Biochemical Pharmacology, Vol. 24, pp. 199–204. Pergamon Press, 1975. Printed in Great Britain.

EFFECT OF ETHANOL ON THE METABOLISM OF ALANINE, GLUTAMIC ACID, AND PROLINE IN RAT LIVER

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(*Received* 22 *November* 1973; *accepted* 18 *June* 1974)

Abstract—The metabolism of amino acids in liver slices and homogenates is influenced by changes in redox balance during the catabolism of ethanol. The net formation of alanine is decreased in incubated liver homogenates from ethanol-treated rats, presumably because of the deficiency of pyruvic acid. The ratio of α-ketoglutaric acid to glutamic acid is shifted very markedly in favour of the latter in all experimental systems tested. Administration of ethanol causes an increase in free liver proline *in vivo*. Likewise the net synthesis of proline is increased during the incubation of liver homogenates obtained from ethanol-treated rats or of liver slices to which ethanol was added *in vitro*. Although ethanol administration increases the rate of proline synthesis from glutamic acid, the ratio of proline to glutamic acid does not change. Data are given for the dependence of proline formation on the incubation time and on the concentration of the added glutamic acid and lactic acid. The results are discussed with special reference to the activation of connective tissue that ethanol induces in liver.

The catabolism of ethanol is accompanied by a shift in the redox balance towards the reducing side [1–3]. This change should also be reflected in the metabolism of the nitrogenous compounds, i.e. the oxidative deamination of glutamic acid, the catabolism of γ-aminobutyric acid to succinic acid, the deamination of biogenic amines, and the synthesis of proline from glutamic acid.

In earlier studies on the effect of ethanol on the amino acid metabolism of the brain, we observed, among other changes, higher levels of γ-aminobutyric acid and glutamic acid [4–6], both of which could be explained by a change in the redox balance.

The purpose of this work was to find out whether ethanol would cause analogous changes in the levels of amino acids in the liver. We were especially interested in proline because the cells of connective tissue depend on the concentration of extracellular proline for the synthesis of collagen and other proteins [7] relevant in the development of cirrhosis. A preliminary report has appeared [8].

EXPERIMENTAL

Tissue samples

Various experimental systems were used: ethanol was given either *in vivo* or *in vitro*, and both slices and homogenates were employed because from earlier work ethanol is known to affect brain slices after application *in vitro* but homogenate only when given *in vivo*.

Male rats of the Wistar strain, weighing 250–430 g, were deprived of food for about 40 hr before the experiments. For pretreatment *in vivo*, when applied, 550 mg ethanol/100 g of body wt was given by stomach tube as a 33 per cent (v/v) aqueous solution. The respective control animals received an equal volume of water. The rats were killed by decapitation after 60 min.

Immediately after killing, the livers were dissected out, chilled on crushed ice, and weighed on a torsion balance. Then they were either sliced to 0·5 mm thickness with a Stadie–Riggs hand-microtome, or homogenized with a Potter–Elvehjem apparatus in 4 volumes of cold isotonic potassium chloride–potassium phosphate solution, pH 7·4 [6]. To facilitate the handling of the homogenate the coarse particles were removed by centrifugation at 0° for 10 min at 700 *g*, and the post-nuclear fraction so obtained was used for incubation.

Incubations

Liver slices and whole homogenates. Krebs–Ringer bicarbonate medium [9] containing additionally 20 mM glucose, 1·4 mM lactate, and 0·14 mM pyruvate was used. Varying amounts of either glutamate or α-ketoglutarate (with 0·40 mM NH_4Cl) were added as substrate (Table 2; Figs. 1 and 2). When ethanol was added *in vitro*, its concentration in the medium was usually 20 mM. One ml of medium was used per 100 mg of liver slices. The incubation temperature was always 37° and the time usually 2 hr. All incubations were carried out in duplicate. The incubation was terminated by adding an equal volume of cold 0·6 M perchloric acid. Samples were then homogenized and centrifuged at 24,000 *g* for 60 min at 0°, and the supernatant was used for the determinations.

