Biochemical Pharmacology, Vol. 29, pp. 685–688.

0006-2952/80/0301-0685 $02.00/0

DIFFERENTIAL EFFECTS OF ETHANOL AND OTHER INDUCERS OF DRUG METABOLISM ON THE TWO FORMS OF HAMSTER LIVER MICROSOMAL ANILINE HYDROXYLASE*

G. DAVID MCCOY

Division of Molecular Biology and Pharmacology, Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY 10595, U.S.A.

(*Received* 28 *July* 1979; *accepted* 4 *October* 1979)

Abstract—The aniline hydroxylase activity of microsomes isolated from hamster liver can be differentiated kinetically into high affinity (low K_m, form I) and low affinity (high K_m, form II) forms. Microsomes isolated from uninduced animals contain slightly more form I activity. The activity of the low affinity form (form II) is preferentially enhanced by Aroclor or 3-methylcholanthrene treatment, while phenobarbital treatment increases the activity of both forms. Chronic ethanol consumption results in enhancement of only the high affinity form (form I).

The existence of two forms of aniline hydroxylase activity in isolated microsomes was first suggested by Wada *et al.*[1]. More recently, Kitada *et al.* [2] have shown that aniline hydroxylase activity is inhibited by cyanide ions and that the inhibition is biphasic, suggesting the presence of two activities with differing inhibitor affinities.

The effects of the common inducers (phenobarbital, Aroclor 1254 and 3-methylcholanthrene) of mixed function oxidase activity on aniline hydroxylase activity have been determined in several animal species. In general, all inducers have the ability to increase the specific activity of aniline hydroxylase, though the degree of induction varies [3–5]. Since different inducers have been shown to give rise to distinct species of cytochrome P-450 [6–11], the failure to observe qualitative differences in the inducibility of aniline hydroxylase might be attributable to the use of aniline concentrations which are high enough to allow both activities to be expressed [12, 13].

This communication presents kinetic evidence for the existence of two forms of aniline hydroxylase in isolated hamster liver microsomes and shows that the activities of the two forms exhibit independent induction patterns depending on the inducer.

MATERIALS AND METHODS

Chemicals. Aniline, nicotinamide adenine dinucleotide phosphate (monosodium salt), tricine (*N*-tris-[hydroxymethyl]-methyl glycine), Tris (tris[hydroxymethyl]-amino-methane), glucose-6-phosphate (monosodium salt), and glucose-6-phosphate dehydrogenase (Type XII) were obtained from the Sigma Chemical Co., St. Louis, MO. 3-Methylcholanthrene and *p*-aminophenol were obtained from the Eastman Kodak Co., Rochester, NY. Aroclor 1254 was from Analabs, North Haven, CT. Phenobarbital (sodium salt) was from Mallinckrodt, St. Louis, MO. Sucrose, special enzyme grade, was from Schwarz-Mann, Orangeburg, NY.

Animals and treatments. Male Syrian golden hamsters (ARS/Sprague-Dawley, Madison, WI) were housed three per cage and allowed free access to NIH-07 lab chow and water. At 8 weeks of age, hamsters were placed on control Lieber–DeCarli liquid diet No. 711 + added fiber obtained from Bio-Serv, Inc., Frenchtown, NJ). At 9 weeks of age, animals were either continued on the control diet or placed on the ethanol-containing diet, in which ethanol isocalorically replaced carbohydrate. Thirty-five per cent of the total caloric intake was ethanol. The daily diet intake was restricted to 40 ml/animal. Animals were killed after 4 weeks.

A second group of hamsters was randomized by weight and given free access to NIH-07 lab chow and water. Induction began at 9 weeks of age. Sodium phenobarbital (PB) was dissolved in saline, and the animals were given intraperitoneal injections of 80 mg/kg body wt/day for 4 days. Animals receiving 3-methylcholanthrene (3-MC) were injected daily for 4 consecutive days. 3-Methylcholanthrene was suspended in corn oil, and an intraperitoneal dose of 20 mg/kg body wt/day was administered for 4 days. Aroclor 1254-treated animals were given a single intraperitoneal injection of 500 mg/kg body wt. PB- and 3-MC-treated animals were killed 24 hr after the last injection, following an overnight fast. Aroclor-treated animals and control animals that received an intraperitoneal injection of corn oil equivalent in volume to that given to Aroclor-treated animals were killed 5 days after treatment following an overnight fast. All animals were killed by CO_2 anesthesia.

