Mechanisms for the Formation of Alanine and Aspartate on Rat Liver *in vivo* after Administration of Ammonium Chloride

By JOHN T. BROSNAN* and DERMOT H. WILLIAMSON
*Metabolic Research Laboratory, Nuffield Department of Clinical Medicine,
Radcliffe Infirmary, Oxford OX2 6HE, U.K.*

(Received 5 October 1973)

1. The time-course of the changes in the concentrations of hepatic metabolites in response to a non-toxic load of NH_4Cl were measured in fed and starved rats. 2. There was a rapid increase (after 2 min) in [alanine] and [aspartate] which remained high for 10–15 min; the absolute increase in [alanine] was smaller in starved rats. 3. These changes were accompanied by a decrease in [oxoglutarate] and in the [3-hydroxybutyrate]/[acetoacetate] ratio. 4. Prior administration of L-arginine to fed rats resulted in smaller increases in [alanine] and [aspartate] after the ammonia load. This is presumably due to stimulation of the urea cycle. 5. Increased formation of alanine in starved rats occurred after prior administration of dihydroxyacetone to increase the availability of pyruvate. 6. Administration of L-cycloserine, an inhibitor of glutamate–alanine aminotransferase, completely prevented the increase in [alanine] after the ammonia load; in this case the absolute increase in [aspartate] was higher. 7. [Oxoglutarate], [citrate] and [isocitrate] at 25 min after the ammonia load were higher than the initial concentrations, but returned to normal by 50 min. It is suggested that this ‘overshoot’ may be due to temporary compartmentation of oxoglutarate. 8. The mechanisms and physiological significance of alanine and aspartate formation in these experiments are discussed.

The formation of alanine and aspartate, through the mediation of glutamate dehydrogenase and alanine and aspartate aminotransferases, can serve to remove excess of NH_3 in ischaemic liver (Brosnan *et al.*, 1970). In the present work the operation of this system *in vivo* has been investigated by intraperitoneal injection of a load of ammonia sufficient to exceed temporarily the capacity of the urea cycle and to measure the timecourse of the changes in metabolite concentrations in the liver. Other workers have either used lethal doses of ammonia (Du Ruisseau *et al.*, 1957) or have only measured the concentrations of metabolites at one point in time after the ammonia load (Nordmann *et al.*, 1972).

Increased concentrations of NH_3 , an oxidant in the glutamate dehydrogenase system, shift the redox state of the mitochondrial free NAD^+ – NADH system in rat liver in favour of oxidation (Williamson *et al.*, 1967a) and therefore decrease the concentration of total NADH (Clifford *et al.*, 1969). These changes might be expected to cause rapid alterations in the concentrations of other reactants linked via the network of near-equilibrium reactions existing in rat liver (Krebs & Veech, 1970) and thus test the behaviour of these systems when upset by temporary alteration of the concentration of one of the components. Some

of these findings have been briefly reported (Brosnan *et al.*, 1967).

Materials and Methods

Reagents

3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) and L-alanine dehydrogenase (EC 1.4.1.1) were prepared as described by Williamson *et al.* (1967a,b) respectively. All other enzymes and coenzymes used for the determination of metabolites were purchased from Boehringer Corp (London) Ltd., London W.5, U.K., except for urease (type III) and glutaminase (type III), which were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Florisil (60–100 mesh) was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., L-cycloserine was from Calbiochem, Los Angeles, Calif., U.S.A., dihydroxyacetone and L-arginine hydrochloride were from British Drug Houses Ltd., Poole, Dorset, U.K., and Norit (activated charcoal) was from Harrington Bros., London S.W.12, U.K.

Animals

Male rats of the Wistar strain, weighing 180–200g, were killed and the liver was freeze-clamped and extracted with HClO_4 as previously described (Brosnan

* Present address: Department of Biochemistry, University of Newfoundland, St. Johns, Newfoundland, Canada.

et al., 1970). Samples of blood were taken, under ether anaesthesia, from the abdominal aorta into a heparinized syringe and immediately added to an equal quantity of 10% (w/v) HClO_4 in a stout-walled test tube. This mixture was then homogenized with a low-speed overhead-drive homogenizer and centrifuged in a bench centrifuge for 10 min. The supernatant was neutralized with 20% (w/v) KOH and, after removal of the KClO_4 was used for metabolite determinations. All injected substances were neutralized to pH 7.0 before administration.

