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EFFECT OF ETHANOL DOSE ON AMINO ACID AND UREA CONCENTRATIONS IN THE FED RAT LIVER *IN VIVO*

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Abstract—In order to assess the role of ethanol oxidation *in vivo* on amino acid metabolism and urea synthesis, the changes in the concentrations of hepatic dicarboxylic amino acids, glutamine, alanine, carbamoylphosphate and urea were measured in fed rats at various times after the administration of 10 and 50 mmoles of ethanol/kg body wt, i.p. (1) The dose dependence of the acute effects of ethanol upon liver dicarboxylic amino acids *in vivo* has been demonstrated. The 10 mmoles/kg ethanol dose determines an accumulation by 30 per cent of aspartate and glutamate in the rat liver (30 and 60 min after ethanol injection) whereas the 50 mmoles/kg ethanol dose induces a 35 per cent fall in the aspartate level at the same time, and only a 20 per cent increase in glutamate concentration during the second hour following ethanol injection. (2) The dose-dependent effects of ethanol upon the hepatic level of urea are also shown. A significant decrease by 20 per cent in the liver content of urea is only observed 60 min after the administration of the 50 mmoles/kg ethanol dose. (3) On the contrary, the ethanol induced alterations in the hepatic alanine and glutamine concentrations are not dose-dependent. A 50 per cent decrease in alanine level and a rise (about 30 per cent) in glutamine concentration are observed 30 and 60 min after the administration of the two ethanol doses. The comparison between the effects of either 10 or 50 mmoles/kg ethanol doses leads to the conclusion that the rate of ethanol oxidation related to the malate–aspartate shuttle activity is directly connected with the glutamate (and aspartate) concentration. These findings suggest that ethanol reduces the hepatic urea concentration *in vivo* only when the ethanol dose injected to rats induces a rapid decrease in the aspartate/glutamate ratio (about 40 per cent within 15 min following the injection of the 50 mmoles/kg ethanol dose).

Urea synthesis from ammonia or amino acids requires a stoichiometric production of carbamoylphosphate and aspartate. It was taken for granted that the formation of these two nitrogen precursors of urea was a main factor controlling the rate of urea synthesis [1].

Krebs *et al.* [2] have shown that ethanol causes an accumulation of aspartate in the perfused liver when excess nitrogen in the form of alanine or ammonium lactate was present, thereby upsetting the expected stoichiometry. These authors came to the same conclusion working on isolated hepatocytes [3]. Their experiments demonstrate that ethanol diverts ammonia formed from alanine or added in presence of lactate from the synthesis of urea to the synthesis of aspartate, glutamate and glutamine.

However, our findings on the aspartate concentration in the rat liver obtained *in vivo* after acute ethanolic intoxication are conflicting. We did find an increase of aspartate hepatic level after administration of 10 mmoles of ethanol/kg body wt, i.p., to fed rats [4], but we observed a strong decrease of this same amino acid after administration of 50 mmoles of ethanol/kg body wt, i.p., to fasted rats [5].

In order to explore the reasons for these opposite disturbances and the role played by ethanol oxidation *in vivo* on amino acid metabolism and urea synthesis, we have therefore studied the effects of the 10 and 50 mmoles/kg ethanol doses on aspartate, glutamate, glutamine, alanine and urea concentrations in the fed rat liver. Changes in the hepatic level of carbamoylphosphate after administration of the smallest ethanol dose are also reported.

MATERIALS AND METHODS

Animals. Female Sprague–Dawley rats, weighing 215 ± 5 g, were maintained on a standard laboratory diet *ad lib.* No fasting period preceded the experiments.

Treatment. The animals were injected intraperitoneally with equal volumes (10 ml/kg) of either physiological saline or 1 or 5 M ethanol in physiological saline giving ethanol doses of 10 and 50 mmoles/kg body wt, respectively.

Determination of metabolites. For the determinations of liver metabolites, animals were lightly anaesthetized with diethylether at various times after the administration of ethanol, as indicated in the results, and livers were promptly freeze-clamped by the method of Wollenberger *et al.* [6].

