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QUANTITATIVE CORRELATION OF ETHANOL ELIMINATION RATES *IN VIVO* WITH LIVER ALCOHOL DEHYDROGENASE ACTIVITIES IN FED, FASTED AND FOOD-RESTRICTED RATS*

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Abstract—The effects of nutritional states upon liver alcohol dehydrogenase (ADH) activity and ethanol elimination rate *in vivo* have been examined in the rat. Male Sprague–Dawley rats, 250–280 g, were studied in the fed state, after fasting for 24, 48 and 72 hr, and after 9 days of food restriction (5 g food/day). Total ADH activity per liver or per animal (2.20 m-moles/hr in fed rats) decreased after a 24-hr fast and was 1.32 and 0.94 m-moles/hr after a 48-hr fast and food restriction respectively. Cytosolic protein and liver wet weight decreased in parallel with total ADH activity, but DNA content exhibited only a 10% decrease with fasting and a 20% decrease with food restriction. Ethanol elimination rate *in vivo* per animal after intraperitoneal injection of 2 g ethanol/kg was 1.92, 1.14 and 0.84 m-moles/hr in the fed, 48 hr-fasted and food-restricted rats, respectively. These data indicate that the decrease in the ethanol elimination rate with fasting and food restriction may be caused by decreasing ADH activity, since the cytosolic free NAD^+/NADH in liver after acute administration of alcohol *in vivo* has been reported to be nearly identical in the fed and 48 hr-fasted rats. The close agreement between liver ADH activity and ethanol elimination rate *in vivo* suggests that the total enzymatic activity of liver ADH is an important rate-limiting factor in ethanol metabolism under the nutritional conditions examined.

The conversion of ethanol to acetaldehyde is the rate-limiting step in the pathway of ethanol metabolism [1]. The reaction occurs principally in the liver, catalyzed by alcohol dehydrogenase (ADH), an NAD^+-dependent enzyme [2, 3]. It is still a matter of debate whether this reaction is rate-limited *in vivo* by the maximal activity of ADH or by the capacity of liver to regenerate cytosolic NAD^+ [4]. Plapp [5] concluded earlier that both these components may have rate-determining roles since neither appears to be in large excess. In fact, a literature review revealed that the maximal activities of liver ADH in several species may be about equal to or less than the corresponding rates of ethanol elimination *in vivo* [5]. Crow *et al.* [6] reported recently that the maximal activity of rat liver ADH at pH 7.2 is only 35% higher than the published rates of ethanol elimination *in vivo* and that the level of liver ADH is the major rate-determining factor for ethanol oxidation in hepatocytes isolated from 48 hr-fasted rats and incubated with pyruvate.

The present paper extends the observations of Plapp [5] and Crow *et al.* [6] by correlating in the rat the total activities of hepatic ADH *in vitro* with rates of ethanol elimination *in vivo* during feeding, fasting and food restriction. Comparison of these states is important because it is generally agreed that fasting decreases the rate of ethanol metabolism [7–13]. However, there is disagreement on the mechanism responsible for this decrease. Smith and Newman [12] reported that the maximal activity of rat liver ADH did not change with 24 or 48 hr of fasting. By contrast, Büttner [14] found that the maximal activity of rat liver ADH decreased 53 and 70% after 19 and 92 hr of fasting respectively. To our knowledge, these divergent findings remain unresolved.

A number of recent studies with isolated rat hepatocytes also have examined the effects of feeding and fasting on changes in pyridine nucleotide metabolism and their roles in regulating the rate of ethanol metabolism [15–18]. The findings indicate that the concentration of the intermediates of the malate-aspartate shuttle for mitochondrial hydrogen transport is rate-limiting in ethanol oxidation, particularly in hepatocytes isolated from fasted rats. The relationship of such findings to ethanol oxidation rates *in vivo*, however, is unclear, because there is extensive depletion of these intermediates in hepatocytes from both fed and fasted rats as a result of the cell isolation procedure [19–21]. Thus, the concentrations of shuttle components seen in isolated rat hepatocytes are much lower than those of freeze-clamped liver even after 48 hr of starvation [20, 21]. At present, there is no evidence that extensive depletion of shuttle components occurs with fasting *in vivo*. Indeed, Veech *et al.* [22] have shown that the cytosolic ratio of free NAD^+ to NADH in liver after acute administration of alcohol *in vivo* is nearly identical in fed and in 48 hr-fasted rats.

