The ornithine requirement of urea synthesis

Formation of ornithine from glutamine in hepatocytes

Patricia LUND and David WIGGINS

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

In hepatocytes, urea synthesis from glutamine is independent of added ornithine, even when rates are high after stimulation of glutamine metabolism by dibutyryl cyclic AMP, phenylephrine or vasopressin. Incubation with glutamine increases tissue [ornithine]. The increases parallel those of [*N*-acetylglutamate] under different conditions. The ornithine requirement of urea synthesis increases with increasing supply of ammonia. A function of the unique, highly regulated, glutaminase of liver may be to regulate ornithine synthesis.

INTRODUCTION

The experiments described in this paper were designed to clarify some aspects of the regulation of urea synthesis. It is accepted that formation of urea *in vitro* from NH_4Cl requires added ornithine and a carbon source for the generation of aspartate. However, urea synthesis from glutamine, but not from alanine or asparagine, was found to be independent of added ornithine (Krebs *et al.*, 1976). It was not known at the time that glutamine degradation is initiated by a complex, allosterically regulated, glutaminase that is found only in liver (see McGivan *et al.*, 1984). In searching for a function for this enzyme, we investigated the possibility that it controls the supply of glutamate for synthesis of *N*-acetylglutamate, the proposed short-term regulator of urea synthesis (see Meijer & Hensgens, 1982). Although incubation of hepatocytes with glutamine does increase the *N*-acetylglutamate content of the tissue, we came to the conclusion that this was not the primary regulatory function of glutaminase (Lund & Wiggins, 1984). We now show that the ornithine content of hepatocytes increases in parallel with *N*-acetylglutamate, and that the ornithine requirement of urea synthesis increases with increasing ammonia supply. These observations suggest that an important function of glutaminase *in vivo* may be in the regulation of ornithine synthesis.

EXPERIMENTAL

Tissue preparations

Hepatocytes were isolated from livers of female Wistar rats (about 200 g) as described by Farrell & Lund (1983). The normal diet was PRM [Dixon & Sons (Ware), Ware, Herts., U.K.]. Animals were then starved for 48 h. Incubations of freshly prepared hepatocytes were carried out in 25 ml Erlenmeyer flasks containing 2 ml of Krebs–Henseleit (1932) saline including additions, in the absence of albumin, plus 2 ml of hepatocyte suspension (containing approx. 100 mg wet wt. of tissue unless stated otherwise). The final concentration of CaCl_2 was 1.6 mM. Reactions were stopped either by addition of 0.4 ml of 20% (v/v) HClO_4 to the flask or by rapidly separating cells from medium by centrifugation followed by careful draining of the tube and deproteinization of

tissue with 0.4 ml of 6% HClO_4 (see Lund & Wiggins, 1984). The assumption was made that 80% of the wet weight of tissue contributed to the volume.

The preparation of samples for determination of the ornithine requirement of urea synthesis was modified to allow accurate correction for ornithine in the incubation medium. Micro-centrifuge tubes (1.5 ml) were prepared with 0.45 ml of silicone fluid [Dow Corning 550:200/lcs (4.9:1, v/v) to give sp.gr. 1.03] layered over 0.25 ml of 6% HClO_4 . At exactly 10 min of incubation, 0.8 ml of cell suspension was pipetted on top of the silicone, and the tube was centrifuged immediately (10000 g for 15 s) in an Eppendorf microfuge. The supernatant was removed for assay of ammonia and urea, and the silicone layer was discarded. The pellet was resuspended in the acid and the tube re-centrifuged. The acid supernatant was weighed, neutralized with KOH and re-weighed before determination of ornithine. Further samples were taken from the flasks at 20 and 30 min to obtain the time course of urea synthesis.

Correction for ‘carrying-down’ of extracellular ornithine was made by using data obtained in other experiments. By means of dual-labelling ($^3\text{H}_2\text{O}$ / [^{14}C]inulin) we found that the mean carry-down of medium, on centrifugation of hepatocytes from 48 h-starved rats through silicone, was 28% of the total water and that the intracellular volume was 1.99 ± 0.07 ml/g dry wt., from ten determinations (P. Lund & D. Wiggins, unpublished work). After measurement of the ornithine concentration in the incubation medium, these factors were used to obtain the ornithine concentration per ml of intracellular water.

Metabolite assays

Glucose, ammonia, urea, glutamate and aspartate were determined essentially as described by Cornell *et al.* (1974). Ornithine was measured by h.p.l.c. after treatment of the neutral tissue extract with *N*-(4-dimethylamino)azobenzene-4'-sulphonyl chloride (from Fluorochem, Glossop, Derbyshire, U.K.) as described by Chang *et al.* (1982).