Subcellular fractionation. Livers were rapidly removed, weighed, minced, rinsed three times with 10 vol. of ice-cold SET (0.3 M sucrose, 0.5 mM EDTA, and 5 mM tricine, pH 7.4), and suspended

* This work was supported by National Cancer Institute Grant CA 12376.

in 5 vol. of ice-cold SET. Homogenization of livers was performed as described previously using a Polytron homogenizer (Willems type, made by Kinematica, Lucerne, Switzerland [14]). The homogenate was diluted to 9 vol. with SET and centrifuged at 10,000 *g* for 10 min. The decanted supernatant fraction was then centrifuged at 105,000 *g* for 60 min. The surface of the pellet was rinsed carefully with 0.15 M KCl and suspended in SET to give a microsomal protein concentration of 4–6 mg/ml.

Aniline hydroxylase assay. Microsomal fractions were assayed for aniline hydroxylase activity using a modification of the procedure described by Mazel [12]. The standard assay mixtures contained in a final volume of 1.0 ml: 0.125 μmole $NADP^+$, 2.5 μmoles glucose-6-phosphate, 6.0 μmoles $MgCl_2$, 12.5 μmoles nicotinamide, 50 μmoles Tris-acetate buffer, pH 8.0, 4 units glucose 6-phosphate dehydrogenase, and from 0.5 to 2 mg of microsomal protein. The aniline concentrations used are indicated in the figure and table legends. Complete assay mixtures minus microsomes were incubated for 5 min at 37°. Reactions were initiated by the addition of microsomes. Following incubation for 15 min at 37°, reactions were quenched by the addition of 1.0 ml of ice-cold 10% trichloroacetic acid. After centrifugation, aliquots of the supernatant fraction were assayed for *p*-aminophenol, as outlined by Mazel [12]. Using these conditions, the rate of product formation at both low (0.1 mM) and high (20 mM) aniline concentrations was linear with respect to both time and microsomal protein. Protein was determined by the method of Lowry *et al.* [15]. Statistical significance was evaluated using Student's *t*-test.

RESULTS

When the effect of different aniline concentrations on the rate of *p*-aminophenol formation was examined, two hyperbolic changes in rate were observed (Fig. 1). The rate of product formation shows an initial hyperbolic increase from 0.01 to 0.05 mM substrate. As the concentration of substrate is increased from 0.05 to 1.0 mM, only a slight further increase in rate is observed. Increasing substrate concentration beyond 1 mM gives rise to a second hyperbolic increase in the rate of product formation. A comparison of substrate titrations shows that liver microsomes isolated from ethanol-treated hamsters have increased activity of the high affinity form of aniline hydroxylase (form I). The data in Fig. 2 are replotted from Fig. 1 and show more clearly the effects of ethanol on the two forms of aniline hydroxylase.

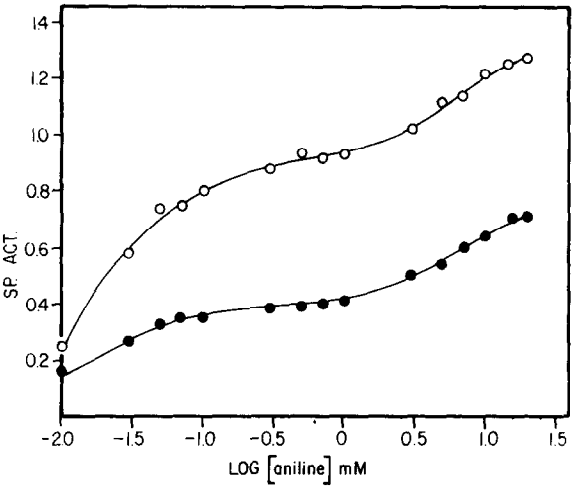

Fig. 1. Effect of aniline concentration on aniline hydroxylase activity of microsomes isolated from control and ethanol-consuming hamsters. Key: (●——●) control; and (○——○) ethanol.

