Dependence on Dose of the Acute Effects of Ethanol on Liver Metabolism In Vivo

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ABSTRACT The dose dependence of the acute effects of ethanol upon liver intermediary metabolism in vivo has been demonstrated in rats. Ethanol was given i.p. in doses of 0.69, 1.7, and 3.0 g/kg in equal volumes (20 ml/kg). The liver was freeze-clamped 120 min after injection, and multiple metabolites were measured in the perchloric acid extract of the tissue. Each group showed a significantly different pattern of metabolites, redox states, and phosphorylation potentials although the rate of ethanol disappearance, at least between the two highest dose groups, was not significantly different. The mitochondrial free [NAD^+]/[NADH] ratios and the cytoplasmic free [$NADP^+$]/[NADPH] ratio were paradoxically most reduced with the lowest dose of ethanol and became progressively more oxidized with increasing dose. Once established, the differences in these ratios between the groups tended to persist with time, relatively independent of the concentration of ethanol. In a somewhat different pattern, the phosphorylation potential ([ATP]/[ADP][P_i]) remained at the control level in the low-dose group but was significantly elevated in the two higher-dose groups.

The results, therefore, show distinct and complicated dose-dependent patterns of intermediary metabolism that cannot be explained completely by any one hypothesis but that imply significant dose-dependent effects of ethanol upon intermediary metabolism not directly related to NADH production.

INTRODUCTION

The liver damage seen clinically in alcoholism seems to be the result both of nutritional factors and of a direct toxic effect of ethanol on liver, independent of diet (1, 2). Although chronic exposure to ethanol is clearly an essential feature in the production of the damage, the possibility that dose might be a separate issue has received little attention. Even with acute biochemical studies, in only a minority of investigations has more than one dose of ethanol been tested. For example, though ethanol is well known to produce extensive changes acutely in the liver metabolite pattern, redox states, and phosphorylation potential (3-8), there doesn't seem to be a previous study comparing the effects of several different doses upon these variables in the unanesthetized animal in vivo.

Prediction of the effect of dose on liver metabolism from previous in vivo and in vitro studies is not possible since extrapolations of these studies lead to conflicting conclusions. Liver, which can metabolize ethanol, shows a profound alteration in intermediary metabolism upon exposure to ethanol (3-8), while tissues, such as brain, that do not participate significantly in the metabolism of ethanol show relatively minor disturbances in the metabolite pattern even with high doses or lengthy exposure (9). A common and logical conclusion from these observations has been that the profound and widespread changes seen during ethanol metabolism in liver are ultimately understandable as consequences of the rate of NADH production and the reduction of the cytoplasmic free [NAD^+]/[NADH] ratio by the reactions of alcohol dehydrogenase (EC 1.1.1.1) (Eq. 1) and aldehyde dehydrogenase (EC 1.2.1.3) (Eq. 2) (10, 11).

$$CH_3CH_2OH + NAD^+ \rightleftharpoons CH_3CHO + NADH + H^+ \quad (1)$$

$$CH_3CHO + NAD^+ \rightarrow CH_3COO^- + NADH + 2H^+ \quad (2)$$

The disappearance of ethanol from the intact rat (even corrected for small respiratory and urinary losses)

Received for publication 28 May 1975 and in revised form 30 July 1975.

is linear over a wide range of concentrations (12, 13) and shows in vivo kinetics (K_m, V_{max}) similar to alcohol dehydrogenase itself (14) (Eq. 1). If the observed metabolism in vivo were entirely via the alcohol dehydrogenase pathway, the rate of cytoplasmic NADH production would be independent of the ethanol concentration above approximately 5 mM in the rat (12). Therefore, to the extent that the effects of ethanol on liver are NADH-dependent, these effects would also be independent of the ethanol concentration above a very low level.

