Effects of Ischaemia on Metabolite Concentrations in Rat Liver

By J. T. BROSNAN,* H. A. KREBS AND D. H. WILLIAMSON

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

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1. Changes in the concentrations of ammonia, glutamine, glutamate, 2-oxoglutarate, 3-hydroxybutyrate, acetoacetate, alanine, aspartate, malate, lactate, pyruvate, NAD^+ , NADH and adenine nucleotides were measured in freeze-clamped rat liver during ischaemia. 2. Although the concentrations of most of the metabolites changed rapidly during ischaemia the ratios $[\text{glutamate}]/[2\text{-oxoglutarate}]$, $[\text{NH}_4^+]$ and $[3\text{-hydroxybutyrate}]/[\text{acetoacetate}]$ changed equally and the value of the expression $[3\text{-hydroxybutyrate}][2\text{-oxoglutarate}][\text{NH}_4^+]/[\text{acetoacetate}][\text{glutamate}]$ remained approximately constant, indicating that the 3-hydroxybutyrate dehydrogenase and glutamate dehydrogenase systems were at near-equilibrium with the mitochondrial NAD^+ couple. 3. The value of the expression $[\text{alanine}][\text{oxoglutarate}]/[\text{pyruvate}][\text{glutamate}]$ was about 0.7 *in vivo* and remained fairly constant during the ischaemic period of 5 min, although the concentrations of alanine and oxoglutarate changed substantially. No explanation can be offered why the value of the ratio differed from that of the equilibrium constant of the alanine aminotransferase reaction, which is 1.48. 4. Injection of L-cycloserine 60 min before the rats were killed increased the concentration of alanine in the liver fourfold and decreased the concentration of the other metabolites measured, except that of pyruvate. During ischaemia the concentration of alanine did not change but that of aspartate almost doubled. 5. After treatment with L-cycloserine the value *in vivo* of the expression $[\text{alanine}][\text{oxoglutarate}]/[\text{pyruvate}][\text{glutamate}]$ rose from 0.7 to 2.4. During ischaemia the value returned to 0.8. 6. The effects of L-cycloserine are consistent with the assumption that it specifically inhibits alanine aminotransferase. 7. Most of the alanine formed during ischaemia is probably derived from pyruvate and from ammonia released by the deamination of adenine nucleotides and glutamine. The alanine is presumably formed by the combined action of glutamate dehydrogenase and alanine aminotransferase. 8. The rate of anaerobic glycolysis, calculated from the increase in the lactate concentration, was $1.3 \mu\text{mol}/\text{min}$ per g fresh wt. 9. Although the concentrations of the adenine nucleotides changed rapidly during ischaemia, the ratio $[\text{ATP}][\text{AMP}]/[\text{ADP}]^2$ remained constant at 0.54, indicating that adenylate kinase established near-equilibrium under these conditions.

In the normal rat liver the reactants of the 3-hydroxybutyrate dehydrogenase and glutamate dehydrogenase systems are at near-equilibrium with the mitochondrial NAD^+ -NADH couple. This follows from the approximate constancy of the term:

$$\frac{[3\text{-hydroxybutyrate}][\text{oxoglutarate}][\text{NH}_4^+]}{[\text{acetoacetate}][\text{glutamate}]} \quad (1)$$

an expression derived from the combination of the equilibrium equations of the two dehydrogenase

systems. This expression therefore equals the ratio of the equilibrium constants. The values for these are known, and at 38°C and I 0.25 the ratio, as defined above, is 7.9×10^{-2} mm (Williamson, Lund & Krebs, 1967a).

Under ischaemic conditions the concentrations of several components of expression (1) are liable to change rapidly. Ammonia concentration has been reported to rise (Brown, Duda, Korkes & Handler, 1957; Thorn & Heimann, 1958), and acetoacetate undergoes reduction to 3-hydroxybutyrate (Berry, Williamson & Wilson, 1965). In addition pH is liable to decrease owing to the formation of lactate

* Present address: Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, Toronto 5, Ont., Canada.

and the hydrolysis of ATP, which cause a disappearance of bicarbonate and a rise in the CO_2 pressure. The object of the present work was to test whether equilibrium between the mitochondrial dehydrogenase systems is maintained in ischaemia. The reactants of related systems catalysed by alanine aminotransferase and adenylate kinase were also measured during ischaemia. Some aspects of this work have been reported in a preliminary form (Brosnan, Krebs & Williamson, 1967).

