The Accumulation of Aspartate in the Presence of Ethanol in Rat Liver

By MARION STUBBS and HANS A. KREBS

Metabolic Research Laboratory, Nuffield Department of Clinical Medicine,
Radcliffe Infirmary, Oxford OX2 6HE, U.K.

(Received 27 January 1975)

1. Isolated hepatocytes were used to establish the reasons for the accumulation of aspartate, previously observed when the isolated rat liver was perfused with ethanol in the presence of alanine or ammonium lactate. 2. The isolated cells did not form aspartate when incubated with alanine and ethanol, but much aspartate was formed on incubation with ammonium lactate and ethanol. 3. Urea was the main nitrogenous product on incubation with alanine, in contrast with the perfused liver, where major quantities of NH_4^+ are also formed. When the formation of urea was nullified by the addition of urease, alanine plus ethanol caused aspartate formation, indicating that aspartate formation depends on the presence of critical concentrations of NH_4^+ . 4. The accumulated aspartate was present in the cytosol. Ethanol halved the content of 2-oxoglutarate in the cytosol and more than trebled that of glutamate in the mitochondria. 5. The findings support the assumption that 2-oxoglutarate formed by the mitochondrial aspartate aminotransferase is not translocated to the cytosol in the presence of ethanol and NH_4^+ , because it is rapidly converted into glutamate, the dehydrogenation of ethanol providing the required NADH. Aspartate, however, is translocated to the cytosol and accumulates there because of the lack of stoichiometric amounts of oxoglutarate.

In previous work (Krebs *et al.*, 1973) it was found that ethanol causes an accumulation of aspartate in the perfused rat liver when excess of nitrogen in the form of alanine or ammonium lactate is present. The tissue concentration of aspartate after 90 min perfusion with alanine and ethanol was about 11 mM, against 2 mM with alanine alone. The present paper is concerned with an analysis of the reasons for this effect of ethanol. Isolated hepatocytes were used as the experimental material.

Materials and Methods

Rats

Female rats of the Wistar strain were obtained from Charles River U.K. Ltd., Manston Road, Margate, Kent, U.K. They were starved for 48 h.

Preparation and treatment of isolated hepatocytes

Hepatocytes were prepared essentially as described by Berry & Friend (1969) incorporating the modifications described by Cornell *et al.* (1973) and Krebs *et al.* (1974). Incubations were in 25 ml Erlenmeyer flasks containing about 80 mg of cells in 4 ml, except where stated otherwise. Cytosolic and mitochondrial components of the cells were separated by the method of Zuurendonk & Tager (1974), which depends on making the plasma membrane permeable by short treatment (40 s at about 5°C) with digitonin (BDH

Chemicals Ltd., Poole, Dorset, U.K.) and centrifuging the suspension. The mitochondria, plasma membranes, nuclei and other insoluble material appear in the pellet and all soluble contents of the cytosol appear in the supernatant. Lactate dehydrogenase and glutamate dehydrogenase were used as cytosolic and mitochondrial markers respectively and were assayed by the methods of Bergmeyer *et al.* (1963) and Schmidt (1963). If more than 90% of the lactate dehydrogenase and less than 10% of the glutamate dehydrogenase were found in the cytosol fraction, adequate separation was assumed to have taken place. When 2-oxoglutarate was to be measured larger amounts of cells were needed because of the low 2-oxoglutarate concentration. About 400 mg wet wt. of cells was incubated in 4 ml of medium for 10 min. A short period of incubation was necessary to avoid exhaustion of the added substrates. Rates of glucose synthesis at this concentration of cells were similar to those found with 80 mg, indicating that the oxygenation was adequate.

Corrections for adhering fluid in separation experiments

Up to 10% of lactate dehydrogenase was found in the pellet in the separation experiments. Corrections were made for this cytosolic contamination of the pellet, based on the assumption that the metabolites measured shared the same cytosolic space as the lactate dehydrogenase.

Reagents

Urease tablets (type 111-20) were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; other enzymes and coenzymes were from Boehringer Corp. (London) Ltd., London W.5, U.K.