This straightforward view, on the other hand, is challenged by much in vitro data. For example, a clear dose-dependent effect of ethanol on gluconeogenesis from lactate in perfused rat liver has been documented (15), an effect that tends to parallel the in vitro substrate inhibition of alcohol dehydrogenase by ethanol at concentrations above about 8 mM (16). These in vitro data suggest that the rate of ethanol metabolism through the alcohol dehydrogenase pathway and, therefore, the rate of cytoplasmic NADH production would decrease with increasing concentrations of ethanol above 8 mM and that, therefore, the effects of high doses of ethanol on intermediary metabolism in liver might actually be less than those of lower doses. The predictions of the effect of dose are further complicated by in vitro evidence for alternate pathways of ethanol metabolism operative at high ethanol concentrations (17, 18) and by nonlinear ethanol metabolism curves also at high levels (19).

Since there has not been an extensive study documenting the effect of ethanol at several doses upon the pattern of metabolites, redox states, and phosphorylation potentials in vivo in the livers of unanesthetized rats, the current study was undertaken to test the predictions of the various hypotheses of the effects of ethanol dose. The results show distinct and complicated dose-dependent patterns of intermediary metabolism that cannot be explained completely by any one hypothesis but that imply significant dose-dependent effects of ethanol upon intermediary metabolism not directly related to NADH production.

METHODS

Treatment of animals. Male Wistar rats, weighing between 190–210 g, were obtained from Carworth Div. (Becton, Dickinson & Co., New City, N. Y.) and maintained on Wayne Lab-Blox diet (4% fat, 24% crude protein, and 50.8% carbohydrate) (Allied Mills, Inc., Chicago, Ill.) and tap water ad lib. The animals, fasted 24 h before use, were injected i.p. with equal volumes (20 ml/kg) of either 0.15 M NaCl or 0.75, 1.8, or 3.3 M ethanol in 0.15 M NaCl, giving ethanol doses of 0.69, 1.7, and 3.0 g/kg, respectively. In one set of experiments (injection schedule A) all animals were killed between 10:00 and 11:00 a.m., 120±1 min after injection. In separate experiments (injection schedule B) comparing only the 1.7 and 3.0 g/kg ethanol doses, all animals were killed between 3:00 and 4:00 p.m., 120±1 min after injection of the 1.7 g/kg ethanol and 445±1 min after injection of the 3.0 g/kg ethanol. In the latter experiments, the animals given the 3.0 g/kg ethanol had the same ethanol concentration in body water at 445 min as animals given 1.7 g/kg ethanol had after 120 min (see Results).

To compare the rates of ethanol disappearance from the blood of animals given 1.7 or 3.0 g/kg ethanol, injections were carried out as usual (injection schedule A), but 50-μl blood samples were obtained in heparinized micropipets from free-flowing blood obtained by repeatedly clipping the tip of the tail. After injection with 1.7 g/kg ethanol, blood specimens were taken at 60, 120, 150, and 180 min (±1 min); after 3.0 g/kg ethanol, the blood was sampled at 240, 300, 360, and 420 min (±1 min). The blood (50 μl) was diluted with 250 μl deionized H_2O (0°C) and mixed immediately with 250 μl 8% (wt/vol) $HClO_4$ (0°C). After the blood stood capped on ice for 15 min, 5 ml 0.2 M Tris-acetate buffer, pH 9.5, was added, and the mixture was centrifuged 3,000 *g* at 0°C for 15 min in a Sorvall RC-2B refrigerated centrifuge (Sorvall-Dupont Instruments, Sorvall Operations, Newtown, Conn.). The Tris (Sigma Chemical Co., Inc., St. Louis, Mo.) had been previously recrystallized from acetone to remove traces of ethanol. Ethanol was determined spectrophotometrically on the clear supernate, with yeast alcohol dehydrogenase (Sigma) (20). Recovery of ethanol added to blood was 98%. The rate of ethanol disappearance was determined for each animal by linear regression analysis.

Treatment of liver. The rats were killed by cervical dislocation, and the liver was freeze-clamped within 8 s with aluminum tongs cooled in liquid nitrogen (21). Perchloric acid extracts of the frozen liver powder were prepared as described previously (22). Before treatment with Florisil (activated magnesium silicate, 100–200 mesh, Fisher Scientific Co., Pittsburgh, Pa.), a sample of the supernate was saved for ATP, ADP, AMP, P_i, and NH_4^+ determinations. The portion of the extract used for the NH_4^+ determination was treated with Norit A (decolorizing charcoal, alkaline, Fisher Scientific Co.) (23).

