Pharmacology Biochemistry & Behavior, Vol. 18, Suppl. 1, pp. 449–454, 1983. © Ankho International. Printed in the U.S.A.

Zonal Redox Changes As a Cause of Selective Perivenular Hepatotoxicity of Alcohol

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BARAONA, E., P. JAUHONEN, H. MIYAKAWA AND C. S. LIEBER. *Zonal redox changes as a cause of selective perivenular hepatotoxicity of alcohol.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 449–454, 1983.—Alcohol-induced lesions predominate in perivenular zones of the liver. To test the hypothesis that ethanol aggravates hypoxia in this zone by stimulating oxygen consumption, we measured hepatic venous pO_2 after ethanol-administration to both alcohol-fed baboons (with perivenular lesions) and their controls, and found no changes: the stimulation of oxygen consumption was fully offset by a parallel increase in blood flow. Despite the lack of hypoxia, ethanol increased lactate/pyruvate (L/P) 15-fold in hepatic venous blood and only 3-fold in liver tissue. Addition of lactate to increase arterial L/P several-fold produced no changes in the hepatic venous ratio, indicating that the equilibrium between this ratio and the cytosolic redox state was reached in one passage through the liver. Thus, the higher L/P in hepatic venous blood most likely reflects an enhanced redox shift in perivenular zones. In isolated hepatocytes, a pO_2 comparable to that normally prevailing in perivenular zones mimicked the exaggeration of the ethanol-induced redox shift and aggravated inhibition of protein synthesis. We therefore propose an alternate mechanism for the selective perivenular injury; namely, that the low pO_2 normally prevailing at this site aggravates the redox-linked toxicity of ethanol.

Alcoholism Liver Oxygenation Redox Protein synthesis Intraacinar heterogeneity

ALCOHOLIC liver injury starts and predominates in the perivenular region (also called centrolobular zone or area 3 of the hepatic acinus) [19], which has the lowest oxygen tension. The prevailing hypothesis for the mechanism of this selective perivenular hepatotoxicity is that the injurious effects of ethanol at this site may be due to hypoxia, resulting from stimulation of hepatic oxygen consumption [10]. Since in the normal liver and probably at early stages of alcoholic liver disease, channels other than sinusoids do not contribute significantly to the hepatic venous blood, the partial pressure of oxygen (pO_2) in the hepatic venous blood can be considered to approximate that existing in perivenular regions. Therefore, to assess this hypothesis, we studied whether acute or chronic ethanol administration to non-human primates (baboons) alters splanchnic oxygen consumption and/or hepatic venous pO_2.

EFFECTS OF ETHANOL ON HEPATIC OXYGENATION

To assess the effects of ethanol on hepatic oxygenation *in vivo*, we used baboons pair-fed nutritionally adequate liquid diets containing 50% of energy either as ethanol or as additional carbohydrate [16]. In these animals, chronic administration of alcohol-containing diets reproduces the spectrum of alcoholic liver disease (from fatty liver to cirrhosis) under controlled nutritional conditions. To focus on the early events that lead to the development of cirrhosis, we studied 3 baboons fed alcohol-containing diet for 5 months (in which only fatty liver was present) and 5 fed for 14 months and 1 for 3 years (in which perivenular fibrosis accompanied the steatosis) together with the corresponding pair-fed controls. Animals with septal fibrosis or cirrhosis were excluded because the possibility of intrahepatic shunts is very likely at these advanced stages of liver disease. In all alcohol-fed baboons, there were degenerative changes of perivenular hepatocytes, consisting in enlargement, hydropic swelling and irregular cytoplasmic clumping, that characterize the so-called "ballooning" of the hepatocytes.

To study the effects of chronic alcohol consumption initially in the absence of ethanol in the blood, the alcohol-containing diet was replaced by control diet (from 4 to 10 p.m. the day prior to the experiments), followed by overnight administration of dextromaltose. Animals were anesthesized with Ketamine and a polyurethane catheter (Copra type I, size 7F, USCI, Billerica, MA) was introduced percutaneously through the femoral vein into the hepatic vein under fluoroscopy. Arterial and peripheral venous cannula were also inserted.

