Evidence for the Participation of Aspartate Aminotransferase in Hepatic Glucose Synthesis in the Suckling Newborn Rat

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Inhibition of liver aspartate aminotransferase by L-2-amino-4-methoxy-*trans*-3-butenic acid in the suckling newborn rat causes a decrease in all gluconeogenic precursors from phosphoenolpyruvate to glucose and an accumulation of lactate but not of pyruvate. This suggests that the aspartate shuttle is operative and confirms the quantitative importance of lactate as a gluconeogenic precursor at this time during development.

In the rat the location of pyruvate carboxylase (EC 4.1.1.1) and phosphoenolpyruvate carboxykinase (EC 4.1.1.32) in the mitochondria and cytosol respectively means that during glucose synthesis the oxaloacetate formed from pyruvate has to be transferred from one cell compartment to the other. However, the mitochondrial membrane is not readily permeable to oxaloacetate, and therefore various shuttles have been postulated to facilitate this transfer depending on the substrate used for gluconeogenesis. If the substrate is in a reduced state, such as lactate, oxaloacetate is transferred as aspartate to the cytosol via mitochondrial and cytosolic aspartate aminotransferase (EC 2.6.1.1), whereas if the substrate is in a more oxidized state, such as pyruvate or alanine, oxaloacetate is transferred as malate via mitochondrial and cytosolic malate dehydrogenase (EC 1.1.1.37) (Krebs *et al.*, 1967; Rognstad & Katz, 1970; Williamson *et al.*, 1970; Rognstad & Clark, 1974; Williamson, 1976). Evidence for the operation of these shuttles has been obtained in the adult rat *in vitro*, by using an inhibitor of the aspartate aminotransferase such as amino-oxyacetate or an inhibitor of malate transport across the mitochondrial membrane such as D-malate or butylmalonate. In the suckling newborn rat, there are no data on the operation of these shuttles in liver during gluconeogenesis, which is the normal situation in this animal, which receives a high-fat low-carbohydrate diet, namely the milk. In the present study we have used an inhibitor of aspartate aminotransferase, L-2-amino-4-methoxy-*trans*-3-butenic acid (Rando *et al.*, 1976), which may be more effective than amino-oxyacetate (Smith *et al.*, 1977), to test whether the aspartate shuttle operates *in vivo* in the suckling rat. No detailed study has, however, been made of the absolute specificity of this inhibitor. It has been suggested previously that lactate could be an important gluconeogenic precursor in the suckling newborn

rat (Ferré *et al.*, 1977; Pégorier *et al.*, 1977), and a further aim of this study was to use the inhibitor to provide additional evidence on this point.

Experimental

Rat pups of the albino Wistar strain were born between 15:00 and 18 h on day 22:00 of gestation. They were left with the mother for 24 h in a room in which the external temperature was 22°C.

Each litter of 24 h-old suckling rats was divided into two groups after separation from the mother. One group was injected intraperitoneally with 50 µl of a solution of L-2-amino-4-methoxy-*trans*-3-butenic acid, pH 7.4, at a concentration of 60 mg/ml and the other was injected with the same volume of NaCl (0.9%, w/v). The pups were then immediately placed at 37°C and 70% relative humidity for 10, 30 or 90 min before blood or liver sampling. Blood and liver were sampled in different animals as the small size of the pups does not allow blood sampling without interfering with the metabolic state of the liver. Blood was sampled via an incision across the axillary vessels and deproteinized in 10% (w/v) HClO₄. The liver was removed rapidly from decapitated newborn rats and freeze-clamped between metal blocks previously cooled in liquid N₂ (Wollenberger *et al.*, 1960). The average time elapsing between decapitation and freezing of liver was 5 s. The frozen liver was weighed, pulverized in a mortar and neutralized as described by Williamson *et al.* (1967). The following metabolites were determined in the neutralized extracts: alanine (Williamson, 1974); aspartate (Pfleiderer, 1963); citrate (Dagley, 1974); glucose (Slein, 1963); glycerol (Eggstein & Kuhlman, 1974); glycerol 3-phosphate (Hohorst, 1963a); D-3-hydroxybutyrate and acetoacetate (Williamson *et al.*, 1962); L-lactate and pyruvate (Hohorst *et al.*, 1959); L-malate (Hohorst,

1963b); phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate (Czok & Eckert, 1963).

All enzymes and coenzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. L-2-Amino-4-methoxy-*trans*-3-butenic acid was a kind gift from Dr. J. Scannell, Hoffman-La Roche, Basle, Switzerland.

Results are expressed as mean $\pm$ S.E.M.; significant differences were determined by using the Wilcoxon rank order test (Wilcoxon, 1947).