To test the effect of ethanol on anoxic changes in liver ATP levels, the animals were injected with either 0.15 M saline or one of the ethanol doses 120±1 min before sacrifice (injection schedule A), but the freezing of the liver after cervical dislocation was delayed. Part of each liver was freezed-clamped at 12 s, and part at 24 s.

Determination of metabolites. Standard analytical-grade laboratory reagents were obtained from Mallinckrodt Chemical Works, St. Louis, Mo. Hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27), and pyruvate kinase (EC 2.7.1.40) were obtained from Calbiochem, San Diego, Calif. All other enzymes and the substrates and cofactors for the assays were obtained from Boehringer Mannheim Corp., New York.

Pyruvate and isocitrate were determined fluorimetrically (24). All other metabolites were measured spectrophotometrically by enzymatic assays. Glutamate was determined by the method of Lowry and Passoneau (24). Ammonia was determined by the method of Folbergrova et al. (25); phosphate was measured by the method of Guynn et al. (22). Other metabolites were measured by methods cited previously (8). The fluorimetric assays were carried out with a Farrand ratio fluorometer (Farrand Optical Co., Inc., Valhalla, N. Y.); the spectrophotometric assays were performed on a Zeiss PMQ III spectrophotometer modified to contain 10 cuvettes (Carl Zeiss, Inc., New York). Recoveries of all metabolites added to frozen liver powder were between 91 and 105%.

Calculations. The cytoplasmic free [NAD^+]/[NADH] ratio was calculated from the lactate dehydrogenase reaction (EC 1.1.1.27) (26). The cytoplasmic free [$NADP^+$]/[NADPH] ratio was calculated from the isocitrate dehydrogenase reaction (EC 1.1.1.42) (27); this ratio has not been calculated from the malic enzyme reaction (EC 1.1.1.50), since this enzyme goes out of equilibrium in the presence of ethanol (8). The cytoplasmic phosphorylation potential was calculated directly from the total concentrations of ATP, ADP, and P_i (28). It should be noted that total P_i rather than HPO_4^{2-} has been used to calculate the phosphorylation potential. The mitochondrial free [NAD^+]/[NADH] ratio was calculated from both the 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) glutamate dehydrogenase reactions (EC 1.4.1.2) (29).

Since the object of this study was to examine differences among the various ethanol doses, it was felt that selection of the middle dose for comparison with the remaining groups would be the most informative statistical comparison. Accordingly, in Tables I–V, the *P* values refer to differences between the 1.7 g/kg dose and the other groups, as judged by Student's *t* test.

RESULTS

Rate of disappearance of ethanol from blood. The rate of ethanol disappearance from the blood of animals receiving the 3.0 g/kg ethanol was 6.19±0.38 μmol/ml blood/h (247±15 mg/kg/h; 2.49±0.15 μmol/g liver/min) (six animals), 8% higher but not significantly different from a rate of 5.75±0.45 μmol/ml blood/h (227±18 mg/kg/h; 2.28±0.18 μmol/g liver/min) (eight animals) found for animals receiving the 1.7 g/kg ethanol dose. For the calculations an average of 36 g liver/kg body weight has been taken. The individual points for each animal readily fit the equation for a straight line, the average correlation coefficient for each group being 0.992±0.002. Extrapolation of the straight