Chemicals and enzymes

Enzymes, coenzymes and dibutyryl cyclic AMP were obtained from Boehringer (London). L-Glutamine was

Table 1. Urea synthesis from ammonia or glutamine; comparison of effects of ornithine and dibutyl cyclic AMP

Hepatocytes were from 48 h-starved rats. Substrate concentrations were 10 mM-NH₄Cl, 10 mM-lactate, 0.5 mM-oleate, 5 mM-glutamine, 0.05 mM-dibutyl cyclic AMP and 0.1 mM-ornithine. Values are means $\pm$ S.E.M. for four separate hepatocyte preparations. $^{**}P \leq 0.005$ (paired *t* test) compared with control without added ornithine or cyclic AMP.

Conditions of incubation	Urea synthesis (μ mol/min per g wet wt.)		
	0–20 min	20–40 min	40–60 min
NH ₄ Cl, lactate, oleate	0.60 $\pm$ 0.20	0.80 $\pm$ 0.16	1.31 $\pm$ 0.15
NH ₄ Cl, lactate, oleate, ornithine	1.68 $\pm$ 0.14 ^{**}	2.37 $\pm$ 0.29 ^{**}	2.87 $\pm$ 0.27 ^{**}
Glutamine	0.60 $\pm$ 0.15	1.07 $\pm$ 0.25	1.61 $\pm$ 0.30
Glutamine, ornithine	0.65 $\pm$ 0.13	1.17 $\pm$ 0.31	1.48 $\pm$ 0.20
Glutamine, cyclic AMP	0.74 $\pm$ 0.13	1.93 $\pm$ 0.19 ^{**}	2.48 $\pm$ 0.02
Glutamine, ornithine, cyclic AMP	0.85 $\pm$ 0.19	2.07 $\pm$ 0.35 ^{**}	2.40 $\pm$ 0.11

Table 2. Parallelism between intracellular [ornithine] and [N-acetylglutamate] on incubation with glutamine

Intracellular ornithine was measured as described in the Experimental section after incubation of hepatocytes for 40 min. 'Unincubated hepatocytes' refers to the freshly prepared suspension. Substrate concentrations were 5 mM-glutamine, 0.05 mM-dibutyl cyclic AMP, 0.1 mM-phenylephrine, 10 mM-NH₄Cl, 10 mM-lactate and 0.5 mM-oleate. Data for N-acetylglutamate and urea synthesis are taken from Lund & Wiggins (1984). Results are means $\pm$ S.E.M. for the numbers of observations shown in parentheses or are individual observations. $^{*}P \leq 0.01$ compared with no substrate; $^{\dagger}P \leq 0.01$ compared with glutamine alone (Student's *t* test).

Conditions of incubation	Ornithine (nmol/g wet wt.)	N-acetylglutamate (nmol/g wet wt.)	Urea synthesis (μ mol/min per g wet wt.) during 20–40 min
Unincubated hepatocytes	49.7 $\pm$ 13.2 (3)	116 $\pm$ 5.4 (6)	
No substrate	88.8 $\pm$ 22.5 (4)	77 $\pm$ 9.2 (4)	0.13 $\pm$ 0.01 (4)
Glutamine	202 $\pm$ 24 (5) [*]	126 $\pm$ 7.8 (6) [*]	0.79 $\pm$ 0.08 (6) [*]
Glutamine, cyclic AMP	326 $\pm$ 26 (6) ^{*†}	206 $\pm$ 17 (5) ^{*†}	1.74 $\pm$ 0.10 (5) ^{*†}
Glutamine, phenylephrine	194 $\pm$ 18 (4) [*]	145 $\pm$ 11 (5) [*]	1.52 $\pm$ 0.11 (5) ^{*†}
NH ₄ Cl, lactate, oleate	106 $\pm$ 16 (3)	118, 107 (2)	0.80 $\pm$ 0.16 (4) [*]

from Sigma Chemical Co. Phenylephrine hydrochloride was from the Boots Co., Nottingham, U.K. Other chemicals were reagent grade.

