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Effect of Ornithine and Lactate on Urea Synthesis in Isolated Hepatocytes

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1. In hepatocytes isolated from 24h-starved rats, urea production from ammonia was stimulated by addition of lactate, in both the presence and the absence of ornithine. The relationship of lactate concentration to the rate of urea synthesis was hyperbolic. 2. Other glucose precursors also stimulated urea production to varying degrees, but none more than lactate. Added oleate and butyrate did not stimulate urea synthesis. 3. Citrulline accumulation was largely dependent on ornithine concentration. As ornithine was increased from 0 to 40mM, the rate of citrulline accumulation increased hyperbolically, and was half-maximal when ornithine was 8–12mM. 4. The rate of citrulline accumulation was independent of the presence of lactate, but with pyruvate the rate increased. 5. The rate of urea production continued to increase as ornithine was varied from 0 to 40mM. 6. It was concluded that intermediates provided by both ornithine and lactate are limiting for urea production from ammonia in isolated liver cells. It was suggested that the stimulatory effect of lactate lies in increased availability of cytosolic aspartate for condensation with citrulline.

Ever since the discovery by Krebs & Henseleit (1932) of the urea cycle, there has been continuous investigation into the steps, mechanisms and regulation of urea synthesis (reviews by Ratner, 1954, 1973). Improved techniques for organ perfusion and cell isolation have led to further characterization of the urea cycle during the past decade. It has been shown both in perfused liver (Hems *et al.*, 1966) and in isolated hepatocytes (Meijer *et al.*, 1975) that added ornithine increases the rate of urea production when ammonia is the major nitrogen donor. The importance of ornithine was also apparent in studies attempting to correlate urea-cycle enzyme activities, which can vary according to diet and hormonal status (Freedland & Sodikoff, 1962; Schimke, 1962), with rate of urea production. In the absence of added ornithine, urea production from ammonia was much greater in the perfused liver of rats fed on a high-protein diet than in livers of those fed on normal amounts of protein. However, when ornithine was added to the perfusion medium, the difference between diet groups disappeared (Kramer & Freedland, 1972). Thus it appears that factors other than enzyme activity may regulate urea synthesis *in vivo*, in liver perfusion and in isolated cell preparations. Preliminary work in our laboratory (Briggs & Freedland, 1975) indicated that lactate stimulates urea production from ammonia in isolated hepatocytes, although no such stimulation is observable in perfused liver. The purpose of the present work is to elucidate further the effects and roles in urea synthesis in isolated hepatocytes of ornithine, lactate and other precursors of C_4 intermediates.

Experimental

Male Sprague–Dawley rats (150–250g) were fed on a commercial laboratory diet. Food was removed 24h before animals were used for liver-cell isolation.

Isolation of rat hepatocytes was performed by a modification of the method of Berry & Friend (1969). Hanks solution was replaced by calcium-free Krebs–Ringer bicarbonate solution (Krebs & Henseleit, 1932) for perfusion and for incubation with enzymes. After filtration, cells and medium were centrifuged at approx. 30*g* for 2min. The supernatant was carefully removed by suction and discarded. The cells were resuspended in about 30ml of Krebs–Ringer bicarbonate solution, containing 1.22% Ca^{2+} and 2.5% (w/v) bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.) and again centrifuged. Undefatted, undialysed albumin was used. The washing was repeated once. After the final washing, cells were weighed and suspended in approx. 17vol. of Krebs–Ringer bicarbonate solution containing 2.5% bovine serum albumin. Cell suspension (2.0ml) was added to flasks containing 2.0ml of substrates, all dissolved in Krebs–Ringer bicarbonate solution and at pH7.35–7.4. After incubation, the contents of flasks were acidified with 2.5ml of 7% (w/v) $HClO_4$. Before the determination of urea and citrulline, these solutions were centrifuged and the supernatant was neutralized with 3M-K_2CO_3/0.5M-triethanolamine. The sum of urea and citrulline was determined by a colorimetric method (Foster & Hochholzer, 1971). Citrulline was determined by the same method after incubation of samples with 1.4 units of urease/ml of neutralized sample (jack-

bean urease, type III; Sigma) for 1h at 37°C. Urea was determined by difference.

Results

In the experiments reported here, comparisons will be made between treatments performed on cells from a single preparation. Measurements from separate cell preparations cannot meaningfully be compared, since no reliable index of cell number was available to us at the time these experiments were completed. The average percentage deviation from the mean of duplicate measurements within single experiments was 5.2±0.8 (S.E.M.). Differences owing to treatments were much greater.

When ornithine concentration was varied from zero to 40mM, urea production continued to increase as ornithine increased. This differs from the finding of Hems *et al.* (1966) that 2.7mM-ornithine is optimal for urea production from ammonia in the perfused liver. Compared with the control (no ornithine), the 10mM-ornithine treatment produced a 3-fold increase in the rate of urea production, in both the absence and the presence of lactate.