Determination of metabolites

References to the methods used for the determination of lactate, pyruvate, 3-hydroxybutyrate, acetoacetate, 2-oxoglutarate, glutamate, alanine, aspartate, malate, ATP, ADP and AMP are given by Brosnan *et al.* (1970). Glutamine was determined as glutamate after treatment with *Escherichia coli* glutaminase by the method of Lund (1970). Urea was hydrolysed with urease to NH_3 which was measured by Nesslerization after micro-diffusion into 0.1M-HCl as described by Conway (1962). Citrate was determined with citrate lyase (Gruber & Möllering, 1966) and isocitrate by the method of Veech *et al.* (1969). NH_3 was determined by the method of Kirsten *et al.* (1963) as modified thus. Samples (2 ml) of neutralized tissue extracts (equivalent to about 0.3 g of liver) were pipetted into centrifuge tubes which contained 0.1 g of activated charcoal (Norit). These tubes were mixed by means of a Vortex mixer and centrifuged at 30000g for 30 min. Blanks containing HClO_4 were treated identically. The supernatant was used for NH_3 determination. This procedure was necessary because the non-enzymic oxidation of NADH interfered with the NH_3 assay; charcoal treatment decreased the blank NADH oxidation to negligible proportions and the recovery of known amounts of NH_3 added to tissue extracts averaged 93%. Norit was the only charcoal found to be effective for this assay; other charcoals were not suitable, because they gave either poor recoveries of NH_3 or large blanks. Particularly important was the use of deionized water in preparing all reagents and making up the NADH solution freshly each day. Florisil was also used, as described by Brosnan *et al.* (1970), to decrease the non-enzymic oxidation of NADH. It could not be used, however, for determinations of NH_3 or adenine nucleotides because of poor recoveries. It was not necessary to treat blood extracts with Florisil or Norit, as they caused negligible non-enzymic oxidation of NADH.

Calculations and expression of results

Calculations of $[\text{free NAD}^+]/[\text{free NADH}]$ and $[\text{free NADP}^+]/[\text{free NADPH}]$ ratios were as described

by Veech *et al.* (1969). Cytoplasmic oxaloacetate concentrations were calculated by assuming that lactate dehydrogenase and cytoplasmic malate dehydrogenase are in equilibrium with the same pool of nicotinamide nucleotides (Hohorst *et al.*, 1959). All equilibrium constants used for the calculations are for pH 7.0, 38°C $I = 0.25$ (Veech *et al.*, 1969). The results of metabolite determinations are expressed as mean values ($\pm$ s.d.) in $\mu\text{mol/g}$ fresh wt. of liver. No correction has been made for the extracellular space because the concentrations of the metabolites were lower in blood, and consequently any correction would have been minor.

Results

Effect of an ammonia load in fed rats

The concentrations of metabolites in freeze-clamped livers from fed rats after intraperitoneal injection of 2.5 mmol of $\text{NH}_4\text{Cl/kg}$ body wt. are given in Table 1. Control experiments in which NaCl was injected instead of NH_4Cl showed no appreciable changes except for a slight tendency for [lactate] to rise [from 0.87 ± 0.27 (s.d.; five observations) $\mu\text{mol/g}$ to 1.20 ± 0.40 2 min after the injection]. The ammonia load caused a threefold increase in both [alanine] and [aspartate], with the maximum increase at 5 min; the values returned to the initial range within 20–25 min. There was a biphasic change in [glutamate]; within 2 min the value fell to 60% of the initial value (by $0.8 \mu\text{mol/g}$) and then increased to a peak at 15 min; it had returned to the starting value at 25 min. There was a rise ($0.8 \mu\text{mol/g}$) in [glutamine] after 2 min, similar in magnitude to the decrease in glutamate at this time. The relatively small increases in liver [urea] do not represent the full extent of the increased rate of urea synthesis because of the rapid discharge of urea into the circulation. The value for [malate] decreased immediately after the ammonia injection and returned to the normal range within 15 min. This is the inverse of the behaviour of [aspartate], as expected if aspartate aminotransferase and malate dehydrogenase share a common pool of oxaloacetate.

The value for [2-oxoglutarate] fell to about one-third after ammonia injection and the ratio of [3-hydroxybutyrate]/[acetoacetate] decreased. Normal values were re-established within 15–25 min. As NH_3 is an oxidant in the glutamate dehydrogenase system, a shift of the 3-hydroxybutyrate dehydrogenase system towards oxidation is to be expected (Williamson *et al.*, 1967a).

The mass-action ratio of alanine aminotransferase ([alanine] [2-oxoglutarate]/[glutamate] [pyruvate]) was almost constant throughout the period of observation despite the large variations in the concentrations of individual metabolites, and it was close to the equilibrium constant for this system (1.51; Krebs,

Table 1. Effects of injection of NH_4Cl on concentrations of metabolites in livers of fed rats

NH_4Cl (2.5 mmol/kg body wt.) as a 0.25 M solution was injected intraperitoneally into fed rats. The rats were killed at the times indicated. The values for 0 min refer to control rats, not injected. Results are expressed as $\mu\text{mol/g}$ fresh wt. $\pm$ s.d. (four animals in each group). Oxaloacetate concentrations were calculated as described in the text. Values which are statistically different from the control rats by the Student's t test are indicated by *; $P < 0.05$.

