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EFFECT OF EPINEPHRINE ON ETHANOL METABOLISM BY ISOLATED RAT HEPATOCYTES

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Abstract—The effect of epinephrine on ethanol metabolism was determined in isolated rat hepatocytes. Epinephrine (10 μM) enhanced an initial rapid rate of ethanol elimination observed in the first 5 min. Thereafter, between 5 and 90 min, the rate of ethanol elimination was slower and not affected by epinephrine. Epinephrine resulted in higher acetaldehyde concentrations at 2 min, but not thereafter. Acetaldehyde production in the presence and absence of epinephrine was inhibited by 4-methylpyrazole, by a low free extracellular calcium concentration, and by the α_1-adrenergic blocker prazosin. Ethanol alone and epinephrine alone increased oxygen consumption, but the effects were not additive. The ethanol-induced decreases in the cytosolic NAD^+/NADH and $NADP^+$/NADPH ratios and in the mitochondrial NAD^+/NADH ratio were delayed by the presence of epinephrine. An accelerated initial alcohol dehydrogenase activity sufficient to account for the rapid initial rate of ethanol elimination shown with epinephrine was demonstrated by coupling ethanol oxidation with lactaldehyde reduction, a system which increases the rate of dissociation of NADH from the enzyme and its oxidation back to NAD^+. The findings in this study indicate that an increased reoxidation of NADH during ethanol oxidation by alcohol dehydrogenase is the basis for the rapid transient increase in ethanol elimination produced by epinephrine.

Epinephrine has been shown to stimulate ethanol oxidation in isolated hepatocytes [1] and in the perfused liver [2]. In hepatocyte culture, a rapid initial rate of ethanol elimination was observed during the first 5 min after the addition of ethanol [3]. In our previous study, epinephrine enhanced this initial rate of ethanol disappearance during the first 5 min, but thereafter the rate of ethanol disappearance was not affected by epinephrine. Alcohol dehydrogenase activity was not changed by the acute addition of epinephrine. The rapid initial rate of ethanol elimination in both control hepatocytes and those exposed to epinephrine was several times higher than the measured alcohol dehydrogenase activity *in vitro*. The purpose of the present study was to investigate the mechanism of the acute effect of epinephrine on enhancing ethanol elimination.

MATERIALS AND METHODS

Animals and materials. Male Sprague–Dawley rats were obtained from the Charles River Breeding Laboratories, Wilmington, MA. Collagenase (Type I), ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'* tetraacetic acid (EGTA) trypan blue, and 4-methylpyrazole were purchased from the Sigma Chemical So., St. Louis, MO. Prazosin was a gift of Pfizer Inc., New York, NY. Insulin and penicillin were purchased from E. R. Squibb & Sons, Inc., Princeton, NJ. Streptomycin was obtained from Eli Lilly, Indianapolis, IN. Epinephrine was purchased from Elkins-Sinno Inc., Cherry Hill, NJ. Dexamethasone was purchased from Lypho Med, Inc., Melrose Park, IL. Propranolol was obtained from Ayerst Laboratories, Inc., New York, NY. Dulbecco's modified Eagle's Medium (MEM) was obtained from Flow Laboratories, Inc., McLean, VA. Supplementary growth factor (SGF-7) was purchased from B & B Research Laboratory, Fiskeville, RI. L-Lactaldehyde was synthesized from L-threonine by the method of Zagalak *et al.* [4]. The concentration of L-lactaldehyde was determined by condensation with 2,4-dinitrophenylhydrazine [5].

Hepatocyte isolation. Rats weighing 200–250 g were anesthetized with ether. The livers were perfused *in situ* through the portal vein with Hanks' balanced salt solution containing 0.5 mM EGTA and 2.5 mM tricine, followed by the complete culture medium containing 0.03% collagenase. The livers were removed and the hepatocytes separated from non-parenchymal cells by centrifugation at 60 *g* for 2 min, a process that was repeated three times, each time after washing and resuspending the cells in cool collagenase-free medium. The collagenase-free medium was Dulbecco's MEM with the following supplemental growth factors: insulin (250 units/L), transferrin, selenium, epidermal growth factor, fetuin, and bovine serum albumin–oleic acid and –linoleic acid complexes. It also contained penicillin (1×10^6 units/L) and streptomycin (0.13 mM). The cell suspensions were saturated with 95% O_2 and 5% CO_2. Only hepatocyte suspensions with a viability greater than 95% determined by trypan blue exclusion were used in the experiments.

