Biochemical Pharmacology, Vol. 33, No. 16, pp. 2623–2628, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

RATE-DETERMINING FACTORS FOR ETHANOL METABOLISM IN FASTED AND CASTRATED MALE RATS

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(*Received* 24 *August* 1983; *accepted* 20 *December* 1983)

Abstract—The effects of castration and fasting upon the alcohol elimination rate, liver alcohol dehydrogenase (LADH) maximum activity (V_{max}), and hepatic concentrations of ethanol, acetaldehyde, and free NADH during ethanol oxidation were examined in male Wistar rats. Castration increased the V_{max} of LADH and, to a lesser extent, the alcohol elimination rate *in vivo*. On the other hand, fasting reduced the V_{max} of LADH and the alcohol elimination rate in sham-operated and castrated rats but it did not nullify the effect of castration. Castration produced small but significant changes in the hepatic concentrations of ethanol, acetaldehyde and free NADH in fed rats during ethanol oxidation. Fasting also caused significant increases in the concentration of free NADH during alcohol oxidation in both the sham-operated and castrated groups. The ratio of the steady-state velocities of LADH *in situ* to the maximum velocities of LADH (v/V_{max}) under the different experimental conditions was calculated by using the steady-state rate equation for the enzyme mechanism of rat LADH and its kinetic constants. The calculated v/V_{max} ratios were 50–62%, indicating that LADH activity was limited to about the same extent by its substrates and products under these conditions and that the changes in alcohol elimination rates produced by fasting and castration mainly reflected changes in the V_{max} of LADH. The calculated steady-state velocities *in situ* (v) were 14–28% lower than the measured rates of alcohol elimination *in vivo*. The extent of agreement is probably acceptable in view of the assumptions needed to determine the free NADH concentration in liver and the existence of non-LADH-related processes for alcohol elimination *in vivo*.

The oxidation of ethanol to acetaldehyde by liver alcohol dehydrogenase (LADH, EC 1.1.1.1) is the rate-determining step in the metabolism of ethanol [1, 2]. Purich and Fromm [3] and Plapp [4] have suggested that the rate of ethanol oxidation mediated by LADH *in vivo* can be predicted by using the steady-state rate equation for LADH based on its kinetic mechanism and by determining the maximum activity of LADH, the kinetic constants of the enzyme, and the concentrations of substrates and products of the LADH reaction *in situ* during ethanol oxidation. Cornell *et al.* [5] measured the kinetic constants of rat LADH using a homogenate-supernatant fraction at physiologic pH, temperature and ionic strength, and then employed the steady-state rate equation for an ordered Bi–Bi mechanism to calculate and predict the velocity of LADH *in vivo*. They compared the calculated activity of ethanol oxidation mediated by LADH with the measured rates of alcohol elimination *in vivo* published in the literature and observed that the calculated activity and measured rates agreed reasonably well. Because the maximum activity of LADH is present at only 1.5 times the activity required to account for the rate of ethanol metabolism *in vivo*, they concluded that the level of LADH could be a major rate-determining factor.

Recently, we [6] purified the two major LADH enzyme forms from fed and fasted rats and found that their kinetic properties are similar. Additionally, studies on the kinetic mechanism for these LADH forms indicated that it best fits the Theorell–Chance mechanism with the added features of dead-end ternary complex formation and substrate inhibition by ethanol. By using the steady-state rate equation and measuring all the needed variables, we were able to predict by calculation quite accurately the alcohol elimination rate *in vivo* for male Sprague–Dawley rats in both the fed and fasted states. Furthermore, we observed that the change in ethanol elimination rate between fed and fasted rats could be attributed mainly to a decrease in the total LADH activity of rats in the fasted state. We therefore concluded that the total LADH activity (or the liver content of enzyme) is the major rate-determining factor of ethanol metabolism in transition from the fed to the fasted state.