Metabolites or metabolite ratios	Concentration (μmol/g fresh wt.)						
	Time (min)	0	2	5	10	15	25
[Lactate]	0.61 ± 0.24	1.31 ± 0.25*	0.68 ± 0.10	0.54 ± 0.08	0.68 ± 0.11	0.70 ± 0.24	
[Pyruvate]	0.073 ± 0.024	0.103 ± 0.042	0.081 ± 0.012	0.060 ± 0.011	0.087 ± 0.056	0.093 ± 0.040	
[Malate]	0.33 ± 0.09	0.21 ± 0.08*	0.18 ± 0.06*	0.22 ± 0.08*	0.33 ± 0.02	0.35 ± 0.06	
[Glutamate]	2.05 ± 0.62	1.29 ± 0.13*	1.95 ± 0.19	2.80 ± 0.25*	3.16 ± 0.27*	2.19 ± 0.71	
[2-Oxoglutarate]	0.175 ± 0.040	0.058 ± 0.014*	0.055 ± 0.020*	0.062 ± 0.017*	0.184 ± 0.123	0.228 ± 0.130	
[3-Hydroxybutyrate]	0.232 ± 0.072	0.184 ± 0.100	0.179 ± 0.173	0.167 ± 0.077	0.208 ± 0.148	0.217 ± 0.228	
[Acetoacetate]	0.090 ± 0.014	0.094 ± 0.020	0.143 ± 0.072	0.123 ± 0.039	0.112 ± 0.039	0.126 ± 0.062	
[Glutamine]	5.03 ± 0.66	5.82 ± 0.66	5.59 ± 0.97	5.74 ± 1.02	5.64 ± 0.74	5.16 ± 1.26	
[Alanine]	1.07 ± 0.36	2.50 ± 0.33*	3.24 ± 1.00*	2.75 ± 1.40*	1.57 ± 0.64	0.68 ± 0.18	
[Aspartate]	1.05 ± 0.20	1.69 ± 0.38*	3.11 ± 0.55*	2.70 ± 0.44*	1.86 ± 0.82	0.90 ± 0.59	
[Urea]	5.52 ± 0.84	6.80 ± 0.89*	6.34 ± 0.31	6.20 ± 1.50	6.82 ± 1.22	6.04 ± 1.74	
[Oxaloacetate] (calculated)	0.010	0.004	0.005	0.006	0.010	0.012	
[Alanine] [2-Oxoglutarate]	1.25	1.09	1.13	1.02	1.05	0.77	
[Glutamate] [Pyruvate]							
[Aspartate] [2-Oxoglutarate]	9.0	18.5	16.6	10.0	10.8	7.7	
[Glutamate] [Oxaloacetate]							

Table 2. *Effect of injection of NH_4Cl on the concentrations of metabolites in arterial blood*

The dose and method of administration of NH_4Cl was as described in Table 1. Blood was collected from the abdominal aorta 5–10 min afterwards. The control animals were injected with NaCl . Results are expressed as $\mu\text{mol/ml}$ of whole blood, $\pm\text{s.d.}$. There were at least five animals in each group. Values which are statistically different from the control rats by the Student's *t* test are indicated by *: $P < 0.05$.

Metabolites or metabolite ratios	Concentration ($\mu\text{mol/ml}$ of whole blood)	
	Control	NH_4Cl -injected
[Ammonia]	0.020	0.679 ± 0.166
[Alanine]	0.369 ± 0.039	0.376 ± 0.067
[Aspartate]	0.026 ± 0.012	0.024 ± 0.003
[Glutamate]	0.091 ± 0.006	0.089 ± 0.012
[Glutamine]	0.556 ± 0.044	$0.615 \pm 0.039^*$
[Pyruvate]	0.123 ± 0.040	0.101 ± 0.024
[2-Oxoglutarate]	0.042 ± 0.008	0.035 ± 0.004
[3-Hydroxybutyrate]	0.101 ± 0.035	0.090 ± 0.019
[Acetoacetate]	0.027 ± 0.018	$0.050 \pm 0.016^*$
[3-Hydroxybutyrate] [Acetoacetate]	3.7	1.8

1953). The mass-action ratio of aspartate aminotransferase was initially close to the equilibrium constant (6.61) but deviated from equilibrium immediately after the ammonia injection. It returned to the normal range within 10 min. Total adenine nucleotides and the $[\text{ATP}]/[\text{ADP}]$ ratio did not alter throughout the experiment (results not shown).

Measurements of metabolite concentrations in blood taken from the abdominal aorta under ether anaesthesia at a time after the ammonia injection when the hepatic changes were maximal (5–10 min) show a more than 30-fold increase in $[\text{NH}_3]$ (Table 2). The blood values for [alanine], [aspartate] and [glutamate] did not change; thus no significant quantities of these amino acids were released from the liver after ammonia injection, but there was a slight increase in the concentration of [glutamine]. Although the sum of [3-hydroxybutyrate] and [acetoacetate] was almost constant the value for [3-hydroxybutyrate] fell slightly and that of [acetoacetate] increased significantly. Hence there was a decrease in the ratio $[\text{3-hydroxybutyrate}]/[\text{acetoacetate}]$, reflecting the corresponding changes in the liver.

Effect of ammonia load in starved rats

The behaviour of [glutamate], [aspartate], [lactate], [pyruvate], [3-hydroxybutyrate] and [acetoacetate] after ammonia loading was similar to that in fed rats. There were, however, the following differences (Table 3). The initial value for [alanine] was lower in the livers of starved rats and the increase after ammonia loading was also less. The mass-action ratio of alanine aminotransferase was less than that observed in fed rats. The value for [malate] rose 2.5-fold to a peak at 10 min (in contrast with the

fall in fed rats) and then gradually returned to the initial value. There was a decrease of [2-oxoglutarate] immediately after the injection of ammonia (as in fed rats) but at 15 min and 25 min the values were higher (2- to 3-fold) than the initial one.