RESULTS

Ornithine requirement: difference between NH₄Cl and glutamine as precursor of urea

High rates of urea synthesis from NH₄Cl have an absolute requirement for ornithine; 1 mM is optimal, and is routinely added to incubations. Urea synthesis from glutamine is independent of added ornithine (Krebs *et al.*, 1976). This phenomenon was re-examined in the light of more recent knowledge of the complexity of liver glutaminase. The results are shown in Table 1. The same batch of hepatocytes was tested under each condition to allow direct comparisons to be made. Firstly, a low concentration of ornithine (0.1 mM) dramatically increases urea synthesis from NH₄Cl, but has no accelerating effect when glutamine is substrate. However, urea synthesis from alanine or asparagine is accelerated by ornithine (see Krebs *et al.*, 1976), but addition of concentrations higher than 0.1 mM has no further accelerating effect (results not shown). Rates of urea synthesis from glutamine, especially after the initial lag in glutamine metabolism (see Table 1), are higher than

from NH₄Cl in the absence of added ornithine. Moreover, ornithine has no effect even after glutamine metabolism is accelerated by dibutyl cyclic AMP (Table 1). In other experiments (P. Lund & D. Wiggins, unpublished work), ornithine was shown to have no effect on urea synthesis when glutamine metabolism was increased by vasopressin or phenylephrine.

It is important to note that the lag during the first 20 min in urea synthesis from 10 mM-NH₄Cl at 0.1 mM-ornithine is not found at higher concentrations of ornithine (see Fig. 1), or at low concentrations of NH₄Cl (see below).

Increase of [ornithine] on incubation of hepatocytes with glutamine

As shown in Table 2, intracellular ornithine concentration increases on incubation of hepatocytes with glutamine. The concentration is further increased by dibutyl cyclic AMP, but not by phenylephrine. This pattern parallels our previous observations (Lund & Wiggins, 1984) on increases in N-acetylglutamate. Briefly, urea synthesis, and concentrations of N-acetylglutamate and ornithine, are increased by dibutyl cyclic AMP in the presence of glutamine, a correlation that does not hold when phenylephrine replaces cyclic AMP. Phenylephrine increases urea synthesis without any corresponding increase in [N-acetylglutamate] or [ornithine].

Fig. 1. Effect of ornithine on urea synthesis from different concentrations of NH_4Cl

For details, see the Experimental section. In this representative experiment each flask contained 15 mg wet wt. of hepatocytes/ml, 2 mM-lactate, 0.5 mM-oleate and 1 mM- NH_4Cl (a), 2 mM- NH_4Cl (b) or 10 mM- NH_4Cl (c). Added concentrations of ornithine were: $\circ$, none; in (a), $\blacksquare$, 0.1, 0.5 and 1 mM; in (b) $\bullet$, 0.1 mM, $\blacksquare$, 0.5 and 1 mM; in (c) $\bullet$, 0.1 mM, $\square$, 0.5 mM, $\blacksquare$, 1 mM.

NH_4Cl , which Saheki *et al.* (1978) had shown to increase [ornithine] and [*N*-acetylglutamate] in rat liver *in vivo*, is less effective than glutamine in hepatocytes (Table 2), even though glutamate can be formed readily from NH_4Cl and lactate, especially in the presence of oleate (D. Wiggins, unpublished work).

Ornithine requirement of urea synthesis

The difference in ornithine requirement in hepatocytes at low ammonia concentrations, as when glutamine is the precursor of urea, and at high concentrations, as when added NH_4Cl is the precursor, led us to investigate the ornithine requirement in more detail. Experiments were carried out at a range of NH_4Cl (1, 2 and 10 mM) and ornithine (0–1 mM) concentrations. Short incubation times and low tissue concentrations were used to maintain substrate supply at 1 mM- NH_4Cl . At low concentrations of substrate, the rate of urea synthesis is essentially linear and independent of added ornithine. In the absence of added ornithine, rates are virtually the same between 1 and 10 mM- NH_4Cl . Ornithine-dependence develops as substrate concentration increases (Fig. 1). Since the measurements of the ornithine content of the incubation medium show that there is no degradation of ornithine under these conditions, it can be assumed that the intracellular concentration at 10 min represents the steady state. If these values and the 10–20 min rates of urea synthesis are used in Lineweaver–Burk plots, values are obtained for the ornithine required to give $0.5 V_{\text{max}}$ at different concentrations of extracellular total ammonia ($\text{NH}_3 + \text{NH}_4^+$). At 10 mM- NH_4Cl (approx. 9 mM remaining during kinetic measurements), this value is 0.31 ± 0.03 mM-ornithine (mean $\pm$ S.E.M. for five determinations), and at 2 mM- NH_4Cl (approx. 1 mM actual concentration remaining) the value is 0.11 ± 0.01 mM (four determinations). At 1 mM- NH_4Cl (< 0.4 mM actual concentration), urea synthesis is virtually independent of added ornithine, and increases over the endogenous

concentration (0.09 ± 0.02 mM for three determinations) have little accelerating effect. Although no attempt was made to measure the intracellular distribution of ornithine, these data provide an explanation for the apparent anomaly that phenylephrine increases urea synthesis from glutamine independently of its effects on [ornithine], in that the increases in [ornithine] provoked by glutamine alone are adequate to cope with the hormonally induced increase in flux of ammonia.