MATERIALS AND METHODS

Animals and diets. Male Sprague–Dawley rats (Cox Laboratory Supply, Indianapolis, IN) were housed in

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wire-bottomed cages and a controlled temperature and humidity environment with fixed light–dark cycles (7.00 a.m. to 7.00 p.m., light and 7.00 p.m. to 7.00 a.m., dark). In studies of liver ADH activity, the animals were divided into five groups: fed, fasted for 24, 48 or 72 hr, or food-restricted. The initial weight of these animals was 265 ± 8 g (mean ± S.E.M.). Fasting was initiated at 8.00 a.m. and all animals were killed at 8.00 a.m. The food-restricted group was given 5 g food (Wayne Lab Blox; Allied Mills, Inc., Chicago, IL) per day for 9 days. In the study of rates of ethanol elimination *in vivo*, the rats were divided into three groups: fed, 48 hr-fasted or food-restricted. The initial weight of these rats was 280 ± 6 g.

Measurement of liver ADH activity. After exsanguination, the livers were rinsed with cold homogenization medium, blotted and weighed. The livers were minced with scissors and then homogenized with 4 vol. of 0.05 M HEPES (Na^+), pH 8.4, containing 0.33 mM dithiothreitol [6]. The presence of dithiothreitol significantly protected the ADH from inactivation [23]. Homogenization was performed in a Potter–Elvehjem vessel, fitted with a teflon pestle, by 12 strokes of the pestle to the bottom of the vessel at 400 rev/min and 4°. This procedure insured complete extraction of ADH activity. The homogenates were centrifuged at 100,000 *g* for 60 min at 4°. The supernatant fractions were assayed at 37° for ADH activity in the direction of ethanol to acetaldehyde as described by Crow *et al.* [6]. The reaction mixture, in a final volume of 3 ml, contained 0.5 M Tris–HCl buffer, pH 7.2 and I = 0.2, 2.8 mM NAD^+, 5 mM ethanol and 0.025 ml of rat liver cytosol. Tris–HCl served as a trapping agent for acetaldehyde, and thus the presence of aldehyde dehydrogenase in the cytosolic fraction did not interfere with the ADH activity assay [24]. One unit of ADH activity is defined as the amount of enzyme that catalyzes an initial rate of reduction of 1 μmole NAD^+/min.

The effects of other buffer mixtures upon liver ADH activity were also examined. The K_m values for ethanol determined in (1) 0.5 M Tris–HCl; (2) 0.25 M Tris–HCl, pH 7.2 and I = 0.1; (3) 0.1MNa_2HPO_4–NaH_2PO_4(NaP_i), pH 7.2 and I = 0.2; and (4) 5.4 mM K_2HPO_4–KH_2PO_4(KP_i) buffer containing 129 mM KCl, 4.7 mM NaCl and 5.9 mM $MgCl_2$, pH 7.2 and I = 0.17, were 0.86, 0.78, 0.35 and 1.32 mM respectively. The extrapolated V_{max} values in these buffers were 0.035, 0.032, 0.019 and 0.030 μmole min^{-1} mg^{-1} protein respectively. With 5 mM ethanol and 2.8 mM NAD^+, and assuming the kinetic constants for an ordered BiBi mechanism reported by Cornell *et al.* [21], the ADH activity measured in 0.5 M Tris–HCl, pH 7.2, would be 80% of the calculated V_{max} value. In addition, liver ADH activities of fed and 48 hr-fasted rats were assayed as described by Smith and Newman [12].