Whereas fasting decreases LADH activity and reduces the rate of alcohol elimination *in vivo* [6–8], castration in fed male rats increases LADH activity and accelerates the ethanol elimination rate [9–11]. In this paper, we report studies which examine whether fasting nullifies the effects of orchiectomy on LADH activity and alcohol elimination rate. By studying the combined effects of fasting and orchiectomy, we further test the hypothesis that the total LADH activity or the level of ADH in liver is a major rate-determining factor of ethanol elimination.

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MATERIALS AND METHODS

Animals and diets. Male Wistar rats (Harlan

Industries Inc., Indianapolis, IN) were housed individually in wire-bottomed cages and in a controlled temperature and humidity environment with fixed light–dark cycles (7:00 a.m.–7:00 p.m. light, 7:00 p.m.–7:00 a.m. dark). They were maintained on Wayne Lab Blox (Allied Mills, Inc., Chicago, IL) and water *ad lib*. The animals underwent a sham-operation or orchiectomy 10–12 days before use. They weighed about 150 g at the time of the operation. The sham-operated and castrated animals weighed 230 ± 5 (mean value ± S.E.M.) and 207 ± 4 g, respectively, at the time of measurement of alcohol elimination rates and intrahepatic metabolite concentrations. To determine the effect of fasting, half of each group was fasted 48 hr before making these measurements. After 48-hr of fasting, the sham-operated and castrated rats weighed 181 ± 3 and 166 ± 3 g respectively.

Determination of LADH activity. Liver homogenate-supernatant fractions were prepared as previously described [7]. The activity of alcohol dehydrogenase in the homogenate-supernatant fraction was measured by following the reduction of NAD^+ to NADH spectrophotometrically. The assay buffer contained 0.5 M Tris–HCl (pH 7.2 and I = 0.2), 2.8 mM NAD^+, and 5 mM ethanol, and the determination was performed at 37° [7]. These substrate concentrations were calculated to saturate the enzyme approximately 88% [6]; therefore, the measured enzyme activity was multiplied by 1.14 to calculate the maximum velocity (or V_{max}). This correction was obtained from the equation shown in Table 1 which also takes into consideration substrate inhibition by ethanol.

Measurement of alcohol elimination rate in vivo. Ethanol (2 g/kg) was administered by the intraperitoneal injection of a 10% (v/v) solution, and the ethanol concentration in samples of tail blood was determined with a gas–liquid chromatograph by a headspace method [7]. The rate of ethanol disappearance was pseudo-zero order, and the elimination rate was calculated using Widmark's equation [12, 13].

Measurement of intrahepatic metabolite concentrations. One hour after intraperitoneal administration of ethanol, rats were killed by cervical dislocation and their livers were freeze–clamped [8]. The 1-hr time point was chosen because it is known that by this time ethanol absorption is complete and tail blood ethanol concentration reflects overall blood and tissue ethanol concentration after total body distribution [14]. The concentration of ethanol and acetaldehyde in the freeze-clamped liver was determined by gas–liquid chromatography [8]. Lactate and pyruvate were assayed by enzymatic methods [15, 16]. The free NAD^+/NADH ratio was calculated from the pyruvate/lactate ratio, assuming $K_{eq} = 1.11 \times 10^{-11}$ M at 38° [17]. The free NAD^+ concentration was assumed to be 0.5 mM [18]. Total NAD^+ and NADH were measured by the method described by Klingenberg [19].

Calculations. The steady-state rate equation for LADH shown in Table 1 was used to calculate the steady-state velocity of LADH *in situ*. This equation was written to account for the Theorell–Chance mechanism with formation of a dead-end enzyme–NADH–ethanol ternary complex [6]. The kinetic constants of rat LADH needed for this equation are those already published [6] and are listed in Table 2. The equilibrium constant for the LADH reaction at 37° is 1.94×10^{-11} M [5]. The K_{eq} values for lactate dehydrogenase and LADH are pH-independent terms.

Statistical analysis. Unpaired Student's *t*-test was used to analyze the significance of difference between two means. Results are expressed as mean values ± standard error.