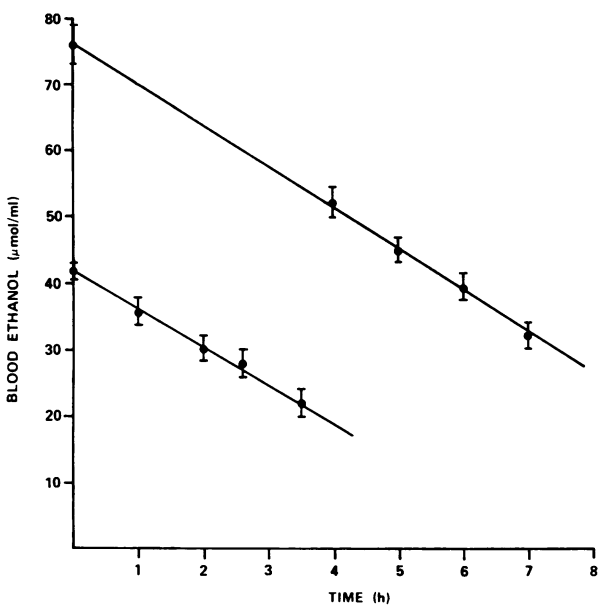

FIGURE 1 Effect of ethanol dose on disappearance of ethanol from blood. Animals were injected with equal volumes (20 ml/kg) with either 3.0 g/kg (upper curve) or 1.7 g/kg (lower curve) ethanol in 0.15 M NaCl. 50-μl samples of blood were obtained from the tail, deproteinized, and assayed enzymatically with yeast alcohol dehydrogenase.

TABLE I

Effect of Ethanol Dose upon Concentration of Metabolites in Freeze-Clamped Liver

Ethanol dose, *g/kg*	0 (9)	0.69 (9)	1.7 (6)	3.0 (11)
	μmol/g wet wt±SEM			
L-Lactate	0.20 ±0.02	0.47 ±0.05	0.52 ±0.02	0.37 ±0.02
P				<0.001
Pyruvate	0.011 ±0.001	0.008 ±0.001	0.008 ±0.001	0.008 ±0.001
P	<0.02			
L-Malate	0.20 ±0.02	0.82 ±0.04	0.91 ±0.10	0.62 ±0.03
P	<0.001			<0.001
α-Ketoglutarate	0.070 ±0.011	0.060 ±0.005	0.085 ±0.011	0.120 ±0.016
P		<0.005		<0.001
Citrate	0.32 ±0.04	0.43 ±0.02	0.45 ±0.03	0.52 ±0.06
P	<0.01			
Ls-Isocitrate	0.014 ±0.001	0.025 ±0.001	0.029 ±0.001	0.029 ±0.004
P	<0.005			
L-Glutamate	2.34 ±0.20	3.79 ±0.22	3.70 ±0.24	2.88 ±0.13
P	<0.001			<0.001
Acetoacetate	0.32 ±0.02	0.17 ±0.02	0.25 ±0.03	0.38 ±0.04
P	<0.001	<0.005		<0.01
D-3-Hydroxybutyrate	0.91 ±0.05	1.17 ±0.06	1.29 ±0.07	1.12 ±0.09
P	<0.001			
ATP	2.23 ±0.06	1.95 ±0.07	2.30 ±0.09	2.46 ±0.06
P		<0.005		<0.02
ADP	1.40 ±0.04	1.50 ±0.06	1.26 ±0.08	1.22 ±0.03
P	<0.01	<0.005		
AMP	0.38 ±0.03	0.46 ±0.02	0.46 ±0.03	0.43 ±0.02
P	<0.05			
P_i	4.37 ±0.17	4.42 ±0.17	3.68 ±0.20	3.77 ±0.11
P	<0.005			
NH_4^+	0.51 ±0.03	0.58 ±0.11	0.51 ±0.02	0.53 ±0.04
Ethanol		1.00 ±0.31	24.1 ±1.1	51.3 ±1.8
P		<0.001		<0.001

All animals were fasted 24 h and were killed 120 min after injection. The *P* values refer to differences in means between the 1.7 g/kg group and other experimental groups as determined by Student's *t* test. The ethanol solutions were prepared in 0.15 M NaCl and injected in equal volumes (20 ml/kg). Numbers of observations are in parentheses.

lines to zero time (Fig. 1) gave intercepts of 76.0±3.0 and 42.0±1.8 μmol/ml for the 3.0 and 1.7 g/kg doses, respectively. The ratio of these intercepts is 1.81, extremely close to the theoretical ratio of 1.83 expected from the two concentrations of ethanol. The rate of ethanol disappearance after the 0.69 g/kg dose was not studied, since the kinetics of the removal of low blood levels of ethanol is known to be logarithmic rather than linear (14).