From the dilution used, an estimate of 0.12g of liver cells per flask can be used to calculate the approximate magnitude of our results on a basis of liver weight. On the basis of this estimate, urea production from ammonia in the presence of 10mM-ornithine and 10mM-lactate was approx. 1.8μmol/min per g of liver, within the range of values reported by other researchers from perfused-liver experiments (Hems *et al.*, 1966; Kramer & Freedland, 1972).

A representative example of the effect of lactate on urea production from ammonia in the presence of 10mM-ornithine is shown in Fig. 1. With the addition of 1mM-lactate, the rate of urea production was nearly doubled. With 10mM-lactate, the rate was tripled. A plot of increases (compared with control) in the rate of urea production (calculated from the 15 and 45min values), expressed as percentages of the rate with no lactate (control), produced a hyperbolic curve (Fig. 2) and indicated that the rate of urea production could be stimulated by lactate to a theoretical maximum of 570% of the control rate. The concentration of lactate required to obtain half the maximal increase was calculated to be 5.2mM.

This stimulatory effect of lactate on urea production from ammonia was observed repeatedly and also with other concentrations of ornithine. When no ornithine was added to the incubations, however, 5 and 10mM-lactate often produced less stimulation than did lower concentrations of lactate. The plots of increase in urea production rate versus lactate concentration when ornithine was provided were consistently hyperbolic, but the theoretical maximum stimulation and the lactate concentration for half-maximal stimulation differed considerably from experiment to experiment (Table 1). Small errors in measurement when determining rates can result in rather discrepant values for the parameters of the hyperbolic equation.

The stimulatory effect of lactate on urea production could result from many possible mechanisms. Since urea production from ammonia is an energy-requiring process and since the cells are undoubtedly depleted of many metabolites in the washings during preparation, we thought that the lactate might be serving as an energy source. Since fatty acids should be better than lactate as an energy source for liver (Krebs *et al.*, 1974), this hypothesis was tested by incubating cells with ammonia, ornithine, and either oleate (2mM) or butyrate (5mM). That the fatty acids do enter the

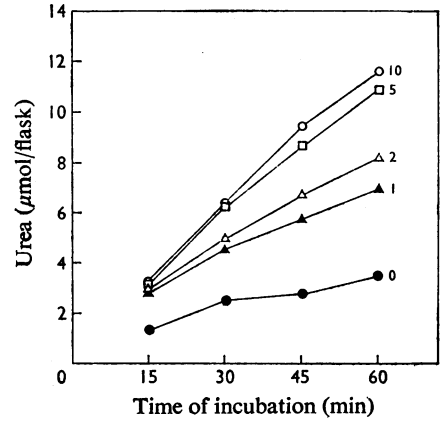

Fig. 1. *Stimulation of urea production rate by lactate*

Cells were incubated with 10mM-NH_4Cl, 10mM-ornithine and lactate at concentrations (mM) as indicated.

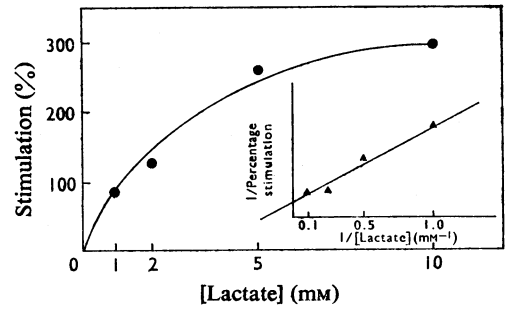

Fig. 2. *Dependence of stimulation of urea production on lactate concentration*

Cells were incubated with 10mM-NH_4Cl, 10mM-ornithine and lactate as indicated. Rates of urea formation were based on 15 and 45min values.

Table 1. *Kinetic constants for the effect of lactate on urea production from ammonia in isolated rat hepatocytes*

Rates of urea production were based on the 15–45 min incubation period (see Fig. 1). All flasks contained 10mM-NH_4Cl. Values are given for the calculated maximum rate of urea production by lactate (V_M) with saturating lactate, expressed as a percentage of the control (no-lactate) rate, the concentration (mM) of lactate that stimulates urea production to give half the maximal increase (K), and the correlation coefficient from fitting data points to the double-reciprocal plot (r).

Ornithine (mM)	V_M	K	r
10	360	1.7	0.999
10	570	5.2	0.996
10	440	4.3	0.982
1	620	1.3	0.996
1	440	0.6	0.985
1	560	1.1	0.984

Table 2. *Effect of various metabolites on urea production from ammonia in isolated hepatocytes*

All flasks originally contained 10mM-NH_4Cl and 10mM-ornithine. The initial concentration of added metabolitef was 10mM, except for butyrate, which was 5mM. Rates of urea formation were based on the 15–45 min incubation.

