REFERENCES

1. B. S. McEwen, *Rev. Neurosci.* **4**, 1 (1979).
2. A. S. Heritage, W. E. Stumpf, M. Sar and L. D. Grant, *Science* **207**, 1377 (1980).
3. H. R. Wagner, K. A. Crutcher and J. N. Davis, *Brain Res.* **171**, 147 (1979).
4. S. E. Robinson, P. L. Mobley, H. E. Smith and F. Sulser, *Naunyn-Schmiedeberg's Archs Pharmac.* **303**, 175 (1978).
5. P. L. Mobley and F. Sulser, *Eur. J. Pharmac.* **60**, 221 (1979).
6. F. Sulser, *Trends pharmac. Sci.* **1**, 92 (1979).
7. J. B. Blumberg, J. Vetulani, R. J. Stawarz and F. Sulser, *Eur. J. Pharmac.* **37**, 357 (1976).
8. A. G. Gilman, *Proc. natn. Acad. Sci. U.S.A.* **67**, 305 (1970).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. chem.* **193**, 265 (1951).
10. H. R. Wagner and J. N. Davies, *Brain Res.* **201**, 235 (1980).
11. S. M. Paul, J. Axelrod, J. M. Saavedra and P. Skolnick, *Brain Res.* **178**, 499 (1979).
12. L. L. Zschaeck and R. J. Wurtman, *Neuroendocrinology* **11**, 144 (1973).
13. B. B. Wolfe, T. K. Harden, J. R. Sporn and P. B. Molinoff, *J. Pharmac. exp. Ther.* **207**, 446 (1978).
14. J. W. Schweitzer, R. Schwartz and A. J. Friedhoff, *J. Neurochem.* **33**, 377 (1979).
15. D. A. Kendall, G. M. Stancel and S. J. Enna, *Science* **211**, 1183 (1981).

Biochemical Pharmacology, Vol. 30, No. 22, pp. 3128–3131, 1981.
Printed in Great Britain.

0006-2952/81/223128-04 $02.00/0

Conversion of alpha-ketobutyrate to alpha-amino-*n*-butyric acid by isolated rat liver cells: effect of ethanol

(*Received* 27 *November* 1980; *accepted* 1 *June* 1981)

Chronic alcohol consumption results in increased plasma alpha-amino-*n*-butyric acid (AANB) in man [1] as well as in experimental animals (rats and baboons) [2]. According to Shaw and Lieber [3, 4], this rise is due, at least in part, to excess hepatic production possibly associated with decreased peripheral utilisation. The mechanism of increased net hepatic production of AANB is unknown. The breakdown of methionine being increased [5] and citric cycle activity depressed [6] during chronic alcohol intoxication, a decreased oxidation and an increased availability of a α-ketobutyrate (α-KB) (the precursor of AANB) may be postulated. However, a possible effect of ethanol on the conversion of α-KB to AANB could also take place. We used therefore freshly isolated hepatocytes to define the metabolic pathway involved in the conversion of α-ketobutyrate into AANB and the effects of ethanol on this conversion.

Materials and methods

Isolation and incubation of cells. Hepatocytes were isolated according to Krebs *et al.* [7] from 18 hr fasted female Sprague–Dawley rats of approx 200 g weight. The cells were incubated at a final concentration of 2×10^6 cells/ml in Krebs–Ringer bicarbonate buffer at pH 7.4 and 37°. Incubations were carried out for 60 min in a Metabolyte gyrotatory shaker (New Brunswick) at 90 oscillations/min under 95% O_2 + 5% CO_2. After storage on ice up to use, hepatocytes were first preincubated for 10 min in the presence or absence of 2 mM α-KB and either 4 mM L-glutamine or L-asparagine or 2 mM ammonium chloride. When indicated, 2 mM aminooxyacetate was added.

Sample preparation and amino acid analysis. For amino acid analysis, the samples were deproteinised with sulphosalicylic acid (4% w/v, final concentration). After centrifugation at 4° during 10 min at 3000 *g*, the clear supernatant was collected and adjusted to pH 2.2 with lithium hydroxide (10%, w/v). Free amino acids in the cell extracts (kept at −90° until analysed) were measured with an automatic amino acid analyser (Liquimat, Labotron, West Germany) [8].

Ethanol determinations. Ethanol (final concentration 10 mM) was added after the preincubation period. The reaction was stopped 60 min later by adding ice-cold perchloric acid (3% w/v, final concentration) and the supernatant used for ethanol determination according to Bernt and Gutman [9].

