Inhibition of Urea-Cycle Activity by High Concentrations of Alanine

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1. Conditions are described in which high intracellular alanine concentrations inhibit urea-cycle flux in isolated hepatocytes. 2. Inhibition of urea-cycle flux by added alanine is DL-cycloserine-insensitive and is accompanied by an increase in intracellular citrulline and a decrease in ornithine. 3. Argininosuccinate synthetase (EC 6.3.4.5) activity in rat liver cytosol is inhibited by alanine in a competitive manner with respect to citrulline. It is concluded that this effect is the primary cause of inhibition of urea-cycle flux by alanine.

During the course of an investigation of the factors involved in the regulation of urea-cycle activity in isolated rat liver cells with amino acids or NH_3 as nitrogen source (Meijer *et al.*, 1975, 1978; Hensgens *et al.*, 1978; Hensgens & Meijer, 1979) it was observed that alanine, which has an important function in glucose and nitrogen metabolism (Felig, 1975), inhibited urea-cycle activity under certain conditions. The mechanism by means of which alanine exerts this rather unexpected effect is the subject of the present paper. An abstract describing this work has appeared (Hensgens & Meijer, 1978).

Materials and Methods

Hepatocytes from 18–24h-starved male Wistar rats were prepared and incubated as described previously (Meijer *et al.*, 1975).

For determination of the intracellular contents of metabolites the cells were centrifuged through a layer of silicone oil into HClO_4 as described by Meijer *et al.* (1978).

Liver mitochondria were prepared as described by Myers & Slater (1957), with 250mM-mannitol as the isolation medium.

Rat liver cytosol was obtained by centrifuging the postnuclear supernatant of a rat liver homogenate at 100000g for 45 min at 0°C. The basal reaction medium for the measurements of the activities of argininosuccinate synthetase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1) consisted of the following components: 15mM-KCl, 10mM-MgCl₂, 2mM-EDTA, 50mM-Tris/HCl, pH7.4, 10mM-ATP and 5mM-potassium phosphate, pH7.4. The activities were measured at 25°C. At the times indicated in Fig. 2, samples of the reaction mixture were removed and added to HClO_4 (final concentration 3%, w/v). Urea was determined in the neutralized protein-free extract.

Citrulline, NH_3 and urea were determined as described by Hensgens *et al.* (1978). Alanine was determined enzymically (Williamson, 1970).

Results

During the course of a study in which proline was the precursor of aspartate for urea synthesis (Hensgens *et al.*, 1978) it was found that addition of alanine to isolated rat liver cells, incubated with NH_3 plus proline, inhibited the synthesis of urea (Table 1). The inhibition was completely abolished by ornithine. This suggests a direct interaction of alanine (or one of its metabolic products) with one or more of the urea-cycle enzymes. In other experiments (not shown) it was found that the inhibition by alanine was not affected by 0.5mM-DL-cycloserine, a concentration sufficient to inhibit intracellular alanine aminotransferase (EC 2.6.1.2) completely (Meijer *et al.*, 1978). Thus the inhibition was due to alanine itself and not to one of its metabolic products. In the presence of DL-cycloserine, proline and NH_3 , addition of alanine led to a crossover between ornithine and citrulline. In a representative experiment intracellular ornithine decreased from 0.4 to 0.2 $\mu\text{mol/g}$ dry wt., and intracellular citrulline increased from 0.2 to 0.7 $\mu\text{mol/g}$ dry wt. upon alanine addition. This suggests a site of interaction of alanine with the urea cycle at the level of argininosuccinate synthetase.

Table 1. Effect of added alanine on urea synthesis from NH_3 and proline in isolated rat liver cells

For experimental details, see the Materials and Methods section. Proline (10mM) and NH_4Cl (10mM) were present in all incubations. Ornithine and alanine were added where indicated, both at a concentration of 10mM. Means $\pm$ S.E.M. for four experiments (without ornithine) or three experiments (with ornithine).

Addition	Urea formation ($\mu\text{mol/h}$ per g dry wt. of cells)	
	Control	+Ornithine
None	398 $\pm$ 53	716 $\pm$ 94
Alanine	190 $\pm$ 26	730 $\pm$ 98

The intracellular concentration of alanine under the conditions of the experiments in Table 1 was about $5\text{ }\mu\text{mol/g}$ dry wt. in the absence, and $45\text{--}75\text{ }\mu\text{mol/g}$ dry wt. in the presence, of added alanine after correction for extracellular alanine.

Alanine generated intracellularly from either lactate or pyruvate also inhibited urea-cycle flux from proline plus NH_3 , unless ornithine was present (Table 2). The inhibition was associated with a large increase in intracellular alanine. Fig. 1 shows that high alanine concentrations also inhibit urea production from alanine itself, and that NH_3 accumulates. These effects were not observed in the presence of ornithine (see also Krebs *et al.*, 1973).

The effect of alanine on argininosuccinate synthetase was tested directly in rat liver cytosol. Alanine inhibited urea formation from citrulline and aspartate, but only at low citrulline concentrations (Figs. 2a and 2b). Concomitantly, the production of fumarate plus malate was inhibited (results not shown). Urea synthesis from argininosuccinate was insensitive to alanine (Fig. 2c), as was the formation of fumarate plus malate (results not shown). These results clearly demonstrate that alanine directly inhibits argininosuccinate synthetase, and that the inhibition is competitive with respect to citrulline.

