KINETIC STUDIES FOR ANILINE HYDROXYLASE AFTER PROLONGED ETHANOL TREATMENT

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In the previous paper, we reported of rats treated chronically with ethanol; that side-chain oxidation of hexobarbital, N-demethylation of aminopyrine and *p*-hydroxylation of aniline in vitro from 9,000 g supernatant of liver homogenates were identical with those of control rats when ethanol was withdrawn and substituted for tap water 24 hours prior to sacrifice and that, in contrast, the activity of aniline hydroxylase of the rats which continued to ingest ethanol ad libitum up to the time of sacrifice was about 2-fold increased, compared with that of controls, in spite of no change detected in hexobarbital oxidase and aminopyrine demethylase (1).

We also reported that the addition of ethanol in a concentration of 8.5 mM to the incubation medium caused an inhibition of *p*-hydroxylation of aniline and that the type of inhibition was, at least superficially, competitive (2).

The present experiments were conducted to examine the kinetic behavior of aniline hydroxylase of rat liver microsomes when rats continued to drink ethanol ad libitum up to the time of sacrifice.

METHODS

Male Wistar rats weighing 80 g initially were used. All the rats had free access to adequate solid food (CLEA, CE-2), and the ethanol treatments were the same as in the previous experiments (1). Each animal housed in an isolated room of individual cages, with controlled temperature (22°C) and illumination (6:00 a.m. to 6:00 p.m.). In the present experiments, rats were killed 2 months after the experiments began (from July 29 to September 30) and the hepatic aniline hydroxylase activity was assayed. According to the drinking fluid, the animals were divided into two groups, one drinking tap water (control group), and the other only ethanol (ethanol group). The rats of ethanol group were given 10 v/v% ethanol for final one week prior to sacrifice. The mean ethanol intake of rats a day was about 10 g per kg of body weight. For the enzyme assay, the latter was further divided into two subgroups, in one of which ethanol was withdrawn and substituted for tap water 24 hours prior to sacrifice (EtOH-1), whereas in the other the animals continued to drink ethanol ad libitum up to the time of sacrifice (EtOH-2).

Tissue preparation

In the previous experiments, 9,000 g supernatant from the liver homogenates of the

rats in group EtOH-2 was found to contain ethanol in concentrations of 5 to 20 mg/dl. Accordingly, the present experiments kept only to washed microsomes as enzyme source.

Immediately the animals were sacrificed by decapitation between 9 and 10 a.m., the livers were totally removed, washed in ice-cold isotonic KCl until clear of blood, minced, weighed and the homogenized in 2 vols of ice-cold isotonic KCl containing 0.02 M Tris-HCl buffer, pH 7.4, by the use of a Potter homogenizer fitted with a loose Teflon pestle. The livers of 3 or 4 animals were pooled. The homogenate was centrifuged in a refrigerated centrifuge at 700 g for 10 minutes at 0°C, the supernatant at 9,000 g for 20 minutes at 0°C and the 9,000 g supernatant at 105,000 g for 70 minutes in succession. The microsomes obtained were resuspended in an isotonic KCl containing 0.02 M Tris-HCl buffer, pH 7.4, and recentrifuged at 105,000 g for 60 minutes. The washed microsomes did not contain any ethanol detectable by gas chromatography and were used as enzyme source in the amount of 5 to 6 mg protein.

Enzyme assay

Complete reaction mixtures of 3.0 ml as a total volume were incubated in 30 ml Erlenmeyer flasks in an atmosphere of air in a Dubnoff type metabolic shaker at 37°C for 10 minutes. Duplicate incubation was carried out for each substrate concentration. The mixtures consisted of: $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 15 μmoles ; NADP, 2 μmoles ; glucose-6-phosphate Na_2 , 15 μmoles ; Tris-HCl, pH 7.4, 50 mM; cell sap from control animals 0.5 ml (about 13 mg protein); washed microsomes 0.5 ml (5 to 6 mg protein); aniline 0.5 ml (0.02 to 4 mM). In the experiment with optimum pH shift pH 6.1 or 8.1 was used for incubation medium. After termination of incubation, when the reaction was stopped by the addition of 1.2 ml of a saturated aqueous solution of NaCl the *p*-aminophenol production, formed for 10 minutes, was measured, to determine the *p*-hydroxylation of aniline, by the method of Guarino et al. (3) under minor modification. After a 2 ml aliquot was transferred to a 60 ml glass-stoppered centrifuge tube containing 0.6 g of solid NaCl and 20 ml of diethylether with 1.5 v/v% isoamyl-alcohol, the tubes were mechanically shaken for 20 minutes. When the phases had been allowed to separate, a 10 ml aliquot of the ether phase was transferred to a 60 ml centrifuge tube of 4.0 ml of 0.5 M K_3PO_4 containing 1 w/v% aqueous phenol, and the tubes were mechanically shaken for 10 minutes and centrifuged. Sixty minutes after the