The kinetic parameters obtained from substrate titrations of microsomes from control and ethanol-treated animals are presented in Table 1. No differences in substrate affinity between the two groups for either activity were observed; however, a large

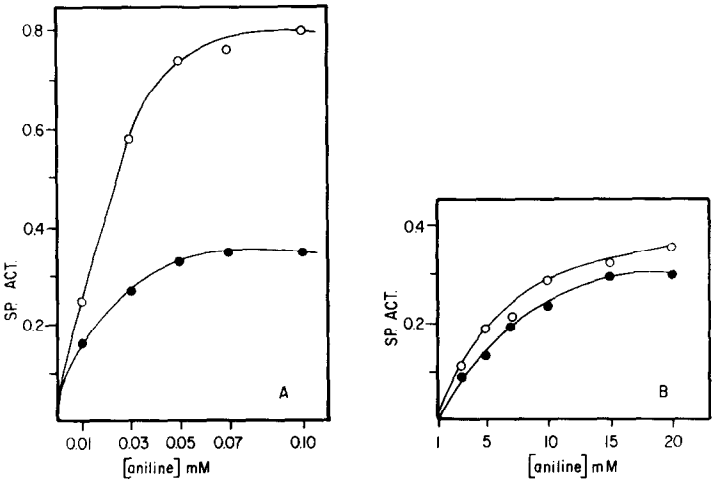

Fig. 2. Effect of aniline concentration on (A) low and (B) high affinity forms of aniline hydroxylase. Key: (●——●) control; and (○——○) ethanol. For purposes of clarity, rates obtained at 1 mM aniline were subtracted in part B. The rates at 1 mM aniline were 0.41 and 0.93 nmoles·min^{-1}·mg $protein^{-1}$ for control and ethanol preparations, respectively.

Table 1. Comparison of kinetic parameters for the two forms of microsomal aniline hydroxylase

Control		Ethanol	
Form I	Form II	Form I	Form II
	K_m (mM)		
0.019	4.65	0.022	5.84
0.011	4.85	0.022	5.96
0.032	3.13		
	Specific activity (nmoles·min^{-1}·mg protein^{-1})		
0.43	0.39	1.01	0.41
0.44	0.38	1.10	0.41
0.67	0.41		

increase in the maximal rate of form I activity was observed in liver microsomal preparations isolated from ethanol-consuming hamsters. Form II activity remained unchanged.

Because of the large differences in substrate affinity between the two forms of aniline hydroxylase, the contribution of each form to the total microsomal activity can be readily estimated by the appropriate choice of substrate concentration. The data in Table 2 show that, when the rate of product formation is determined for liver microsomal preparations from control and ethanol-treated hamsters at low (0.1 mM) and high (20 mM) aniline concentrations, results similar to those from full substrate titrations are obtained.

The effects of three inducers of microsomal metabolic activity on the two forms of aniline hydroxylase activity are shown in Table 3. All of the inducers increase total aniline hydroxylase activity; however, the pattern of induction varies. Phenobarbital treatment resulted in small increases in both forms I and II, whereas treatment with Aroclor 1254 or 3-methylcholanthrene increased only form II.

Table 2. Effect of chronic ethanol consumption on the specific activity of the two forms of microsomal aniline hydroxylase*

	Form I	Form II	Total
Control	0.57 ± 0.07	0.62 ± 0.11	1.16 ± 0.17
Ethanol	1.52 ± 0.52†	0.76 ± 0.17	2.28 ± 0.67†

* Form I activity was determined using 0.1 mM aniline. Total activity was determined using 20 mM aniline. Form II activity is calculated by subtracting form I activity from total activity. Values are expressed as nmoles · min^{-1} · mg^{-1} and are the means ± S.D. for six separate preparations.

† Significance from control, $P \leq 0.01$.