Measurement of ethanol elimination rate in vivo. Ethanol, 2 g/kg, was injected intraperitoneally as a 10% (v/v) solution. Tail blood samples were collected in heparinized capillary tubes at 30-min intervals 1 hr after injection. Blood ethanol concentrations were determined with a Hewlett–Packard 5734A gas–liquid chromatograph and a 3380A reporting integrator. The glass columns were packed with 50% Poropak Q and 50% Poropak R (100/120 mesh). An aliquot (0.3 ml) of the tail blood was mixed with 0.3 ml of 1.2 M perchloric acid containing 1.5 mg/ml of *n*-propanol, as internal standard, and centrifuged. The deproteinated supernatant fluid (0.5 ml) was injected into a sealed 25-ml serum vial. The vial was incubated at 65° for at least 30 min, and four headspace samples were obtained from each vial for analysis. The rates of blood ethanol disappearance were pseudo-zero order, and ethanol elimination rates were calculated according to Widmark's equation, as described in Ref. 25.

Other measurements. Protein concentrations were determined by the procedure of Lowry *et al.* [26] with bovine serum albumin as standard. DNA concentrations were assayed by the method of Croft and Lubran [27].

Materials. All chemicals were reagent grade. The coenzymes and other reagents were purchased from the Sigma Chemical Co., St. Louis, MO.

Statistical analysis. Student's *t*-test was employed to determine the significance of a difference between two means. The results are expressed as means ± standard error.

RESULTS

The effects of fasting and food restriction on body weight, liver weight and liver cytosolic protein and DNA content were examined (Table 1). Body weight, liver weight and cytosolic protein all decreased markedly with fasting and food restriction, but the extent to which these occurred varied. In the fed state, the liver wet weight/body weight ratio was 4.0%. With fasting

Table 1. Effects of fasting and food restriction on body weight and on hepatic wet weight, cytosolic protein and DNA

			Liver			
Group	N	Body wt (g)	Wet wt (g)	Cytosolic protein (mg)	DNA (mg)	Liver wet wt/body wt (g/g, %)
Fed	9	256 ± 3*	10.2 ± 0.2	877 ± 15	31.8 ± 0.8	4.00 ± 0.05
Fasted						
24 hr	10	213 ± 5+	5.9 ± 0.2+	570 ± 17+	28.2 ± 1.0+	2.8 ± 0.04+
48 hr	10	200 ± 4+	5.3 ± 0.1+	520 ± 12+	28.0 ± 1.1+	2.7 ± 0.03+
72 hr	9	192 ± 3+	4.9 ± 0.1+	470 ± 9+	28.7 ± 1.6	2.6 ± 0.08+
5 g Food/day	10	148 ± 3+	3.6 ± 0.2+	372 ± 14+	25.0 ± 1.4+	2.4 ± 0.08+

* Mean ± S.E.M.

+ $P < 0.001$, compared with fed group.

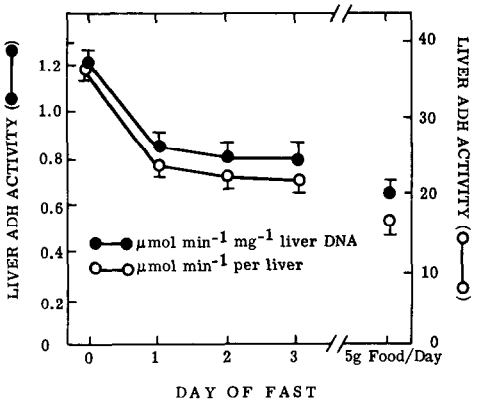

Fig. 1. Effects of nutritional state on total ADH activity and on ADH specific activity, expressed as μmoles min^{-1} per liver and per mg of liver DNA respectively. All the values for the fasted and food-restricted groups are significantly ($P < 0.001$) lower than those for the fed group.