MATERIALS AND METHODS

Reagents. 3-Hydroxybutyrate dehydrogenase was prepared as described by Williamson *et al.* (1967a) and L-alanine dehydrogenase as described by Williamson, Lopes-Vieira & Walker (1967b). All other enzymes and coenzymes used for the determination of metabolites were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Florisil (60–100 mesh) was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and L-cycloserine from Calbiochem, Los Angeles, Calif., U.S.A.

Treatment of liver. Male rats of the Wistar strain weighing 180–200 g and fed *ad libitum* were used. The rats were killed by dislocation of the neck. A portion of the liver was rapidly removed and freeze-clamped (Wollenberger, Ristau & Schoffa, 1960). The average time between dislocation of the neck and deep-freezing the tissue was 8 s. Cervical dislocation does not stop the heart beat and the interval between cessation of circulation and freezing was 1–2 s. Immediately after the freezing of this portion of the liver the hepatic blood vessels were severed and the rest of the liver was allowed to remain *in situ*. Further portions of the same liver were frozen 2 and 5 min later. The frozen liver was pulverized in a mortar, extracted with HClO_4 and neutralized with KOH, as described by Williamson *et al.* (1967a). A separate sample of frozen liver powder

was extracted with hot ethanolic KOH by the method of Klingenberg (1963b) for the determination of total NADH.

Determination of metabolites. Lactate, pyruvate, 3-hydroxybutyrate, acetoacetate, 2-oxoglutarate, glutamate, alanine, aspartate and glutamine were determined as previously described (Williamson *et al.* 1967a,b). Ammonia was determined by the method of Kirsten, Gerez & Kirsten (1963), malate by the method of Hohorst (1963), NAD^+ and NADH by the methods of Klingenberg (1963a,b), ATP by the method of Lamprecht & Trautschold (1963) and ADP and AMP by the method of Adam (1963). The liver extracts were shaken with Florisil, as described by Williamson *et al.* (1967a), to eliminate the slow non-enzymic oxidation of NADH observed with untreated samples. As Florisil interfered with the recovery of the nicotinamide–adenine dinucleotides and adenine nucleotides and of ammonia it was not employed for the extracts used for the determination of these substances. The blank oxidation of NADH was particularly troublesome in the ammonia determination. To avoid artifacts simulating high ammonia values two identical cuvettes were set up for each liver extract but glutamate dehydrogenase was added to only one; the amount of NADH oxidation in the other cuvette was used to correct the blank.

RESULTS

Effect of ischaemia on the concentration of metabolites in rat liver. The concentration of metabolites in rat liver and their changes during a 5 min period of ischaemia are shown in Table 1. There were no appreciable changes in the tissue concentrations of ammonia, glutamate or aspartate up to 5 min after the hepatic blood supply had been cut. The concentration of 2-oxoglutarate fell considerably so that the $[\text{glutamate}]/[2\text{-oxoglutarate}][\text{NH}_4^+]$ ratio increased. The concentration of total ketone bodies increased by about 40% and the [3-hydroxybuty-

Table 1. Concentrations of metabolites in rat liver during ischaemia

Fed rats (male; 180–200 g) were killed by cervical dislocation and a portion of liver of each was freeze-clamped immediately (0 min). The blood vessels were then severed and each liver was allowed to remain *in situ* for the times indicated and then freeze-clamped. The results are mean values ($\pm$ s.d.) of five rats in each group, except for the nicotinamide–adenine dinucleotides (two rats).