Liver slices with [^{14}C]glutamic acid. Marsh–Drabkin standard incubation medium [10], with O_2 + CO_2 as the gas phase was used, 7 ml/1 g of liver slices (Table 3). Four μCi of uniformly labelled [^{14}C]glutamic acid (CFB 10, The Radiochemical Centre, Amersham, England) was added, corresponding to 2 μmoles/sample. When ethanol was given *in vivo* the amount was, exceptionally, 750 mg/100 g of body wt as a 45 per cent aqueous solution. The incubation lasted 3 hr, and was terminated by adding 3 vol. cold absolute ethanol to each flask. The samples were then homogenized, kept overnight at 4°, and centrifuged at 24,000 *g* for 60 min at 0°; the precipitates were suspended in 5 ml of cold 75 per cent (v/v) ethanol and recentrifuged for 30 min. The combined supernatants were evaporated to dryness in an air stream at room temperature, the dry residues were dissolved in water, and used for the determinations of amino acids.

Postnuclear fraction of the homogenate. The medium, slightly modified from that of DiPietro and Weinhouse [11], contained 1·4 mM ATP, 10 mM NAD^+, 0·06 mM cytochrome *c*, 0·2 mM fumaric acid, 10 mM glucose, 8 mM $MgSO_4$, 77 mM KCl, and 40 mM Na_2HPO_4–NaH_2PO_4 buffer, pH 7·4. Other substances added to the incubation medium as substrates are mentioned in Table 1 and Figs. 1 and 2. One ml of the medium corresponded to 40 mg of the original tissue. The incubation time was usually 60 min, and the treatment of samples after incubation was the same as above.

Analytical methods

Proline. Proline was determined according to the photometric method of Troll and Lindsley [12]. Amberlite IRC-50 resin was used instead of Permutit for removal of the interfering amino acids.

Alanine. Alanine was separated by means of high-voltage paper electrophoresis according to a method described earlier [13] and measured spectrophotometrically [13]. The solvent used in electrophoresis was 80% formic acid–acetic acid–acetone–water (3:12:15:70, by vol.), pH 1·9 [14].

Glutamic acid. Glutamic acid was determined enzymatically with glutamate dehydrogenase, a method based on the enzymatic determination of α-ketoglutaric acid [15]. Radioactive glutamic acid was separated by means of ion exchange chromatography [16] and determined colorimetrically according to the ninhydrin method of Rosen [17].

α-Ketoglutaric acid, pyruvic acid, and lactic acid. Enzymatic methods based on instructions for the test combinations produced by Boehringer & Soehne, GmbH (Mannheim, Germany) were used for the determination of α-ketoglutaric acid [15], pyruvic acid [18], and lactic acid [19].

Measurement of radioactivities. Free [^{14}C]glutamic acid and [^{14}C]proline were isolated from the ethanol-soluble fraction of incubated liver slices according to Rojkind and Diaz de León [16]. The corresponding ethanol-insoluble precipitate contained the "total protein" activity. The material was hydrolysed overnight in 6 N HCl at 105–110°, and the acid removed by evaporation on the water bath.

For the assay of the radioactivities the samples were dissolved in water and 200 μl of the solution was mixed with 10 ml of hydrophilic scintillation fluid, containing 15 g 2,5-diphenyloxazole (PPO) and 100 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 1000 ml of toluene which solution was mixed with 600 ml of ethylene glycol monomethyl ether [20].

The radioactivities were measured with Packard Tri-Carb Model 2420 Liquid Scintillation Spectrometer.