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TABLE 1

EFFECTS OF ACUTE AND CHRONIC ETHANOL ADMINISTRATION ON HEPATIC OXYGENATION IN BABOONS*

	Pair-Fed Controls		Alcohol-Fed Baboons	
	No acute ethanol	Plus acute† ethanol	No acute ethanol	Plus acute† ethanol
Splanchnic oxygen consumption (μmoles/kg body wt./min)	58.4 ± 7.0	82.8 ± 12.1‡	59.9 ± 6.3	65.4 ± 7.7
Estimated hepatic blood flow (ml/kg body wt./min)	25.6 ± 3.2	39.5 ± 5.7§	20.6 ± 1.8	27.5 ± 2.9
Hepatic venous oxygen tension (mmHg)	39.2 ± 2.9	41.2 ± 3.7	31.2 ± 2.9¶	33.1 ± 2.8
Hepatic venous lactate/pyruvate	11.9 ± 1.7	166 ± 15§	15.3 ± 1.9	184 ± 36§

*Means ± standard error of the means of 18 baboons pair-fed liquid diets containing 50% of energy either as ethanol or as additional carbohydrate for 5 months to 3 years.

†A steady level of blood ethanol (26 ± 2 mM) was achieved by intravenous injection of 1.1 g of ethanol per kg body weight (given in 30 minutes) followed by constant infusion at a rate similar to that of ethanol elimination: 2.43 ± 0.09 mmoles/kg body wt/hr in the controls vs. 3.24 ± 0.10, in the alcohol-fed baboons; $p<0.01$.

‡$p<0.02$, compared to before acute ethanol administration (paired comparisons).

§$p<0.01$, compared to before acute ethanol administration (paired comparisons).

¶$p<0.05$, compared to the pair-fed controls (paired comparisons).

Hepatic blood flow was monitored from the extraction of indocyanine green (Cardio-Green, Hanson, Wescott & Dunning Inc., Baltimore, MD), according to the Fick principle [5]. Plasma volume was determined with Evans blue dye (Harvey Laboratories, Inc., Philadelphia, PA) by the dilution method [9]. pO_2 was measured using a Radiometer acid-base analyzer (model BMS3Mk2, The London Co., Cleveland, OH). Splanchnic oxygen consumption was calculated from the arterio-hepatic venous difference in oxygen content (calculated from pO_2 and hemoglobin content and saturation with oxygen) and the hepatic blood flow. Hematocrit, as well as lactate and pyruvate [18] were measured in arterial and hepatic venous blood at frequent intervals.

After a basal period of 1–2 hours, ethanol was administered intravenously as a loading dose of 1.1 g per kg body weight in 30 minutes to create a blood ethanol concentration of 26±2 mM, which was maintained constant for 3 hours by continuous infusion of ethanol at a rate equal to the rate of oxidation previously determined in these animals: 3.24±0.11 mmoles per kg body wt. per hour in the alcohol-fed baboons and 2.43±0.09 in the pair-fed controls ($p<0.01$). The blood ethanol concentrations achieved were comparable to those found during continuous and spontaneous feeding of the alcohol-containing diets. Indeed, the mean blood ethanol concentrations were 38±5 mM at the middle of the feeding period and 20±6 prior to the serving of a fresh ration of diet. Thus, unless imposed by experimental protocols, there were no periods of alcohol withdrawal in these animals. The same procedures performed in the absence of ethanol were repeated 2–3 hours after starting the ethanol infusion.

The infusion of ethanol increased splanchnic oxygen consumption in the control baboons (Table 1). However, this effect was associated with a parallel increase in hepatic blood flow that fully offset possible hypoxic effects in perivenular zones, as reported previously [23]. In fact, hepatic venous pO_2 remained unchanged. Moreover, these acute effects of ethanol disappeared or became greatly attenuated in animals chronically fed alcohol-containing diets. Thus, our findings do not support the hypothesis that ethanol-induced stimulation of hepatic oxygen consumption leads to hypoxia in perivenular zones of the liver.