Results and Discussion

Gluconeogenesis

The injection of L-2-amino-4-methoxy-*trans*-3-butenic acid into 1-day-old suckling rats resulted in a decreased concentration of all the hepatic metabolites of gluconeogenesis from phosphoenolpyruvate to glucose (Fig. 1), suggesting an inhibition of glucose synthesis at a step after the formation of oxaloacetate. Evidence for the effectiveness of the inhibition of gluconeogenesis is the marked hypoglycaemia at 90min after injection of the inhibitor (Table 1). A large accumulation of liver lactate occurred 10min after the injection and before any increase in blood lactate (Fig. 1 and Table 1). This indicates that decreased lactate utilization by the liver is the primary cause for its accumulation in liver rather than increased production by the peripheral tissues; at this time there was no increase in liver alanine or pyruvate. These results are comparable with those obtained *in vitro* in the adult rat with amino-oxoacetate in isolated liver cells (Rognstad & Clark, 1974; Smith *et al.*, 1977) or in the perfused liver (Anderson *et al.*, 1971), namely an inhibition of gluconeogenesis from lactate, but not from alanine or pyruvate, at a step involving the aspartate aminotransferase. It suggests that the aspartate shuttle is operative in the suckling newborn rat. The fact that pyruvate does not accumulate in the present study, whereas it does after the injection of 3-mercaptopycolinate which inhibits the gluconeogenic flux by 80% under similar experimental conditions (Ferré *et al.*, 1977), indicates that an additional system for the transport of reducing equivalents and carbon from the mitochondria to the cytosol exists in the newborn rat and this is presumably the malate shuttle.

Mechanism of lactate accumulation

The accumulation of lactate after injection of L-2-amino-4-methoxy-*trans*-3-butenic acid may be explained as follows. Lactate oxidation to pyruvate in the cytosol produces a reducing equivalent that cannot be utilized in the gluconeogenic pathway, as the aspartate shuttle is inhibited. The result is an excess of cytosolic NADH, as shown by

Fig. 1. Metabolite profile after injection of L-2-amino-4-methoxy-*trans*-3-butenic acid in suckling newborn rats. Suckling newborn rats (24h-old) were injected intraperitoneally with L-2-amino-4-methoxy-*trans*-3-butenic acid and the livers removed at 10min (○), 30min (■) or 90min (△) after the injection. As there was no significant difference between the control groups, 10, 30 or 90min after the injection of 0.9% NaCl, the values have been pooled in one group (●). Control values (μ mol/g wet wt.): alanine, 0.87; lactate, 0.60; pyruvate, 0.04; malate, 0.36; phosphoenolpyruvate, 0.14; 2-phosphoglycerate, 0.05; 3-phosphoglycerate, 0.33; aspartate, 1.0; glucose, 4.3. Values shown are the means $\pm$ S.E.M. for 5–15 determinations.

the [lactate]/[pyruvate] ratio, which increased from 14 before injection to 27 at 10min and 95 at 30min after injection, and the increase in glycerol 3-phosphate (Table 1). However, pyruvate can still enter the mitochondria for carboxylation to oxaloacetate and subsequent reduction to malate (Fig. 1). Increased transport of malate to the cytosol will then contribute to the excess of reducing equivalents in this compartment and this in turn will slow down the oxidation of lactate to pyruvate, leading to the accumulation of the former, but will allow gluco-

Table 1. Concentrations of metabolites in blood and liver of suckling newborn rats at various times after the injection of L-2-amino-4-methoxy-trans-3-butenoic acid

Suckling newborn rats (24h-old) were injected intraperitoneally with L-2-amino-4-methoxy-trans-3-butenoic acid or NaCl (0.9%), and blood or liver was sampled at 10, 30 or 90 min after the injection. As there was no significant difference between the control groups at the different times, they have been pooled in a single group (0 min). The results are mean values $\pm$ S.E.M. expressed as μ mol/g wet wt. for the liver and μ mol/ml for the whole blood. The numbers of rats are shown in parentheses. Values statistically different from the control as determined by the Wilcoxon's rank order test are shown: * $P < 0.05$ and ** $P < 0.01$.