RESULTS

Formation of alanine. In incubation experiments with various substrates as amino group donors, the net

Table 1. Effect of ethanol on the net formation of alanine in the incubation of liver homogenate

Addition to the medium		Concn (mM)	Net formation of alanine (μmoles/100 g of liver) Control rats (C)	Ethanol-treated rats (E)	E/C
None	(3)	—	25	−44	—
Glutamic acid	(2)	9·6	18	−3	—
Glutamine	(1)	9·6	147	33	0·22
Aspartic acid	(2)	2·4	289	96	0·33
	(1)	9·6	1110	215	0·19
Tyrosine	(1)	3·2	68	5	0·08
Adrenaline	(3)	2·4	226	100	0·44
Serotonin	(1)	4·8	140	−27	—
Ammonium chloride	(1)	9·6	258	25	0·10

The preparation of the postnuclear fraction of the homogenate, the composition of the incubation medium, and other experimental conditions are described in the text. The values represent the means from 1 to 3 independent experiments (number in parentheses), each with pooled livers from 2 control and 2 ethanol-treated rats. The respective value of alanine obtained from non-incubated homogenate is subtracted in order to get the net formation during incubation.

Table 2. Effect of ethanol on the formation of glutamic acid and proline from added α-ketoglutaric acid in rat liver

Administration of ethanol	Preparation	Addition of α-ketoglutaric acid	Experiment	Consumption of α-ketoglutaric acid Contr.	Consumption of α-ketoglutaric acid Eth.	Formation of glutamic acid Contr.	Formation of glutamic acid Eth.	Formation of proline Contr.	Formation of proline Eth.	Ratios of final concentrations: Lactic acid / Pyruvic acid Contr.	Lactic acid / Pyruvic acid Eth.	Glutamic acid / α-Ketoglutaric acid Contr.	Glutamic acid / α-Ketoglutaric acid Eth.	Proline / Glutamic acid Contr.	Proline / Glutamic acid Eth.
In vitro,	slices	0·16 mM	1	0·51	1·60	2·21	3·38	0·53	0·82	15·5	65·8	6·6	84·4	0·11	0·12
20 mM			2	0·32	1·51	(−0·22)	(1·48)	0·47	0·72	10·8	48·1	3·4	28·2	0·18	0·17
		1·60 mM	1	10·55	12·38	2·21	3·05	0·53	0·70	10·6	35·6	1·4	2·3	0·10	0·11
			2	10·27	12·21	2·31	3·24	0·57	0·73	10·4	35·3	1·3	2·1	0·13	0·14
In vitro,	homogenate	1·60 mM	1	8·44	16·33	2·95	2·95	1·31	1·41	7·0	44·8	0·8	10·3	0·28	0·29
20 mM			2	7·97	16·29	2·95	2·73	1·33	1·16	6·7	40·3	0·7	9·3	0·28	0·26
In vivo,	homogenate	1·60 mM	1	8·88	14·92	2·91	4·50	1·12	1·66	8·4	67·9	1·0	17·6	0·26	0·32
5·5 mg/g body wt			2	8·99	14·76	2·97	4·04	1·18	1·13	8·0	38·8	1·0	12·4	0·27	0·27
P for the effect of ethanol*				<0·05†		<0·02‡		<0·05§							
				Concentrations at killing											
In vivo, 5·5 mg/g body wt	whole liver			0·11	0·05	0·84	1·93	0·60	1·04	10·4	11·3	7·1	39·4	0·71	0·54

One g of liver was incubated in 10 ml of medium. Other experimental conditions are described in the text. Amounts are expressed in μmoles/g of fresh liver. The experiments were performed *in vitro* each with one animal, *in vivo* each with one control and one ethanol-treated animal. All incubations and analyses were made in duplicate. The respective values obtained without added α-detoglutaric acid are subtracted in order to get the net formations of the substances.

* Treated as non-independent pairs and expressed in per cent of the respective control. Data in the parentheses not included.

† Data from experiments with 1·60 mM α-ketoglutarate, treated as independent samples, yield $P < 0.001$.

‡ Data treated as independent samples yield $P < 0.02$. Data in the parentheses not included.