Ethanol metabolism. The rate of ethanol metabolism was determined by incubating freshly isolated hepatocytes in 2 mL of medium in the presence of

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8 mM ethanol at 37° for various periods of time in sealed flasks with teflon-lined septa. Epinephrine (10 μM) or an isovolumetric amount of medium was added to the flasks at the same time as the ethanol. At zero time and at each time thereafter, the metabolism of ethanol was stopped by precipitation of the cell suspension with 0.5 mL of 70% trichloroacetic acid (TCA) injected into the flasks. Ethanol and acetaldehyde concentrations in the flasks were then determined by head-space gas chromatography. Ethanol elimination was calculated from the decrease of ethanol concentration by the method of least squares. The area under the acetaldehyde concentration time curve over a 15-min period of sampling was calculated by the trapezoidal rule using computer software.

Alcohol dehydrogenase. Alcohol dehydrogenase activity was assayed by coupling the oxidation of ethanol to the reduction of lactaldehyde as described by Gupta and Robinson [6]. The reaction mixture consisted of 0.5 M Tris–HCl buffer (pH 7.2), 18 mM ethanol, 2.8 mM NAD^+, 1.4 mM L-lactaldehyde and 0.03 mL of homogenates of hepatocyte suspensions after sonication in a total volume of 1.0 mL. The assay was carried out in sealed flasks with teflon-lined septa at 37° for 0, 2, 3, 10, and 15 min, and stopped at each of the times with 0.25 mL of 70% TCA injected into the flasks. The acetaldehyde formed was determined by head-space gas chromatography. Protein was determined by the method of Lowry *et al.* [7] with bovine serum albumin used as a standard.

Oxygen uptake. Samples of three million cells were suspended in 2 mL of medium, shaken at 37°, and gassed with 95% O_2 and 5% CO_2. Oxygen uptake was measured at 37° in the presence of ethanol and/or epinephrine for 5 min with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). The oxygen electrode was calibrated based on the stoichiometry of the xanthine oxidase reaction as described by Billiar *et al.* [8].

Hepatic free NAD^+/NADH and $NADP^+$/NADPH ratios. These determinations were done by assay of oxidized and reduced substrates of NAD^+(P)-linked dehydrogenases located in the cytosol and mitochondria and calculation of the redox ratios from the equilibrium constants of the dehydrogenases as described by Veech *et al.* [9]. Suspensions of one million cells in 2 mL of medium were incubated in the presence of 8 mM ethanol with and without 10 μM epinephrine at 37° in sealed flasks with teflon-lined septa. At various intervals of time, 1.0 mL of 6% perchloric acid was added to the cell suspensions followed by sonication and freezing with liquid nitrogen and storage at −70°. Further treatment of the samples consisted of centrifugation to separate precipitated protein and adjustment of the supernatant fraction to pH 6.0 with 2% KOH, followed by centrifugation to remove $KClO_4$ and treatment with Florisil (Floridin Co., Hancock, WV) as described by Williamson *et al.* [10]. The supernatant fraction obtained after centrifugation to remove Florisil was used for the determination of metabolites. The cytosolic NAD^+/NADH ratio was determined from the lactate dehydrogenase reaction, while the mitochondrial NAD^+/NADH ratio was calculated from the β-hydroxybutarate dehydrogenase reaction. The cytosolic $NADP^+$/NADPH ratio was determined from the malic enzyme reaction. Lactate was determined by the method of Hohorst [11]. Pyruvate was determined by the fluorometric method of Lowry and Passonneau [12]. β-Hydroxybutyrate was determined by the method of Williamson *et al.* [13] and acetoacetate by the method of Mellanby and Williamson [14]. Malate was determined by the method of Mollering [15].

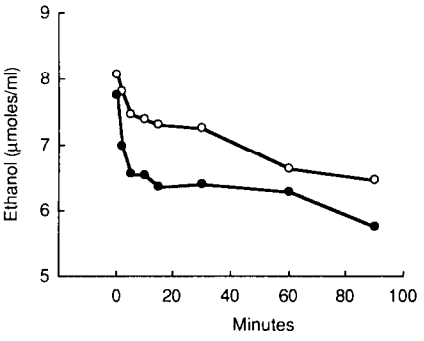

Fig. 1. Effect of epinephrine (10 μM) on the disappearance of ethanol from a suspension of 3 million hepatocytes in 2 mL of medium. Values are the means of 4–5 determinations in the presence (●—●—●) and absence of epinephrine (○—○—○).