Dicarboxylic amino acids, glutamine and alanine were determined enzymatically as described previously [7]. Ammonia and urea were assayed consecutively using glutamate dehydrogenase followed by the addition of urease [8].

Liver extracts for the measurement of carbamoylphosphate were prepared as described by Raijman [9]. The estimation of carbamoyl phosphate was carried out according to the method used by Yashphe [10]. In each experiment, separate homogenizer tubes were run at the bottom of which a known amount of carbamoylphosphate was frozen prior to adding a portion of frozen liver. The amount of carbamoylphosphate recovered during the assay procedure corresponded to 47 ± 8 per cent of the carbamoylphosphate initially added to sample tissue. However, our standard curves obtained

for the estimation of carbamoylphosphate and urea indicated that the conversion of carbamoylphosphate into urea at the first step of the assay was about 90 per cent, value similar to that reported by Yashphe [10].

To compare the rates of ethanol disappearance from the blood of animals given 10 or 50 mmoles/kg ethanol, heparinized blood samples from the ophthalmic plexus were collected under light anaesthesia 10, 30, 60, 90 and 120 min after ethanol administration, and deproteinized. Ethanol was determined spectrophotometrically, with yeast alcohol dehydrogenase, according to Bernt and Gutmann [11].

Chemicals. Carbamoylphosphate (dilithium salt) and L-alanine dehydrogenase from *Bacillus subtilis* were purchased from Boehringer Mannheim; 2,3-butanedione monoxime and *p*-diphenylamine sulfonic acid sodium salt were obtained from Eastman Organic Chemicals; other enzymes and coenzymes were from Sigma Chemical Co., and all chemicals used were commercial products of reagent grade.

Expression of results. The results of metabolite determinations are expressed as mean values ± S.E.M. (standard error of the mean) in nmoles/g fresh wt of liver. Blood ethanol concentration is expressed as μmoles/ml of whole blood. Statistical comparisons were determined by Student's *t* test.

RESULTS AND DISCUSSION

Effect of ethanol dose upon concentrations of dicarboxylic amino acids in the fed rat liver. The administration of the 10 mmoles/kg ethanol dose, i.p. (corresponding to 0.46 g/kg body wt), determined a significant increase (about 30%, $P < 0.01$) in both aspartate and glutamate concentrations 30 min and 60 min after ethanol injection (Fig. 1).

On the contrary, the administration of the 50 mmoles/kg ethanol dose, i.p. (corresponding to 2.3 g/kg body wt) affected in the opposite direction the aspartate level; within 15 min the value fell to 60 per cent of the initial value (by 400 nmoles/g) and returned to the normal range at 90 min (Fig. 2). When the aspartate level was depressed, the hepatic glutamate concentration remained unchanged and then it gradually increased by about 20 per cent during the second hour following the ethanol injection, whereas the initial values for aspartate were re-established (Fig. 2). Thus, the 50 mmoles/kg ethanol dose induced a fall in the aspartate/glutamate ratio, with the maximum decrease at 30 min (0.30 instead of 0.54 at zero time) followed by a gradual return to control value (Fig. 2). In contrast, the aspartate/glutamate ratio showed no change throughout the 60 min following the injection of the 10 mmoles/kg ethanol dose (Fig. 1).

These results indicate that ethanol effects on the hepatic levels of dicarboxylic amino acids *in vivo* depend on the dose of alcohol injected to rats. As a matter of fact, these differences are entirely due to ethanol dose and not to local effects on the liver after i.p. administration of two different concentrations of ethanol (1 M or 5 M), because the experiments have been repeated by administering different volumes (2 or 10 ml/kg) of the same ethanol concentration (as a 5 M solution in physiological saline) and similar results have been obtained (not shown).