Effect of ethanol dose on liver metabolites and redox ratios 120 min after injection. The measured concentrations of the individual metabolites for each group are shown in Table I. Although in general the experimental groups showed changes qualitatively similar to those usually associated with ethanol administration (4–8), there were significant differences among the groups. An important finding is that even though there was no significant difference between the two highest dose groups in the rate of disappearance of ethanol from blood, nevertheless there were significant differences between the two groups in the concentrations of lactate, malate, glutamate, acetoacetate, and ATP (Table I). Also, it can be noted that the effect of ethanol at the lowest dose on acetoacetate and ATP was greater than the effect of the other two doses.

The distinction among the groups is more striking comparing the redox ratios and phosphorylation states (Table II). The mitochondrial free [NAD^+]/[NADH] ratio as determined by the 3-hydroxybutyrate dehydrogenase reaction was decreased by a factor of 1.8 by the 1.7 g/kg ethanol with respect to the saline controls. The ratio in the low-dose group was significantly more reduced than the ratio in the middle-dose group, by a factor of 1.4, while the ratio in the high-dose group was significantly more oxidized than that of the middle-dose group by a factor of 1.8. The effects of dose on the mitochondrial free [NAD^+]/[NADH] ratios calculated from the glutamate dehydrogenase reaction paralleled the ratios calculated from the 3-hydroxybutyrate dehydrogenase reaction.

A similar pattern was found for the cytoplasmic free [$NADP^+$]/[NADPH] ratio. This ratio fell by a factor 1.8 from the control value with the middle dose of ethanol. The ratio in the low-dose group again was significantly more reduced than the middle-dose group (by a factor of 1.3), while the ratio for the high dose was more oxidized than that of the middle-dose value by a factor of 1.3.

The cytoplasmic free [NAD^+]/[NADH] ratio decreased by a factor of 3.6 from the control value with

TABLE II

Effect of Ethanol Dose upon Redox and Phosphorylation Ratios in Freeze-Clamped Rat Liver

Ethanol dose, g/kg	0 (9)	0.69 (9)	1.7 (6)	3.0 (11)
[NAD^+]/[NADH] from lactate dehydrogenase (cytoplasm)	495 ±36	154 ±14	139 ±8	200 ±13
P	<0.001			<0.001
[$NADP^+$]/[NADPH] from isocitrate dehydrogenase (cytoplasm)	0.0068 ±0.0004	0.0029 ±0.0002	0.0037 ±0.0005	0.0049 ±0.0004
P	<0.001	<0.005		<0.02
[NAD^+]/[NADH] from 3-hydroxybutyrate dehydrogenase (mitochondria)	7.31 ±0.55	2.92 ±0.15	3.95 ±0.58	7.04 ±0.64
P	<0.001	<0.001		<0.001
[NAD^+]/[NADH] from glutamate dehydrogenase (mitochondria)	4.07 ±0.17	2.29 ±0.28	3.18 ±0.58	5.84 ±0.87
P		<0.02		<0.02
[ATP]/[ADP][P_i](M^{-1}) (cytoplasm)	376 ±28	311 ±37	532 ±84	547 ±39
P	<0.001	<0.001		
[ATP][AMP]/[ADP]^2	0.42 ±0.02	0.43 ±0.06	0.65 ±0.07	0.72 ±0.03
P	<0.001	<0.01		

Ratios are calculated from the metabolite values given in Table I and given as mean±SEM. The *P* values refer to differences between the means of the 1.7 g/kg ethanol group and remaining groups, as determined by Student's *t* test. See text for methods of calculation. Numbers of observations are in parentheses.

administration of the intermediate dose of ethanol. With the highest dose, this ratio was significantly more oxidized than that of the intermediate dose by a factor of 1.4. The values for the cytoplasmic free [NAD^+]/[NADH] ratio in the control groups in the current report were more reduced than reported previously (8). The difference was due solely to the use of a fluorimetric assay for pyruvate. At the low concentrations in the tissue in the starved animal, the fluorimetric assay of the current study is considered more nearly correct.