Chemicals. Collagenase (grade II), other enzymes and coenzymes were from Boehringer, Mannheim, West Germany; α-KB (sodium salt), L-glutamine and L-asparagine. H_2O from Sigma Chemical Co., St. Louis, MO. The other chemicals used were of analytical grade.

Expression of results. The results are expressed as μmoles of substrate removed or produced per gram wet weight of liver (on the basis of 10^8 cells per g wet weight of liver) and per time unit. The results are given as the values from at least two representative experiments from different liver cell preparations.

Results and discussion

Cell viability. All preparations of cells satisfied the following criteria: (a) the ATP content was 20–25 nmoles/10^6 cells; (b) the lactate dehydrogenase activity in the supernatant was less than 10% of the total activity; (c) the rate of ethanol oxidation during incubation with 10 mM ethanol (at which concentration ethanol is mainly metabolised by alcohol dehydrogenase reaction [10]) was about 0.9 μmoles/min/g liver wet weight as reported by several authors for similar preparations [10–12].

Formation of alpha amino-n-butyric acid (AANB) from α-ketobutyrate (α-KB) in isolated rat liver cells. As shown in Table 1, isolated rat liver cells were able to synthesise about 1.60 μmoles of AANB per g liver wet wt when

Table 1. Formation of alpha amino-*n*-butyric acid (AANB) from α-ketobutyrate (α-KB) in isolated rat liver cells incubated in the absence or presence of ethanol

	Additions		Substrates			
	Ethanol	A O A	None	L-glutamine	L-asparagine	NH_4Cl
Exp. 1	–	–	1.61 (100)*	†(100) 10.44 (648)*	†(100) 6.23 (387)*	†(100) 1.87 (116)*
	+	–	1.85 (115)*	†(109) 11.38 (707)*	†(132) 8.53 (530)*	†(131) 2.46 (153)*
	–	+	N.D.	†(002) 0.20 (012)*	0.00	N.D.
	+	+	N.D.	†(005) 0.55 (034)*	0.00	N.D.
Exp. 2	–	–	1.59 (100)*	†(100) 6.64 (418)*	†(100) 3.50 (220)*	†(100) 2.25 (141)*
	+	–	2.80 (176)*	†(101) 6.73 (423)*	†(131) 4.60 (289)*	†(150) 3.37 (212)*
	–	+	N.D.	†(002) 0.13 (008)*	0.00	N.D.
	+	+	N.D.	†(007) 0.47 (029)*	0.00	N.D.

Rat liver cells (20–40 mg of wet wt/ml) were incubated for 60 min in the presence of 2 mM α-ketobutyrate (α-KB) and, when indicated, 4 mM L-glutamine or L-asparagine, 2 mM ammonium chloride (NH_4Cl) in the absence (−) or presence (+) of 10 mM ethanol or/and 2 mM aminooxyacetate (AOA).

* Numbers in parentheses indicate percentage of the AANB production obtained in the presence of 2 mM α-KB alone.

† Numbers in parentheses indicate percentage of the AANB formation obtained without ethanol and aminooxyacetate.

N.D. = not indicated.

incubated during 60 min in the presence of α-KB alone (2 mM). This AANB formation was associated with a marked decrease in the L-glutamine concentration (about 75 per cent) whereas L-aspartate level was significantly increased by about 60 per cent (Table 2).

The decrease in the L-glutamine level in hepatocytes incubated in the presence of α-KB suggested a possible role of L-glutamine as donor of the amino group for AANB synthesis from α-KB. Consequently, we studied the influence of the addition into the medium of 4 mM glutamine (at which concentration L-glutamine is metabolised in isolated hepatocytes [13]) on AANB production from α-KB. Such an addition resulted in a 4.5–6.5-fold enhancement in AANB production (Table 1); at the same time, L-glutamate was considerably increased, reaching 12 times the control values (without any addition), and a 4-fold increase in the L-aspartate level was observed (Table 2).