The data were analyzed by Student's *t*-test when appropriate or by analysis of variance when comparing means of more than two groups. The Dunnett procedure was used to compare multiple treatments with control [16].

RESULTS

Exposure of hepatocytes to 10 μM epinephrine resulted in a very rapid initial rate of ethanol elimination of 111.5 ± 14.7 nmol/mg protein/min in the first 5 min (Fig. 1). This compares with an initial rate of ethanol elimination of 58.9 ± 16.0 nmol/mg protein in the control hepatocytes ($P < 0.05$). Thereafter, between 5 and 90 min slower rates of ethanol elimination of 4.00 ± 1.34 nmol/mg protein/min for hepatocytes exposed to epinephrine and 6.03 ± 1.22 nmol/mg protein/min for controls were not significantly different.

Acetaldehyde concentration increased linearly during the first 10 min after addition of ethanol to the hepatocytes and then decreased (Fig. 2). Epinephrine resulted in an initial higher acetaldehyde concentration at 2 min of 93.6 ± 2.6 μM as compared with a value of 81.0 ± 4.0 μM in the control ($P < 0.05$). Thereafter, there were no differences in the acetaldehyde concentrations attained in the presence or absence of epinephrine. The areas under the acetaldehyde concentration time curve were 7.9 and 7.8 $\mu mol \cdot mL^{-1} \cdot min$ in the presence and absence of epinephrine respectively. Acetaldehyde production was inhibited markedly by 4-methylpyrazole (Fig. 3) and by EGTA. The addition of 3 mM EGTA was calculated by means of a computer program, to decrease the free calcium concentration in the

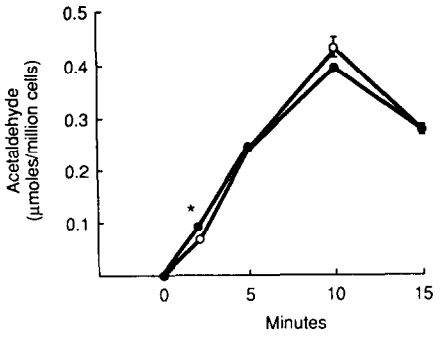

Fig. 2. Acetaldehyde accumulation from the metabolism of 8 mM ethanol by isolated hepatocytes (2 million/2 mL of medium) in the presence (●—●—●) and absence of 10 μM epinephrine (○—○—○). Values are the means ± SE of 4 determinations. Key: (*) statistically significant versus control, $P < 0.05$.

medium from 1.8 mM to 0.37 μM [17]. Acetaldehyde concentrations were lower in the presence of prazosin. Propranol resulted in a delayed increase in acetaldehyde, but not in the total amount of acetaldehyde accumulated over 15 min. The actions of the above inhibitors were similar in the presence and absence of epinephrine.

The cytosolic $NAD^+/NADH$ and $NADP^+/NADPH$ ratios decreased in hepatocytes incubated in medium without ethanol or epinephrine (Table 1). The decrease was apparent within 5 min and became more marked after 30 min of incubation. Epinephrine (10 μM) delayed the decreases in the cytosolic $NAD^+/NADH$ and $NADP^+/NADPH$ ratios with the decrease in $NAD^+/NADH$ becoming apparent only after 10 min of incubation. The mitochondrial $NAD^+/NADH$ ratio was not affected by incubation of the hepatocytes in the presence or absence of epinephrine. Ethanol (8 mM) resulted in a more marked fall in cytosolic $NAD^+/NADH$ and $NADP^+/NADPH$ ratios during 5 min of incubation (Table 2) than found in hepatocytes not exposed to ethanol (Table 1). A further gradual decrease in both cytosolic $NAD^+/NADH$ and $NADP^+/NADPH$ ratios occurred at later times of incubation of the hepatocytes in the presence of ethanol. A decrease in the mitochondrial $NAD^+/NADH$ ratio occurred at 15 min (Table 2). Epinephrine delayed the decreases in both cytosolic $NAD^+/NADH$ and $NADP^+/NADPH$ ratios to 10 min after the addition of ethanol, whereas the decreases in the mitochondrial $NAD^+/NADH$ ratio became apparent only at 60 min.