§ Data from experiments with slices yield $P < 0.001$ when treated as independent samples.

Table 3. Effect of ethanol on the conversion of [^{14}C]glutamic acid to proline in liver slices

Administration of ethanol		Glutamic acid (cpm/mg liver, wet wt)	Glutamic acid (cpm/μg Glu)	Proline (cpm/mg liver, wet wt)	Proline (cpm/μg Pro)	Total protein (cpm/mg liver, wet wt)	Radioactivity recovered (cpm/mg liver, wet wt)
In vivo	7·5 mg/g body wt	1340 ± 134	630 ± 19	2210 ± 89	23,100 ± 754	450 ± 97	4000
	none (controls)	1150 ± 21	540 ± 37	2130 ± 5	24,800 ± 54	450 ± 6	3730
In vitro	50 mM	2880 ± 304	1350 ± 134	4170 ± 95	28,700 ± 1418	600 ± 18	7650
	20 mM	2170 ± 11	1290 ± 18	4380 ± 172	27,600 ± 655	640 ± 24	7190
	none (controls)	1630 ± 259	830 ± 130	3010 ± 134	28,700 ± 1857	530 ± 30	5170

In all experiments 4 μCi [^{14}C]glutamic acid was added to the medium; from non-incubated sample was recovered 7900 cpm/mg liver, wet wt. Experimental details are described in the text. The values are means of two rats, each analysed in duplicate.

formation of alanine (Table 1) was always lower in the postnuclear fraction of liver homogenate prepared from ethanol-treated rats than in the respective control preparation ($P < 0.02$), presumably as a result of the deficiency in pyruvic acid. The final concentration of alanine at the end of incubation (actual figures not presented) was decreased as the effect of ethanol on average by 31·8 per cent (t paired = 8·04, $P < 0.001$, $n = 13$).

Formation of glutamic acid from α-ketoglutaric acid. Administration of ethanol increased the consumption of added α-ketoglutaric acid during incubation both of homogenates and slices (Table 2). Correspondingly, when ethanol was given to the rats *in vivo*, the concentration of α-ketoglutaric acid in liver was decreased. This accelerated disappearance can, in part, be accounted for by the formation of glutamic acid, which is accumulated in liver during ethanol intoxication. Glutamic acid was accumulated in incubated slices and homogenates prepared from ethanol-treated rats, but not in homogenates to which ethanol had been added *in vitro*. Thus, an integrated cell structure is necessary for this effect to occur. Added ethanol markedly decreased the conversion of added glutamic acid

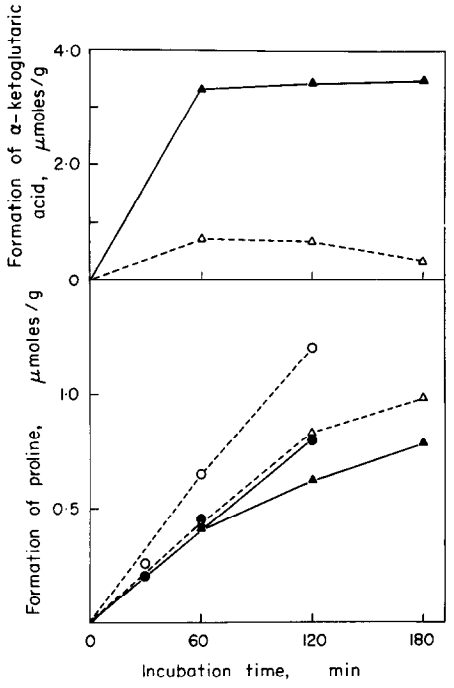

Fig. 1. Formation of α-ketoglutaric acid and proline in liver preparations as functions of the incubation time. The concentration of glutamic acid was 3 mM in the incubation medium of liver slices and 2·4 mM in that of the postnuclear fraction of liver homogenate. Other experimental details are described in the text. The values are means from 2 to 4 independent experiments. Liver slices incubated without ethanol (▲), in the presence of ethanol (△). Postnuclear fraction of homogenate from control rats (●), from ethanol-treated rats (○).