Metabolite	Concn. of metabolite ($\mu\text{mol/g}$ fresh wt.)		
	0 min	2 min	5 min
Ammonia	0.71 $\pm$ 0.27	0.77 $\pm$ 0.20	0.69 $\pm$ 0.26
Glutamate	2.35 $\pm$ 0.73	2.84 $\pm$ 0.57	2.65 $\pm$ 0.61
Oxoglutarate	0.16 $\pm$ 0.016	0.068 $\pm$ 0.002	0.041 $\pm$ 0.001
3-Hydroxybutyrate	0.105 $\pm$ 0.043	0.187 $\pm$ 0.028	0.201 $\pm$ 0.010
Acetoacetate	0.053 $\pm$ 0.020	0.028 $\pm$ 0.010	0.026 $\pm$ 0.015
Alanine	0.87 $\pm$ 0.65	1.53 $\pm$ 0.93	2.26 $\pm$ 0.61
Aspartate	1.19 $\pm$ 0.24	1.01 $\pm$ 0.31	0.92 $\pm$ 0.22
Malate	0.42 $\pm$ 0.18	0.55 $\pm$ 0.14	0.58 $\pm$ 0.08
Lactate	0.97 $\pm$ 0.43	3.08 $\pm$ 1.73	5.13 $\pm$ 2.19
Pyruvate	0.080 $\pm$ 0.031	0.052 $\pm$ 0.022	0.055 $\pm$ 0.14
Total NAD^+	0.83	0.67	
Total NADH	0.16	0.19	

Table 2. Calculation of $[free\ NAD^+]/[free\ NADH]$ ratios and the alanine aminotransferase reactant ratio in rat liver *in vivo* and during ischaemia

The data used for the calculations are from Table 1. The method of calculation of the $[free\ NAD^+]/[free\ NADH]$ ratio was described by Williamson *et al.* (1967a).

Duration of ischaemia (min)	Calculated $[free\ NAD^+]/[free\ NADH]$ ratio			$\frac{[Alanine][oxoglutarate]}{[pyruvate][glutamate]}$
	From lactate dehydrogenase	From glutamate dehydrogenase	From hydroxybutyrate dehydrogenase	
0	738	12.9	10.2	0.77
2	152	4.7	3.0	0.70
5	96	2.8	2.6	0.63

rate]/[acetoacetate] ratio also rose. There was a large rise in the concentration of lactate, owing to anaerobic glycolysis, and, as reported by Hohorst, Kreutz & Reim (1961), a great increase in the [lactate]/[pyruvate] ratio. The liver content of alanine doubled after 2 min of ischaemia and almost trebled after 5 min. As expected there was an increase in the concentration of total NADH and a fall in that of total NAD^+ during ischaemia (Table 1).

Effect of ischaemia on the $[free\ NAD^+]/[free\ NADH]$ ratio in mitochondria and cytoplasm. The ratios $[3\text{-hydroxybutyrate}]/[acetoacetate]$ and $[glutamate]/[2\text{-oxoglutarate}][NH_4^+]$ may be used to calculate the mitochondrial $[free\ NAD^+]/[free\ NADH]$ ratio (Williamson *et al.* 1967a). The agreement between the two calculations, previously found *in vivo*, also applies to the ischaemic conditions, when the ratios fell (Table 2).

One of the factors that is liable to affect the values of the $[free\ NAD^+]/[free\ NADH]$ ratio during ischaemia is the acidification of the tissue by the formation of lactate from glucose and the hydrolysis of ATP. However, $[H^+]$ affects both dehydrogenase systems equally and the calculated ratios should therefore be the same irrespective of the intracellular pH, provided that the two systems are in equilibrium. Expression (1) is therefore independent of pH and at equilibrium has a theoretical value of $7.9 \times 10^{-2} \text{ mM}$ (38°C and $I\ 0.25$). The calculated values for this expression from the data in Table 1 are $9.9 \times 10^{-2} \text{ mM}$, $12.3 \times 10^{-2} \text{ mM}$ and $8.3 \times 10^{-2} \text{ mM}$ after 0 min, 2 min and 5 min of ischaemia respectively. These values indicate that the two dehydrogenase systems are at near-equilibrium *in vivo* and during post-mortem ischaemia.