FIG. 1. The development of the colour of the aqueous phase with time.
 solid line : aniline 1 mM
 dotted line : aniline 0.1 mM
 open circle : ether phase 10 ml
 closed circle : ether phase 20 ml

shaking ended, the optical density of the aqueous phase was measured at 620 m μ in a Beckman spectrophotometer. The colour of the aqueous phase, developing with time, became constant 60 minutes after the shaking (Fig. 1). Protein contents in cell sap and microsomes were estimated by the method of Lowry et al. (4), crystalline bovine serum albumin serving as the standard. The ethanol concentration in 9,000 g supernatant of liver homogenates was determined by gas chromatography according to the method of Duritz and Truitt (5), after the samples were treated only with 2/3 N H₂SO₄.

For the determination of kinetic values for aniline hydroxylase, the graphycal procedures of Lineweaver and Burk (6) and of Dixon (7) were used.

RESULTS

1. Growth rate and the ratio of liver weight to body weight

It was found that there was difference in the amounts of solid food taken by animals between the control group and the ethanol group. Though a control rat ate about 24 g of solid food a day, a rat of ethanol group, fed with 10% ethanol instead of water, ate about 19 g, and, with 20% ethanol, only about 8 g, a day.

TABLE 1. The ratio of liver weight to body weight in ethanol-treated rats (2 months).

Group	Body weight (g)	Liver weight (g)	Liver weight/body weight
Control (21)	320 $\pm$ 29	11.9 $\pm$ 1.6	0.037 $\pm$ 0.003
EtOH-1 (15)	259 $\pm$ 27**	10.9 $\pm$ 1.6	0.042 $\pm$ 0.004*
EtOH-2 (15)	255 $\pm$ 30**	9.8 $\pm$ 1.4**	0.039 $\pm$ 0.004

Values are the mean values $\pm$ standard deviation.

The number of animals used for each value is in parentheses.

* significantly different from control and group EtOH-2, $p < 0.01$

** significantly different from control group $p < 0.01$

In the present experiments the growth rate of chronically ethanol-treated rats was significantly lower than that of controls ($p < 0.01$). The average body weight of a rat in group EtOH-1 and EtOH-2 was 259 g and 255 g respectively, though that of control group was 320 g (Table 1). The average liver weight of group EtOH-1 and EtOH-2 was 10.9 g and 9.8 g, whereas that of control group was 11.9 g. The value of group EtOH-2 was significantly different from that of control group ($p < 0.01$). There is a significant difference in the ratio of liver weight to body weight between group EtOH-1 and group EtOH-2, the ratio of the former higher than that of the latter ($p < 0.01$), whereas no difference was found between control rats and group EtOH-2 (Table 1).

There is no difference in protein contents of microsomes and cell sap between three groups.

2. Kinetic values for aniline hydroxylase

We measured the apparent values for the maximal velocity (V_{max}) and Michaelis constant (K_m) of the enzyme, to determine whether or not the increased activity of aniline hydroxylase resulted from an increase in protein synthesis.

I) Our previous study revealed that the addition of ethanol in concentrations of 8.5 to 66 mM to the incubation medium caused an inhibition of *p*-hydroxylation of aniline, and the type of inhibition was competitive (2).

Since our preliminary studies showed that the kinetic values obtained from Lineweaver and Burk curves of the *p*-hydroxylation of aniline were the same whether washed microsomes or 9,000 g supernatant was used, we used washed microsomes as the enzyme source this time in order to avoid the inhibitory effect of ethanol *in vitro*. The washed microsomes did not contain any detectable ethanol.

TABLE 2. Kinetic values for aniline hydroxylase at pH 7.4 (washed microsomes).

Group	Apparent K_m (mM)	Apparent V_{max} (μ moles <i>p</i> -aminophenol formed/ 100 mg protein/10 min)
Control	0.057 ± 0.004	2.12 ± 0.10
EtOH-1	0.060 ± 0.008	$1.90 \pm 0.15^*$
EtOH-2	0.054 ± 0.010	$2.95 \pm 0.11^{**}$

Each mean represents six experiments.

Values are the mean values $\pm$ standard deviation.

* significantly different from control, $p < 0.05$

** significantly different from control, $p < 0.01$

FIG. 2. A typical Lineweaver and Burk curve for aniline hydroxylase.

S : aniline mM

V : μ moles *p*-aminophenol formed/100 mg protein/10 minutes

The apparent K_m and apparent V_{max} obtained are presented in Table 2. No difference was seemingly found in the apparent K_m between the three groups (control rats, EtOH-1 and EtOH-2), the apparent V_{max} of group EtOH-2 was about 1.5-fold increased in comparison with those of the other two groups (controls and EtOH-1). These results suggest that induction of aniline hydroxylase may occur in the livers of group EtOH-2.