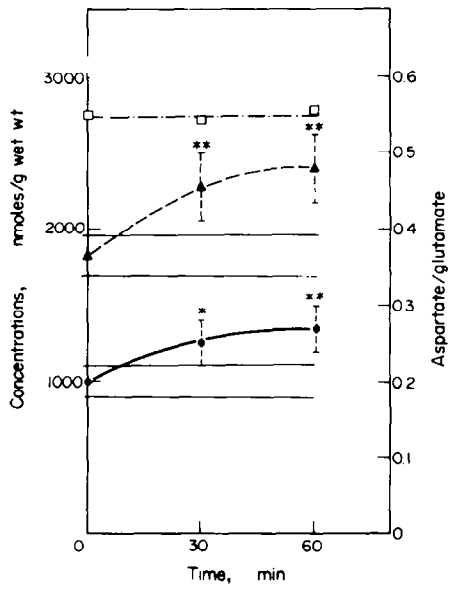

Fig. 1. Effect of 10 mmoles/kg ethanol dose upon concentrations of dicarboxylic amino acids in the fed rat liver. The rats were injected intraperitoneally with 10 mmoles of ethanol/kg body wt at zero time. They were sacrificed 30 or 60 min after ethanol administration. (—●—●—) Aspartate concentration. (—▲—▲—) Glutamate concentration. (·□—□·) Aspartate/glutamate ratio. Each point represents the mean value ± S.E. for ten rats at least. The statistical significance refers to the values found with saline: *$0.05 > P > 0.01$, **$P < 0.01$. The two horizontal lines represent the mean ± S.E.M. determined in the control animals.

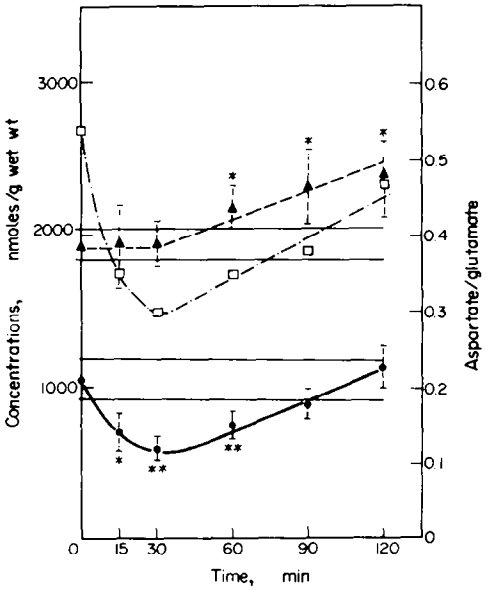

Fig. 2. Effect of 50 mmoles/kg ethanol dose upon concentrations of dicarboxylic amino acids in the fed rat liver. The rats were injected intraperitoneally with 50 mmoles of ethanol/kg body wt at zero time. They were sacrificed 15, 30, 60, 90 or 120 min after ethanol administration. Standard symbols, representation of results and statistical significance as in Fig. 1.

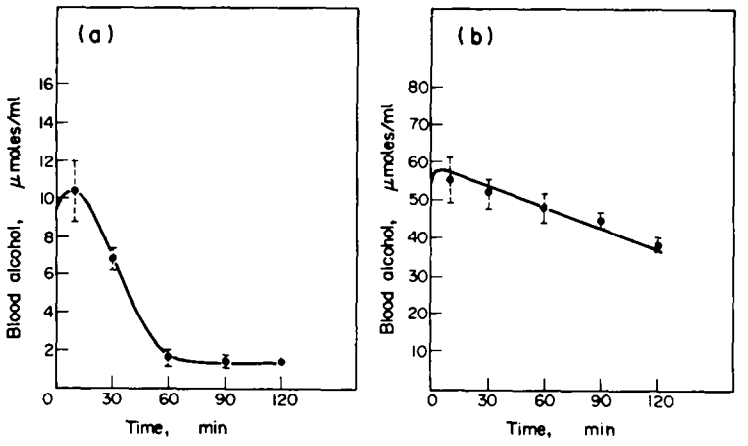

Fig. 3. Effect of ethanol dose on disappearance of ethanol from blood. The rats were injected i.p. with equal volumes (10 ml/kg) of either 10 mmoles/kg (a) or 50 mmoles/kg (b) ethanol in physiological saline. Samples of blood were collected from the ophtalmic plexus 10, 30, 60, 90 and 120 min after ethanol administration. Each point is the mean of four animals. Vertical bars represent 2 S.E.M.

