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EFFECT OF STRESS BY REPEATED IMMOBILIZATION ON HEPATIC ALCOHOL DEHYDROGENASE ACTIVITY AND ETHANOL METABOLISM

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Abstract—The hepatic activity of alcohol dehydrogenase was increased after 7, 14 and 42 days of stress induced by immobilization of rats for 2.5 hr/day. A single immobilization had no effect on the activity of alcohol dehydrogenase. Immobilization for 14 days resulted in increases in the rates of ethanol metabolism. This was not associated with changes in the activity of the microsomal ethanol-oxidizing system, microsomal catalase, cytochrome P-450, or NADPH cytochrome *c* reductase. A decrease in the hepatic phosphorylation potential ([ATP]/[ADP][P_i]) was found to be due to a decrease in [ATP] and an increase in [P_i]; however, there were no changes in O_2 consumption by liver slices or in hepatic ($Na^+ + K^+$)-stimulated adenosine triphosphatase activity. The increased rate of ethanol metabolism after stress remains unexplained since alcohol dehydrogenase activity is not rate-limiting in ethanol oxidation and there were no increases in ethanol oxidation by microsomes or in mitochondrial oxidative rate.

The hepatic activity of alcohol dehydrogenase (EC 1.1.1.1) was elevated in rats made uremic by removal of 85 per cent of the renal mass [1]. Also, recently, higher activities of alcohol dehydrogenase were demonstrated in the livers of patients dying suddenly from trauma or acute illness than in those dying from chronic illness or cancer [2, 3]. In both cases, the increases in alcohol dehydrogenase activity may have been the result of stress. Different types of stress have been shown to result in either increases [4] or decreases [5] in drug-metabolizing enzymes and in decreases in sleeping times after the administration of some barbiturates [6, 7]. Exposure of rats to cold resulted in increases in rates of ethanol metabolism [8–10], but no changes in liver alcohol dehydrogenase activity occurred [10]. Repeated immobilization is a well-defined method for the production of chronic stress [11, 12]. Hence, in the present study the effect of stress, induced by repeated immobilization, on alcohol dehydrogenase activity and on rates of ethanol metabolism was determined.

MATERIALS AND METHODS

Materials. Adenosine 5¹-[γ-^{32}P]triphosphate ($AT^{32}P$) was obtained from New England Nuclear, Boston, MA. Hydrolyzed starch for gel electrophoresis was purchased from Connaught Medical Research Laboratories, Toronto, Canada. Silicotungstic acid was obtained from ICN Pharmaceuticals, Plainview, NY. All other chemicals were purchased from either Sigma Chemical Co., St. Louis, MO, or Fisher Scientific Co., Pittsburgh, PA.

Animals and treatment. Male Sprague–Dawley rats (specific pathogen free from Taconic Farm, Inc., Germantown, NY) weighing between 250 and 300 g were stressed by immobilization for 2.5 hr/day as described previously [11]. Control animals were kept in their cages in a separate room. All the animals were provided with water and Purina Chow *ad lib.* In an initial experiment the hepatic activity of alcohol dehydrogenase was determined after 1, 7 and 42 days of immobilization. In additional experiments, the effect on alcohol dehydrogenase of immobilization for 14 days was determined as well as the effects on the activities of other ethanol-oxidizing enzymes and microsomal enzymes, rates of ethanol metabolism, O_2 consumption by liver slices, the activity of ($Na^+ + K^+$)-stimulated adenosine triphosphatase (ATPase), and the concentrations of ATP, ADP, AMP and P_i in liver homogenates. In all the experiments the immobilized animals and their respective controls were of the same age at the time of sacrifice.

Enzyme determinations. The animals were killed by decapitation immediately after the last immobilization and blood was collected in heparinized tubes for the determination of catecholamines.

The livers were removed, rinsed in 1.15% KCl and weighed. Liver tissue was minced and homogenized in a Potter–Elvehjem homogenizer with 4 vol. of 0.25 M sucrose in 0.1 M Tris–HCl buffer, pH 7.4. The homogenate was centrifuged at 9000 *g* for 10 min. The resulting precipitate was discarded and the supernatant fraction centrifuged at 106,000 *g* for 60 min. The supernatant fraction obtained at this point was separated for assay of alcohol dehydrogenase activity. For the determination of enzymes in the microsomal fraction, the microsomal pellet was washed with 8 ml of the above buffer and then recentrifuged at 106,000 *g* for 60 min. The washed microsomal pellet was finally resuspended in 8 ml of 0.1 M NaH_2PO_4–K_2HPO_4 buffer, pH 7.4.