Prior to the infusion of ethanol, hepatic venous pO_2 in the baboons withdrawn from alcohol was 20% lower than in the pair-fed controls (Table 1). However, there were no differences in splanchnic oxygen consumption between these two groups. The lower hepatic venous pO_2 in alcohol-fed animals was associated with a reduction of hepatic blood flow, which becomes very significant when considering that the liver size, measured by the radiographic procedure of Walk [26], increased by 30% (31±2 cm^3 per kg body weight in the alcohol-fed baboons versus 21±1 in the pair-fed controls; $p<0.01$). The lack of parallelism between the blood flow and the hepatomegaly may reflect increased resistance to sinusoidal flow by swollen hepatocytes [11], perivenular fibrosis [17] or both. In any event, the decrease in liver perfusion cannot be considered a primary action of ethanol on oxygen consumption, but rather a secondary consequence of the liver damage.

EFFECTS OF ETHANOL ON REDOX STATE

Despite the lack of changes in hepatic oxygenation, acute alcohol administration produced a 15-fold elevation of the lactate/pyruvate ratio in the hepatic venous blood of both alcohol-fed and pair-fed control baboons (Table 1). To study whether similar changes occur in liver tissue, we performed needle biopsies and the tissue obtained was homogenized immediately in perchloric acid. The weight of the liver sample was assessed from its protein content and previous knowledge of the hepatic protein composition in these animals. While, in the absence of ethanol, the lactate/pyruvate ratio and the concentration of these metabolites were similar in hepatic venous blood and in total liver tissue, the

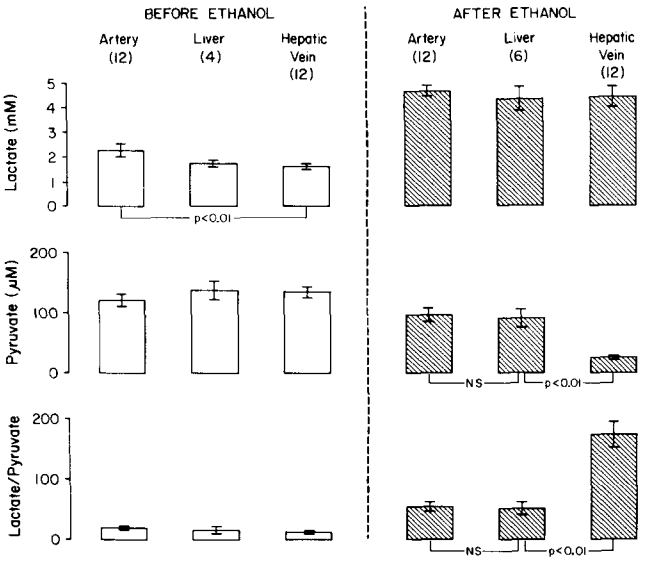

FIG. 1. Effects of ethanol (26±2 mM) on hepatic lactate (L) and pyruvate (P). Numbers in parentheses indicate numbers of baboons. Since there were no significant differences between the alcohol-fed and the pair-fed controls, they are treated here as a single group. During ethanol infusion, the hepatic removal of lactate was inhibited and its concentration rose both in blood and liver tissue, increasing L/P ratios. In addition, in hepatic venous blood, there was a marked depletion of pyruvate, leading to an elevation of L/P ratio much greater than in total liver tissue. From [12], with permission.

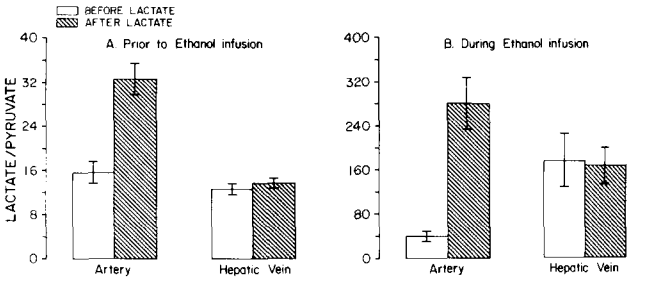

FIG. 2. Intravenous administration of lactate markedly increased lactate/pyruvate ratios in the arterial blood, whereas it produced no changes in the hepatic venous ratio, regardless of the presence of ethanol. Thus, the equilibrium between the lactate/pyruvate ratio of the blood perfusing the liver and the cytosolic redox state of the hepatocytes occurs in one single passage through the organ.