The 10 mmoles/kg ethanol dose produced a 12 mM concentration in the blood of fed rats (Fig. 3a), as we have previously found [4]. Maximum rates of alcohol oxidation by alcohol dehydrogenase occurred *in vivo* at such a value for blood ethanol. Indeed, the concentration of ethanol fell to 50 per cent, 40 min after injection (i.e. about 5 mM) and approx. 10 mM ethanol was metabolized in 60 min by the rat liver (Fig. 3a). The administration of the 10 mmoles/kg ethanol dose induced also, 30 min after injection, an increase (3-fold) in the lactate/pyruvate ratio (Table 1), but we have previously observed [4] that the 3-hydroxybutyrate/acetoacetate ratio failed to be modified significantly at 30 min in the fed rat. Veech *et al.* [12] had shown that the maximum increase in the lactate/pyruvate ratio was obtained at 15 min and that the 3-hydroxybutyrate/acetoacetate ratio was increased 5 min after ethanol administration. Thus, the latter ratio as well as the $NADP^+/NADPH$ ratio, which was immediately decreased after the ethanol injection, returned nearly to control values at 15 min [12]. It is noteworthy that this return of the mitochondrial $NAD^+/NADH$ and cytosolic $NADP^+/NADPH$ ratios to normal coincided with the rise of the dicarboxylic amino acids in the liver.

These ethanol-induced alterations in liver redox state associated with an active metabolism of alcohol are in favour of reductive amination of oxoglutarate by mitochondrial glutamate dehydrogenase reaction, forming the glutamate used up in the mitochondrial aspartate aminotransferase reaction for the increased production of aspartate. Our findings extend *in vivo* the observations of Krebs *et al.* [1, 2], Stubbs and Krebs [3] and Henley *et al.* [13] in their experiments on perfused liver or isolated hepatocytes. It confirms that the concentrations of dicarboxylic amino acids are related to changes in the redox state and provides evidence for the *in vivo* involvement of glutamate dehydrogenase as a factor to regulate the levels of pyridine nucleotide coenzymes in the liver [14].

The decrease in aspartate concentration associated with an unchanged level of glutamate found 30 min after the 50 mmoles/kg ethanol dose is in discrepancy with the conclusion drawn from our data after the 10 mmoles/kg ethanol dose. An important finding is that, even though there was no significant difference between the two ethanol doses in the lactate/pyruvate ratio (as well as in malate and 3-glycerophosphate concentrations at the same time); (Table 1), neverthe-

Table 1. Effect of ethanol dose upon concentrations of 3-glycerophosphate, L-malate, L-lactate and pyruvate in the fed rat liver

Metabolite (nmoles/g wet wt) or metabolite ratio	Ethanol dose, mmoles/kg body wt		
	0	10	50
3-Glycerophosphate	159 ± 21 (9)	435 ± 104† (7)	586 ± 126† (3)
L-Malate	409 ± 88 (10)	741 ± 123† (6)	559 ± 126 (3)
L-Lactate	1023 ± 197 (12)	773 ± 225 (9)	688 ± 234 (3)
Pyruvate	142 ± 26 (12)	40 ± 7† (9)	29 ± 10† (3)
L-Lactate/pyruvate	7.5 ± 1.3 (12)	20.5 ± 6.7† (9)	25.0 ± 6.7† (3)

The rats were injected i.p. with ethanol either 10 or 50 mmoles/kg body wt at zero time. All animals were sacrificed 30 min after injection.

Results are expressed as the mean ± S.E. with the number of determinations in parentheses. Values which are statistically different from the control rats are indicated by: †$P < 0.01$.