Similar experiments concerning the influence of the addition into the medium of L-asparagine at the same concentration (4 mM) showed that L-asparagine determined also an enhancement of AANB production from α-KB, but the magnitude of this effect is smaller than that obtained with L-glutamine (Table 1). A decrease in L-glutamine concentration (about 70 per cent) is observed under this experimental condition, suggesting either that L-glutamine contributes to AANB synthesis from α-KB in

Table 2. Amino acid concentrations in isolated rat liver cells incubated with α-ketobutyrate (α-KB) in the absence or presence of ethanol

Amino acid concentrations (μmoles/g liver wet wt.)	Additions		Substrates			
	Ethanol	A O A	None	L-glutamine	L-asparagine	NH_4Cl
L-glutamine						
†0.83 ± 0.19 (4)	–	–	0.22 ± 0.04*(3)	–	0.235 (0.14 – 0.33)	0.32 (0.20 – 0.44)
	+	–	0.24	–	0.31 (0.26 – 0.36)	0.23
	–	+	N.D.	–	0.75	N.D.
	+	+	N.D.	–	1.14	N.D.
L-glutamate						
†0.78 ± 0.11 (5)	–	–	0.69 ± 0.08 (4)	9.07 (8.53 – 9.61)	–	0.89 (0.78 – 1.00)
	+	–	0.845 (0.83 – 0.86)	7.15	–	1.235 (0.82 – 1.65)
	–	+	N.D.	–	–	N.D.
	+	+	N.D.	16.9 (10.5 – 23.3)	–	N.D.
L-aspartate						
†0.30 ± 0.04 (5)	–	–	0.49 ± 0.08* (4)	1.12 (0.63 – 1.41)	–	0.95 (0.65 – 1.25)
	+	–	0.785 (0.60 – 0.92)	1.145 (0.83 – 1.46)	–	1.14 (0.72 – 1.56)
	–	+	N.D.	0.48 (0.45 – 0.50)	–	N.D.
	+	+	N.D.	0.81 (0.66 – 0.96)	–	N.D.
L-ornithine						
†0.76 ± 0.24 (5)	–	–	0.53 ± 0.34 (4)	0.11 (0.09 – 0.13)	0.32 (0.23 – 0.41)	0.26 (0.16 – 0.36)
	+	–	0.33 (0.24 – 0.42)	0.165 (0.13 – 0.20)	0.42 (0.38 – 0.46)	0.345 (0.31 – 0.38)
	–	+	N.D.	0.685 (0.66 – 0.71)	0.96 (0.86 – 1.06)	N.D.
	+	+	N.D.	0.70 (0.62 – 0.78)	0.845 (0.83 – 0.86)	N.D.

Experimental conditions were identical to those described in Table 1. Each value represents the mean ± S.E.M. or the average of two separate experiments with range in parentheses. Values which are statistically different from the values obtained without any addition (†) are indicated by $^*P < 0.05$; N.D. = not determined.

spite of the presence of L-asparagine or that ammonia resulting from L-asparagine hydrolysis enhanced mitochondrial glutaminase activity [14] (Table 2).

The present data, showing that AANB production from α-KB is enhanced by the addition of L-glutamine or L-asparagine, are likely to result from transamination reactions using L-glutamine or L-asparagine aminotransferase. However, the role of transamination reactions invòlving L-glutamate or L-aspartate produced by hydrolysis of L-glutamine or L-asparagine cannot be ruled out; experiments testing the influence of L-glutamate or L-aspartate addition to the medium on AANB production from α-KB were not undertaken as these dicarboxylic amino acids do not readily enter the hepatocytes [15].

Additional support for the involvement of transamination reactions in AANB production from α-KB in the presence of L-glutamine or L-asparagine were obtained by testing the influence of aminooxyacetate addition on this production (Table 1). We have previously shown that aminooxyacetate, which is known to inhibit alanine and aspartate aminotransferase [16], also inhibits *in vivo* glutamine aminotransferase [17]. As shown in Table 1, aminooxyacetate addition reduced by about 98 per cent AANB production in the presence of L-glutamine and supressed it completely in the presence of L-asparagine.

Whereas the previous results favour the involvement of transamination reactions using L-glutamine (or L-glutamate) and L-asparagine (or L-aspartate) as nitrogen donors, L-ornithine could also contribute to AANB formation from α-KB through the ornithine aminotransferase reaction; as a matter of fact, a decrease in the cellular ornithine level was found to occur when hepatocytes are incubated with α-KB either alone or in the presence of L-glutamine or L-asparagine and this decrease was suppressed by the addition of aminooxyacetate (Table 2).