Determination of metabolites

Glucose, lactate, pyruvate, aspartate, glutamate, alanine, NH_4^+ and urea were determined on neutralized HClO_4 extracts as described by Cornell *et al.* (1974). Glutamine was determined by the method of Lund (1970), malate by that of Hohorst (1963) and 2-oxoglutarate by that of Bergmeyer & Bernt (1963). Except where stated otherwise, metabolites were measured in the cell suspension. Aspartate, oxoglutarate, malate and glutamate were shown to be mainly intracellular [cell/medium gradients of >70 ; see also Hems *et al.* (1975)]. The number of observations given in the Tables for any given experimental condition refers to results obtained from separate batches of cells.

Results

Comparison of the effects of ethanol on alanine metabolism in liver perfusion and isolated hepatocytes

Alanine carbon metabolism, e.g. rates of glucose, lactate and pyruvate formation and alanine removal, was similar in the perfused organ and in isolated hepatocytes (not shown in Tables). There are, however, differences in the quantities of nitrogenous substances formed (see Table 1). In hepatocytes more aspartate was formed from alanine than in the perfused liver, but whereas ethanol increased the yield of aspartate 5.5-fold in the perfused organ, it had no effect in the hepatocytes. Further, there are differences in the relative amounts of NH_4^+ and urea formed. In the perfused organ 64% of the total nitrogen derived from alanine, in the absence of ethanol, appeared as NH_4^+ , against 17.5% in the hepatocytes. In the presence of ethanol a similar distribution of alanine nitrogen occurred.

These findings raise the question whether the differences in the formation of aspartate between the intact liver and the isolated cells in the presence of ethanol are related to the difference in production of NH_4^+ . Urease (1.5 units/flask) was therefore added to the hepatocyte suspension to maintain a higher NH_4^+ concentration. This caused a sevenfold increase in aspartate formation on the addition of ethanol (from 6 to 43 $\mu\text{mol/h per g}$), indicating that aspartate formation depends on the NH_4^+ concentration. This was further tested by experiments where alanine was replaced by lactate and NH_4Cl .

Effect of ethanol on the metabolism of lactate and NH_4Cl

In the presence of 10mm-lactate and 10mm- NH_4Cl , ethanol caused a 22-fold increase in [aspartate] (see Table 2). Ethanol inhibited glucose synthesis by 73% under these conditions; the high [lactate]/[pyruvate] ratio indicated a highly reduced redox state of the cytosolic NAD couple (see Veech *et al.*, 1972).

The amount of NH_4^+ removed was similar with and without ethanol, but with ethanol more NH_4^+ was converted into aspartate (and glutamate) and less into urea. As a source of both carbon and nitrogen is necessary for the synthesis of aspartate and glutamate, there was no increase in the yields of these amino acids when lactate alone, lactate plus ethanol, NH_4Cl alone, ethanol alone or NH_4Cl plus ethanol were present. These controls also demonstrate that the extra amino acids do not arise from proteolysis.

Effect of ornithine on aspartate accumulation

The addition of ornithine increased urea synthesis in both the absence and presence of ethanol (Table 3). It abolished the accumulation of aspartate by providing an acceptor in the form of citrulline. Glucose synthesis from lactate plus NH_4Cl was depressed in the presence of ornithine, and the addition of ethanol partially overcame this depression.

Table 1. *Comparison of the effects of ethanol on alanine nitrogen metabolism in liver perfusion and isolated hepatocytes*

Experimental conditions for the hepatocytes were as described in the text. Results are expressed as $\mu\text{mol/h per g}$ wet wt. (means $\pm$ s.d. for five observations). The values for the perfusion experiments are calculated from Table 3 in Krebs *et al.* (1973), expressed as μmol (tissue plus medium)/h per g wet wt.

Metabolite formed	Alanine (10mm)		Alanine (10mm)+ethanol (10mm)	
	Hepatocytes	Perfused liver	Hepatocytes	Perfused liver
Urea	42.4 $\pm$ 10.3	17	36.4 $\pm$ 8.8	10
NH_4^+	18.2 $\pm$ 4.3	61	14.4 $\pm$ 4.8	23
Aspartate	7.2 $\pm$ 2.2	2.7	7.3 $\pm$ 2.4	15

Table 2. *Effect of ethanol on gluconeogenesis, urea synthesis and amino acid formation in isolated rat liver cells*

Experimental details were as described in the text. The results are expressed as μmol of product formed or utilized/g wet wt. for a 60 min incubation (means $\pm$ s.d. for eight observations). The initial substrate concentrations were 10 mM. Carbon formed was calculated on the assumption that two molecules of lactate are needed for the synthesis of one molecule each of glucose, glutamate and glutamine. Values that are statistically different by Student's *t* test are indicated by: * $P < 0.05$.