Marshall & Cohen (1972) have shown that ornithine carbamoyltransferase (EC 2.1.3.3) from

Table 2. *Effect of alanine generated intracellularly on urea synthesis from NH_3 plus proline in isolated rat liver cells*
For experimental details see the Materials and Methods section. Proline (10mM) and NH_4Cl (10mM) were present in all incubations. Ornithine, lactate and pyruvate were added where indicated at a concentration of 10mM. The values are from a representative experiment. + and – indicate production and removal respectively.

Additions	Metabolic changes ($\mu\text{mol/h}$ per g dry wt. of cells)			Intracellular concentration ($\mu\text{mol/g}$ dry wt. of cells)
	Urea	NH_3	Alanine	Alanine
Ornithine absent				
None	+384	–613	+18	6
Lactate	+161	–619	+197	72
Pyruvate	+116	–779	+339	79
Ornithine present				
None	+898	–1511	+17	5
Lactate	+767	–1536	+172	39
Pyruvate	+796	–1766	+209	61

Fig. 1. *Inhibition by high concentrations of alanine of urea synthesis from alanine (b) and accumulation of NH_3 (a) in isolated rat liver cells*

For experimental details see the Materials and Methods section. Oleate (1mM) was present in all incubations. ○, Ornithine absent; ●, 3 mM-ornithine present.

Fig. 2. Effect of alanine on urea-cycle enzymes in rat liver cytosol

For experimental details see the Materials and Methods section. The amount of cytosolic protein in the incubation medium was 1.87 mg/ml. The substrate concentrations were: 1 mM-citrulline and 10 mM-aspartate (a); 10 mM-citrulline and 1 mM-aspartate (b); 0.8 mM-argininosuccinate (c). ○, Control; □, 10 mM-alanine; ●, 20 mM-alanine.

Streptococcus faecalis and from bovine liver are inhibited by alanine. There is a similar inhibition of the rat liver mitochondrial enzyme in sonicated mitochondria (results not shown). The inhibition by alanine is competitive with respect to ornithine, the K_i being 11–15 mM.

Discussion

Although alanine inhibits both ornithine carbamoyltransferase and argininosuccinate synthetase, our studies strongly suggest that inhibition of argininosuccinate synthetase was primarily responsible for the observed inhibition of urea synthesis by alanine. Wojtczak *et al.* (1978) observed that the rate of urea synthesis from NH_3 in the absence of ornithine and presence of lactate was higher at low (2 mM) than at high (10 mM) concentrations of NH_3 . This difference in urea-cycle flux is probably due to different degrees of inhibition of argininosuccinate synthetase by intracellular alanine, the concentration of which is several times higher at high than at low NH_3 concentrations under these conditions (not shown).

Inhibition by alanine of argininosuccinate synthetase also explains the finding that the apparent K_m for citrulline of argininosuccinate synthetase in isolated rat liver cells incubated with lactate plus NH_3 is much higher than the reported K_m value for the isolated enzyme (Meijer *et al.*, 1978).

Although the inhibition of argininosuccinate synthetase by alanine has important implications for studies *in vitro*, it is doubtful whether this effect has

any physiological significance under normal conditions. In our experiments intracellular alanine concentrations of 40–70 $\mu\text{mol/g}$ dry wt. of cells caused a 50% inhibition of urea-cycle activity in the absence of added ornithine. Under normal conditions, the intrahepatic alanine concentration *in vivo* ranges from 0.4 to 2.0 mM (Brosnan *et al.*, 1970; Greenbaum *et al.*, 1971; Brosnan & Williamson, 1974). However, high values for the intrahepatic concentrations of alanine (to up to 15 mM) were reported by Brosnan & Williamson (1974) after a non-toxic load of NH_3 .

Recently we have observed that besides alanine other amino acids, such as isoleucine, valine, leucine and lysine, in order of decreasing effectiveness, also inhibit the conversion of citrulline into urea in a rat liver cytosol fraction (H. E. S. J. Hensgens & A. J. Meijer, unpublished results). For example, 5 mM-isoleucine inhibited urea production from 1 mM-citrulline and 10 mM-aspartate by 90%. A mixture of alanine, valine, leucine, lysine and isoleucine, each at a concentration similar to those found in the liver *in vivo* (0.2–0.7 mM), inhibited urea formation by about 50%. These effects were due not only to inhibition of arginase, which is known to be inhibited by these amino acids (Hunter & Downs, 1945; Kaysen & Strecker, 1973), but also to inhibition of argininosuccinate synthetase (H. E. S. J. Hensgens & A. J. Meijer, unpublished observations). It is likely therefore that *in vivo*, even though the cytosolic concentration of each of the amino acids mentioned is low, substantial inhibition of urea-cycle flux by these amino acids can occur because of the additivity of their individual effects.

Under certain pathological conditions, e.g. in patients with inborn errors of amino acid catabolism or urea synthesis, the concentrations of certain amino acids in plasma, such as alanine and lysine, increase (Shih, 1978). It may be expected that under such conditions intrahepatic amino acid concentrations are elevated. However, to our knowledge no experimental data are available on this point. Especially in cases of a deficiency in one of the urea-cycle enzymes, direct inhibitory effects of accumulating alanine and other amino acids may enhance the deleterious effect of the defect.

Note Added after Submission

After submission of this paper an article by Takada *et al.* (1979) appeared, in which the inhibitory effects on purified argininosuccinate synthetase of several amino acids, including alanine and the other four amino acids mentioned in the Discussion section of our paper, was reported. The results of Takada *et al.* (1979) therefore provide independent confirmation of the mechanism suggested by us for inhibition of urea-cycle flux by amino acids.

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