Table 2. Time-course of the effects of ethanol dose upon concentrations of alanine and glutamine in the fed rat liver

Metabolite	Ethanol dose (mmoles/kg body wt)	Concentration (nmoles/g wet wt) (0 min)	(30 min)	(60 min)	(90 min)	(120 min)
Alanine	10	1370 ± 272 (12)	692 ± 189† (12)	654 ± 143† (9)	—	1211 ± 285 (5)
	50	1069 ± 134 (7)	440 ± 85† (8)	553 ± 112† (8)	—	1003 ± 482 (4)
Glutamine	10	2878 ± 219 (16)	3441 ± 516* (13)	4512 ± 515† (8)	—	2819 ± 178 (3)
	50	3998 ± 181 (12)	4565 ± 265† (8)	4680 ± 321† (11)	4288 ± 659 (3)	3893 ± 762 (6)

Ethanol (10 or 50 mmoles/kg body wt) was injected i.p. into fed rats. The animals were sacrificed at the times indicated. The values for 0 min refer to control rats, injected with saline.

Results are expressed as the mean ± S.E. with the number of determinations in parentheses. Values which are statistically different from the 0 min values are indicated by $^{*}0.05 > P > 0.01$; $^{\dagger}P < 0.01$.

Table 3. Time-course of the effects of ethanol dose upon concentration of urea in the fed rat liver

Metabolite	Ethanol dose (mmoles/kg body wt)	Concentration (nmoles/g wet wt) (0 min)	(30 min)	(60 min)	(90 min)	(120 min)
Urea	10	5972 ± 510 (13)	5584 ± 616 (12)	5297 ± 419 (10)	—	5280 ± 838 (3)
	50	5630 ± 566 (12)	5566 ± 323 (14)	4552 ± 474* (14)	5642 ± 529 (7)	5106 ± 684 (8)

Experimental conditions, expression of results and statistical significance as in Table 2.

less there were significant differences between the two doses in the dicarboxylic amino acid concentrations in the fed rat liver.

As shown in Fig. 3b, the 50 mmoles/kg ethanol dose produced approx. a 60 mM concentration in the blood at zero time and no significant changes were observed during the 60 min following the administration of this dose. The value for blood ethanol level fell slightly, close to significance ($0.10 > P > 0.05$), at 90 min and only decreased significantly ($0.010 > P > 0.001$ vs 10, 30 and 60 min values) at 120 min. These results are in agreement with other studies [15] and show that all ethanol oxidizing systems are saturated, because the hepatic ethanol concentration is above 30 μmoles/g liver [16]. Then, the accelerating effect of ethanol on the hepatic dicarboxylic amino acids formation has not to be found under these experimental conditions. The present results support *in vivo* those of Crow *et al.* [17] in isolated hepatocytes showing a correlation between glutamate concentration and rates of ethanol oxidation. This favours the concept that there is a possible dependence of the rate of operation of the malate–aspartate shuttle on the glutamate concentration [17]. It seems that the conversion of glutamate to aspartate by aspartate aminotransferase reaction is not enhanced after the 50 mmoles/kg ethanol dose because this treatment does not induce a rapid increase in glutamate owing to a possible deficiency of the malate–aspartate shuttle. It is not either at all unlikely that acetaldehyde (the concentration of which is in the range of 200 μM in rat liver during such an ethanol oxidation [16]) by displacing pyridoxal 5'-phosphate (PLP) from aspartate aminotransferase, a PLP enzyme [18], inhibits the aspartate production in the liver acting as aminooxyacetate [4].