Fig. 1 shows that there is no lag in urea synthesis from NH_4Cl under optimal conditions, that is when a low concentration of oleate is also present in hepatocytes from 48 h-starved rats. Any increases in *N*-acetylglutamate that occur under these conditions are therefore unrelated to flux through carbamoyl-phosphate synthetase.

DISCUSSION

Possible role of liver glutaminase in the regulation of ornithine synthesis

The existence of a complex, allosterically regulated, glutaminase in liver to initiate degradation of a non-essential amino acid suggests that the enzyme must have a specific function. In view of the importance accorded to *N*-acetylglutamate in the regulation of urea synthesis (see Meijer & Hensgens, 1982), it seemed likely that glutaminase could function to control the supply of glutamate for *N*-acetylglutamate synthesis. In spite of the fact that glucagon (or cyclic AMP) increases both glutaminase activity (Lacey *et al.*, 1981; Corvera & Garcia-Sainz, 1983) and the *N*-acetylglutamate content of liver (Cathelineau *et al.*, 1980; Hensgens *et al.*, 1980; Rabier *et al.*, 1982; Staddon *et al.*, 1984), we came to the conclusion that regulation of *N*-acetylglutamate synthesis was not the primary function of glutaminase (Lund & Wiggins, 1984).

The theory of a specific function for *N*-acetylglutamate

as a short-term regulator of urea synthesis had always seemed inherently unsatisfactory, in that disposal of the compound was assumed to involve its diffusion to the cytosol across the relatively impermeable mitochondrial inner membrane (Meijer *et al.*, 1982) for deacylation by a non-specific deacylase, which is more active in kidney than in liver (Reglero *et al.*, 1977). A more feasible function for glutaminase may be to control the synthesis of ornithine; glutamine appears to be the most effective precursor, as evidenced by the measured increases in ornithine and the fact that urea synthesis *in vitro* from other precursors is dependent on exogenous ornithine. Saheki *et al.* (1978) observed increases in the *N*-acetylglutamate and ornithine contents of liver *in vivo* on injection of NH_4Cl ; these probably occurred as a result of activation of glutaminase (see Petit *et al.*, 1974). It is curious that the changes in ornithine parallel those of *N*-acetylglutamate in a variety of conditions *in vitro* when glutamine is the precursor (Table 2). The close correlation implies that the latter may be an intermediate in the pathway to ornithine. The only known route from *N*-acetylglutamate is via *N*-acetylglutamate phosphate $\rightarrow$ *N*-acetylglutamate semialdehyde $\rightarrow$ *N*-acetylornithine $\rightarrow$ ornithine, and has been shown so far only in micro-organisms. The alternative pathway from glutamate, involving glutamic semialdehyde as an intermediate, has been considered to be irreversible (Strecker, 1965), and Henslee & Jones (1983) found no evidence that it operates in liver. Since the *N*-acetyl group prevents spontaneous cyclization of glutamic semialdehyde, theoretically the acetylated pathway would appear to be a possibility.

Ornithine requirement of urea synthesis

The K_m for ornithine of rat liver ornithine carbamoyl-transferase (EC 2.1.3.3) is reported to be 0.4 mM at pH 7.7 (Lusty *et al.*, 1979), 1.82 at pH 7.5 and 0.23 at pH 8.5 (Raijman, 1974); the ionic species with zero net charge is the substrate (Snodgrass, 1968). The K_m for carbamoyl phosphate is very low (22–26 μM ; Raijman, 1974; Lusty *et al.*, 1979). The kinetic properties of the enzyme conform with classical Michaelis–Menten behaviour and suggest that activity *in situ* is determined mainly by the intramitochondrial concentrations of carbamoyl phosphate and ornithine. Our rather simple experiments show that the rate of the entire pathway of urea synthesis can also be used to determine the ornithine requirement. In the range of extracellular concentrations of ammonia that approach the concentrations reaching the liver in the portal vein (see Rémésy *et al.*, 1978), synthesis of urea in hepatocytes is saturated at the endogenous concentration of ornithine (0.09 ± 0.02 mM). This is in keeping with the general consensus that urea synthesis under normal physiological conditions is regulated by supply of ammonia. On a diet high in protein or in hyperammonaemia the ornithine requirement would be greater. It has been suggested that the hyperammonaemia of organic acidaemia or in patients treated with the anti-epileptic drug sodium valproate is caused by inhibition of *N*-acetylglutamate synthesis (see Coude *et al.*, 1983). Inhibition of the pathway of ornithine synthesis, or an enzymic defect on the pathway, might also explain the susceptibility of these individuals. A similar explanation should be considered for Reye's syndrome. There is no