Effect of arginine on the formation of amino acids after ammonia loading

The rate of urea synthesis from NH_3 can be increased *in vivo* by L-arginine (Greenstein *et al.*, 1956). Preinjection of 3 mmol of L-arginine/kg body wt. 60 min before the injection of NH_4Cl modified the latter's effects on the concentration of amino acids (Table 4). Qualitatively the changes in [alanine], [aspartate] and [glutamate] were similar to those in normal rats but they were much smaller and of shorter duration. Initial values were re-established within 10 min instead of 15–25 min.

Effect of L-cycloserine

L-Cycloserine is an inhibitor of alanine aminotransferase both *in vitro* (Braunstein *et al.*, 1961) and *in vivo* (Otto, 1965; Williamson *et al.*, 1967b). The injection of cycloserine 60 min before ammonia loading modified the changes in [malate] as well as the mass-action ratio of alanine aminotransferase (Table 5). The values for [malate] decreased as rapidly as in normal rats, but this was followed by an overshoot to twice the initial value. Cycloserine raised the initial value of [alanine] 2.5-fold, but on ammonia loading [alanine] did not increase and the mass-action ratio of alanine aminotransferase fluctuated erratically. Alanine aminotransferase is the only reaction by which alanine can be formed

Table 3. Effects of injection of NH_4Cl on concentrations of metabolites in livers of starved rats

Rats (starved for 48 h) were injected with NH_4Cl (2.5 mmol/kg body wt. as a 0.25 M solution). Results are expressed as $\mu\text{mol/g}$ fresh wt. $\pm$ S.D. (four rats in each group). Values which are statistically different from the 0 min values by the Student's *t* test are indicated by *; $P < 0.05$.

Metabolite or metabolite ratio	Concentration ($\mu\text{mol/g}$ fresh wt.)						
	Time (min) ... 0	2	5	10	15	25	
[Lactate]	0.53 $\pm$ 0.42	1.05 $\pm$ 0.35	0.86 $\pm$ 0.70	0.74 $\pm$ 0.03	0.60 $\pm$ 0.20	0.50 $\pm$ 0.15	
[Pyruvate]	0.035 $\pm$ 0.024	0.054 $\pm$ 0.020	0.044 $\pm$ 0.024	0.062 $\pm$ 0.030	0.043 $\pm$ 0.014	0.038 $\pm$ 0.020	
[Malate]	0.28 $\pm$ 0.13	0.35 $\pm$ 0.16	0.40 $\pm$ 0.23	0.76 $\pm$ 0.28*	0.61 $\pm$ 0.16*	0.47 $\pm$ 0.16	
[Glutamate]	2.67 $\pm$ 0.46	1.71 $\pm$ 0.40*	2.50 $\pm$ 0.16	4.34 $\pm$ 1.33*	3.15 $\pm$ 1.49	2.43 $\pm$ 1.43	
[2-Oxoglutarate]	0.053 $\pm$ 0.017	0.033 $\pm$ 0.010*	0.042 $\pm$ 0.035	0.050 $\pm$ 0.010	0.093 $\pm$ 0.073	0.134 $\pm$ 0.086	
[3-Hydroxybutyrate]	1.65 $\pm$ 0.22	0.97 $\pm$ 0.35*	1.34 $\pm$ 0.51	1.04 $\pm$ 0.48	1.68 $\pm$ 0.84	1.34 $\pm$ 0.18	
[Acetoacetate]	0.35 $\pm$ 0.26	0.54 $\pm$ 0.22	0.62 $\pm$ 0.51	0.34 $\pm$ 0.13	0.35 $\pm$ 0.10	0.31 $\pm$ 0.11	
[Alanine]	0.34 $\pm$ 0.21	1.37 $\pm$ 1.03*	1.07 $\pm$ 0.81*	1.62 $\pm$ 0.93*	0.35 $\pm$ 0.23	0.20 $\pm$ 0.04	
[Aspartate]	0.63 $\pm$ 0.36	2.10 $\pm$ 0.85*	2.42 $\pm$ 1.08*	3.02 $\pm$ 2.04*	0.74 $\pm$ 0.50	0.46 $\pm$ 0.28	
[Glutamine]	3.73 $\pm$ 1.15	3.79 $\pm$ 2.36	3.24 $\pm$ 2.16	1.93 $\pm$ 0.77*	1.96 $\pm$ 0.80*	2.23 $\pm$ 1.22	
[Alanine] [2-Oxoglutarate] [Glutamate] [Pyruvate]	0.19	0.49	0.40	0.30	0.24	0.29	

(apart from proteolysis), thus the failure of [alanine] to rise after ammonia loading indicates that this reaction is involved in the accumulation of alanine recorded in Table 1. The net increase in [aspartate] within the first 2 min was twice that observed in normal rats, however, the zero-time values were lower than those for normal rats. Thus on inhibition of alanine aminotransferase accumulation of aspartate appears to be quantitatively more important in the removal of NH_3 .