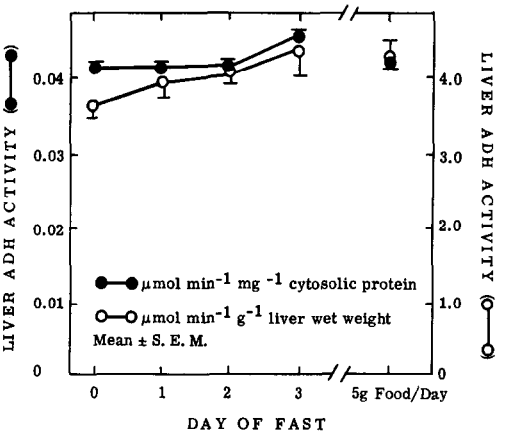

Fig. 2. Effects of nutritional state on specific activities of liver ADH expressed as units per mg of cytosolic protein and per g wet weight. The activities, calculated as μmoles min^{-1} per g wet weight, in the 72 hr-fasted and food-restricted groups are significantly higher than those of the fed group ($P < 0.02$ and 0.001 respectively).

and food restriction, this ratio decreased to 2.8–2.6% and to 2.4% respectively. By contrast, the DNA content of liver decreased only 10% with fasting and about 20% in the food-restricted group.

Figure 1 shows the effects of fasting and food restriction on total liver ADH activity and on ADH specific activity expressed as units per mg of liver DNA. In 24 hr of fasting, total ADH activity and specific activity (units per mg of DNA) decreased 33 and 30% respectively. With longer durations of fasting, no further change was observed. However, after food restriction (5 g/day) for 9 days, the specific activity of liver ADH (units per mg of DNA) decreased 48% and the total activity decreased 58%. Since Smith and Newman [12] reported earlier that the total activity of liver ADH does not change with fasting, studies were also performed using their assay conditions (pH 9.6). Contrary to their report, the total liver ADH activity decreased about 40% in the 48 hr-fasted rats.

Specific activities of liver ADH, expressed as units per mg of protein or per g wet weight, did not change with fasting or food restriction (Fig. 2). This circumstance is the consequence of cytosolic protein content and liver wet weight decreasing in parallel with total ADH activity during fasting and food restriction (cf. Table 1, Fig. 1).

Table 2 summarizes the effect of nutritional state on the rate of ethanol elimination *in vivo*, calculated by Widmark's formula. The *r* value which represents the ratio of the volume of distribution for ethanol to body mass increased with fasting and food restriction. It is apparent from these data that the ethanol elimination rate per rat decreased significantly ($P < 0.001$) with 48-hr fasting and food restriction for 9 days. The decreases are also evident ($P < 0.001$) when the data are expressed as rate per kg body weight. Owing to the change in body weight, however, the decreases are smaller than when the results are calculated as rate per rat. On the other hand, when the data are expressed as rate per g of liver, the rates did not decrease with 48 hr-fasting and food restriction.

Figure 3 shows the quantitative correlation of ethanol elimination rate with the total liver ADH activity in the fed, 48 hr-fasted and food-restricted rats. Both measurements decreased in parallel and total liver ADH activity exceeded the ethanol elimination rate by only 10–15% in each nutritional state.

DISCUSSION

The data presented here agree well with those reported earlier by Büttner [14] but they are at variance

Table 2. Effect of nutritional state on the rate of elimination of alcohol *in vivo*