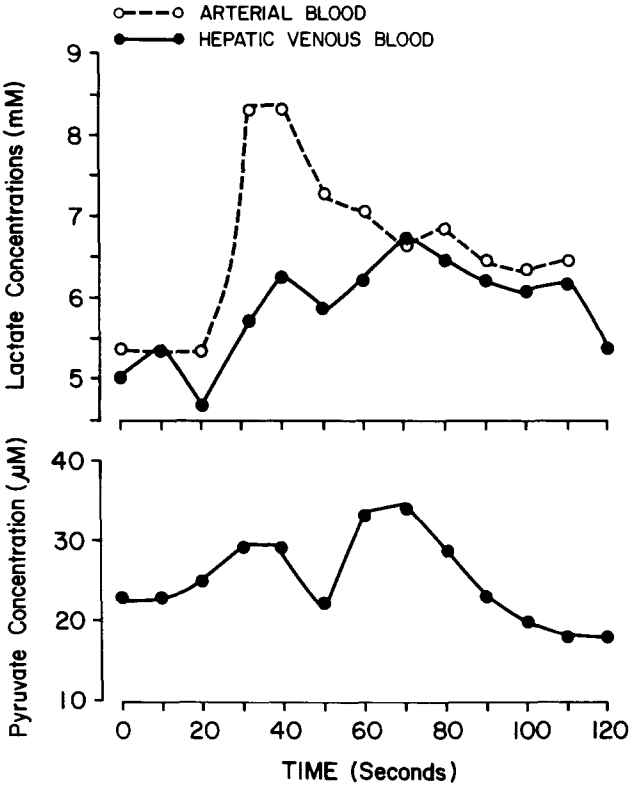

FIG. 3. Effects of a rapid lactate injection on the hepatic venous lactate (L) and pyruvate (P) concentrations in a control baboon infused with ethanol. The rise in hepatic venous lactate was immediately followed by hepatic release of pyruvate, maintaining the L/P ratio essentially unchanged. Arterial pyruvate concentrations did not change during this period. The rapid equilibration suggests that the L/P ratio in the hepatic venous blood reflects the cytosolic redox state of perivenular hepatocytes, with which hepatic venous blood was last in contact before leaving the liver.

infusion of ethanol produced striking differences in the rise of the lactate/pyruvate ratio at these two sites (Fig. 1). The most conspicuous alteration was a marked depletion of pyruvate in the hepatic venous blood, elevating the lactate/pyruvate ratio considerably more than in total liver tissue or arterial blood.

We therefore wondered whether this high L/P ratio in hepatic venous blood was reflecting the cytosolic redox state of the total liver or only that of perivenular hepatocytes in equilibrium with the blood about to leave the liver via the hepatic vein. The rapid equilibrium between intra- and extra-cellular concentrations of these metabolites is fully documented under a wide range of lactate/pyruvate ratios in the absence of ethanol [4]. To determine how rapidly this equilibrium takes place "in vivo" and in the presence of ethanol, we administered intravenous loads of lactate sufficient to increase the lactate/pyruvate ratio several-fold in the arterial blood (Fig. 2). Both in the absence and in the presence of ethanol, the changes in the arterial lactate/pyruvate were associated with no changes in the hepatic venous ratio. In some of these experiments, we administered 1 to 1.5 mmoles of lactate as a rapid intravenous bolus and we collected arterial and hepatic venous blood (0.1 ml per second) at 5 second intervals. As illustrated in one of these experiments (Fig. 3), the dilution curve of lactate collected in the hepatic vein followed that in the artery and had two domes, probably reflecting the hepatic arterial and portal contributions to the liver blood flow. The rise in hepatic venous lactate was accompanied by a parallel increase in pyruvate concentration with practically no delay, leaving the lactate/pyruvate ratio unchanged. Thus, the equilibrium of the lactate/pyruvate ratio of the blood perfusing the liver with the cytosolic redox state of the hepatocytes occurs in one single passage through the organ. Therefore, we conclude that the hepatic venous ratio must reflect the redox state of perivenular hepatocytes, with which the blood was last in contact before leaving the sinusoids.