The cytoplasmic $[free\ NAD^+]/[free\ NADH]$ ratio, as calculated from the [lactate]/[pyruvate] ratios (Table 2), moved in the same direction as the mitochondrial ratio, but the change was greater. The ratio fell to 1:8 after 5 min in the cytoplasm and only to 1:4 in the mitochondria. The greater

change in the cytoplasm may be connected with the circumstance that in ischaemia most of the H^+ is generated in the cytoplasm and that the transfer of the acidity to the mitochondria may be a relatively slow process. Another reason for the smaller change in the mitochondria may be a greater 'buffering' capacity against changes in the redox state, a consequence of the lower value of the $[free\ NAD^+]/[free\ NADH]$ ratio, being about 10 in the mitochondria and 1000 in the cytoplasm (see Table 2).

Effect of ischaemia on the reactants of the alanine aminotransferase system. The value of the expression:

$$\frac{[alanine][oxoglutarate]}{[pyruvate][glutamate]} \quad (2)$$

calculated from the data in Table 1 remained fairly constant at about 0.7 throughout the ischaemic period (Table 2), although [alanine] and [oxoglutarate] changed substantially. The equilibrium constant of this system at 25°C was previously found to be 1.51 (Krebs, 1953) and new measurements at 37°C and $I\ 0.25$ in 0.1 M-potassium phosphate buffer, pH 7.0, gave a similar value (1.48). No satisfactory explanation can be offered for the small deviation of the concentration ratio in rat liver from the equilibrium constant (see also Williamson *et al.* 1967b) and for the constancy of this deviation during the period of ischaemia.

Effects of L-cycloserine on the reactants of the alanine aminotransferase system. The alanine formed during ischaemia (Table 1) could arise either via the action of alanine aminotransferase or via proteolysis. To differentiate between these possibilities L-cycloserine, a powerful inhibitor of alanine aminotransferase (Braunstein, Azarkh & Tin-Sen, 1961-62; Otto, 1965) was injected into rats 60 min before they were killed and the livers freeze-clamped. This treatment increased the initial concentration of alanine fourfold (Table 3), but decreased the concentrations of all other metabolites measured except pyruvate, the fall being between 15 and 40%. This

Table 3. *Concentrations of metabolites and reactant ratio of alanine aminotransferase in livers from L-cycloserine-treated rats during ischaemia*

Fed rats (male; 180–200g) were injected intraperitoneally with L-cycloserine (25mg/kg body wt.). After 60min they were killed by cervical dislocation and a portion of liver of each was freeze-clamped immediately (0min). The other experimental details are as for Table 1. The results are mean values ($\pm$ S.D.) of 5 rats in each group.

Metabolite	Duration of ischaemia ...	Concn. of metabolite (μ mol/g fresh wt.)		
		0min	2min	5min
Ammonia		0.63 $\pm$ 0.21	0.75 $\pm$ 0.23	0.77 $\pm$ 0.39
Glutamate		1.63 $\pm$ 0.42	1.91 $\pm$ 0.53	1.85 $\pm$ 0.40
Oxoglutarate		0.114 $\pm$ 0.061	0.044 $\pm$ 0.014	0.034 $\pm$ 0.017
Alanine		3.40 $\pm$ 0.98	3.46 $\pm$ 0.70	3.44 $\pm$ 0.92
Aspartate		0.74 $\pm$ 0.22	1.09 $\pm$ 0.24	1.30 $\pm$ 0.35
Malate		0.29 $\pm$ 0.15	0.27 $\pm$ 0.14	0.26 $\pm$ 0.12
Lactate		0.65 $\pm$ 0.50	3.11 $\pm$ 2.31	5.87 $\pm$ 2.58
Pyruvate		0.098 $\pm$ 0.083	0.071 $\pm$ 0.05	0.080 $\pm$ 0.03
[alanine]/[oxoglutarate]		2.43	1.12	0.79
[pyruvate]/[glutamate]				

Table 4. *Changes in concentrations of possible ammonia precursors in rat liver during ischaemia*

Experimental details are as for Table 1. The results are mean values ($\pm$ S.D.) of five rats in each group.