RESULTS

As has been observed with the spontaneously hypertensive (SH) [10] and Sprague–Dawley [9] rats, orchiectomy in Wistar rats resulted in an increase of both alcohol elimination rate and LADH activity (Table 3). The changes induced by orchiectomy were significant no matter what reference unit was used for comparison and were observed in both the fed and the fasted animals. However, as discussed previously [7], comparison of alcohol elimination rate and LADH activity in animals of similar initial body weight is least obfuscated by concomitant changes in body weight and liver weight or protein content produced by experimental procedures if alcohol elimination rate and LADH activity are expressed simply on the basis of rate or total activity per liver (or per animal) instead of activity per kg body weight or activity per g liver or mg protein. The increase in LADH activity (expressed as mmoles/hr/rat) after orchiectomy for the fed and fasted rats was 33 and 28% respectively. By comparison, the increase in

Table 1. Steady-rate equation for rat LADH based on its kinetic mechanism

$$v/V_{max} = \left(\frac{([A]\,[B] - [P]\,[Q]/K_{eq})}{K_{ia}K_b + K_b[A] + K_a[B] + [A]\,[B] + K_{ia}K_bK_q[P]/K_pK_{iq}} + (K_{ia}K_b[Q]/K_{iq}) + \frac{K_bK_q[A]\,[P]}{K_{iq}K_p} + \frac{K_{ia}K_b[P]\,[Q]}{K_pK_{iq}} + \frac{K_a[B]\,[Q]}{K_{iq}} + \frac{K_{ia}K_b[B]\,[Q]}{K_iK_{iq}} + \frac{K_a[Q]\,[B]^2}{K_iK_{iq}} + \frac{[A]\,[B]^2}{K_i}\right)$$

Steady-state velocity and the maximum velocity in the forward direction are denoted by v and V_{max} respectively. $[A]$ = $[NAD^+]$, $[B]$ = [ethanol], $[P]$ = [acetaldehyde], and $[Q]$ = [NADH]. The kinetic constants are defined in Table 2. K_{eq} is the equilibrium constant for the oxidation of alcohol to acetaldehyde. See Ref. 6 for a detailed discussion of the kinetic mechanism of rat LADH.

Table 2. Michaelis and inhibition constants for rat LADH

Michaelis constant	(μM)	Inhibition constant	(μM)
K_a (NAD^+)	33*	K_{ia} (NAD^+)	58
K_b (ethanol)	480	K_{ib} (ethanol)	810
K_p (acetaldehyde)	37	K_{ip} (acetaldehyde)	12
K_q (NADH)	4	K_{iq} (NADH)	0.9
		K_i (ethanol)	1.7×10^5

* These values have been published previously [6].

alcohol elimination rate (mmoles/hr/rat) for the fed and fasted rats was less, 9 and 15% respectively. The data in Table 3 also demonstrate the effect of fasting on alcohol elimination rate and LADH activity of sham-operated and orchiectomized rats. The decrease in LADH activity due to fasting in the sham-operated and orchiectomized rats was 26 and 29% respectively. On the other hand, the decrease in alcohol elimination rate due to fasting in the sham-operated and castrated rats was 44 and 41%. The lack of perfect correlation between changes in LADH activity and alcohol elimination rate due to castration and fasting indicates that factors other than the maximum activity of LADH are also likely to influence the steady-state rate of LADH. Accordingly, the substrates and products of LADH *in situ* were measured, and the ratio of the velocity of LADH *in situ* to the maximum velocity of LADH was calculated for each of the experimental conditions (cf. Tables 4 and 5).

The measured concentrations of the substrates and products of the LADH reaction in the livers of control and castrated rats after ethanol administration are shown in Table 4. The intrahepatic concentration of ethanol was lower while that of acetaldehyde and free NADH during ethanol oxidation was significantly higher in the fed castrated than in the fed sham-operated animals. The intrahepatic concentration of free NADH also increased during ethanol oxidation as a result of fasting in both the sham-operated and castrated rats. Although fasting in the sham-operated rats produced a large (44%) decrease in alcohol elimination rates (Table 3), the hepatic ethanol concentration in the fasted rats was only slightly higher (not significant) than in the fed rats. This discrepancy could be explained partly by a difference in the volume of distribution of ethanol which was higher in the fasted rats (i.e. 0.72 ml/g in the fed rats vs 0.75 ml/g in the fasted). The total liver content of nicotinamide adenine dinucleotides was measured in the four different conditions but they did not differ from one another (data not shown).