reason why inborn errors of ammonia metabolism should be limited to the enzymes of the urea cycle. As Raijman (1976) pointed out, with reference to inborn errors of the urea cycle, decreased availability of ornithine *in vivo* may underlie the observation that the extent of a defect in a urea-cycle enzyme can be disproportionately small in relation to the degree of hyperammonaemia.

We are grateful to R. Hems for preparing the hepatocyte suspensions and to Deutsche Ajinomoto G.m.b.H., who contributed during 1985 to the running costs of setting up amino acid analysis by h.p.l.c. The work was supported by the Medical Research Council.

REFERENCES

- Cathelineau, L., Rabier, D., Petit, F. & Kamoun, P. (1980) C.R. Hebd. Seances Acad. Sci. Ser. D **291**, 625–627
- Chang, J. Y., Knecht, R. & Braun, D. G. (1982) *Biochem. J.* **203**, 803–806
- Cornell, N. W., Lund, P. & Krebs, H. A. (1974) *Biochem. J.* **142**, 327–337
- Corvera, S. & Garcia-Sainz, J. A. (1983) *Biochem. J.* **210**, 957–960
- Coude, F. X., Grimber, G., Parvy, P., Rabier, D. & Petit, F. (1983) *Biochem. J.* **216**, 233–236
- Farrell, R. & Lund, P. (1983) *Biosci. Rep.* **3**, 539–543
- Hensgens, H. E. S. J., Verhoeven, A. J. & Meijer, A. J. (1980) *Eur. J. Biochem.* **107**, 197–205
- Henslee, J. G. & Jones, M. E. (1983) *Arch. Biochem. Biophys.* **219**, 186–197
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Krebs, H. A., Lund, P. & Stubbs, M. (1976) in *Gluconeogenesis* (Hanson, R. W. & Mehlman, M. A., eds.), pp. 269–291, John Wiley and Sons, New York
- Lacey, J. H., Bradford, N. M., Joseph, S. K. & McGivan, J. D. (1981) *Biochem. J.* **194**, 29–33
- Lund, P. & Wiggins, D. (1984) *Biochem. J.* **218**, 991–994
- Lusty, C. J., Jilka, R. L. & Nietsch, E. H. (1979) *J. Biol. Chem.* **254**, 10030–10036
- McGivan, J. D., Bradford, N. M., Verhoeven, A. J. & Meijer, A. J. (1984) in *Glutamine Metabolism in Mammalian Tissues* (Häussinger, D. & Sies, H., eds.), pp. 122–137, Springer-Verlag, Berlin and Heidelberg
- Meijer, A. J. & Hensgens, H. E. S. J. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 259–286, Academic Press, London and New York
- Meijer, A. J., Van Woerkom, G. M., Wanders, R. J. A. & Lof, C. (1982) *Eur. J. Biochem.* **124**, 325–330
- Petit, M., Nordmann, J. & Nordmann, R. (1974) *Biochimie* **56**, 1155–1157
- Rabier, D., Briand, P., Petit, F., Parvy, P., Kamoun, P. & Cathelineau, L. (1982) *Biochem. J.* **206**, 627–631
- Raijman, L. (1974) *Biochem. J.* **138**, 225–232
- Raijman, L. (1976) in *The Urea Cycle* (Grisolia, S., Baguena, R. & Mayor, F., eds.), pp. 243–254, John Wiley and Sons, New York
- Reglero, A., Rivas, J., Mendelson, J., Wallace, R. & Grisolia, S. (1977) *FEBS Lett.* **81**, 13–17
- Rémésy, C., Demigné, C. & Aufrère, J. (1978) *Biochem. J.* **170**, 321–329
- Saheki, T., Ohkuno, T. & Katsunuma, T. (1978) *J. Biochem. (Tokyo)* **84**, 1423–1430
- Snodgrass, P. J. (1968) *Biochemistry* **7**, 3047–3051
- Staddon, J. M., Bradford, N. M. & McGivan, J. D. (1984) *Biochem. J.* **217**, 855–857
- Strecker, H. J. (1965) *J. Biol. Chem.* **240**, 1225–1230