Metabolite or metabolite ratio	Metabolite changes in the presence of:	
	Lactate+ NH_4Cl	Lactate+ NH_4Cl +ethanol
Glucose	63.4 $\pm$ 15.5	17.0 $\pm$ 10.2*
Lactate	-204 $\pm$ 61	-129 $\pm$ 51
Pyruvate	19.5 $\pm$ 7.33	1.59 $\pm$ 0.62*
[Lactate]/[pyruvate]	11.3 $\pm$ 3.3	203 $\pm$ 35*
Urea	59.5 $\pm$ 4.1	44.4 $\pm$ 3.4*
NH_4^+	-151 $\pm$ 19.9	-158 $\pm$ 19.7
Glutamate	6.92 $\pm$ 1.25	10.6 $\pm$ 1.83*
Glutamine	5.32 $\pm$ 1.26	5.98 $\pm$ 0.78
Alanine	20.7 $\pm$ 5.3	17.5 $\pm$ 7.85
Aspartate	2.77 $\pm$ 1.45	60.2 $\pm$ 21.5*
Sum of carbon formed (as C_3)	+194	+146
Sum of nitrogen formed	+160	+189

Table 3. *Effect of ethanol on glucose synthesis, urea synthesis and aspartate formation in the presence of ornithine*

Experimental details were as described in the text. The results are expressed as $\mu\text{mol/g}$ wet wt. for a 60 min incubation (means $\pm$ s.d. for three observations). The initial concentrations were 10 mM for lactate, NH_4Cl and ethanol and 2 mM for ornithine.

Metabolite	Metabolite changes in the presence of:			
	Lactate+ NH_4Cl	Lactate+ NH_4Cl +ornithine	Lactate+ NH_4Cl +ethanol	Lactate+ NH_4Cl +ethanol+ornithine
Glucose	50.5 $\pm$ 5.3	27.3 $\pm$ 2.9	18.2 $\pm$ 3.0	41.4 $\pm$ 14
Urea	58.8 $\pm$ 12.4	183 $\pm$ 32	45.5 $\pm$ 15.6	243 $\pm$ 82
Aspartate	3.5 $\pm$ 1.9	0.71 $\pm$ 0.2	49.6 $\pm$ 10	3.2 $\pm$ 1.9

Effect of ethanol on the distribution of aspartate, glutamate and 2-oxoglutarate between cytosol and mitochondria

For elucidation of the reasons for the accumulation of aspartate it was thought necessary to establish the distribution of aspartate, glutamate and 2-oxoglutarate between cytosol and mitochondria. Cytosol and mitochondria were separated by the method of Zurendonk & Tager (1974). The measurements provide information of the amounts but not of the concentrations of the metabolites in the two compartments.

Ethanol increased the amount of aspartate in the cytosol by a factor of 10, whereas the mitochondrial content (when corrected for adhering fluid) did not change (Table 4). The glutamate content increased in both compartments on the addition of ethanol by a

factor of 1.9 in the cytosol and 3.7 in the mitochondria. The 2-oxoglutarate content of the cytosol was halved in the presence of ethanol, but the mitochondrial content was not affected. This result is in accordance with additional measurements in cell suspensions (where the analytical accuracy is greater). These show that the amounts of oxoglutarate in the suspension were decreased by ethanol from 0.22 ± 0.11 s.d. (3) μmol per g wet wt. to 0.10 ± 0.02 s.d. (3) μmol per g wet wt. ($P < 0.05$ by Student's *t* test for paired observations). The mass-action ratio for the aspartate aminotransferase reaction $\frac{[\text{aspartate}][2\text{-oxoglutarate}]}{[\text{glutamate}][\text{oxaloacetate}]}$ in the cytosol was 11.8 in the absence of ethanol and 43.5 in its presence. Thus in the absence of ethanol the system is near equilibrium [the equilibrium constant being 6.61 (Krebs & Veech, 1970)], whereas ethanol causes a displacement from equilibrium.