Duration of ischaemia ...	Concn. of possible NH ₃ precursor (μ mol/g fresh wt.)		
	0min	2min	5min
Glutamine	4.68 $\pm$ 0.29	4.57 $\pm$ 0.50	4.26 $\pm$ 0.51
ATP	2.45 $\pm$ 0.36	1.44 $\pm$ 0.57	0.86 $\pm$ 0.30
ADP	1.17 $\pm$ 0.23	1.42 $\pm$ 0.16	1.39 $\pm$ 0.11
AMP	0.30 $\pm$ 0.11	0.78 $\pm$ 0.31	1.18 $\pm$ 0.50
Δ Glutamine	—	–0.11	–0.42
Δ ANP*	—	–0.28	–0.49
[ATP]/[AMP]			
[ADP] ²	0.54	0.55	0.54

* ANP, sum of adenine nucleotides.

rise in the alanine concentration *in vivo* in the L-cycloserine-treated animal can be explained by the inhibition of the removal of alanine entering the liver from the circulation and of that formed within the liver by protein degradation. During the ischaemic period the concentration of alanine did not change but that of aspartate almost doubled (Table 3). The absolute increase in the aspartate concentration (0.56 μ mol/g) was considerably smaller than the rise in alanine concentration (1.39 μ mol/g) in normal rats. There was no appreciable increase in the concentrations of NH₄⁺ or glutamate.

A striking effect of L-cycloserine *in vivo* is the change in the value of expression (2) from 0.7 to 2.4 before ischaemia. During ischaemia the value returned to 0.8. The changes in the concentrations of metabolites *in vivo* and during ischaemia in the livers from L-cycloserine-treated rats (Table 3) are all consistent with an inhibition of alanine aminotransferase.

Origin of the increase of nitrogenous substances during ischaemia. As shown in Table 4, the concentrations of the adenine nucleotides and of glutamine fell during ischaemia and ammonia derived from these compounds could account for a major part (0.91 μ mol/g in the normal liver at 5min) of the increase of the sum of [alanine] + [aspartate] + [glutamate] + [NH₃] (1.40 μ mol/g at 5min). This refers to the nitrogen balance, and increase in carbon must have been in the main derived from pyruvate generated by glycolysis. The ratio [ATP]/[AMP]/[ADP]² remained remarkably constant during ischaemia at 0.54 in spite of major changes in the amounts of the reactants (Table 4).

DISCUSSION

Changes in amino acid content during ischaemia. The content of alanine, in contrast with that of glutamate and aspartate, showed a striking change

during ischaemia, rising to two to three times the normal value within 5 min (see also Williamson *et al.* 1967b). The findings are consistent with the assumption that alanine formation was brought about by the reactions:

The sum of these reactions is an 'alanine dehydrogenase' with an equilibrium constant favouring alanine formation:

$$K = \frac{[\text{Pyruvate}][\text{NH}_4^+][\text{NADH}]}{[\text{alanine}][\text{NAD}^+]} = 2.61 \times 10^{-6} \text{ M } (38^\circ\text{C}; I \ 0.25; \text{pH } 7.0)$$

The physiological role of this system in the liver is probably to dispose of ammonia when the capacity of the urea cycle is exceeded or when the urea cycle is inhibited (i.e. in anoxia).

Changes in amino acid content after L-cycloserine injection. The accumulation of alanine in the liver *in vivo* after L-cycloserine treatment indicates that the disposal of alanine arising from protein degradation in the liver and from the uptake by the liver of alanine discharged into the circulation by other tissues is inhibited by L-cycloserine. On the other hand the non-accumulation of alanine during ischaemia after L-cycloserine treatment is accounted for by the inhibition of alanine formation from pyruvate and glutamate. It appears that during ischaemia (when the synthesis of urea diminishes) the formation of alanine represents an alternative way of disposing of ammonia. Thus during ischaemia the alanine aminotransferase would react in the direction of alanine formation whereas in the normal liver it reacts mainly in the direction of alanine disposal. In livers from L-cycloserine-treated rats aspartate accumulates instead of alanine during ischaemia. The oxaloacetate required could be formed from malate or citrate. The failure of the malate concentration to rise during ischaemia in the L-cycloserine-treated rats (Table 3) compared with the normal rats (Table 1) supports this assumption. During ischaemia the supply of oxaloacetate is more restricted than that of pyruvate arising from glycolysis, and this is presumably an important factor in deciding the relative amounts of alanine and aspartate formed in the normal rats.