TABLE III

Effect of Ethanol Dose upon Concentration of Metabolites in Freeze-Clamped Liver

Ethanol dose, *g/kg*	1.7	3.0
	μmol/g wet wt ±SEM	
L-Lactate	0.546 (7) ±0.039	0.550 (8) ±0.034
Pyruvate	0.011 (7) ±0.001	0.012 (8) ±0.001
α-Ketoglutarate	0.084 (12) ±0.007	0.159 (11) ±0.014 $P < 0.001$
Citrate	0.437 (12) ±0.023	0.609 (12) ±0.031 $P < 0.001$
LS-Isocitrate	0.021 (12) ±0.001	0.031 (12) ±0.002 $P < 0.001$
L-Glutamate	3.60 (12) ±0.19	4.15 (11) ±0.25
Acetoacetate	0.300 (8) ±0.020	0.355 (7) ±0.050
D-3-Hydroxybutyrate	1.41 (8) ±0.14	1.14 (7) ±0.09
ATP	2.55 (8) ±0.08	2.95 (7) ±0.12 $P < 0.05$
ADP	1.40 (8) ±0.09	1.34 (7) ±0.06
AMP	0.515 (8) ±0.049	0.380 (7) ±0.039
P_i	3.76 (8) ±0.23	4.08 (7) ±0.17
NH_4^+	0.441 (12) ±0.022	0.435 (11) ±0.020
Ethanol	27.2 (8) ±0.8	26.4 (7) ±1.0

Liver samples were taken at 120 min after i.p. injection for the 1.7 g/kg ethanol group and at 400 min for the 3.0 g/kg ethanol group. The number of animals is in parentheses.

The phosphorylation potential showed a different and very distinctive pattern. That of the middle-dose ratio increased by a factor of 1.4 with respect to the saline controls. The phosphorylation potential in the high-dose group was not significantly different from that of the middle-dose group, but the phosphorylation potential of the low-dose group had a value decreased from the middle-dose value by a factor of 1.7: i.e., below that of the control group.

Comparison of metabolites and redox states 120 min after 1.7 g/kg ethanol and 445 min after 3.0 g/kg ethanol. 445 min after the 3.0 g/kg ethanol dose, the level of ethanol in liver had fallen to the level found 120 min after a dose of 1.7 g/kg ethanol (Table III). Both sets of animals were killed at the same time of day for comparison. In the case of individual metabolites, several originally significant differences between the intermediate and high-dose groups disappeared with time (lactate, glutamate, acetoacetate), whereas other differences emerged or trends became significant (AMP, citrate, isocitrate). Of interest was that the highly significant differences between the intermediate and high-dose groups in α-ketoglutarate concentrations remained (Tables I and III). The elevated α-ketoglutarate concentrations found after the 3.0 g/kg ethanol dose persisted even after 445 min, in spite of the lowering of the tissue ethanol concentration.

The differences between the redox ratios of the two groups is shown in Table IV. Original differences in the cytoplasmic free [NAD^+]/[NADH] ratios (Table II) were no longer evident (Table IV). However, differences in the cytoplasmic free [$NADP^+$]/[NADPH] ratio and in both mitochondrial free [NAD^+]/[NADH] ratios were still present (Table IV).

Effect of ethanol dose on changes of ATP in anoxia. Groups of animals were injected with the usual ethanol doses; however, freeze-clamping of the livers after cervical dislocation was delayed. Part of each liver was frozen 12 s, and part at 24 s after the animal was killed. The rate of change of the ATP concentration between 12 and 24 s was not significantly different among the groups (Table V).

DISCUSSION

The linear rate of disappearance of ethanol from the blood (corresponding to an average of 237 mg ethanol/liter blood/h) is in agreement with other studies (12, 13, 30, 31) and can probably be taken as a good approximation of the actual rate of ethanol metabolism, since urinary and pulmonary losses are thought to be minor (32). Therefore, there is no evidence that the sudden increase in ethanol metabolism reported in isolated liver cells between concentrations of 40 mM and 65 mM ethanol (19) occurs in vivo. If such a nonlinear curve

TABLE IV

Effect of Ethanol Dose upon Redox and Phosphorylation Ratios in Freeze-Clamped Rat Liver