Table 4. *Effect of ethanol on the cytosolic and mitochondrial content of aspartate, glutamate and 2-oxoglutarate formed in the presence of ammonium lactate*

Experimental details were described in the text. Initial concentrations of lactate, NH_4Cl and ethanol were 10 mM. The data are from a representative experiment in which 424 mg wet wt. of cells was incubated for 10 min before separation. The results are expressed as μmol of metabolite present in the named compartment. 'Corrected' values refer to μmol of metabolite present in the mitochondria corrected for adhering cytosol (for details of correction see the text). Oxaloacetate was calculated from the measured cytosolic malate content as described by Williamson *et al.* (1967). Calculation of mitochondrial oxaloacetate is unreliable.

Metabolite	Addition	Cytosol metabolites (μmol)	Mitochondrial metabolites (μmol)	
			Uncorrected	Corrected
Aspartate	Lactate+ NH_4Cl	0.36	0.40	0.36
	Lactate+ NH_4Cl +ethanol	3.40	0.76	0.43
Glutamate	Lactate+ NH_4Cl	0.83	0.17	0.09
	Lactate+ NH_4Cl +ethanol	1.52	0.48	0.33
2-Oxoglutarate	Lactate+ NH_4Cl	0.040	0.015	0.011
	Lactate+ NH_4Cl +ethanol	0.019	0.013	0.012
Oxaloacetate (calculated)	Lactate+ NH_4Cl	0.00151		
	Lactate+ NH_4Cl +ethanol	0.00098		

Discussion

Formation of urea and NH_4^+ from alanine

The finding that urea is the main nitrogenous end-product of alanine in the isolated cells, in contrast with the perfused liver where much NH_4^+ is formed, may be connected with differences in the intracellular concentration of ornithine. As ornithine is a rate-limiting factor in urea synthesis the results suggest that the concentration of ornithine is higher in the isolated cells than in the perfused liver, perhaps because of the action of the proteinase used in the preparation of the cells.

Accumulation of aspartate

The experiments demonstrate that the accumulation of aspartate in the presence of ethanol depends on the presence of relatively high concentrations (several millimolar) of NH_4^+ . As for the mechanism for the accumulation of aspartate the following facts are relevant.

1. The accumulated aspartate is located in the cytosol (except for traces shed by the cells).
2. The concentration of oxoglutarate is low in the cytosol when aspartate accumulates and that of glutamate is raised in the mitochondria.
3. Ornithine abolishes the accumulation of aspartate.
4. The components of the aspartate aminotransferase system are not at equilibrium in the cytosol when the NH_4^+ concentration is high.

The accumulation of aspartate implies that one of the two reactions by which aspartate is removed (the cytosolic aspartate aminotransferase reaction and the argininosuccinate synthetase reaction) must be decreased in relation to the production of cytosolic

aspartate. The aspartate that accumulates in the cytosol must originate from the mitochondrial aspartate aminotransferase reaction because the two precursors (glutamate and oxaloacetate) are both generated in the mitochondrial matrix by the glutamate dehydrogenase and pyruvate carboxylase reactions respectively. As the aspartate aminotransferase is the only enzyme, apart from proteolytic enzymes and asparaginase, which can generate major quantities of aspartate and since the aminotransferase cannot be energy driven, the driving force forming the mitochondrial aspartate must be taken to be the tendency to reach equilibrium in the aspartate aminotransferase system. Cytosolic aspartate can arise only by translocation of the mitochondrial aspartate, either by facilitated diffusion along a concentration gradient or by a mechanism involving an energy-driven pump against the gradient. These are open questions (see La Noue *et al.*, 1973, 1974), but whatever the mechanism there is the fact that aspartate is translocated to the cytosol. Normally, in the presence of lactate, when gluconeogenesis occurs the translocation of aspartate is accompanied by an equimolecular amount of 2-oxoglutarate (for details of the aspartate shuttle, see Borst, 1963; Lardy *et al.*, 1965; Cornell *et al.*, 1974). The finding of low concentrations of 2-oxoglutarate in the cytosol and raised concentrations of glutamate in the mitochondria is in accordance with the assumption that the stoichiometric transfer of aspartate and oxoglutarate is upset in the presence of NH_4Cl and ethanol because the mitochondrial oxoglutarate, instead of being translocated, reacts with NH_4^+ and NADH to regenerate the glutamate used up in the mitochondrial aminotransferase reaction, the oxidation of ethanol and acetaldehyde providing the NADH. This can account for the excess of aspar-

tate in the cytosol, which has no stoichiometric partner for the disposal reaction.