Using the steady-state rate equation for LADH (Table 1), the kinetic constants of the enzyme (Table 2) and the hepatic substrate and product concentrations (Table 4), the ratio of the velocity of LADH *in situ* to the maximum velocity of LADH (v/V_{max}) was calculated for each experimental condition. As can be seen from Table 5, the enzyme is limited to 50–62% of its maximum activity (V_{max}) during ethanol oxidation. The calculated enzyme velocity was within 15% of the measured alcohol elimination rate in the two groups of fasted rats, but it underestimated by 19–28% the elimination rates in the fed rats.

DISCUSSION

Several experimental conditions are associated with substantial changes in the alcohol elimination rate of animals [7–11, 20–22]. The best studied among them is fasting, which was reported earlier to reduce the elimination rate by 40–50% [6–8]. The results of the present study confirm these earlier reports. In both the sham-operated and castrated male rats, the decrease in alcohol elimination rates due to fasting was nearly identical and amounted to 41–44%. The decrease in alcohol elimination rates could be explained mainly by a 26–29% reduction in the activity of LADH even though fasting also decreased the NAD^+/NADH ratio and increased the free NADH level in the livers of sham-operated and castrated rats during ethanol oxidation.

Previously, several investigators [9–11] have shown that orchiectomy produces increased LADH

Table 3. Effects of orchiectomy and fasting on alcohol elimination rates and LADH activities in rats

	Alcohol elimination rate				LADH activity	
	N	(mmoles/hr/rat)	(mmoles/hr/kg)	(mmoles/ml/hr)	N	(mmoles/hr/rat)	(mmoles/hr/kg)
Fed rats							
Sham-operated	23	1.89 ± 0.05	8.89 ± 0.25	12.4 ± 0.3	12	2.20 ± 0.14	8.90 ± 0.53
Orchiectomy	24	2.06 ± 0.04*	10.46 ± 0.13*	15.3 ± 0.3*	12	2.92 ± 0.14*	13.04 ± 0.62*
48-hr Fasted rats							
Sham-operated	22	1.06 ± 0.05†	5.86 ± 0.26†	8.1 ± 0.4†	12	1.62 ± 0.19†	8.29 ± 1.31
Orchiectomy	20	1.22 ± 0.05*‡	6.59 ± 0.21*‡	9.5 ± 0.4*‡	12	2.08 ± 0.17*‡	12.07 ± 0.95*

* $P < 0.05$ to <0.001, compared with the sham-operated group of the same nutritional status.
† $P < 0.05$ to <0.001, compared with the fed, sham-operated group.
‡ $P < 0.005$ to <0.001, compared with the fed, orchiectomy group.

Table 4. Intrahepatic concentrations of the substrates and products of the LADH reaction 1 hr after ethanol administration

	Ethanol (mM)	Acetaldehyde (μM)	Free NADH* (μM)
Fed rats			
Sham-operated	28.6 ± 0.8	15.9 ± 1.9	4.0 ± 0.3
Orchiectomy	24.6 ± 0.5†	21.8 ± 2.1†	5.7 ± 0.5†
48-hr Fasted rats			
Sham-operated	29.6 ± 0.7	13.2 ± 1.9	6.4 ± 0.6‡
Orchiectomy	28.1 ± 0.6	16.8 ± 4.1	8.8 ± 0.7‡

* Free [NADH] was calculated from the pyruvate/lactate ratio, assuming a free [NAD^+] of 0.5 mM [18].
† $P < 0.05$, compared with the sham-operated group.
‡ $P < 0.05$, compared with the respective fed group.

activity and alcohol elimination rate in two strains of rats, i.e. the Sprague–Dawley and the SH (an inbred line derived from the Wistar rat). The results of the present study indicate that this phenomenon is not limited to the SH strain but can be seen more generally among Wistar rats. Because administration of testosterone can reverse these effects of orchiectomy, it is apparent that testosterone regulates the LADH-dependent metabolism of ethanol in the male rat.