Group (N)	Co* (mg ml^{-1})	p† (g)	β_{60}‡ (μmoles ml^{-1} hr^{-1})	*r*§ (ml g^{-1})	Ethanol elimination rate (μmoles hr^{-1} per rat)	(μmoles hr^{-1} per g liver)	(μmoles hr^{-1} per kg body wt)
Fed (7)	2.42 ± 0.05‖	279 ± 14	8.32 ± 0.25	0.83 ± 0.01	1922 ± 108	175 ± 14	6892 ± 185
Fasted, 48 hr (7)	2.32 ± 0.06	244 ± 15	5.49 ± 0.53	0.86 ± 0.02	1138 ± 105	173 ± 14	4713 ± 402
5 g Food/day (5)	2.10 ± 0.08	166 ± 6	5.38 ± 0.66	0.96 ± 0.04	835 ± 56	196 ± 14	5081 ± 426

* Y-intercept.
† Body weight.
‡ Slope.
§ Ao/(Co · p), where Ao is the amount (g) of ethanol injected intraperitoneally.
‖ Mean ± S.E.M.

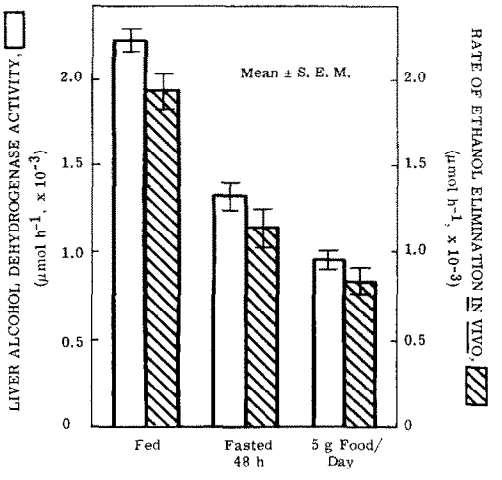

Fig. 3. Correlation of ethanol elimination rate per animal with the total liver ADH activity in the fed, 48 hr-fasted and food-restricted rats.

with those of Smith and Newman [12]. The liver is catabolic during fasting with respect to cytosolic protein, i.e. the rate of degradation is greater than the rate of synthesis [28]. While a recent study concluded that long-term ethanol treatment does not alter rat liver ADH content or degradation rate [29], no studies examining the effect of fasting on ADH synthesis or degradation have been reported. Since total ADH activity in liver decreases within 24 hr of fasting, data in this regard would be of considerable interest. Assuming that the decrease in total ADH activity is due to a decrease in enzyme content in liver, it appears that ADH is degraded more rapidly in the fasted state but at a rate similar to that for total cytosolic protein (Fig. 1, Table 1). Thus, when specific activity of liver ADH is expressed as units per mg of cytosolic protein, it does not change significantly in the fed, fasted and food-restricted rats (Fig. 2). Moreover, because feeding and fasting are linked to hormonal changes, the effect of hormones on the ADH level in liver will also need evaluation. Recent studies have suggested that this enzyme can be affected by hormones, e.g. testosterone and glucocorticosteroids, in some strains of rats [30, 31].

In this study, specific measures were undertaken to insure complete extraction and to prevent inactivation of ADH. Temperature, pH and ionic strength were kept close to that of the intracellular milieu. The endogenous NAD^+ "reductases" in the liver cytosolic fraction were low, as evidenced by the small blank rate in the absence of ethanol. However, both the K_m and V_{max} values of ADH in liver cytosol were dependent upon the composition of the assay buffer. Liver ADH activity assayed in a phosphate buffer was significantly lower than that measured in the Tris–HCl buffer or in the phosphate–KCl medium. These differences, not entirely accountable by the aldehyde trapping action of Tris–HCl, have been explained by a stimulatory effect of chloride ion on the activity of rat liver ADH (N. W. Cornell, personal communication). The use of different buffer systems may explain some of the divergent results in the past on the ethanol oxidizing capacity of liver. Of course, measurement of ethanol oxidation rates at alkaline pH and assays in the direction of acetaldehyde to ethanol [6] or in the presence of lactaldehyde [32] will consistently yield higher activity.