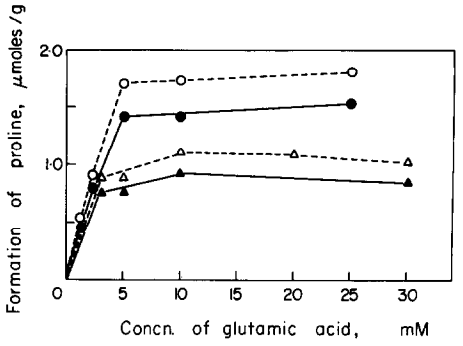

Fig. 2. Formation of proline in incubated liver preparations at various concentrations of added glutamic acid. The incubation time was 2 hr for liver slices and 1 hr for the postnuclear fraction of liver homogenate. Other experimental details are described in the text. The values are means from 2 to 8 independent experiments. Liver slices incubated without ethanol (▲), in the presence of ethanol (△). Postnuclear fraction of homogenate from control rats (●), from ethanol-treated rats (○).

to α-ketoglutaric acid in the slices (Fig. 1). Likewise, when homogenate was incubated with glutamic acid (2·4 mM) the increment in α-ketoglutaric acid in controls was 5·9 m-moles/100 g, but with preparations from ethanol-treated rats, it was only 3·3 m-moles/100 g.

Formation of proline. Ethanol stimulated the formation of proline both in incubated liver slices and in homogenate when ethanol was administered *in vivo* (Table 2). Similarly, a notable increase was observed in the concentration of proline in whole liver *in vivo*. The ratio of proline to glutamic acid was not increased, however. The rate of formation of proline from glutamic acid was almost constant during the first 120 min of incubation and this rate was higher than in control livers (Fig. 1). This increased synthesis was found throughout the concentration range of glutamic acid tested (Fig. 2).

The values for the radioactivity remaining in glutamic acid (Table 3) show that the metabolic dilution of glutamic acid is suppressed by ethanol. This is in agreement with the decreased deamination of glutamic acid to α-ketoglutaric acid (Fig. 1). On the other hand, ethanol causes an increase in the total conversion of [^{14}C]glutamic acid to proline, but the specific radioactivity of proline is not changed. In the experiments *in vitro* there is a slight increase in the radioactivity incorporated into total proteins.

Addition of lactic acid (5 and 25 mM) to the homogenate increased the formation of proline from glutamic acid by 37 and 100 per cent, respectively, but pretreatment of the rats with ethanol had no clear effect.

DISCUSSION

Comments on the methods

The results are generally calculated as net changes during the experiment to emphasize the effects of eth-

anol on specific reactions of the added substrates. Furthermore, the basal values without added substrates differ for the various preparations used. For example, the concentration of proline in liver slices was 0·261 ± 0·028 μmoles/g (n = 6), which is in agreement with the values reported for liver tissue [3] and slices [16]. In fresh homogenate the corresponding value was 0·520 (0·476–0·565) μmoles/g, and in a homogenate incubated without addition of amino acids, it was 1·45 μmoles/g.

In order to ascertain the effect of ethanol on the redox balance, the concentrations of lactic acid and pyruvic acid were determined in most experimental series. After an incubation of both slices and homogenates for 2 hr, the ratio of lactic acid to pyruvic acid varied from 30 to 65 in the presence of ethanol and from 6 to 15 in its absence. This change is mainly due to a decrease in the concentration of pyruvic acid.

Concentration of amino acids and amines

Alanine. Krebs *et al.* [21] have recently studied the accumulation of amino acids by the perfused rat liver in the presence of ethanol and observed that ethanol does not influence the disappearance of alanine added as the sole substrate. Thus, the changes found by us in the metabolism of alanine (Table 1) are due to a decreased synthesis of alanine. The increase in the formation of aspartate from alanine observed by Krebs *et al* [21] supports our earlier results on brain [4–6].