The data in Table 3 indicate that the effects of castration can be observed in both fed and fasted animals. Conversely, the effects of fasting can also be demonstrated in the sham-operated as well as the castrated male rats. These findings imply that modulation of LADH activity and alcohol elimination rate during fasting and after orchiectomy are mediated by independent mechanisms. The effect of orchiectomy in the sexually mature male rat is probably explained by increased LADH synthesis associated with a decrease in serum testosterone [9–11, 23]. On the other hand, the effect of fasting is due in part to increased LADH degradation and may be related to an increased level of circulating glucagon or the glucagon/insulin ratio [24].

In this paper, an attempt was made to define as quantitatively as possible the interrelationship of the maximum velocity of LADH, the calculated steady-state velocity of LADH *in situ*, and the alcohol elimination rate *in vivo*. Based on the steady-state kinetic properties and enzyme mechanism of the rat LADH, the calculated steady-state velocities *in situ* were found to be 50–62% of the measured maximal velocity of the enzyme (Table 5). These calculated steady-state velocities *in situ* were also observed to be 14–28% lower than the measured rates of alcohol elimination *in vivo*. The lack of better agreement between the calculated velocities *in situ* and the measured rates *in vivo* can be explained by at least two factors. First of all, physiologic and metabolic processes other than the LADH pathway can contribute significantly to ethanol elimination *in vivo*. Pulmonary and renal excretion of ethanol can account for as much as 5% of the elimination rate [2, 3, 25, 26]. Additionally, extrahepatic ADH-linked oxidation [27], as well as hepatic non-ADH-mediated metabolism [2, 3], are also likely to contribute to the discrepancy between the calculated velocities of LADH and the measured rates of alcohol elimination *in vivo*. A second reason for the discrepancy may be the difficulty in determining precisely the hepatic free NADH concentration during ethanol oxidation. In the literature [28–31], the hepatic NAD^+/NADH ratios of fed rats during ethanol oxidation have been reported to vary from 139 to 500. Assuming the hepatic free NAD^+ concentration to be 0.5 mM, the free NADH concentration in the liver would vary between 3.6 and 1 μM respectively. In the two studies which have originated from our laboratory, we have reported hepatic free NADH values of 1.6 μM [8] and 4.0 μM (Table 3) in fed rats 1 hr after ethanol administration. A better agreement between the calculated steady-state velocities of LADH and the measured rates of

Table 5. Comparison of alcohol elimination rates measured *in vivo* with steady-state LADH reaction rates calculated from the known kinetics of the enzyme

	Calculated v/V_{max}*	Calculated v (mmoles/hr/rat)	Measured alcohol elimination rate (mmoles/hr/rat)	Measured LADH maximum activity (V_{max}, mmoles/hr/rat)
Fed rats				
Sham-operated	0.62	1.36	1.89 ± 0.05	2.20 ± 0.14
Orchiectomy	0.57	1.66	2.06 ± 0.04†	2.92 ± 0.14†
48-hr Fasted rats				
Sham-operated	0.56	0.91	1.06 ± 0.05	1.62 ± 0.19
Orchiectomy	0.50	1.04	1.22 ± 0.05†	2.08 ± 0.17†

* v/V_{max} was calculated based on the steady-state rate equation shown in Table 1. v and V_{max} denote the steady-state velocity and the maximum velocity in the forward direction respectively.
† $P < 0.05$, compared with the sham-operated group.