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Alcohol dehydrogenase activity was determined in the 106,000 *g* supernatant fraction as described previously [1]. The volume of the reaction mixture was 3 ml and consisted of 0.01 M pyrophosphate buffer, pH 10.3, 18 mM ethanol, 0.41 mM NAD^+, and 0.1 ml of a $\frac{1}{5}$ dilution of the supernatant fraction. A blank reaction without ethanol was run in each case. The change in optical density at 340 nm was recorded for 10 min in each case. The alcohol dehydrogenase activities were then calculated from the molecular extinction coefficient of 6.22 $cm^2/\mu mole$ for NADH [13].

The enzymes and activities determined in the microsomes were catalase by the method of Feinstein [14], the microsomal ethanol-oxidizing activity described by Lieber and DeCarli [15], aniline hydroxylase according to Imai and Sato [16], cytochrome P-450 by the method of Omura and Sato [17], and NADPH-cytochrome *c* reductase by the method of Masters *et al.* [18]. Protein concentration was determined by the method of Lowry *et al.* [19] with bovine serum albumin used as a standard.

Kinetics and electrophoresis of alcohol dehydrogenase. The Michaelis–Menten constant (K_m) for ethanol was calculated from Lineweaver–Burk plots obtained from at least five duplicate determinations of alcohol dehydrogenase activity at non-saturating ethanol concentrations. The intercepts were determined by the method of least squares.

Starch-gel electrophoresis of the 106,000 *g* supernatant fraction was carried out on horizontal starch gels at pH 8.5 and 4° for 20 hr as described by Pietruszko and Theorell [20]. At the end of the electrophoresis the gels were stained for alcohol dehydrogenase activity by incubating them in 0.05 M Tris–HCl buffer, pH 8.6 containing, per 100 ml: NAD^+, 80 mg; nitro blue tetrazolium, 40 mg; phenazine methosulfate, 8.0 mg; and ethanol 100%, 0.4 ml.

Rates of ethanol disappearance. Ethanol (2.5 g/kg of body weight) was given as a 20 per cent solution (w/v) in water by stomach tube immediately prior to starting a 2.5-hr immobilization. Blood was obtained, from the retro-orbital plexus of each animal with a heparinized capillary tube, at the end of the 2.5-hr immobilization and hourly thereafter for 4 hr. After centrifugation at 2000 *g* for 10 min, the separated plasma samples were analyzed for ethanol concentration by gas liquid chromatography [21]. Ethanol concentrations in the plasma, when plotted against time, followed a linear function. The rate of ethanol disappearance from the plasma was obtained from the slope of the regression line calculated by the method of least squares. The ethanol degradation rate expressed in mg/kg of body weight/hr was obtained by first calculating Widmark factor *r*, and then multiplying it by ten times the rate of ethanol disappearance from the blood [22].

Oxygen consumption by liver slices. Liver slices (0.2 mm thick) were cut with a Stadie–Riggs slicer. About 30 mg of tissue slices was preincubated at 37° for 10 min in Ringer bicarbonate buffer, pH 7.4, and gassed continuously with $O_2 + CO_2$ (95:5) in a Dubnoff bath with shaking. The slices were then transferred to an O_2 electrode chamber equilibrated with the above gas mixture, and O_2 consumption was recorded with a Clark oxygen electrode for 2–4 min at 37° [23]. Oxygen consumption was determined also in separate slices from the same lobe of the liver in the presence of either dinitrophenol (0.1 mM) or ouabain (1.0 mM).

Liver ATPase activity. ATPase activity was determined in liver homogenates using $AT^{32}P$ as described by Videla *et al.* [10]. Liver (1 g) was homogenized in 12 ml of 0.32 M sucrose and 1 mM EDTA–Tris buffer, pH 7.4. The homogenate was diluted $\frac{1}{4}$ in water and 0.1 ml was used for the enzyme determination. The released ^{32}P was extracted as phosphomolybdate in isobutanol and measured in a liquid scintillation counter. The $(Na^+ + K^+)$-stimulated ATPase was calculated from the difference between the total ATPase activity minus the Mg^{2+}-stimulated activity determined in the presence of 1.0 mM ouabain and in the absence of NaCl and KCl.