		Metabolite concentrations			
	Time after injection (min) ...	0	10	30	90
Liver	Citrate	0.23 $\pm$ 0.01 (16)	0.33 $\pm$ 0.04* (6)	0.37 $\pm$ 0.03** (6)	—
	Glycerol 3-phosphate	0.16 $\pm$ 0.01 (16)	0.32 $\pm$ 0.02** (5)	1.45 $\pm$ 0.30** (5)	2.2 $\pm$ 0.1** (5)
	Glycerol	0.52 $\pm$ 0.08 (15)	0.56 $\pm$ 0.04 (5)	1.01 $\pm$ 0.05** (5)	—
	Acetoacetate	1.03 $\pm$ 0.05 (15)	0.9 $\pm$ 0.1 (5)	0.33 $\pm$ 0.04** (5)	—
	D-3-Hydroxybutyrate	1.07 $\pm$ 0.06 (15)	1.2 $\pm$ 0.2 (5)	0.58 $\pm$ 0.08** (5)	—
	[D-3-Hydroxybutyrate]/[acetoacetate]	1.04	1.30	1.80	—
Blood	Alanine	0.30 $\pm$ 0.02 (11)	0.41 $\pm$ 0.04* (11)	0.58 $\pm$ 0.04** (11)	—
	L-Lactate	1.5 $\pm$ 0.1 (11)	1.5 $\pm$ 0.1 (11)	2.4 $\pm$ 0.1** (11)	8.5 $\pm$ 0.3** (6)
	Pyruvate	0.16 $\pm$ 0.1 (11)	0.13 $\pm$ 0.1 (11)	0.13 $\pm$ 0.1 (11)	0.02 $\pm$ 0.002** (6)
	Glucose	4.0 $\pm$ 0.3 (11)	3.9 $\pm$ 0.2 (11)	3.1 $\pm$ 0.2* (11)	1.1 $\pm$ 0.2** (6)

neogenesis to continue from alanine or pyruvate entering the liver from peripheral tissues. It is likely that at later stages the cytosol is in such a reduced state (Table 1) that even the cytosolic oxidation of malate to oxaloacetate will be inhibited as is suggested by the greater inhibition of the gluconeogenic pathway and the marked hypoglycaemia at 90 min (Table 1 and Fig. 1). The increase in the [3-hydroxybutyrate]/[acetoacetate] ratio (Table 1), which suggests a block in the transfer of reducing equivalents from the mitochondria to the cytosol, also supports this hypothesis.

The large increase in blood lactate after the injection of L-2-amino-4-methoxy-trans-3-butenoic acid indicates that peripheral tissues are producing lactate at a high rate and confirms the quantitative importance of this substrate as a gluconeogenic precursor in the suckling newborn rat.

An alternative explanation for the hypoglycaemia and hyperlactataemia after the administration of the inhibitor is that inhibition of aspartate aminotransferase in peripheral tissues may decrease the transfer to the mitochondria of reducing equivalents produced during glycolysis in the cytoplasm. This would in turn lead to increased reduction of pyruvate to lactate and a consequent decrease in glucose oxidation. The comparatively slow increase in the blood

[lactate]/[pyruvate] ratio suggests that this alternative mechanism does not play a major role.

Glycerol 3-phosphate and glycerol

The accumulation of glycerol 3-phosphate in the liver after L-2-amino-4-methoxy-trans-3-butenoic acid injection could be secondary to the decreased [NAD⁺]/[NADH] ratio and provides a means of removing some of the excess of reducing equivalents in the cytosol. These could then be transported back to the mitochondria by the glycerol 3-phosphate shuttle (Bücher & Klingenberg, 1958), as the mitochondrial glycerol 3-phosphate dehydrogenase (EC 1.1.99.5) activity is already high in the liver of the newborn rat (Hémon, 1967). The accumulation of glycerol can be explained by the fact that the glycerokinase is inhibited by high concentrations of glycerol 3-phosphate (Robinson & Newsholme, 1969) and this may further contribute to the hypoglycaemia, as glycerol utilization for gluconeogenesis (Vernon & Walker, 1970; Ferré *et al.*, 1977) will be blocked.

Alanine

Although it has been suggested that L-2-amino-4-methoxy-trans-3-butenoic acid does not affect the

liver alanine aminotransferase (EC 2.6.1.12) (Smith *et al.*, 1977), there is a small increase in blood and liver alanine at 30 and 90 min (Fig. 1 and Table 1). This may indicate that L-2-amino-4-methoxy-*trans*-3-butenic acid is not completely specific or alternatively that the mechanism suggested above prevents the operation of the malate shuttle at these later times and removal of alanine is inhibited.

Ketone bodies

The decrease in liver ketone bodies at 30 min after the injection can be attributed to (a) a direct effect of the inhibitor on an enzyme of the ketogenic pathway, (b) an indirect effect via the increased glycerol 3-phosphate which could direct more non-esterified fatty acids towards esterification or (c) the increased availability of oxaloacetate inside the mitochondria which may direct more acetyl-CoA to citrate which increases after L-2-amino-4-methoxy-*trans*-3-butenic acid injection (Table 1).

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