Reductive amination of α-KB through glutamate dehydrogenase as described *in vitro* [18] could also play a role in AANB production from α-KB. The fact that aminooxyacetate almost completely inhibits AANB production from α-KB in the presence of either L-asparagine or L-glutamine (whereas we have previously reported [17] a stimulating effect of aminooxyacetate on glutaminase activity) argues against a major involvement of this pathway in isolated rat liver cells. Further arguments for such a statement result from the finding that NH_4Cl addition (2 mM) to the medium induced only a slight increment in AANB production from α-KB (Table 1). The glutamine level was reduced by 60 per cent in such conditions associated with a 2-fold increase in the aspartate concentration (Table 2); the stimulation of mitochondrial glutaminase activity by ammonia [14] is likely to contribute to these alterations in amino acid concentrations.

Effect of ethanol on AANB production from α-KB. Table 1 shows that the addition of 10 mM ethanol enhanced the rate of AANB production from α-KB added either alone or in the presence of L-asparagine or NH_4Cl by isolated liver cells. This stimulatory effect of ethanol was about 40 per cent under whatever conditions, but large variations in AANB production were observed when α-KB was added alone with ethanol (Table 1). On the other hand, ethanol did not affect the AANB formation from α-KB before strongly enhanced by L-glutamine (Table 1). The fact that aminooxyacetate addition in the presence of L-glutamine or L-asparagine suppressed almost completely AANB production from α-KB even when ethanol was added, favours the concept that reductive amination of α-KB does not play a major role in the hepatic AANB synthesis. Table 2 shows that the ethanol-induced enhancement in AANB formation from either α-KB alone or in the presence of NH_4Cl was associated with a slight increase in L-glutamate and L-aspartate concentrations. So, it can be suggested that ethanol acts upon the hepatic conversion of α-KB to AANB by increasing the availability of glutamate as a nitrogen donor for α-KB transamination [19, 20]. However, another possible explanation is a decrease in the oxidative breakdown of α-KB in favour of transamination to AANB by ethanol [6]. The precise mechanism whereby ethanol increases AANB formation from α-KB remains to elucidate. In the presence of L-glutamine, it might be that the stimulation of AANB production from α-KB was maximal and ethanol could not exert its effect. This finding supports the role playing by the availability of L-glutamate for the conversion of α-KB to AANB.

Effect of α-ketobutyrate on ethanol elimination rate by isolated rat liver cells. As previously reported [21, 22], the rate of ethanol removal by isolated liver cells was accelerated by the addition of 4 mM L-asparagine (3-fold) or 2 mM ammonium chloride (about 2-fold) or 4 mM L-glutamine at a lesser extent (64 per cent) (Table 3). Table 3 shows that the addition of 2 mM α-KB alone also induced

Table 3. Effect of α-ketobutyrate (α-KB) on the ethanol elimination rate by isolated rat liver cells

Additions			Ethanol elimination rate (μmoles/min/g liver wet wt.)
Nitrogen substrates	α KB	AOA	
None	-	-	0.80 ± 0.12 (3)
	+	-	1.10 (0.90 - 1.30)
L-Glutamine	-	-	1.31 (0.87 - 1.75)
	+	-	1.85 (1.52 - 2.17)
	+	+	0.98 (0.65 - 1.30)
L-Asparagine	-	-	2.39 (2.17 - 2.61)
	+	-	1.63 (1.52 - 1.74)
	+	+	2.18 (1.75 - 2.61)
NH_4Cl	-	-	1.42 (1.10 - 1.74)
	+	-	0.98 (0.65 - 1.30)

Experimental conditions were identical to those described in Table 1. Each value represents the mean ± S.E.M. or the average of two separate experiments with range in parentheses.

an increased rate of ethanol elimination (about 40 per cent). When α-KB was added with L-glutamine, a 2.3 increase in ethanol oxidation was observed (Table 3). On the contrary, when α-KB was added with L-asparagine or ammonium chloride, it reduced by about 30 per cent the stimulatory effect exerted by these substances added alone on the rate of ethanol removal (Table 3). A possible explanation for this finding is that the increased utilisation of glutamate for AANB synthesis (Table 1) results in a decreased availability of aspartate for the malate–aspartate shuttle. In contrast, the finding that addition of L-glutamine in the presence of α-KB is more effective on ethanol elimination than the addition of L-glutamine or α-KB alone is probably due to a stimulation by α-KB of glutamine uptake and metabolism in hepatocytes. The effects of α-KB on ethanol metabolism in the presence of L-glutamine or L-asparagine are almost completely suppressed by aminooxyacetate addition (Table 2). This result suggests a relationship between the role played by L-glutamine or L-asparagine in AANB production from α-KB and the effects exerted by α-KB on ethanol metabolism.