Ethanol dose, *g/kg* Time after injection, *min*	1.7 120	3.0 445
[NAD^+]/[NADH] from lactate dehydrogenase (cytoplasm)	181 (8) ±13	196 (7) ±14
[$NADP^+$]/[NADPH] from isocitrate dehydrogenase (cytoplasm)	0.0039 (12) ±0.0002	0.0051 (12) ±0.0005 ($P < 0.05$)
[NAD^+]/[NADH] from 3-hydroxybutyrate dehydrogenase (mitochondria)	4.53 (8) ±0.66	6.26 (8) ±0.42 ($P < 0.05$)
[NAD^+]/[NADH] from glutamate dehydrogenase (mitochondria)	2.71 (12) ±0.24	3.82 (10) ±0.35 ($P < 0.02$)
[ATP]/[ADP][P_i](M^{-1}) (cytoplasm)	531 (8) ±75	557 (7) ±51

Ratios are calculated from the metabolite values given in Table III and are given as the mean±SEM. The number of animals is in parentheses.

did occur, one would have expected a significant downward shift of the extrapolated intercept of the high dose of ethanol (Fig. 1).

The decreasing reduction of the cytoplasmic and mitochondrial redox ratios with increasing ethanol dose (Table II) is the most striking finding of the current study. Moreover, this finding is not restricted to the current conditions. Similar results have been obtained in the 48-h starved animal,[1] and a failure of reduction of the mitochondrial ratio with a high dose of ethanol has also been reported in fed animals (7, 33). The findings might be compatible with increasing substrate inhibition of alcohol dehydrogenase. However, if the rat liver and horse liver enzymes react similarly, one would expect only about 20% inhibition at 50 mM ethanol and about 27% inhibition at 80 mM ethanol (16). Such an explanation, therefore, cannot easily be reconciled either with the magnitude of the differences among the groups (Tables I and II) or with the linear alcohol disappearance curves (Fig. 1) unless an alternative pathway of ethanol metabolism, such as the microsomal ethanol oxidizing system (17) or catalase (18), is functional in vivo and able to compensate virtually exactly for concentration-dependent inhibition of alcohol dehydrogenase. Although previous studies in vivo have been unable to demonstrate the presence of alternative ethanol oxidative pathways (34, 35), the increasing oxidation of the cytoplasmic free [$NADP^+$]/[NADPH] ratio and the mitochondrial free [NAD^+]/[NADH] ratios from the lowest to highest doses in the current study (Table II) is compatible with a significant in vivo contribution by the microsomal ethanol oxidizing system, especially since it has been shown that animals exposed to chronic ethanol (which presumably induced the microsomal ethanol-oxidizing system) had a decreased reduction of cytoplasmic and mitochondrial free [NAD^+]/[NADH] ratios when given acute ethanol (36). Caution, however, must be taken with this interpretation since the cytoplasmic free [$NADP^+$]/[NADPH] ratio is dependent upon the dose of ethanol (Table II) but not necessarily upon the concentration of ethanol (Table IV), and since the microsomal ethanol oxidizing system is saturated above 20 mM ethanol (17). Therefore, further work will be necessary before the role of alternative pathways of ethanol metabolism in vivo can be established.

Another factor contributing to the observed decreasing reduction of the redox ratios with increasing dose might be related to the observation in the perfused liver that at low concentrations of ethanol, the acetaldelyde produced from ethanol oxidation is nearly completely further oxidized in the liver, whereas a large percentage of the acetaldelyde leaves the liver at higher concentrations of ethanol (37). The result would be a decreasing reduction, perhaps especially in the mitochondria where the acetaldelyde is mainly utilized (38). Unfortunately, the effect is not concentration-dependent above 16 mM (37) and therefore cannot explain the results of the current study, unless there is a significant difference in dose response between the in vitro and in vivo conditions.

[1] Unpublished results.