Ethanol increased oxygen consumption by the hepatocytes (Fig. 4). Epinephrine (10 μM) also increased oxygen consumption (Table 3). The combined effect of 8 mM ethanol and 10 μM epinephrine on oxygen consumption was not significantly greater than that found with 10 μM epinephrine alone.

Lactaldehyde resulted in a marked stimulation of alcohol dehydrogenase activity in the hepatocyte homogenates (Fig. 5). Alcohol dehydrogenase activity was 295.5 ± 62.0 nmol/mg protein/min in the first 2 min in the presence of lactaldehyde compared with a rate of 42.0 ± 1.9 nmol/mg protein/min in the control ($P < 0.01$). Thereafter, between 2 and 15 min, the alcohol dehydrogenase activities were 63.2 ± 6.9 and 19.8 ± 0.9 nmol/mg protein/min in the presence and absence of lactaldehyde ($P < 0.001$).

DISCUSSION

This study confirms and extends prior observations of an effect of epinephrine in stimulating ethanol elimination by hepatocytes [1, 3]. In agreement with our prior observations in hepatocyte culture [3], epinephrine in the isolated hepatocytes produced a transient enhancement of ethanol elimination in the first 5 min with no significant effect thereafter. Epinephrine also resulted in a higher initial acetaldehyde

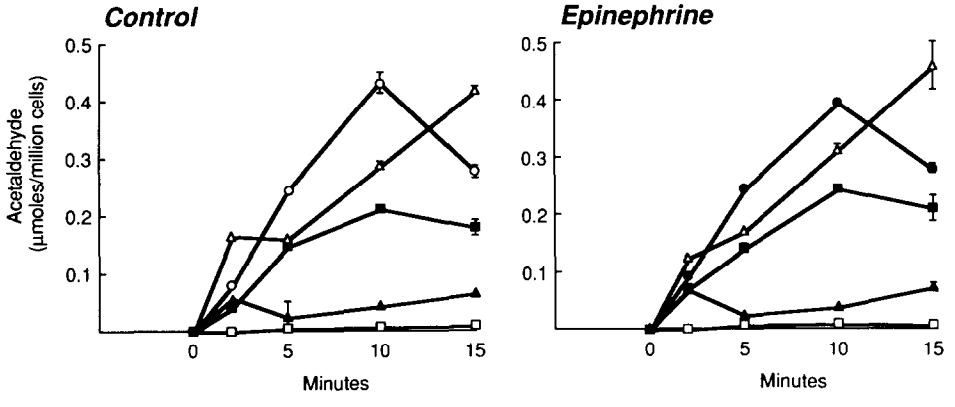

Fig. 3. Effects of inhibitors on acetaldehyde accumulation from the metabolism of 8 mM ethanol by isolated hepatocytes in the absence (○—○—○) and presence of 10 μM epinephrine (●—●—●). The hepatocytes were incubated with the inhibitors prior to the addition of ethanol and epinephrine. The inhibitors used were: 4-methylpyrazole, 8 mM (□—□); prazosin, 10 mM (■—■); propranolol, 10 mM (△—△) and EGTA, 3 mM (▲—▲). All values are the means ± SE of 4 determinations. The effects of all inhibitors were statistically significant at all time points at either $P < 0.05$ or $P < 0.01$ (all values after 5 min).

Table 1. Changes in redox state by isolated hepatocytes in the presence and absence of epinephrine

Time (min)	Cytosol NAD+/NADH	Cytosol NADP+/NADPH	Mitochondria NAD+/NADH
Control			
0	1374 ± 53	0.0262 ± 0.0037	8.8 ± 1.8
5	813 ± 67*	0.0122 ± 0.0007†	6.9 ± 0.7
10	960 ± 98*	0.0140 ± 0.0010†	12.8 ± 1.6
30	142 ± 20*	0.0036 ± 0.0006*	14.9 ± 2.3
90	122	0.0034	19.7
Epinephrine			
0	995 ± 86	0.0218 ± 0.0051	10.5 ± 1.9
5	1199 ± 56	0.0223 ± 0.0013	11.7 ± 1.2
10	642 ± 82†	0.0231 ± 0.0014	13.3 ± 0.9
30	257 ± 104*	0.0036 ± 0.0009*	12.3 ± 2.2
90	131	0.0054	15.7

Values are means ± SE of 3 determinations, except for the single 90-min values.
*† Significantly different from zero time values: *$P < 0.01$, and †$P < 0.05$.