Glutamic acid and its derivatives. The present findings on glutamic acid and α-ketoglutaric acid are in agreement with the previous results of Williamson *et al.* [22] and Rawat [3], who utilized isolated perfused liver. Williamson *et al.* found ethanol to decrease the concentration of α-detoglutaric acid from 1118 to 663 m-moles/g (dry wt) and to increase that of glutamic acid from 27·64 to 34·04 μmoles/g.

Ethanol increases the ratio of glutamic acid to α-ketoglutaric acid. We were surprised to find that the ratio of final concentration of proline to that of glutamic acid was not increased by ethanol treatment (Table 2) although the rate of the conversion of glutamic acid to proline was enhanced (Figs 1 and 2). The consumption of proline in perfused liver was inhibited by ethanol [23].

Ethanol influences the amino acid levels in tissues through transaminases, too: in brain we have found pretreatment with ethanol to cause a slight decrease in GPT (alanine aminotransferase, EC 2.6.1.1) and an increase in GOT (aspartate aminotransferase, EC 2.6.1.2) activity [24]. Furthermore, the elimination of nitrogen is changed also by a small (6·4 per cent) but consistent decrease in glutamine synthetase (EC 6.3.1.2) activity in the soluble protein fraction of brain ($t = 8{\cdot}55$, $P < 0{\cdot}001$; unpublished).

All these changes should influence the composition of the amino acid pool accumulated for protein synthesis in the cells.

Amines. Ethanol depresses the catabolism of biogenic amines because there is less pyruvic acid available for transamination (Table 1). Indeed, it has been observed by several authors that ethanol retards the catabolism of serotonin [25–27]. In earlier studies (unpublished) we found the amount of hydroxyindoleacetic acid formed from added serotonin during the incubation of liver homogenate to decrease after ethanol pretreatment.

Relevancy of the results to alcoholic cirrhosis

In liver cirrhosis the proliferation of the hepatocytes and fibroblasts leads to disturbed lobular architecture and fibrosis. The shift of the redox balance to the reduced side enhances the production of connective tissue (i) by increasing the synthesis of collagen-precursor proline through the pathway α-ketoglutaric acid → glutamic acid → glutamic semialdehyde → proline or from arginine → glutamic semialdehyde → proline [28], (ii) by providing reduced cofactors for the hydroxylation of protocollagen proline and lysine to hydroxyproline and hydroxylysine [29], respectively, as exemplified by the effects of ascorbic acid on the connective tissue [30, 31], and (iii) by facilitating the epimerization reactions which are involved in the production of galactosamine and iduronic acid for the synthesis of chondroitin sulfates [32, 33]. The addition of lactate has been shown to activate protocollagen proline hydroxylase in cell culture [34] and to increase the incorporation of glutamic acid to collagen in granulation-tissue slices [35].

The synthesis of proline from glutamic acid is augmented in liver slices from carbon tetrachloride-treated rats [16]. The concentration of free proline is high in the samples of cirrhotic human livers [36]. In fibroblasts the intracellular concentration of proline and, hence, its incorporation to collagen and other proteins depend on the extracellular supply of proline [7, 35].

The factors which activate the connective tissue in liver to develop alcoholic cirrhosis may include (i) agents active in the reparation of inflammatory damage in general, (ii) stimulatory peptides like that demonstrated by McGee *et al.* [37] in liver of CCl_4-injured mice, but also (iii) the increased supply of free proline which supports the synthesis of proteins in fibroblasts responding to any stimulus.

Acknowledgements—We thank Prof. H. Suomalainen and Dr. O. Forsander for the interest and advice and Mrs. Ritva Veini for technical assistance. E.K. has been supported in part by Finnish Research Council for Medical Sciences and the Sigrid Jusélius Foundation.

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