In summary, isolated rat hepatocytes have been shown to synthesise alpha-amino-*n*-butyric acid (AANB) when incubated in the presence of α-ketobutyrate (α-KB). (1) This AANB production is strongly increased when L-glutamine or L-asparagine are also added to the incubation medium, whereas ammonium chloride addition results only in a slight enhancement of AANB formation. The changes in the levels of some amino acids which are observed at the same time as well as the effects of aminooxyacetate suggest that AANB formation from α-KB involves mainly transamination reactions with glutamine and/or glutamate, asparagine and/or aspartate aminotransferase. L-Ornithine could also contribute to AANB formation from α-KB. In contrast, the reductive amination of α-KB does not appear to play a significant role. (2) Ethanol added to 10 mM exerts a stimulatory effect on AANB production from α-KB either alone or in the presence of L-asparagine or ammonium chloride, but it has no effect on the AANB formation enhanced by L-glutamine. (3) The ethanol elimination rate is slightly increased when α-KB is added alone, but α-KB reduces the accelerating effect of L-asparagine or ammonium chloride, whereas it enhances this exerted by L-glutamine. These findings suggest a stimulation of hepatic AANB production by ethanol resulting from an enhanced availabilty of L-glutamate, L-glutamine + L-asparagine for the transamination reaction with α-KB and a possible contribution of this phenomemon to the increased plasma AANB level frequently observed during chronic alcohol intoxication.

Acknowledgements—This research was supported by the Institut National de la Santé et de la Recherche Médicale and the Scientific Council of the Faculté de Médecine Paris Ouest.

Groupe de Recherches sur le Métabolisme Intermédiaire (I.N.S.E.R.M. U72) 45, rue des Saints-Pères 75270 Paris Cedex 06, France

M. A. PETIT
I. BARRAL-ALIX
J. NORDMANN
R. NORDMANN

I.N.S.E.R.M. U30 Hôpital Necker—Enfant Malades 75015 Paris, France

G. JEAN

REFERENCES

1. S. Shaw and C. S. Lieber, *Gastroenterology* **74**, 677 (1978).
2. S. Shaw and C. S. Lieber, *Clin. Res.* **25**, 499a (1977).
3. S. Shaw and C. S. Lieber, *Gastroenterology* **73**, 1246 (1977).
4. S. Shaw and C. S. Lieber, *Gastroenterology* **78**, 108 (1980).
5. J. D. Finkelstein, J. P. Cello and W. E. Kyle, *Biochem. biophys. Res. Comm.* **61**, 525 (1974).
6. A. I. Cederbaum, C. S. Lieber, D. S. Beattie and E. Rubin, *J. biol. Chem.* **250**, 5122 (1975).
7. H. A. Krebs, N. W. Cornell, P. Lund and R. Hems, in *Regulation of Hepatic Metabolism* (Eds. F. Lundquist and N. Tygstrup), p. 726. Academic Press, New York (1974).
8. C. Delaporte, G. Jean and M. Broyer, *Am. J. clin. Nutr.* **31**, 1647 (1978).
9. E. Bernt and I. Gutman, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), Vol. 3, p. 1499. Verlag Chemie, Weinheim and Academic Press, New York (1974).
10. N. Grunnet, B. Quistorff and H. I. D. Thieden, *Eur. J. Biochem.* **40**, 275 (1973).
11. M. Sjöblom and J. Mørland, *Biochem. Pharmac.* **28**, 3417 (1979).
12. D. C. Fanning, M16. S. Hopper and H. L. Segal, *Archs Biochem. Biophys.* **105**, 501 (1964).
17. M. A. Petit, J. Nordmann and R. Nordmann, *Archs int. Physiol. Biochim.* **84**, 527 (1976).
18. W. Greenaway and F. R. Whatley, *FEBS Lett.* **75**, 41 (1977).
19. M. A. Petit and I. Barral-Alix, *Biochem. Pharmac.* **28**, 2591 (1979).
20. M. Stubbs and H. A. Krebs, *Biochem. J.* **150**, 41 (1975).
21. H. A. Krebs and M. Stubbs, in *Alcohol Intoxication and Withdrawal* (Ed. M. M. Gros), p. 149. Plenum Press, New York (1975).
22. M. Mangeney, F. Beaugé, J. Nordmann and R. Nordmann, *Archs int. Physiol. Biochim.* **87**, 603 (1979).