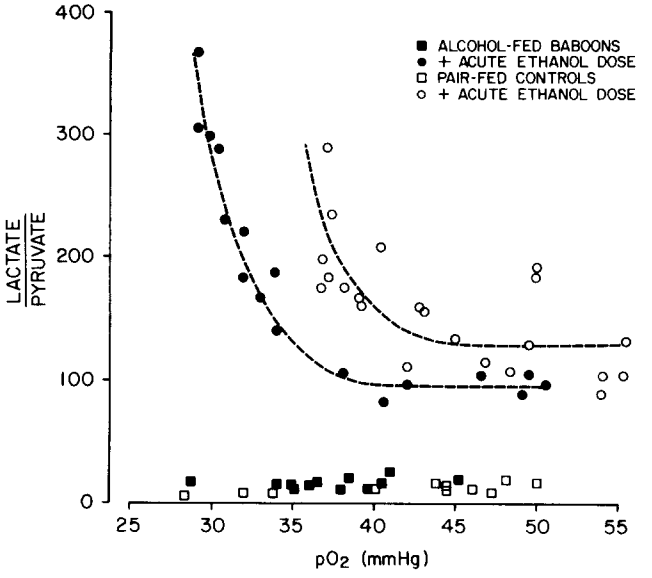

FIG. 4. Relationships between hepatic venous pO_2 and lactate/pyruvate (L/P) ratio after acute and chronic alcohol administration. In the absence of ethanol, the L/P ratio is not affected even at the lowest oxygen tensions found in hepatic venous blood, suggesting that the availability of oxygen is sufficient for oxidation of the normally produced reducing equivalents. In the presence of ethanol, oxygen tensions normally encountered in hepatic venous blood are associated with exaggerated rises of the L/P ratio, suggesting that the availability of oxygen is insufficient for the oxidation of the excessive production of reducing equivalents during ethanol oxidation. Chronic alcohol consumption attenuates the ethanol-induced redox shift; therefore, oxygen tensions lower than in naive animals are required for similar rises in L/P ratio. However, alcohol-fed baboons had lower pO_2 (due to impaired circulation), which offset the latter effect.

The striking difference between hepatic venous blood and liver biopsy tissue in the rise of the lactate/pyruvate ratio after ethanol suggests an enhanced ability of ethanol to shift the cytosolic redox state in the perivenular zones of the liver. To validate the comparison of tissue and blood, the possibility that ethanol could interfere with the diffusion of pyruvate from cell to blood had to be assessed. To this effect, we incubated isolated rat hepatocytes in Krebs-Ringer bicarbonate medium under 95% O_2 and 5% CO_2, with 10 mM lactate to stimulate intracellular production of pyruvate. After one hour of incubation with 50 mM ethanol (a time sufficient for equilibrium between intra- and extra-cellular lactate/pyruvate ratios), the hepatocytes were separated from their media by a 15 second-12,000 × g centrifugation through a silicone oil (d=1.030) layer into 21% perchloric acid [25]. Compared to the 10:1 intra- to extra-cellular gradient of glucose-6-phosphate (which indicates intact membranes), there was no gradient of pyruvate concentration between cells (147 nmoles per ml of cell water) and the medium (146 nmoles per ml). Thus, the comparison between cell and blood lactate/pyruvate ratios appears justified.

The increased ability of ethanol to produce a more reduced state of the cytosol in perivenular zones was also noted by Quistorff *et al.* [20] in two- and three-dimensional recordings of the redox state in freeze-clamped samples of hemoglobin-free perfused rat livers. However, the possibility that the increase in oxygen consumption produced by ethanol [24] might have produced perivenular hypoxia under conditions of fixed flow and perfusates with low oxygen-carrying capacity was not excluded.