Effect of dihydroxyacetone

The decreased formation of alanine on ammonia loading in starved rats and the lower mass-action ratio for the alanine aminotransferase system (Table 3) suggested a connexion with the low [pyruvate] in starved livers. The results of Hopper & Segal (1962) indicate that at a glutamate concentration of 2.05 mM (as found in livers of fed rats) the K_m for pyruvate of alanine aminotransferase is about 0.09 mM, i.e. close to the [pyruvate] value *in vivo*. At the glutamate concentration found in starved liver the K_m for pyruvate is almost three times the [pyruvate] *in vivo*. Thus the activity of the enzyme *in vivo* may be decreased in starvation because of the low [pyruvate]. This suggestion can be tested by the administration of dihydroxyacetone, which increases hepatic [pyruvate] (Williamson *et al.*, 1969; Burch *et al.*, 1970). Fasted rats were injected with dihydroxyacetone and 10 min later were given an ammonia load (Table 6). The values for [2-oxoglutarate], [lactate], [pyruvate] and in particular [alanine] rose substantially, and the value for the mass-action ratio of alanine aminotransferase was nearer to the equilibrium constant. The rise in [aspartate] was smaller than that found without dihydroxyacetone treatment, whereas the increase in [alanine] was much greater. These results support the assumption that the small increase in [alanine] after an ammonia load in starved rats is connected with the low hepatic [pyruvate].

2-Oxoglutarate 'overshoot'

In all ammonia-loading experiments reported so far there was an 'overshoot' of [2-oxoglutarate], i.e. after an early decrease values obtained at 25 min were greater than the initial values, though because of the spread of the results the finding is not statistically significant. This rather erratic effect was especially striking in some experiments on liver of starved rats (Table 3) and was therefore further investigated in these animals by including determinations of $[\text{NH}_3]$, [citrate] and [isocitrate] and by extending the time of observation to 50 min after injection (Table 7). The values for the concentrations of all intermediates, including 2-oxoglutarate, returned to the initial range within 50 min. $[\text{NH}_3]$ increased 5-fold after 5 min

Table 4. *Effect of pre-treatment with L-arginine on the changes in the concentrations of amino acids in rat liver after an NH₄Cl load*

L-Arginine (3 mmol/kg body wt. as a 0.3 M solution) was injected intraperitoneally into fed rats. After 60 min the rats were given NH₄Cl (2.5 mmol/kg body wt. as a 0.25 M solution). The other experimental details were as for Table 1. Results are expressed as $\mu\text{mol/g}$ fresh wt. $\pm$ s.d. (four rats in each group). Values which are statistically different from the 0 min values by the Student's *t* test are indicated by*: $P < 0.05$.

Metabolites	Time (min)	...	Concentration ($\mu\text{mol/g}$ fresh wt.)					
			0	2	5	10	15	25
[Glutamate]			2.42 $\pm$ 1.20	1.48 $\pm$ 0.37	2.18 $\pm$ 0.79	2.23 $\pm$ 0.52	1.78 $\pm$ 0.57	1.38 $\pm$ 0.16
[Alanine]			0.73 $\pm$ 0.27	1.79 $\pm$ 0.69*	1.57 $\pm$ 0.46*	1.00 $\pm$ 0.18	0.72 $\pm$ 0.22	0.59 $\pm$ 0.33
[Aspartate]			1.12 $\pm$ 0.25	1.71 $\pm$ 0.64	1.80 $\pm$ 0.45*	1.11 $\pm$ 0.27	0.87 $\pm$ 0.18	0.68 $\pm$ 0.17*

Table 5. *Effects on hepatic metabolites of an NH₄Cl load to L-cycloserine-pretreated rats*

L-Cycloserine (25 mg/kg body wt.) as a 0.1 M solution was injected intraperitoneally into fed rats 60 min before the NH₄Cl load (2.5 mmol/kg body wt.). Other details are as in Table 1. Results are expressed as $\mu\text{mol/g}$ fresh wt. $\pm$ s.d. (four rats in each group).

Metabolites or metabolite ratios	Time (min) ...	Concentration ($\mu\text{mol/g}$ fresh wt.)					
		0	2	5	10	15	25
[Lactate]		0.63 $\pm$ 0.39	1.36 $\pm$ 0.54	0.75 $\pm$ 0.33	0.61 $\pm$ 0.22	0.42 $\pm$ 0.11	0.55 $\pm$ 0.21
[Pyruvate]		0.104 $\pm$ 0.088	0.125 $\pm$ 0.057	0.099 $\pm$ 0.063	0.082 $\pm$ 0.064	0.054 $\pm$ 0.045	0.057 $\pm$ 0.026
[Malate]		0.19 $\pm$ 0.07	0.12 $\pm$ 0.06	0.13 $\pm$ 0.07	0.24 $\pm$ 0.05	0.38 $\pm$ 0.05	0.41 $\pm$ 0.10
[Glutamate]		1.77 $\pm$ 0.33	1.38 $\pm$ 0.33	2.15 $\pm$ 0.28	2.89 $\pm$ 0.60	2.97 $\pm$ 0.83	1.34 $\pm$ 0.64
[2-Oxoglutarate]		0.196 $\pm$ 0.085	0.041 $\pm$ 0.018	0.051 $\pm$ 0.035	0.059 $\pm$ 0.027	0.092 $\pm$ 0.016	0.313 $\pm$ 0.190
[Alanine]		2.56 $\pm$ 0.08	2.57 $\pm$ 0.36	2.39 $\pm$ 0.08	2.31 $\pm$ 0.17	2.94 $\pm$ 0.66	2.55 $\pm$ 0.77
[Aspartate]		0.57 $\pm$ 0.13	1.78 $\pm$ 0.28	2.21 $\pm$ 0.73	2.78 $\pm$ 0.80	2.19 $\pm$ 1.55	0.52 $\pm$ 0.24
[Alanine] [2-Oxoglutarate] [Glutamate] [Pyruvate]		2.72	0.61	0.57	0.57	1.69	10.5

Table 6. *Effects on hepatic metabolites of an NH₄Cl load to starved dihydroxyacetone-pretreated rats*

Rats (starved for 48 h) were injected intramuscularly with dihydroxyacetone (2 g/kg body wt.) and 10 min later were given an NH₄Cl load (2.5 mmol/kg body wt.). Other experimental details are as in Table 1. Results are expressed as $\mu\text{mol/g}$ fresh wt. and are the means of two experiments.