ATP, ADP, AMP and P_i. The animals were killed by a blow on the neck. A portion of the liver was removed, rinsed in Ringer bicarbonate buffer, pH 7.4, and rapidly frozen in liquid nitrogen. Less than 10 sec elapsed between removal of the liver tissue and its freezing. The frozen liver was homogenized at high speed in a Potter–Elvehjem homogenizer with cold $HClO_4$ (2 ml/g of liver weight). The nucleotides were isolated and determined by the methods of Adam [24, 25]. P_i was determined by the method of Chen *et al.* [26].

Blood catecholamines. Catecholamines were determined in the plasma of rats after 1, 7 and 42 days of

Table 1. Effect of repeated daily immobilization on liver alcohol dehydrogenase activity*

Immobilization (days)	Alcohol dehydrogenase activity (μmoles/mg protein/hr)	Alcohol dehydrogenase activity (μmoles/g liver/hr)
0	1.28 ± 0.078	91.5 ± 5.30
1	1.28 ± 0.047	89.2 ± 2.16
7	1.59 ± 0.079+	114.7 ± 4.94+
42	1.97 ± 0.233+	137.7 ± 12.32‡

* Rats were immobilized for 2.5 hr/day as described previously [11]. The activity of alcohol dehydrogenase was determined as described in Materials and Methods. The enzyme activities are shown as means ± S. E. M. of eight animals in each group.

+ Significance of the differences (as compared to non-immobilized control rats) was $P < 0.05$.

‡ Significance of the differences (as compared to non-immobilized control rats) was $P < 0.01$.

Table 2. Body and liver weights and protein concentrations in the supernatant fraction of liver homogenates after repeated immobilization*

Immobilization (days)	Body wt (g)	Liver wt (g)	Liver wt (g/100 g body wt)	Supernatant fraction protein (mg/g)
0	373.7 ± 6.10	11.8 ± 0.34	3.2 ± 0.09	71.5 ± 2.81
1	369.9 ± 13.48	10.6 ± 0.46	2.9 ± 0.09	69.2 ± 1.65
7	337.1 ± 4.16†	8.8 ± 0.16†	2.6 ± 0.08†	73.0 ± 1.22
42	303.8 ± 5.69†	9.4 ± 0.32†	3.1 ± 0.12	71.4 ± 2.87

* Rats were immobilized for 2.5 hr/day as described previously [11]. The data are shown as means ± S. E. M. of eight animals in each group.

† Significance of the differences (as compared to non-immobilized control rats) was $P < 0.001$.

immobilization and in control non-immobilized rats by the radiometric–enzymatic assay of Coyle and Henry [27].

Statistical analysis. The results are presented as means ± S. E. M. The data were analyzed by Student's *t* test.

RESULTS

The activity of hepatic alcohol dehydrogenase was increased after 7 and 42 days of immobilization whether expressed per mg of protein or per g of liver (Table 1). A single immobilization had no effect on the activity of alcohol dehydrogenase. The animals immobilized for 7 and 42 days had lower final body and liver weights than the control animals (Table 2). The liver/body weight ratio was lower in the animals after 7 but not after 42 days of immobilization. The concentration of protein in the supernatant fraction of the liver homogenate was not affected by immobilization. Plasma catecholamines were elevated after 1, 7, and 42 days of immobilization (Table 3). The catecholamine levels were higher after 7 days than after 1 and 42 days of immobilization ($P < 0.02$).

In a separate experiment, immobilization for 14 days resulted in increases in the activity of hepatic alcohol dehydrogenase, as well as in the rates of ethanol metabolism (Table 4). Also, in this experiment, the final body and liver weights were lower in the immobilized animals, while the liver/body weight ratios and the concentration of protein in the supernatant fractions of the liver homogenates were similar to those found in the control animals.

Table 3. Plasma catecholamines after repeated daily immobilization (2.5 hr/day)*

Immobilization (days)	Plasma catecholamines (ng/ml)
0	12.4 ± 0.65
1	22.0 ± 1.41†
7	33.2 ± 3.03†‡
42	23.5 ± 1.64†

* The animals were killed by decapitation and blood was collected in heparinized tubes. Catecholamines were measured as described by Coyle and Henry [27]. Results are shown as means ± S. E. M. of eight animals in each group.

† Significance of the differences (as compared to non-immobilized control rats) was $P < 0.001$.

‡ Significance of the differences from values after 1 and 42 days of immobilization was $P < 0.02$.