Table 2. Changes in redox state during ethanol elimination by isolated rat hepatocytes in the presence and absence of epinephrine

Time after ethanol addition (min)	Cytosol NAD+/NADH	Cytosol NADP+/NADPH	Mitochondrial NAD/NADH
Control			
0	1175 ± 45	0.0244 ± 0.0035	10.4 ± 0.9
5	568 ± 41*	0.0051 ± 0.0014*	9.5 ± 1.1
10	357 ± 50*	0.0040 ± 0.0006*	11.0 ± 1.8
15	335 ± 62*	0.0051 ± 0.0008*	6.0 ± 0.4*
30	310 ± 70*	0.0023 ± 0.0005*	5.2 ± 1.4*
60	115 ± 16*	0.0028 ± 0.0006*	3.1 ± 0.5*
90	89 ± 6*	0.0010 ± 0.0001*	6.4 ± 0.8*
Epinephrine			
0	960 ± 63	0.0301 ± 0.0067	10.8 ± 0.9
5	820 ± 35	0.0163 ± 0.0091	11.6 ± 0.7
10	453 ± 46*	0.0040 ± 0.0012*	8.5 ± 1.5
15	519 ± 64	0.0033 ± 0.0008*	8.2 ± 0.5
30	213 ± 80*	0.0018 ± 0.0002*	10.7 ± 1.8
60	89 ± 6*	0.0032 ± 0.0012*	5.0 ± 0.3*
90	65 ± 4*	0.0013 ± 0.0002*	6.0 ± 1.2*

All values are means ± SE of 4 determinations.
* Significantly different from value without ethanol (0 time) at $P < 0.01$.

concentration in the first 2 min of ethanol metabolism, but this effect was smaller in magnitude and not sustained. The lack of a larger and more prolonged difference in the accumulation of acetaldehyde between hepatocytes exposed to epinephrine and control hepatocytes is because the capacity of aldehyde dehydrogenase to metabolize acetaldehyde exceeds its generation [18]. An additional factor is reduction of acetaldehyde back to ethanol at high acetaldehyde concentrations. In this study, decreases in ethanol concentrations of 940 and 506 nmol per million hepatocytes after 15 min incubation in the presence and absence of epinephrine (Fig. 1) were associated with accumulations of acetaldehyde of 297 and 277 nmol respectively. The acetaldehyde concentrations obtained in this study were higher than concentrations usually obtained in hepatocytes during ethanol oxidation [19], because they represent acetaldehyde that was released from the hepatocytes and accumulated in a sealed vapor phase at 37°. The marked inhibition of acetaldehyde accumulation by 4-methylpyrazole of greater than 97% demonstrates that the metabolism of ethanol by the hepatocytes is catalyzed by alcohol dehydrogenase. Prasozin inhibited acetaldehyde accumulation in the presence and absence of epinephrine, suggesting that the effect of epinephrine on ethanol metabolism was mediated by the α_1-adrenergic receptor. The inhibition by prasozin of acetaldehyde accumulation by control hepatocytes

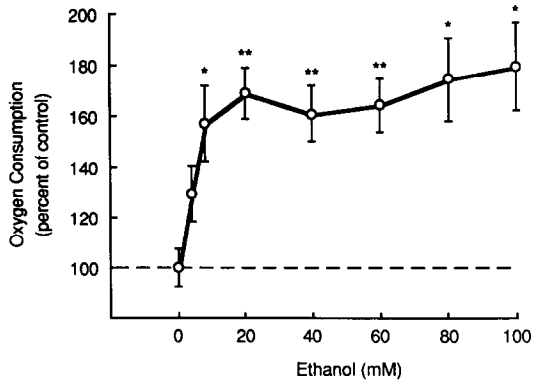

Fig. 4. Effect of ethanol on oxygen consumption by hepatocytes. Oxygen uptake was determined with a Clark oxygen electrode for 5 min after the addition of various concentrations of ethanol to samples of 3 million hepatocytes suspended in 2 mL of medium. Values are expressed as mean (±SE) percent of control of 4–7 observations. The mean oxygen uptake in control hepatocytes (no addition of ethanol) was 9.51 nmol/mg protein/min. Statistically significant increase from value without ethanol: (*) $P < 0.05$, and (**) $P < 0.01$.