Metabolites or metabolite ratios	Time (min)	Concentration ($\mu\text{mol/g}$ fresh wt.)					
		± 0	2	5	10	15	25
[Lactate]		1.66	1.90	2.68	1.95	2.57	1.49
[Pyruvate]		0.14	0.13	0.17	0.15	0.28	0.13
[Malate]		0.34	0.34	0.32	0.41	0.48	0.55
[Glutamate]		2.63	1.77	2.35	3.70	3.14	2.13
[2-Oxoglutarate]		0.17	0.06	0.06	0.06	0.15	0.28
[Alanine]		1.88	3.01	5.36	7.56	4.94	0.98
[Aspartate]		0.61	1.21	1.36	2.58	1.11	0.95
[Alanine] [2-Oxoglutarate]		0.88	0.77	0.83	0.76	0.82	1.01
[Glutamate] [Pyruvate]							

Table 7. *Extended time-course of effects of an NH₄Cl load in livers of starved rats*

NH₄Cl (2.5 mmol/kg body wt.) was administered intraperitoneally to rats which had been starved for 48 h. Other details are as in Table 1. Results are expressed as $\mu\text{mol/g}$ fresh wt. $\pm$ s.d. (five rats in each group). Calculations of [free NAD⁺]/[free NADH] and [free NADP⁺]/[free NADPH] were as described by Veech *et al.* (1969).

Metabolites or metabolite ratios	Time (min)	Concentration ($\mu\text{mol/g}$ fresh wt.)			
		... 0	5	25	50
[Glutamate]		2.88 $\pm$ 0.25	3.02 $\pm$ 0.59	1.74 $\pm$ 0.79	2.47 $\pm$ 0.30
[2-Oxoglutarate]		0.073 $\pm$ 0.028	0.033 $\pm$ 0.017	0.325 $\pm$ 0.098	0.077 $\pm$ 0.037
[Ammonia]		0.88 $\pm$ 0.40	4.25 $\pm$ 0.93	0.86 $\pm$ 0.38	0.71 $\pm$ 0.36
[3-Hydroxybutyrate]		1.62 $\pm$ 0.41	1.15 $\pm$ 0.78	1.45 $\pm$ 0.28	1.34 $\pm$ 0.25
[Acetoacetate]		0.87 $\pm$ 0.15	1.23 $\pm$ 0.60	0.89 $\pm$ 0.14	0.79 $\pm$ 0.09
[Isocitrate]		0.0094 $\pm$ 0.0026	0.0054 $\pm$ 0.0016	0.035 $\pm$ 0.015	0.0098 $\pm$ 0.0015
[Citrate]		0.18 $\pm$ 0.05	0.15 $\pm$ 0.04	1.05 $\pm$ 0.43	0.27 $\pm$ 0.06
[Malate]		0.17 $\pm$ 0.03	0.25 $\pm$ 0.07	0.53 $\pm$ 0.14	0.18 $\pm$ 0.05
[Pyruvate]		0.023 $\pm$ 0.004	0.036 $\pm$ 0.020	0.028 $\pm$ 0.002	0.024 $\pm$ 0.007
[Lactate]		0.36 $\pm$ 0.14	0.51 $\pm$ 0.53	0.54 $\pm$ 0.15	0.33 $\pm$ 0.11
[Aspartate]		0.89 $\pm$ 0.21	2.83 $\pm$ 1.80	0.73 $\pm$ 0.09	0.84 $\pm$ 0.18
[Oxaloacetate] (calculated)		0.0027	0.0044	0.0069	0.0033
[Aspartate] [2-Oxoglutarate]		8.4	7.1	19.9	7.9
[Glutamate] [Oxaloacetate]					
[Free NAD ⁺] from glutamate		5.8	12.0	41.3	5.8
[Free NADH] dehydrogenase					
[Free NAD ⁺] from 3-hydroxybutyrate		10.9	21.7	12.5	12.0
[Free NADH] dehydrogenase					
[Free NADP ⁺] from 'malic' enzyme		0.0048	0.0048	0.0018	0.0045
[Free NADPH]					
[Free NADP ⁺] from isocitrate		0.0077	0.0061	0.0092	0.0078
[Free NADPH] dehydrogenase					

but returned to normal within 25 min. [Citrate] and [isocitrate], like [2-oxoglutarate], fell immediately on injection but rose to four to five times the initial value at 25 min and returned to the normal range within 50 min.