The K_m of alcohol dehydrogenase for ethanol was of similar magnitude in the immobilized (2.3×10^{-3}M) and control (1.1×10^{-3}M) animals. Starch gel electrophoresis of the liver supernatant fractions revealed a single band of alcohol dehydrogenase activity migrat-

Table 4. Effect of repeated daily immobilization (2.5 hr/day) for 14 days on the activity of hepatic alcohol dehydrogenase and on rates of ethanol metabolism*

Determination	Control	Immobilized
Alcohol dehydrogenase (μmoles/mg protein/hr)	1.32 ± 0.126	2.19 ± 0.110†
Alcohol dehydrogenase (m-moles/kg body wt/hr)	2.24 ± 0.184	3.96 ± 0.185†
Ethanol metabolism (mg/kg body wt/hr)	153.9 ± 17.1	332.7 ± 29.5†
Ethanol metabolism (m-moles/kg body wt/hr)	3.35 ± 0.372	7.23 ± 0.641†
Body weight (g)	363.3 ± 5.78	290.5 ± 5.75†
Liver weight (g)	11.5 ± 0.56	8.9 ± 0.63†
Liver weight (g/100 g body wt)	3.2 ± 0.06	3.1 ± 0.09

* The activity of alcohol dehydrogenase was determined as described in Materials and Methods. For the determination of the rates of ethanol metabolism, ethanol (2.5 g/kg of body weight) was administered orally prior to starting the 2.5-hr immobilization. Blood samples for ethanol concentration were obtained at the end of the immobilization and hourly thereafter for 4 hr. Results are shown as means ± S. E. M. for eight animals in each group.

† Significance of the differences (as compared to non-immobilized control rats) was $P < 0.001$.

Table 5. Effect of repeated daily immobilization (2.5 hr/day) for 14 days on the activity of microsomal enzymes*

Determination	Control	Immobilized
MEOS† (μmoles/mg/min)	7.6 ± 0.99	8.1 ± 1.27
Microsomal catalase (mequiv/mg/hr)	7.4 ± 0.81	8.2 ± 0.45
Aniline hydroxylase (nmoles/mg/min)	8.4 ± 0.81	7.1 ± 0.94
Cytochrome P-450 (nmoles/mg)	0.67 ± 0.177	0.79 ± 0.286
Cytochrome *c* reductase (μmoles/mg/min)	0.12 ± 0.009	0.12 ± 0.008

* Enzyme assays were carried out as described in Materials and Methods. All the enzyme activities are expressed per mg of microsomal protein. Results are shown as means ± S. E. M. for eight animals in each group. No significant changes were found.

† Microsomal ethanol-oxidizing system.

ing towards the cathode in both the immobilized and control animals.

There were no changes in the hepatic activities of the microsomal ethanol-oxidizing system, microsomal catalase, aniline hydroxylase, and NADPH-cytochrome *c* reductase or in the concentration of cytochrome P-450 after immobilization (Table 5).

Oxygen consumption by liver slices of animals immobilized for 14 days was not significantly increased over that found in control animals (Table 6); however, dinitrophenol resulted in a 15 per cent increase in oxygen consumption of liver slices in the control animals, but in only a 2 per cent increase in the immobilized animals. Ouabain reduced oxygen consumption of liver slices by 15 per cent in both the control and immobilized animals.

Table 6. Effect of repeated daily immobilization (2.5 hr/day) for 14 days on respiration by liver slices*

Additions	Control	Immobilized
	(μmoles O_2/g liver/min)	
None	0.48 ± 0.034	0.52 ± 0.029
Dinitrophenol	0.55 ± 0.024	0.53 ± 0.022
Ouabain	0.41 ± 0.049	0.44 ± 0.041

* Oxygen consumption by liver slices was determined as described in Materials and Methods. The slices were preincubated with dinitrophenol (0.1 mM) or ouabain (1.0 mM) for 10 min prior to the determination of oxygen consumption with a Clark oxygen electrode. The values are shown as means ± S. E. M. of eight animals in each group. No significant changes were found.

The activity of $(Na^+ + K^+)$-stimulated ATPase was not changed by immobilization (Table 7). The activity of the Mg^{2+}-stimulated ATPase was increased significantly by immobilization only when expressed per g of liver.

Immobilization for 14 days resulted in a decrease in the phosphorylation potential, $[ATP]/[ADP][P_i]$ due to a decrease in the hepatic concentration of ATP, and an increase in the hepatic concentration of P_i (Table 8). The hepatic concentration of AMP was also increased by immobilization. No significant changes were found in the concentration of total nucleotides or in the adenylate kinase mass action ratio $[ATP][AMP]/[ADP]^2$.