TABLE V

Effect of Anoxia upon the ATP Concentration of Liver Treated with Several Doses of Ethanol

Time after cervical dislocation	[ATP]			Δ[ATP]
	8 s	12 s	24 s	
	μmol/g wet wt			*μmol/g wet wt/12 s*
0.15 M NaCl	2.23 (9) ±0.06	2.36 (5) ±0.10	1.76 (5) ±0.08 ($P < 0.005$)	0.60 (5) ±0.12
0.69 g/kg ethanol	1.95 (9) ±0.07	2.07 (7) ±0.06	1.47 (7) ±0.07 ($P < 0.005$)	0.60 (7) ±0.06
1.7 g/kg ethanol	2.30 (6) ±0.09	2.16 (6) ±0.08	1.51 (6) ±0.05 ($P < 0.001$)	0.65 (6) ±0.06
3.0 g/kg ethanol	2.46 (11) ±0.06	2.48 (8) ±0.05	1.83 (8) ±0.06 ($P < 0.001$)	0.65 (8) ±0.05

Freeze-clamping of the liver was delayed for 12 and 24 s after cervical dislocation. The samples at 12 and 24 s are from the same animals; the 8-s samples are those from Table I and are listed for comparison. The values for Δ[ATP] are calculated between the 12 and 24-s samples. For statistical purposes, the [ATP] at other times were compared with the 8-s values and the Δ[ATP] values were compared with the 0.15 M NaCl controls by Student's *t* test. The number of animals is in parentheses. Zero time is 120 min after i.p. injection.

Though bearing on the observed redox ratios, the above considerations cannot explain all the findings. For example, unlike the redox ratios, which tend to move back towards the control values with increasing dose, the ATP (Table I) and the phosphorylation potentials (Table II) surpass the control values with the two highest doses. This elevation of the phosphorylation potential with higher doses might be compatible with a direct membrane effect. Anesthetics, for example, decrease the metabolic rate of brain, as evidenced by a lowered rate of ATP utilization with anoxia in animals pretreated with anesthetics (39). The effect seems to be nonspecific and has been suggested to be part of the effect of ethanol on brain (9). Support for such a mechanism is found in the observation that ethanol inhibits the membrane $(Na^+ + K^+)$-stimulated ATPase activity in vitro (40), an effect that has previously been used to explain part of the effect of chronic ethanol on rat liver (41). In the current study, elevation of the tissue ATP content (Table I) and the phosphorylation potential (Table II) with high ethanol would likewise be compatible with a similar acute effect in liver in vivo. However, an attempt to demonstrate a dose-dependent effect of ethanol on ATP utilization in liver directly under our conditions was not successful, although this technique has been useful in demonstrating the effects of anesthetics in brain (39). The rate of fall of the ATP concentration with anoxia did not significantly differ among the groups (Table V). However, at the concentrations of ethanol used, the degree of inhibition of the $(Na^+ + K^+)$-stimulated ATPase would be expected to be very low, on the order of 5% or less, if rat liver and rat brain respond similarly (40).

It is apparent that no one explanation can fully explain the results. In contrast to in vitro studies, the in vivo state is complicated and undoubtedly modified by such factors as blood levels of amino acids, fatty acids, and hormones. The final effect of dose upon intermediary metabolism is certainly a summation of several factors. However, even though the important factors operative in vivo are yet to be resolved, it can at least be concluded from the differential effects of dose of the current study that the rate of NADH production alone cannot explain the findings. The data give in vivo evidence for the presence of other significant factors. Since the redox ratios and the phosphorylation potential are interrelated through near-equilibrium reactions (8), the pattern of metabolites at a given dose would be the result of overlapping effects. Further in vivo evidence will be necessary, however, to identify and quantitate the contribution of each factor better. Of clinical importance, however, is the finding of qualitative as well as quantitative differences among doses of alcohol. Some of the differences, once established, show a tendency to

persist over relatively prolonged periods of time, independent of the actual ethanol concentrations (Tables III and IV). The clinical implications are straightforward. The results suggest that in the consideration of the factors contributing to the pathology of alcoholism, the amount of ethanol ingested at one time may be an important variable, distinct from chronicity of intake.

ACKNOWLEDGMENTS

Appreciation is expressed for the excellent technical assistance of David K. Merrill and Ellen Eschenbrenner.

This investigation was supported by a research grant from the National Council on Alcoholism.

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