There was reasonable agreement between the two estimates of the mitochondrial [free NAD⁺]/[free NADH] ratio as calculated from either the glutamate dehydrogenase system or the 3-hydroxybutyrate dehydrogenase system, except at 25 min when the values obtained from the glutamate dehydrogenase system were higher. There was also reasonable agreement between the two estimates of the cytoplasmic [free NADP⁺]/[free NADPH] except at 25 min, when the ratio as calculated from isocitrate dehydrogenase was higher than that calculated from the 'malic' enzyme system. The mass-action ratio for aspartate aminotransferase was near eight (equilibrium value 6.61) except at 25 min, when it was 19.9. Thus in the dehydrogenase and transaminase systems investigated there was near equilibrium except at 25 min. A metabolite common to the three systems is 2-oxoglutarate, the concentration of which was high owing to the 'overshoot' at 25 min.

Discussion

Mechanism of removal of NH₃ by formation of alanine and aspartate, and its physiological significance

The findings reported in the present paper extend the observations of Du Ruisseau *et al.* (1957) and Nordmann *et al.* (1972) that [aspartate] and [alanine] increase in liver after administration of a lethal or sublethal dose of ammonia. Use of a non-lethal dose of ammonia and measurement of the time-course of the metabolite changes in the present study have provided more information on the mechanisms involved.

The increase in [alanine] on ammonia loading can be accounted for by the occurrence of the following reactions:

An analogous increase in [aspartate] can be accounted for by reactions (1) and (2), plus:

Since the formation of alanine and aspartate can substantially exceed the amounts of lactate or malate initially present in the liver it is necessary to postulate

that lactate is supplied by glycolysis (either in the liver or in peripheral tissues). The above five reactions do not represent all the intermediary stages. Thus in reaction (1) NAD⁺ and NADH react within the mitochondrial matrix while reaction (2) takes place in the cytoplasm. Hence a transfer of reducing equivalents between mitochondria and cytoplasm is required.

Reactions (1), (2) and (3) are readily reversible and are fast enough to establish near-equilibrium in the liver. This means that the formation of alanine occurs automatically when [NH₄⁺] and/or [pyruvate] rises. This also holds for aspartate as long as the carbon skeleton is provided by preformed malate. If reaction (4) is involved ATP is required (see below).

Nordmann *et al.* (1972) have put forward the following alternative explanation for the rise in [aspartate] after an ammonia load:

This hypothesis is based on their observation that [glutamine] decreased 10 min after ammonia administration and that NH₃ activates the glutaminase of rat liver (Charles, 1968). The time-course of the changes in metabolite concentrations presented in Table 1 does not support this hypothesis, because [aspartate] rose without change in [glutamine]. In starved rats (Table 3) there was a decrease in [glutamine], but this occurred after the major increase in [aspartate].

A rise of hepatic [NH₄⁺] occurs when the rate of supply of NH₄⁺ is more rapid than the rate of NH₃ removal by the synthesis of urea. In the present experiments such a situation was created by the loading with ammonia. *In vivo* this may occur in partial ischaemia because of the ATP requirements for urea synthesis, or in situations in which there are specific hepatic deficiencies of urea-cycle enzymes. It also occurs in extrahepatic tissues where, because of the absence of urea synthesis, the rate of NH₃ removal is low. The reactions (1), (3) and (5) may thus be looked on as a metabolic buffer system that can keep [NH₄⁺] approximately constant in the face of relatively high rates of NH₃ production. As the reactions involving the formation of alanine are all readily reversible the free-energy changes accompanying NH₃ binding are minimal. For aspartate formation one pyrophosphate bond of ATP is required to synthesize the oxaloacetate from pyruvate for each molecule of NH₃ bound.

As for the quantitative importance of amino acid formation (alanine + aspartate) in NH₃ binding the

amount of NH_3 removed by the liver of normal fed rats at 5 min was on average $42.3 \mu\text{mol}/100 \text{ g body wt.}$ or 8.1% of the administered load. In rats pretreated with arginine only 2.9% of the ammonia load was recovered in the amino acids. In starved rats 3.3% of the administered ammonia was recovered in the form of aspartate plus alanine, and pretreatment of starved rats with dihydroxyacetone raised this to 5.6%. These calculations are based on the values for [amino acids] given in Tables 1, 3, 4 and 6 and the values for liver wt./100 g body wt. published by Williamson *et al.* (1968).

The fact that [alanine] is an indicator of the available pyruvate is of importance in metabolic regulation because alanine is an inhibitor of pyruvate kinase (Seubert *et al.*, 1968). Its physiological role in this respect has been somewhat in doubt because hepatic [alanine] decreases in gluconeogenic situations (Williamson *et al.*, 1967b) where the enzyme might be expected to be inhibited. Inhibition of pyruvate kinase by alanine may be of importance in the fed state after exercise, where there is a large influx of lactate into the liver and a consequent rise in [pyruvate]. In this situation it would be advantageous to inhibit pyruvate kinase and allow maximum rates of conversion of lactate into glucose. Indeed, as might be expected, hepatic [alanine] raises in fed rats after exercise (C. R. S. Houghton & D. H. Williamson, unpublished work).