DISCUSSION

This study demonstrates increases in hepatic alcohol dehydrogenase activity in rats stressed by immobilization. Recently, increases in hepatic alcohol dehydrogenase activity were demonstrated in rats made uremic by partial nephrectomy [1]. It is suggested that the increases in hepatic alcohol dehydrogenase in experimental uremia may be caused by the stress of the surgery and the uremic state. Previously, increases in

Table 7. Effect of repeated daily immobilization (2.5 hr/day) on hepatic ATPase activity*

Determination	Control	Immobilized
$(Na^+ + K^+)$-stimulated ATPase		
(μmoles of P_i/mg protein/hr)	0.16 ± 0.055	0.21 ± 0.050
(μmoles of P_i/g liver/hr)	32.76 ± 11.51	44.82 ± 10.78
Mg^{2+}-stimulated ATPase		
(μmoles of P_i/mg protein/hr)	4.05 ± 0.261	4.63 ± 0.245
(μmoles of P_i/g liver/hr)	799.6 ± 49.96	966.5 ± 46.15†

* ATPase activity was determined in liver homogenates using $AT^{32}P$ as described by Videla *et al.* [10]. The $(Na^+ + K^+)$-stimulated ATPase was calculated from the difference between the total ATPase activity determined in the presence of 1.0 M ouabain and in the absence of NaCl and KCl. The values are shown as means ± S. E. M. of eight animals in each group.

† Significance of the differences (as compared to non-immobilized control rats) was $P < 0.05$.

Table 8. Effect of repeated daily immobilization (2.5 hr/day) on concentrations of adenine nucleotides and inorganic phosphorus in the liver*

Determination	Control	Immobilized
	(μmoles/g liver)	
ATP	2.96 ± 0.168	2.06 ± 0.211†
ADP	1.46 ± 0.112	1.82 ± 0.128
AMP	0.57 ± 0.082	1.16 ± 0.103‡
Total nucleotides	4.98 ± 0.206	4.96 ± 0.155
P_i	5.76 ± 0.348	7.48 ± 0.571§
$[ATP]/[ADP][P_i](M^{-1})$	4.10 ± 0.751	1.63 ± 0.232†
$[ATP][AMP]/[ADP]^2$	0.81 ± 0.115	0.65 ± 0.082

* The animals were killed by a blow on the neck. The livers were removed and rapidly frozen in liquid nitrogen. The nucleotides and P_i were determined as described in Materials and Methods. The values are means ± S. E. M. of eight animals in each group.

† Significance of the differences (as compared to non-immobilized control rats) was $P < 0.01$.

‡ Significance of the differences (as compared to non-immobilized control rats) was $P < 0.001$.

§ Significance of the differences (as compared to non-immobilized control rats) was $P < 0.05$.

hepatic alcohol dehydrogenase were reported to occur after ethanol administration [28, 29]. However, more recently a number of investigators have been unable to find increases in hepatic alcohol dehydrogenase activity after acute and chronic ethanol administration [15, 30, 31]. In humans, increased hepatic alcohol dehydrogenase activity has been demonstrated in patients dying suddenly from trauma or acute illness [2, 3]. Starch gel electrophoresis of specimens with high alcohol dehydrogenase activity revealed, in addition to the usual isoenzymes, an anodic form [3]. This anodic form was subsequently isolated and found to have many of the characteristics of other forms of alcohol dehydrogenase, but a much higher K_m for ethanol [32]. In the rat, in contrast to the human, starch gel electrophoresis yields only one band migrating toward the cathode [33, 34], which was the finding in both the stressed and control animals in this study. Furthermore, in this study, the K_m for ethanol was not found to be altered by stress. The increase in hepatic alcohol dehydrogenase produced by stress may be mediated by hormones. The peak rise in plasma catecholamines obtained after 7 days of immobilization coincides with maximum increases in adrenal catecholamine-synthesizing enzymes demonstrated previously [12], and precedes the maximum increase in hepatic alcohol dehydrogenase activity. The plasma catecholamine levels obtained in the control animals killed by decapitation in this study are higher than values obtained in undisturbed rats by arterial catheter [35].

The finding of increased rates of ethanol metabolism after repeated immobilization is in agreement with prior studies showing that chronic stress induced by chronic exposure of rats to cold at 5° for various days resulted in increases in both the rate of ethanol matabolism *in vivo* [8–10] and in the rate of ethanol oxidation by liver slices *in vitro* [10]. By contrast, acute stress produced by hind leg ligation for 1 hr did not change rates of ethanol metabolism [36].

