2). Treatment with phenobarbital caused about a doubling in both these "constants." The V_{max} was also doubled in phenobarbital-treated animals, whereas A_{max} was about 5 times as great in phenobarbital-treated as in NaCl-treated animals.

DISCUSSION

Previous reports have led to the general conclusion that induction of hepatic microsomal enzymes by phenobarbital is characterized by an increase in V_{max} without concomitant changes in K_m (3, 4). Thus, in studying the metabolism of ethylmorphine, chlorpromazine, and hexobarbital by hepatic microsomes, Rubin *et al.* (3) reported that phenobarbital treatment increased V_{max} but did not significantly change the K_m of any of the substrates. Similar findings were reported by Gram *et al.* (4) in studies of aminopyrine *N*-demethylase in microsomes from control and phenobarbital-treated rats and rabbits. Since these substrates all produce type I difference spectra when added to microsomal suspensions whereas aniline produces type II changes (9, 13), it is possible that induction by phenobarbital may have no effect on K_m for type I compounds but may alter this value for type II substances.

The use of inducers other than phenobarbital has revealed that other qualitative factors may alter the activity of microsomal enzymes. For example, Alvares *et al.* (14) reported that prior treatment of rats with phenobarbital had no significant effect on the K_m of hepatic microsomal benzpyrene hydroxylase, whereas prior administration of 3-methylcholanthrene caused a marked decrease in this parameter. These observations indicate that drug-induced changes in the kinetic properties of microsomal enzymes are complex functions of the inducing agent employed and the drug substrate investigated.

It is interesting that phenobarbital treatment produced a disproportionately greater effect on A_{max} than on V_{max} (Table 2); this suggests that part of the cytochrome P-450–aniline complex measured spectrophotometrically is not related to the metabolism of aniline. In accord with this view, K_s values were several times greater than the K_m values. Likewise, Krantz and Staudinger (15) found that K_s for the binding of coumarin (a type I substrate) to rabbit liver microsomes is the same in both control and phenobarbital-treated animals, but is about 100 times higher than K_m for the hydroxylation of coumarin. Phenobarbital thus may induce significant quantities of hemoprotein which is active

in the binding of aniline and coumarin as measured spectrophotometrically, but not in the metabolism of these substrates.

REFERENCES

1. T. E. Gram, A. M. Guarino, F. E. Greene, P. L. Gigon and J. R. Gillette, *Biochem. Pharmacol.* **17**, 1769 (1968).
2. D. Y. Cooper, S. Levin, S. Narasimhulu, O. Rosenthal and R. W. Estabrook, *Science*, **147**, 400 (1965).
3. A. Rubin, T. R. Tephly and G. J. Mannering, *Biochem. Pharmacol.* **13**, 1007 (1964).
4. T. E. Gram, J. T. Wilson and J. R. Fouts, *J. Pharmacol. Exp. Ther.* **159**, 172 (1968).
5. J. R. Fouts, R. L. Dixon and R. W. Shultice, *Biochem. Pharmacol.* **7**, 265 (1961).
6. J. M. Peters, C. J. Krijnen and H. D. McEwen, *Experientia* **23**, 70 (1967).
7. G. M. Higgins and R. M. Anderson, *Arch. Pathol.* **12**, 186 (1931).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
9. J. B. Schenkman, I. Frey, H. Remmer and R. W. Estabrook, *Mol. Pharmacol.* **3**, 516 (1967).
10. D. S. Davies, P. L. Gigon and J. R. Gillette, *Biochem. Pharmacol.* **17**, 1865 (1968).
11. C. E. Inturrisi and E. Titus, *Mol. Pharmacol.* **4**, 591 (1968).
12. G. W. Snedecor, "Statistical Methods," Ed. 5. Iowa State University Press, Ames, 1956.
13. H. Remmer, J. Schenkman, R. W. Estabrook, H. Sasame, J. Gillette, S. Narasimhulu, D. Y. Cooper and O. Rosenthal, *Mol. Pharmacol.* **2**, 187 (1966).
14. A. P. Alvares, G. R. Schilling and R. Kuntzman, *Biochem. Biophys. Res. Commun.* **30**, 588 (1968).
15. F. Krantz and H. J. Staudinger, *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 455 (1968).