This assumption does thus explain the disequilibrium in the aspartate aminotransferase system of the cytoplasm. It is necessary to assume that at the lowered concentration of oxoglutarate the activity of the transferase is too weak to maintain equilibrium, [oxoglutarate] of the order of 0.045 mM being far below the K_m value of 0.3–0.4 mM [reported for ox liver by Wada & Morino (1964); there are no values for rat liver].

Effect of ornithine on aspartate accumulation

The non-accumulation of aspartate in the presence of ornithine might be taken to indicate that the argininosuccinate synthetase reaction can keep pace with the rate of supply of cytosolic aspartate when ornithine promotes the rate of supply of citrulline. An alternative explanation is the assumption that the intracellular concentration of NH_4^+ may be kept low when ornithine is present because the rate of penetration of NH_4^+ may be balanced by the rate of removal.

Comment on other findings

There are two observations, the inhibition of urea synthesis by ethanol in the presence of ammonium lactate (Table 2), and the inhibition of glucose synthesis from lactate by ornithine in the presence of NH_4Cl and the partial reversal of this inhibition by ethanol, for which no satisfactory explanation can as yet be offered.

This work was supported by grants from the Medical Research Council, the National Institute on Alcohol Abuse and Alcoholism (no. 1 R01 AA00381) and from Schering AG, Berlin.

References

- Bergmeyer, H. U. & Bernt, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 324–327, Academic Press, New York and London
- Bergmeyer, H. U., Bernt, E. & Hess, B. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 736–741, Academic Press, New York and London
- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Borst, P. (1963) in *Funktionelle und Morphologische Organisation der Zelle*, pp. 137–158, Springer-Verlag, New York
- Cornell, N. W., Lund, P., Hems, R. & Krebs, H. A. (1973) *Biochem. J.* **134**, 671–672
- Cornell, N. W., Lund, P. & Krebs, H. A. (1974) *Biochem. J.* **142**, 327–337
- Hems, R., Lund, P. & Krebs, H. A. (1975) *Biochem. J.* **150**, 47–50
- Hohorst, H. J. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 328–332, Academic Press, New York and London
- Krebs, H. A. & Veech, R. L. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), pp. 413–438, Springer-Verlag, Berlin
- Krebs, H. A., Lund, P. & Hems, R. (1973) *Biochem. J.* **134**, 697–705
- Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) in *Regulation of Hepatic Metabolism: Alfred Benzon Symposium VI* (Lundquist, F. & Tygstrup, N., eds.), pp. 457–479, Munksgaard, Copenhagen
- La Noue, K. F., Walajts, E. I. & Williamson, J. R. (1973) *J. Biol. Chem.* **248**, 7171–7183
- La Noue, K. F., Bryla, J. & Bassett, D. J. P. (1974) *J. Biol. Chem.* **249**, 7514–7521
- Lardy, H. A., Paetkau, V. & Walter, P. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **53**, 1410–1415
- Lund, P. (1970) in *Methoden der Enzymatischen Analyse*, 2nd edn. (Bergmeyer, H. U., ed.), vol. 2, pp. 1670–1673, Verlag Chemie, Weinheim
- Schmidt, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 752–756, Academic Press, New York and London
- Veech, R. L., Guynn, R. & Veloso, D. (1972) *Biochem. J.* **127**, 387–397
- Wada, H. & Morino, Y. (1964) *Vitam. Horm.* **22**, 411–444
- Williamson, D. H., Lund, P. & Krebs, H. A. (1967) *Biochem. J.* **103**, 514–527
- Zuurendonk, P. F. & Tager, J. M. (1974) *Biochim. Biophys. Acta* **333**, 393–399