RELATIONSHIPS BETWEEN pO_2 AND LACTATE/PYRUVATE RATIOS IN HEPATIC VENOUS BLOOD

These relationships (Fig. 4) indicate that, in the absence of ethanol, the hepatic venous pO_2 varies within a wide range, producing no changes in the lactate/pyruvate ratio, both in control and alcohol-fed baboons. This suggests that the oxygen tensions prevailing in perivenular zones are adequate for the oxidation of normal loads of reducing equivalents, even at the lower pO_2 values generally found in alcohol-fed animals. However, in the presence of ethanol, oxygen tensions normally prevailing at this site are insufficient for the oxidation of the excessive amounts of reducing equivalents generated during ethanol oxidation, resulting in severe shifting of the cytosolic redox state.

At similar oxygen tensions, the rise in lactate/pyruvate ratio produced by ethanol was lower in animals chronically fed alcohol-containing diet than in the naive controls. This is consistent with the previously reported [8,21] attenuation of the ethanol-induced redox shift by chronic alcohol consumption. However, the development of alcoholic liver injury impairs circulation and decreases perivenular oxygenation, as discussed above, completely offsetting the benefits of the attenuation of the redox shift.

EFFECTS OF pO_2 ON THE ETHANOL-INDUCED REDOX SHIFT AND INHIBITION OF PROTEIN SYNTHESIS

The results discussed above suggest that the low pO_2 normally prevailing in perivenular zones could be at least one of the mechanisms for the exaggeration of the ethanol-induced redox shift. Moreover, we previously reported [1] that under conditions of exaggerated cytosolic redox shift and pyruvate depletion (similar in magnitude to those found here in hepatic venous blood) ethanol was capable of inhibiting protein synthesis. Since this could be at least one of the reasons why alcoholic liver injury is more severe in perivenular zones, we studied the effects of pO_2 on these ethanol-induced changes in isolated hepatocytes prepared from fed rats by the Seglen [22] modification of the Berry and Friend [3] procedure. Approximately 150 mg of hepatocytes were incubated with and without 50 mM ethanol in 3 ml of medium containing Hepes instead of bicarbonate as a buffer. Incubations were carried out for 30 minutes, either under air or under 47 ml of 4% O_2 and 96% N_2. The media were supplemented with 0.38 mM ^{14}C-leucine, 3 mM lactate and 0.3 mM pyruvate. Lactate and pyruvate were added to provide an initial ratio of 10 at concentrations of these metabolites similar to those found in blood during in vivo ethanol oxidation. Moreover, these additions promote rates of ethanol oxidation closer to those found *in vivo* [6]. The rate of ethanol elimination was determined under the above conditions, except that an initial concentration of 10 mM ethanol was necessary for accurate measurements of the changes in ethanol concentration during incubation. Under air, the rate of ethanol elimination was 1.38±0.20 μmoles/g of packed cells/min. When corrected for the difference in wet weight/dry weight ratios between packed hepatocytes and liver tissue, this rate is equivalent to 2.37 μmoles/g of liver/min, in which is only slightly lower than the 2.9 rates reported *in vivo* in a similar strain of rats [15]. Incubation under 4% O_2

TABLE 2

EFFECTS OF INCUBATION OF RAT ISOLATED HEPATOCYTES WITH AND WITHOUT 50 mM ETHANOL AT TWO OXYGEN TENSIONS ON REDOX STATE AND PROTEIN SYNTHESIS*

	Incubation under 21% O_2		Incubation under 4% O_2	
	No ethanol	Ethanol	No ethanol	Ethanol
Lactate (μmoles/liter)	2950 ± 260	3260 ± 260	3610 ± 390	3210 ± 260
Pyruvate (μmoles/liter)	237 ± 28	43 ± 4†	191 ± 19	25 ± 3†‡
Lactate/pyruvate ratio	14.0 ± 0.1	86.4 ± 5.4†	20.1 ± 1.6‡	161.9 ± 15.7†§
Oxygen consumption (μmoles/g packed cells/min)	1.51 ± 0.1	1.42 ± 0.1	1.07 ± 0.1‡	1.01 ± 0.1
^{14}C-leucine incorporation into cell protein (dpm × 10^{-3}/g packed cells/hr)	350 ± 5	307 ± 9†	357 ± 5	269 ± 13†§