Effect of ethanol dose upon concentrations of alanine and glutamine in the fed rat liver. As shown in Table 2, the 10 and 50 mmoles/kg ethanol doses induced a strong decrease (50%, $P < 0.001$) in the hepatic alanine level 30 and 60 min after injection. This finding suggests an increased rate of alanine utilization in the fed rat liver after ethanol treatment at whatever dose. As reported on perfused liver [2] and isolated hepatocytes [3], ethanol could induce *in vivo* an excess formation of aspartate (and glutamate) not only from ammonia but also from alanine in the liver of rats receiving a low ethanol dose (i.e. the 10 mmoles/kg ethanol dose). Oxalacetate (resulting from the pyruvate carboxylase reaction) would be diverted either to the formation of aspartate after the low ethanol dose or to the formation of citrate after the high ethanol dose, which induced a 2-fold increase in the citrate level [15]. As a matter of fact, the inhibition of gluconeogenesis from alanine by ethanol [19] could be explained *in vivo* by the diversion of oxalacetate to these pathways instead of glucose synthesis.

At the opposite, the 10 and 50 mmoles/kg ethanol doses caused a significant rise in the glutamine concentration at 30 and 60 min (Table 2). This ethanol-induced alteration in glutamine level results probably from a reduced glutamine utilization by the mitochondrial glutaminase I reaction (related to a decreased ammonia/glutamine ratio, [7]) rather than from a raised glutamine synthesis by glutamine synthetase reaction [2].

It is noteworthy that the values for the hepatic alanine and glutamine concentrations returned to the initial range within 90–120 min.

Effect of ethanol dose upon concentrations of carbamoylphosphate and urea in the fed rat liver. The 10 mmoles/kg ethanol dose did not induce a significant alteration in the hepatic urea concentration during the hour following ethanol injection (Table 3). The measurement of the carbamoylphosphate level 30 min after the administration of this same ethanol dose, showed, therefore, a tendency to decrease (Table 4), as suggested by Krebs *et al.* [1]. This last modification is not significant because a 35 per cent decrease in liver carbamoylphosphate has been found only in 60 per cent of ethanol treated-rats. So, the aspartate/carbamoylphosphate ratio was significantly increased (about 2-fold) essentially due to the important rise in the aspartate concentration and this slight decrease in the carbamoylphosphate level, 30 min after the 10 mmoles/kg ethanol dose (Table 4). Thus, it is noteworthy that such an imbalance in the formation of two urea precursors fails to modify the hepatic levels of ammonia (not shown) and urea (Table 2).

On the contrary, the 50 mmoles/kg ethanol dose induced a significant decrease (17%, $0.02 > P > 0.01$) in urea content of the liver at 60 min. The fall in the aspartate/glutamate ratio during the first hour following the administration of the 50 mmoles/kg ethanol dose (Fig. 2) could be responsible for this decreased urea level. Indeed, normal values for urea concentration were re-established at 90 min, when the aspartate/glutamate ratio gradually returned to the initial range.

The present observation indicates that ethanol reduced the hepatic urea concentration *in vivo* only when the ethanol dose injected to rats induced a rapid fall in the aspartate/glutamate ratio associated with a deficiency of malate–aspartate shuttle metabolites. This finding favours the concept of Williamson *et al.* [20] according to which the glutamate concentration exerts a key role at the level of intra-mitochondrial aspartate aminotransferase in controlling the rate of aspartate production.

In conclusion, the present report shows dose-dependent effects of ethanol upon concentrations of dicarboxylic amino acids and urea in the fed rat liver; it confirms, *in vivo*, that there is a correlation between dicarboxylic amino acid concentrations and rates of ethanol oxidation [17, 20] and that the aspartate/glutamate ratio plays an important role in the regulation of ureogenesis [21, 22].

Table 4. Effect of 10 mmoles/kg ethanol dose upon concentration of carbamoylphosphate and aspartate/carbamoylphosphate ratio in the fed rat liver

Metabolite or metabolite ratio	Concentration (nmoles/kg wet wt) Control	Ethanol-injected
Carbamoylphosphate	61 ± 14 (14)	47 ± 17 (8)
Aspartate	833 ± 111 (7)	1249 ± 226* (8)
Aspartate/ carbamoylphosphate	15 ± 2 (6)	27 ± 9* (6)

Animals were sacrificed 30 min after the i.p. injection of saline (control) or 10 mmoles of ethanol/kg body wt (ethanol-injected).

Expression of results and statistical significance as in Table 2.

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