The data on the effect of nutritional state on ethanol elimination rate *in vivo* (Table 2) demonstrate clearly that the elimination rate decreases in the fasted and food-restricted rats when expressed per rat or per kg body weight. This observation is in agreement with a number of previous reports [7–10]. Interestingly, when the rate of ethanol elimination per rat or per liver was compared directly with total liver ADH activity, they decreased in parallel after fasting and food restriction (Fig. 3) and, under all conditions examined, total ADH activity in liver was only in slight excess. However, since the ethanol elimination rate *in vivo* also includes contributions by respiratory and urinary excretion and extrahepatic oxidation [33], this comparison probably underestimated to a certain extent the excess of total ADH activity in liver when compared with hepatic ethanol oxidation alone.

Based on the known K_i of NADH for rat liver ADH, it would appear that the concentration of free NADH in hepatic cytosol must serve as a determinant of the rate of liver ADH reaction. However, the limitation imposed by the increase in free NADH concentration during ethanol oxidation is nearly identical in the fed and fasted states [22]. Since total liver ADH activity decreases in parallel with the ethanol elimination rate following fasting and food restriction and is, in each instance, only slightly higher than the elimination rate, we concluded that the content of ADH in liver is a major rate-determining factor for ethanol elimination in the fasted and food-deprived rats. This conclusion does not exclude the possibility that the activities of minor ethanol-oxidizing enzymes, such as the microsomal ethanol-oxidizing system and catalase, also decrease with fasting and food deprivation and contribute as rate-determining factors.

Even in the fed state, it is likely that the level of liver ADH in the rat also has a rate-determining role. Kaiser and Burns [34] and Plapp [5] have emphasized that the rate of any enzymatic step in a series of reactions must affect the overall rate to some extent, such that there may be more than one principal rate-limiting step. Since the level of liver ADH is not in large excess, this factor must influence the overall rate of ethanol elimination. Indeed, several attempts have been made in the rat to increase the rate of ethanol elimination *in vivo* by the administration of pyruvate [35, 36], alanine [37], fructose [37] and dinitrophenol [38]. Unfortunately, two of these studies [36, 37] did not take into account alterations in the distribution of body water (r values Table 2) and did not transform the rates of fall of blood ethanol concentration into rates of ethanol metabolism, as outlined by Widmark [39]. As has been emphasized by Hawkins and Kalant [4], the rate of fall of blood ethanol concentration cannot be equated with the rate of ethanol metabolism, because the former is dependent on the volume of distribution. In the instances where the rates of ethanol metabolism were actually determined, the effect of pyruvate was nil [35] and that of dinitrophenol was only a 20–30% stimulation. Some studies *in vitro* using isolated tissue from rats have demonstrated stimulation of ethanol oxidation rates of similar or greater magnitudes by these compounds [40–47], but in these experiments, the control rates were invaria-

bly lower than the expected rate of ethanol oxidation, 3 μmoles/min/g of liver [6].

In studies of the ethanol–oxidizing capacity of liver and the rate of ethanol elimination *in vivo*, particular care should be taken to apply the proper reference base when calculating results. Since the wet weight and protein content of liver are altered markedly by hormonal and nutritional factors (Table 1), the use of these parameters as reference base can lead to erroneous interpretation (Fig. 2). Comparison of enzyme activity may best be made on the basis of total activity per liver or activity/mg of DNA (Fig. 1). Calculations of ethanol elimination rates per g liver wet weight can be similarly misleading. Rates of ethanol elimination determined *in vivo* are customarily expressed per kg body weight. Here, the underlying assumption is that a constant relationship exists between liver mass and lean body weight. It is evident from this study that the liver weight/body weight ratio does not remain constant (Table 1). Thus, even this manner of expression can be misleading, unless the liver weight/body weight ratios are known. All these complexities can be removed if both enzyme activity and ethanol elimination rate are compared simply on the basis of total activity or rate per liver (or per animal). Data expressed in this manner demonstrates clearly a correlated effect of different nutritional states on both ADH activity and ethanol elimination rate.

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