Equilibrium of the adenylate kinase system. As shown in Table 4, the value of the ratio $[\text{ATP}][\text{AMP}]/[\text{ADP}]^2$ remained remarkably constant during ischaemia although the concentrations of ATP and AMP changed markedly. The value of 0.53 observed is within the range expected for physiological Mg^{2+} concentrations (Bowen & Kerwin, 1956; Blair, 1968; Rose, 1968). Adenylate kinase is located in

the cytoplasm (Adelman, Lo & Weinhouse, 1968) and on the outer mitochondrial membrane (Chappell & Crofts, 1965; Sottocasa, 1967); thus it can maintain near-equilibrium in these compartments. Owing to the existence of permeability barriers for the adenine nucleotides between the outer and inner mitochondrial spaces, any adenine nucleotides in the inner mitochondrial space would not be controlled by adenylate kinase. The constancy of the reactant ratio of the adenylate kinase system in ischaemia indicates that the amounts of adenine nucleotides in the inner mitochondrial space were small under the test conditions.

Rate of anaerobic glycolysis. The data in Table 1 can be used to calculate the minimum rate of anaerobic lactate formation by intact rat liver. During the first 2 min of anaerobiosis the lactate concentration increased by $2.10 \mu\text{mol/g}$. Since anaerobiosis does not start immediately after the severance of the blood vessels the anaerobic period was shorter than 2 min. Calculations based on the known oxygen consumption by the liver ($2.2 \mu\text{mol/min per g}$; Hems, Ross, Berry & Krebs, 1966) and the concentration of oxygen in the liver indicate that most of the hepatic oxygen is used up within the first 30 s of the severance of the circulation. On the assumption, then, that anaerobiosis started 30 s after zero time $2.0 \mu\text{mol}$ of lactate was formed/g of liver in 1.5 min and this gives a rate of anaerobic glycolysis of $1.3 \mu\text{mol/min per g}$, a value that agrees well with rates obtained with the perfused liver (F. H. Woods & H. A. Krebs, unpublished work). It is higher than those found in fresh liver slices ($0.82 \mu\text{mol/min per g}$) but the same as in slices pre-incubated aerobically ($1.46 \mu\text{mol/min per g}$) (Bernelli-Zazzera, Gaja & Ragnotti, 1966).

Equilibrium between 3-hydroxybutyrate dehydrogenase and glutamate dehydrogenase systems. The fact that the value of expression (1) remained constant during ischaemia in spite of major changes in the concentrations of the components implies, as pointed out by Williamson *et al.* (1967a) for the situation *in vivo*, that the two dehydrogenases are at equilibrium with the same mitochondrial NAD pool. It has been suggested (see Tager, Papa, De Haan, D'Aloia & Quagliariello, 1969; Papa, Tager, Francavilla & Quagliariello, 1969) that in its natural environment glutamate dehydrogenase reacts only with the NADP couple, and further that the redox state of the NADP couple, owing to the energy-linked transhydrogenase, can be much more reduced than is expected on the basis of equilibrium with the NAD couple (Klingenberg & Slenczka, 1959; Lee & Ernster, 1964). Although experiments on isolated mitochondria and mitochondrial fragments lend support to these possibilities, the present results argue against their occurrence *in vivo*. If the energy-linked transhydrogenase had been operating *in vivo*

and if glutamate dehydrogenase had been in equilibrium with this couple, then the $[\text{free NAD}^+]/[\text{free NADH}]$ ratio calculated from the glutamate dehydrogenase system should have been lower than the value calculated from the 3-hydroxybutyrate dehydrogenase system and the value of expression (1) should be lower than that calculated from the equilibrium constants. Lee & Ernster (1964) have shown with submitochondrial particles that, in the absence of an energy supply, the energy-linked transhydrogenase equilibrium proceeds rapidly towards the non-energy-linked position. The fact that the value of expression (1) did not alter during ischaemia, when the supply of high-energy intermediates presumably ceases, is further evidence against the mediation of the energy-linked transhydrogenase in the maintenance of the equilibrium between 3-hydroxybutyrate dehydrogenase and glutamate dehydrogenase.

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