Oxoglutarate 'overshoot'

A puzzling finding is that the concentration of oxoglutarate and associated metabolites (citrate and isocitrate) rose to values at 25 min after the ammonia load which exceeded the initial values. This 'overshoot' in [oxoglutarate] occurred at a time when other metabolite concentrations were returning or had returned to their initial values and its erratic nature is presumably due to variations in the time-course of the changes in individual rats. During the period of alanine and aspartate accumulation the direction of flux in the glutamate dehydrogenase system (reaction 1) is towards amination of oxoglutarate, whereas when the ammonia load has been removed the flux must presumably alter in the direction of glutamate deamination. Glutamate dehydrogenase is located within the mitochondrial matrix, but the glutamate-alanine aminotransferase occurs mainly in the cytosol and the glutamate-aspartate aminotransferase in both compartments. Thus it might be expected that transport of glutamate and oxoglutarate across the mitochondrial membrane would occur during the formation and subsequent removal of aspartate and alanine. As there appear to be restraints to the trans-mitochondrial transport of both oxoglutarate (De Haan & Tager, 1968) and

glutamate (Bradford & McGivan, 1973) a possible explanation of the 'overshoot' of [oxoglutarate] lies in its temporary sequestration in a particular compartment owing to some limitation of transport. From the work of Palmieri *et al.* (1972) on the kinetics of the oxoglutarate carrier it can be calculated that the rate of oxoglutarate uptake by rat liver mitochondria is of the order of $4 \mu\text{mol}/\text{min per g of liver}$, and this is somewhat in excess of the maximum required rates of oxoglutarate transport (about $1 \mu\text{mol}/\text{min/g of liver}$) in these experiments. However, this rate of transport *in vitro* was measured under optimum conditions and may have little relation to the rate *in vivo*, therefore it is possible that transport of oxoglutarate is a limiting factor in the present studies.

This work was supported by grants from the Medical Research Council and the U.S. Public Health Service (Grant No. AM 11748). J.T.B. was an Agricultural Institute of Eire Scholar and D.H.W. is a member of the External Staff of the Medical Research Council.

References

- Bradford, N. M. & McGivan, J. D. (1973) *Biochem. J.* **134**, 1023–1029
- Braunstein, A. E., Azarkh, T. & Tin-Sen, S. (1961) *Biokhimiya* **16**, 760–772
- Brosnan, J. T., Krebs, H. A. & Williamson, D. H. (1967) *Biochem. J.* **104**, 43P–44P
- Brosnan, J. T., Krebs, H. A. & Williamson, D. H. (1970) *Biochem. J.* **117**, 91–96
- Burch, H. B., Lowry, O. H., Meinhardt, L., Max, P. & Chyu, K. (1970) *J. Biol. Chem.* **245**, 2092–2102
- Charles, R. (1968) Ph.D. Thesis, University of Amsterdam
- Clifford, A. J., Prior, R. L. & Vissek, W. J. (1969) *Amer. J. Physiol.* **217**, 1269–1272
- Conway, E. J. (1962) *Microdiffusion Analysis and Volumetric Error*, Crosby Lockwood, London
- De Haan, E. J. & Tager, J. M. (1968) *Biochim. Biophys. Acta* **153**, 98–112
- Du Ruisseau, J. P., Greenstein, J. P., Winitz, M. & Birnbaum, S. M. (1957) *Arch. Biochem. Biophys.* **68**, 161–171
- Greenstein, J. P., Winitz, M., Gullino, P., Birnbaum, S. M. & Otey, M. C. (1956) *Arch. Biochem. Biophys.* **64**, 342–354
- Gruber, W. & Möllering, H. (1966) *Biochem. Z.* **346**, 85–88
- Hohorst, H. J., Kreutz, F. H. & Bücher, T. (1959) *Biochem. Z.* **332**, 18–46
- Hopper, S. & Segal, H. L. (1962) *J. Biol. Chem.* **237**, 3189–3195
- Kirsten, E., Gerez, C. & Kirsten, R. (1963) *Biochem. Z.* **337**, 312–319
- Krebs, H. A. (1953) *Biochem. J.* **54**, 82–86
- Krebs, H. A. & Veech, R. L. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), pp. 413–438, Springer-Verlag, Berlin, Heidelberg and New York

- Lund, P. (1970) in *Methoden der enzymatischen Analyse* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1670–1673, Verlag Chemie, Weinheim
- Nordmann, R., Petit, M. A. & Nordmann, J. (1972) *Biochimie* **54**, 1473–1478
- Otto, K. (1965) *Hoppe-Seyler's Z. Physiol. Chem.* **341**, 99–104
- Palmieri, F., Quagliariello, E. & Klingenberg, M. (1972) *Eur. J. Biochem.* **29**, 408–416
- Seubert, W., Henning, H. V., Schoner, W. & L'Age, M. (1968) *Advan. Enzyme Regul.* **6**, 153–180
- Veech, R. L., Eggleston, L. V. & Krebs, H. A. (1969) *Biochem. J.* **115**, 609–619.
- Williamson, D. H., Lund, P. & Krebs, H. A. (1967a) *Biochem. J.* **103**, 514–526
- Williamson, D. H., Lopes-Vieira, O. & Walker, B. (1967b) *Biochem. J.* **104**, 497–502
- Williamson, D. H., Bates, M. W. & Krebs, H. A. (1968) *Biochem. J.* **108**, 353–361
- Williamson, D. H., Veloso, D., Ellington, E. V. & Krebs, H. A. (1969) *Biochem. J.* **114**, 575–584