*Hepatocytes were isolated by a modification of the method of Berry and Friend and incubated in HEPES-buffered saline media supplemented with 3 mM lactate and 0.3 mM pyruvate, either under air (which produces a pO_2 of 150 mmHg) or under 4% oxygen (which produces a pO_2 comparable to that found in hepatic venous blood, namely 30 mmHg) for 30 min. Mean ± SEM of 6 experiments done in quadruplicates.

†$p<0.01$, compared to hepatocytes incubated without ethanol at the same pO_2.

‡$p<0.05$, compared to hepatocytes incubated without ethanol under air.

§$p<0.05$, compared to hepatocytes incubated with ethanol under air.

markedly decreased the rate of ethanol elimination (0.89±0.07 μmoles/g of packed cells/min; $p<0.05$). In spite of the decreased rate of ethanol oxidation, ethanol increased the lactate/pyruvate ratio almost twice as much under 4% oxygen as under air (Table 2). This was associated with more severe depletion of pyruvate. It should be pointed out that, even in the absence of ethanol incubation under 4% oxygen slightly, but significantly, increased lactate/pyruvate ratio. This suggests that the pO_2 was close to the critical hypoxic level [14]. In keeping with this interpretation, parallel incubations in a Gilson submarine differential respirometer showed that oxygen consumption was in fact decreased under 4% oxygen, but ethanol did not produce any further change. Therefore, the exaggeration of the redox shift produced by ethanol under low oxygen tension was not due to further hypoxia (Table 2). Thus, these experiments indicate that low oxygen tensions comparable to those found in perivenular zones increase the ability of ethanol to shift the redox state, probably by diminishing the ability to reoxidize the excess of reducing equivalents generated during alcohol oxidation.

The exaggeration of the cytosolic redox shift produced by ethanol under low oxygen tension was associated with a greater decrease in the incorporation of leucine into cell protein. This inhibition was associated with no changes in the specific activity of intracellular leucine, thereby most likely reflecting inhibition of protein synthesis. The mechanism of the inhibition of protein synthesis by ethanol has not been fully elucidated, but it has been linked to the ability of ethanol to deplete pyruvate [1], the inhibition being prevented by a variety of conditions that spare pyruvate [2,7]. It has been hypothesized that pyruvate depletion may divert non-essential amino acids from protein synthesis [2,7]. The aggravation of this inhibitory effect by low oxygen tensions was also noted by others [13]. In any event, these experiments indicate a potentially very deleterious consequence of the exaggeration of the ethanol-induced redox shift in perivenular zones. It remains to be assessed whether these effects take place in perivenular hepatocytes and *in vivo*.

CONCLUSIONS

The prevailing hypothesis for the pathogenesis of the selective perivenular hepatotoxicity of ethanol, namely that alcohol increases the hepatic oxygen consumption and that the resulting hypoxia of perivenular zones is severe enough to cause cellular damage, is not supported by our findings in an animal model which reproduces the perivenular lesions found in alcoholics. The acute stimulation of oxygen consumption produced by ethanol did not alter hepatic venous pO_2 because of a concomitant increase in blood flow. Moreover, these acute effects were attenuated after prolonged alcohol consumption. As liver damage progresses, hepatic venous pO_2 decreases as a consequence of impaired circulation rather than because of a hypermetabolic state. Therefore, we propose the alternate hypothesis that the conditions normally prevailing in perivenular zones aggravate the toxicity of ethanol. Our findings indicate that oxygen tensions normally prevailing at this site aggravate the redox-linked toxicity of alcohol to the extent of inhibiting protein synthesis. Despite some attenuation of the redox shift after chronic alcohol consumption, the development of perivenular hypoxia due to the progression of the liver damage may aggravate this form of ethanol toxicity even further.

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