Fig. 2 shows a typical Lineweaver and Burk curve. It is of great interest that the rate of *p*-hydroxylation of aniline in group EtOH-1 is mostly lower than in controls.

Some rats, though not so many, of group EtOH-2 did not contain any ethanol de-

detectable by gas chromatography in their 9,000 g supernatant of liver homogenates. The rate of *p*-hydroxylation of aniline in them was slightly lower than in the rats with ethanol in their 9,000 g supernatant, but significantly higher than that of control rats (Table 3).

2) pH shift

Rickert and Fouts (8) reported that the pH optimum for the metabolism in vitro of aniline was shifted from pH 7.0 to pH 8.1 by benzpyrene pretreatment. *K_m* values for aniline hydroxylase were altered with the changes of pH in hepatic microsomes from either controls or benzpyrene-pretreated rats. Thus we experimented with the chronically ethanol-treated rats whether this phenomenon is observed or not.

The pH optimum for metabolism of aniline in vitro was not shifted after chronic ethanol treatment (Fig. 3).

FIG. 3. The pH optimum for metabolism of aniline in vitro.

DISCUSSION

It is very interesting that the ratio of the liver weight to the body weight in the rats of group EtOH-1 is higher than that in the rats of group EtOH-2. However, at present, we have no explanation for this finding. There is no difference in protein contents of microsomes and cell sap between the three groups.

Of the chronically ethanol-treated rats which continued to drink ethanol ad libitum up to the time of sacrifice (group EtOH-2), only the activity of aniline hydroxylase was approximately 2-fold increased, compared with that of control rats (1). In this experiment we used 9,000 g supernatant as an enzyme source. 9,000 g supernatant of the livers from the group EtOH-2 contained ethanol in concentrations of 5 to 20 mg/dl. On the other hand, the addition of ethanol in a concentration of 8.5 mM to the incubation medium caused a competitive inhibition of *p*-hydroxylation of aniline (2). Thus, the microsomes from the livers of group EtOH-2 have still higher activity of *p*-hydroxylation of aniline, in spite of the presence of ethanol, as compared with that of control rats.

In the present experiments for kinetic studies, we used washed microsomes as the enzyme source for aniline hydroxylase in order to avoid the inhibitory effect of ethanol on aniline hydroxylation in vitro. The results obtained show that there is no difference in apparent *K_m* for aniline hydroxylase between the three groups (controls, EtOH-1 and EtOH-2). In contrast, the apparent *V_{max}* for aniline hydroxylase was approximately 1.5-fold increased in group EtOH-2 as compared with that in control rats. Furthermore, in most cases, the apparent *V_{max}* in group EtOH-1 was lower than that in control rats. These findings suggest that the induction of aniline hydroxylase may occur in the rats of group EtOH-2.

Furthermore, the rate of *p*-hydroxylation of aniline of the rats in group EtOH-2 without any ethanol in 9,000 g supernatant was lower than that of the rats in the same group with ethanol in 9,000 g supernatant. This finding also supports the possibility of induction of aniline hydroxylase by ethanol.

The results obtained in the present experiments again appear to disagree with those of Rubin and Lieber (9), as mentioned in the previous paper (1).

Kato and Gillette, and Kato demonstrated that starvation for 72 hours markedly impaired metabolism of aminopyrine and hexobarbital by microsomes from male rats but enhanced metabolism of these substrates by microsomes from female rats. Aniline metabolism was enhanced in both sexes by starvation (10, 11). In the present experiments, a control rat ate about 24 g of solid food a day but a rat fed with 10 v/v% ethanol instead of water ate about 19 g. Though further studies must be conducted in detail, the increase in the apparent V_{max} in group EtOH-2 might be not due to the difference of amounts of food taken.

Recently Rickert and Fouts (8) reported that the pH optimum for the metabolism in vitro of aniline was shifted from pH 7.0 to pH 8.1 by benzpyrene pretreatment. K_m values for aniline hydroxylase were altered by changes in pH with hepatic microsomes from either control rats or benzpyrene pretreated rats in their experiments. In the present experiments chronic ethanol treatment (both group EtOH-1 and EtOH-2) did not change the pH optimum for the metabolism in vitro of aniline.

SUMMARY

The present experiments were conducted to examine the kinetic behavior of aniline hydroxylase of rat liver microsomes after prolonged ethanol treatments.

The results obtained are as follows:

1. There is no difference in the apparent K_m between the three groups (control rats, group EtOH-1 and group EtOH-2).
2. The apparent V_{max} in group EtOH-2 was about 1.5-fold increased, compared with those in control group and group EtOH-1.
3. The pH optimum for metabolism of aniline in vitro was not shifted after chronic ethanol treatment.

These findings suggest that the induction of aniline hydroxylase may occur in the rats of group EtOH-2.

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