Table 3. Effects of ethanol and epinephrine on oxygen consumption by isolated hepatocytes

Treatment	Oxygen consumption (nmol/mg protein/min)
Control	10.9 ± 1.3
Ethanol	14.9 ± 0.9*
Epinephrine	18.6 ± 2.5*
Ethanol + epinephrine	19.8 ± 3.2*

Oxygen uptake was determined with a Clark oxygen electrode in the presence or absence of ethanol (8 mM) and epinephrine (10 μM). All values are means ± SE of 4–6 determinations.

* $P < 0.05$, compared to control value.

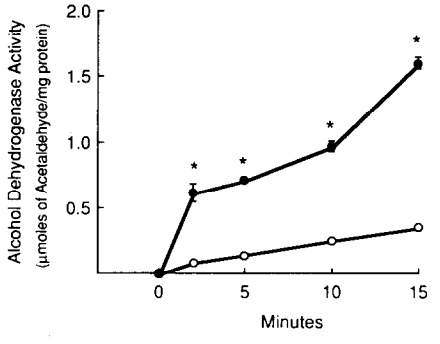

Fig. 5. Alcohol dehydrogenase activity in hepatocyte homogenates assayed in the presence of 1.4 mM L-lactaldehyde (●—●) and in its absence (○—○). Acetaldehyde formation was assayed by head space gas chromatography as described in Materials and Methods. All values are the means ± SE of 4 determinations. Key: (*) statistically significant difference compared to control, $P < 0.01$.

suggests that, in addition, prasozin has an effect on either alcohol dehydrogenase activity or on the reoxidation of the formed NADH. The effect on control hepatocytes was not due to inhibition of residual endogenous catecholamines after hepatocyte isolation, since it also inhibits ethanol elimination by hepatocytes after 4 days in culture in the absence of catecholamines [3]. Propranolol delayed the accumulation of acetaldehyde, but eventually led to a higher concentration. This effect of propranolol is probably multifactorial, since propranolol has been found to inhibit alcohol dehydrogenase [20] and hepatic mitochondrial NADH oxidation [21].

Ethanol was shown in this study to increase oxygen consumption by the isolated hepatocytes. This agrees with one prior study in which 10 mM ethanol increased oxygen consumption in isolated hepatocytes [22]; however, in other studies no such effect could be demonstrated [23, 24]. The reason for the difference in results is unknown; however, in the perfused liver ethanol has been consistently shown to increase oxygen consumption [2, 25, 26]. Epinephrine [2, 27] and other catecholamines [28–30] are well known to increase oxygen consumption in hepatocytes. The effects of ethanol and epinephrine in increasing oxygen consumption in this study were not additive which agrees with similar observations in the perfused liver [25]. The presence of a normal calcium concentration was necessary for optional ethanol metabolism. Both ethanol [31] and epinephrine [32] have been shown to result in a rapid transient increase in intracellular calcium in hepatocytes, which in the case of epinephrine has been shown to precede the increase in oxygen consumption [33].

The effect of epinephrine in increasing the initial rate of ethanol oxidation was most likely due to an increased flux in the mitochondrial respiratory chain resulting in increased reoxidation of NADH and, hence, increased availability of NAD^+. This is supported by the effect of epinephrine in increasing oxygen consumption and producing a delay in the decreases in the cytosolic NAD^+/NADH and $NADP^+$/NADPH ratios and the mitochondrial NAD^+/NADH ratio caused by ethanol oxidation. Electron acceptors such as methylene blue and phenazine methosulfate are well known to stimulate both hepatocyte oxygen consumption and ethanol oxidation [34]. Furthermore, this study shows that rates of ethanol oxidation by hepatocyte alcohol dehydrogenase achieved in the coupled reaction with lactaldehyde [6] were sufficient to account for the high initial rates of ethanol elimination observed in hepatocytes exposed to epinephrine. Indeed, the initial rate of ethanol elimination in the first 5 min after exposure of the hepatocytes to epinephrine of 111.5 nmol/mg protein/min was lower than the initial alcohol dehydrogenase activity recorded in the lactaldehyde coupled assay. The increased rate of ethanol oxidation in the coupled reaction with lactaldehyde implies that the dissociation of bound NADH from the enzyme is the rate-limiting step in the alcohol dehydrogenase reaction. The effects of epinephrine in increasing the rate of ethanol metabolism resemble the swift increase in ethanol metabolism (SIAM) which occurs *in vivo* a few hours after

ethanol administration [25] and which is likewise associated with an increased hepatic oxygen uptake and increased reoxidation of NADH.

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