The principal enzyme responsible for the oxidation of ethanol is alcohol dehydrogenase. Ethanol has also been shown to be oxidized by catalase [37] and a microsomal enzyme system [15]. Some investigators suggested that ethanol oxidation by microsomes was due to contaminating catalase in the presence of hydrogen peroxide generated by NADPH oxidase from NADPH and oxygen [38]. However, recent studies show that ethanol is catalyzed by microsomal preparations free of contaminating alcohol dehydrogenase and catalase [39, 40]. Increases in the rate of ethanol metabolism have been attributed to increases in the activity of the microsomal enzyme system since alcoholics after recent alcohol ingestion [41], and rats, after ethanol administration [42], have increases in both the rates of ethanol metabolism and the activity of the microsomal enzyme. However, in the present study, repeated immobilization resulted in no changes in the activities of microsomal catalase and the microsomal ethanol-oxidizing system, suggesting that neither of these two enzymes is responsible for the observed increases in the rates of ethanol metabolism. In addition, repeated immobilization resulted in no changes in the microsomal activities of aniline hydroxylase or NADPH cytochrome *c* reductase, or in the concentration of cytochrome P-450. Previous studies of the effect of stress on hepatic enzymes have been few. Stress by cold acclimation of rats at 5° for 4 days resulted in increases in hepatic aniline hydroxylase and ethylmorphine demethylase [4]. In another study, however, acute stress induced in rats by unilateral hind limb ligation for 1 hr resulted in decreases in hepatic microsomal cytochrome P-450 concentration and hexobarbital hydroxylase [5].

Although both the hepatic activity of alcohol dehydrogenase and the rates of ethanol metabolism were increased by stress in this study, there is no evidence that changes in the activity of this enzyme control rates of ethanol metabolism. Many studies show a lack of correlation between *in vitro* activity of hepatic alcohol dehydrogenase and *in vivo* rates of ethanol metabolism [10, 41, 43]. Also, in our previous study, increases in the activity of hepatic alcohol dehydrogenase in uremic animals were not associated with increases in

the rates of ethanol metabolism [1]. The rate of ethanol metabolism by alcohol dehydrogenase *in vivo* is principally dependent on the mitochondrial reoxidation of NADH generated during ethanol oxidation [44]. The reoxidation of NADH could be limited by its transfer into the mitochondria or its oxidation by the mitochondrial respiratory chain. Increases in ethanol metabolism *in vivo* after chronic ethanol administration [23], cold-stress [10], and thyroxine administration [45] were shown to occur in association with increased hepatic mitochondrial oxidative capacity attributed to changes in phosphorylation potential as a result of increases in hepatic $(Na^+ + K^+)$-stimulated ATPase activity. Increased oxygen consumption was demonstrated also in association with an increased rate of ethanol utilization by the perfused livers of animals after chronic ethanol feeding [46]. In the present study, the increase in the rate of ethanol metabolism in the stressed animals was not associated with any significant changes in either oxygen consumption by liver slices or in $(Na^+ + K^+)$-stimulated ATPase. Hence, an increased mitochondrial oxidative rate does not appear to be the cause of the increased rate of ethanol metabolism after stress by immobilization. Other investigators have also failed to find increases in oxygen consumption by isolated hepatocytes despite increases in the rate of ethanol metabolism after chronic ethanol administration [47, 48]. In addition, in one of those studies, $(Na^+ + K^+)$-stimulated ATPase was found not to be changed, while the phosphorylation ratio, $[ATP]/[ADP][P_i]$, was decreased, as found in this study [48]. A decreased synthesis, rather than an increased demand, of ATP was postulated to be the cause of the decrease in phosphorylation ratio after chronic ethanol administration, since the decrease in ATP concentration was associated with a decrease in the activity of the adenine nucleotide translocase system, but in no significant increases in $(Na^+ + K^+)$-stimulated ATPase [49]. A decreased synthesis of ATP may also be a mechanism for the decrease in the phosphorylation found in the stressed animals in this study. Possible mechanisms, which remain to be studied, for increased rates of ethanol metabolism in the stressed animals are: an increased transfer of NADH into the mitochondria by either the malate–aspartate or α-glycerophosphate shuttles, or an increased reoxidation of NADH in the cytosol.

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