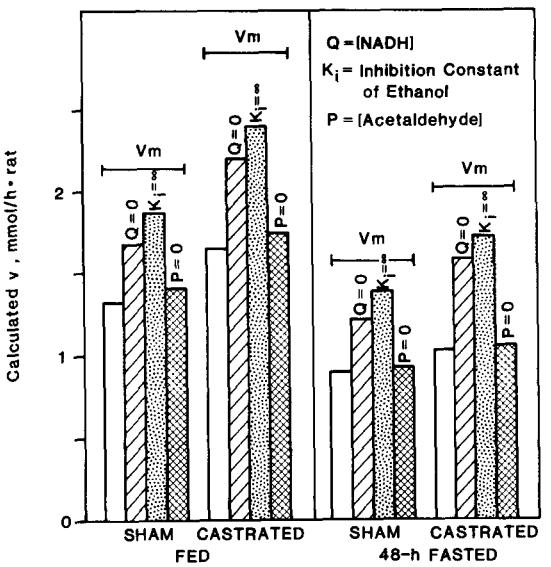

Fig. 1. Relationship of the calculated velocity of LADH *in situ* (v) to the maximum activity (V_{max}) of the enzyme and the effects of removing individually product inhibition by NADH, substrate inhibition by ethanol, or product inhibition exerted by acetaldehyde.

ethanol elimination was obtained in the previous publication [8] because the hepatic free NADH concentration was found to be lower. Why different free NADH concentrations were obtained in these two studies remains obscure. The same freeze-clamping and assay techniques were employed, but Sprague–Dawley rats were used previously [8] while Wistar rats were used here. It is uncertain that the difference in the rat strains alone can explain the discrepancy. It is also uncertain that the hepatic free NAD^+ concentration can always be assumed to be 0.5 mM as was done in the two studies.

The steady-state rate equation for LADH has been utilized previously to predict the rate of alcohol elimination *in vivo* and to evaluate the relative importance of different factors as rate determinants of ethanol metabolism in fed and fasted rats [5, 6, 8]. Accordingly, in this paper, we have also applied this analysis to determine the rate-limiting factors for ethanol metabolism in the sham-operated and castrated rats. It should be pointed out that the electrophoretic pattern and kinetic properties of LADH are not altered by fasting [6, 24] or after orchiectomy [9]. Therefore, the same Michaelis and inhibition constants for rat LADH were used for analysis in the different experimental states. As shown in Fig. 1 (and also Table 5), orchiectomy increased the maximum velocity (V_{max}) of LADH in both the fed and fasted groups of animals. It is also evident that fasting decreased the measured V_{max} of LADH in both the sham-operated and castrated groups. Since the calculated steady-state rate (Table 1) is a fraction of the V_{max} of LADH, it is clear that V_{max} or the content of LADH is always a rate-determining factor for alcohol metabolism. The role of substrate and product inhibition can be assessed by calculating the steady-state rate (v) using the substrate and product concentrations measured *in situ* during ethanol oxidation and removing from consideration each of the factors individually. If one removes product inhibition by NADH, that is by assuming $[Q] = 0$ (Fig. 1), the calculated steady-state rates will approach the V_{max} values. Similarly, if one removes substrate inhibition by ethanol, that is by allowing K_i of ethanol to become very large, the calculated steady-state rates will also increase. However, if one removes the effect of product inhibition by acetaldehyde, $[P] = 0$, this produces only a very small effect on the calculated steady-state rates. Thus, based on these considerations, the maximum activity of LADH, product inhibition by free NADH, and substrate inhibition by ethanol are the major rate determinants of alcohol metabolism. Among these rate determinants, the maximum activity of LADH is probably most important. As shown by Table 5, LADH was limited under all the conditions studied to about 50–62% of its maximum activity. This was true under conditions of low LADH maximum activity (i.e. fasting) as well as high activity (i.e. orchiectomy). Additionally, the alcohol elimination rate was higher in orchiectomized rats even though the hepatic NADH concentration was elevated. Thus, the greater product inhibition exerted by elevated NADH level was more than offset by the increase in the maximum activity of LADH. The importance of NADH accumulation in the cytosol and the rate-determining role of the malate-aspartate shuttle in governing ethanol metabolism have been emphasized by a number of previous studies [32].

Acknowledgements—The authors gratefully acknowledge the excellent technical assistance of R. G. Minter and M. B. Welsh. They also wish to thank Drs. Ting-Kai Li and William Bosron for their helpful suggestions and J. L. McCorkhill for typing the manuscript.

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