DISCUSSION

The distinctive changes in rate in response to substrate concentration, as well as the differential induction of the two forms of aniline hydroxylase, are consistent with the presence of two catalytic sites for the hydroxylation of aniline in isolated hamster liver microsomes. The fact that there is a 100- to 200-fold difference in the affinity of each of the two activities for aniline greatly simplifies study of the two forms since only two substrate concentrations need be employed in order to estimate activities.

It is of interest to compare the results obtained in this study with those reported previously for mouse and rat liver microsomal preparations by Wada *et al.* [1]. Although the authors show biphasic double reciprocal plots which indicate two activities, no attempt was made to estimate the contribution of each form to total aniline hydroxylase activity. We have recalculated their data, and it indicates that form I activity accounts for approximately 60 per cent of the total aniline hydroxylase activity in uninduced rat and mouse microsomal preparations. This compares with the 50–60 per cent as form I which we have observed. The affinity of form II for aniline for their preparations is similar to those reported in this study. The affinity of form I could not be estimated because of insufficient data points.

Taken together, these data indicate that liver microsomes from three of the more common laboratory species contain two kinetically distinct forms of aniline hydroxylase activity and that each activity responds independently, depending on the nature of the inducer of MFO activity. Form I activity appears to be selectively and preferentially enhanced in microsomes from ethanol-consuming hamsters, whereas form II is most responsive to inducers of polycyclic aromatic hydrocarbon metabolism.

REFERENCES

1. F. Wada, H. Shimakawa, M. Takasugi, T. Kotake and T. Sakamoto, *J. Biochem, Tokyo* **64**, 109 (1968).
2. M. Kitada, K. Chiba, T. Kamataki and H. Kitagawa, *Jap. J. Pharmac.* **27**, 601 (1977).

Table 3. Effects of phenobarbital, Aroclor 1254 and 3-methylcholanthrene pretreatment on the two forms of microsomal aniline hydroxylase*

Pretreatment	Form I	Form II	Total
Control	0.34 ± 0.06	0.60 ± 0.07	0.94 ± 0.09
Phenobarbital	0.44 ± 0.03†	0.81 ± 0.09†	1.26 ± 0.11†
Aroclor 125	0.29 ± 0.04	1.15 ± 0.10†	1.45 ± 0.11†
3-Methylcholanthrene	0.26 ± 0.03‡	1.09 ± 0.11†	1.34 ± 0.12†

* Estimations of Form I, Form II and total activity are as described in the legend of Table 2. Values are expressed as nmoles · min^{-1} · mg^{-1} and are the means ± S.D. for six separate preparations.

† Significance from control, $P \leq 0.01$.

‡ Significance from control, $P \leq 0.05$.

3. M. Ikeda, H. Ohtsuji and T. Imamura, *Jap. J. Pharmac.* **22**, 479 (1972).
4. A. Alvares and A. Kappas, *J. biol. Chem.* **252**, 6373 (1977).
5. R. Kato, *Xenobiotica* **7**, 25 (1977).
6. K. Comai and J. L. Gaylor, *J. biol. Chem.* **248**, 4947 (1973).
7. T. Imai and R. Sato, *Biochem. biophys. Res. Commun.* **60**, 8 (1974).
8. W. Leven, D. Ryan, S. West and A. T. H. Lu, *J. biol. Chem.* **249**, 1747 (1974).
9. T. A. van der Hoeven, D. A. Haugen and M. J. Coon, *Biochem. biophys. Res. Commun.* **60**, 569 (1974).
10. E. P. Guengerich, *J. biol. Chem.* **253**, 7931 (1978).
11. C. R. Wolf, M. M. Szutowski, L. M. Ball and R. M. Philpot, *Chem. Biol. Interact.* **21**, 29 (1978).
12. P. Mazel, in *Fundamentals of Drug Metabolism and Drug Disposition* (Eds. B. W. LaDu, H. G. Mandel and E. L. Way), p. 569. Williams & Wilkins, Baltimore (1971).
13. T. Imai and R. Sato, *Biochim. biophys. Acta* **36**, 571 (1959).
14. G. D. McCoy and K. A. Doeg, *Biochem. biophys. Res. Commun.* **46**, 1411 (1972).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).