Additional metabolite	Urea (nmol/min per flask) Expt. 1	Expt. 2
Control	53	89
Lactate	227	298
Pyruvate	162	231
L-Alanine	184	267
L-Glutamine	158	298
L-Asparagine	71	209
L-Aspartate		149
Butyrate		47

cells and are oxidized is evidenced by the accumulation of ketone bodies (Ontko, 1972; O'Donnell & Freedland, unpublished work). In no case did either fatty acid stimulate urea production from ammonia. This is in conflict with the findings reported by Meijer *et al.* (1975), who found that oleate doubled the rate of urea production from ammonia in the presence of ornithine. Nevertheless, our observations suggest that energy is not rate-limiting for urea production in our systems and that the stimulation by lactate is by another mechanism.

Lactate may be enhancing urea production from ammonia by providing substrate for the production of aspartate, which must donate one of the nitrogen groups for the formation of urea. Other potential precursors of oxaloacetate (and therefore of aspartate) were therefore tested. Pyruvate and several

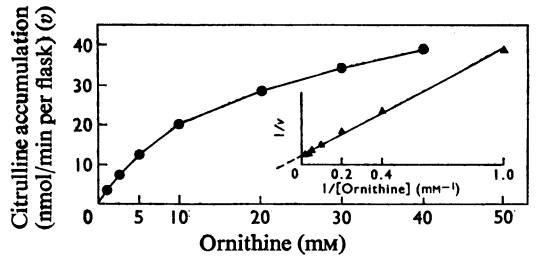

Fig. 3. *Effect of ornithine concentration on accumulation of citrulline*

Cells were incubated with 10mM-NH_4Cl and ornithine as indicated, but without added lactate. Rates of citrulline accumulation are based on 15 and 45 min values.

glucogenic amino acids all stimulated urea production in the presence of ammonia and ornithine, although not equally effectively (Table 2). Permeability of the cell membrane to aspartate is recognized to be greatly limited (Ross *et al.*, 1967), but apparently some is able to enter.

The rate of accumulation of citrulline was linear with time from 15 to 60 min and was related to the amount of ornithine added to the incubation medium. The relationship again appeared to be hyperbolic, with 8–12mM-ornithine stimulating the rate of citrulline accumulation to half the calculated theoretical maximum (Fig. 3). In the first 15 min the rate of citrulline accumulation was lower in the presence of lactate than in its absence, but after 15 min the rate appeared to be independent of the presence or amount of lactate.

Measurements were made on supernatant and cellular material after centrifugation of the incubated cells to determine the relative amounts of citrulline inside and outside the cells. Throughout 1 h of incubation with 10mM-ornithine, about 60% of the citrulline was intracellular. The concentration of citrulline in the flask was approx. 0.6mM after 45 min incubation. With the major part of the citrulline concentrated inside the small volume of cells, the intracellular concentration can be estimated to be in the range of 10–15 μmol/g of tissue, which is very high compared with the citrulline concentration (<0.05 μmol/g) or that of any amino acid *in vivo* (Buttery & Rowsell, 1974).

Though the accumulation of citrulline is dependent mainly on the concentration of added ornithine, it varied somewhat with the gluconeogenic substrate added. With some of the amino acids, particularly glutamine, the rate at which citrulline accumulated was somewhat less than that for the control (Table 3). Perhaps there was some competition between those amino acids and ornithine for entry into the cell. Only with pyruvate did citrulline accumulate at a notably greater rate (about 40%) than in the control

Table 3. *Effect of various metabolites on rate of citrulline accumulation in isolated hepatocyte incubations*

All flasks originally contained 10mM-NH_4Cl and 10mM-ornithine. The initial concentration of added metabolites was 10mM, except for butyrate, which was 5mM. Rates are based on 15 and 45min values.

Additional metabolite	Citrulline (nmol/min per flask) Expt. 1	Expt. 2
Control	30	76
Lactate	31	74
Pyruvate	48	106
L-Alanine	24	84
L-Glutamine	22	60
L-Asparagine	32	86
L-Aspartate		72
Butyrate		81

incubations. We as yet have no explanation for this observation. In no way did pyruvate interfere with the citrulline determinations.

Discussion

The above results show that both ornithine and lactate can stimulate urea production from ammonia. Stimulation of urea synthesis by ornithine has been previously demonstrated by others *in vitro* in liver slices (Krebs & Henseleit, 1932), in the perfused liver system (Hems *et al.*, 1966; Saheki & Katunuma, 1975) and in isolated hepatocytes (Meijer *et al.*, 1975). It has also been observed *in vivo* with ammonia-toxicity studies, when injections of arginine, ornithine or citrulline given to rats before injection with ammonium acetate greatly lowered the mortality rate (Greenstein *et al.*, 1956). From these observations it appears that the concentration of urea-cycle intermediates is one of the limiting factors in the production of urea in the presence of excess of ammonia.

Stimulation of urea synthesis by lactate, on the other hand, is apparent in isolated cell systems but not in the perfused liver (Hems *et al.*, 1966; S. Briggs & R. A. Freedland, unpublished work). In perfusion experiments, variation between animals could mask an effect of lactate, but not one of the magnitude observed in the isolated-cell experiments. More probably, the concentrations of lactate and of other C_3 and C_4 intermediates are greatly lowered in isolated cells as compared with the perfused liver, and the stimulatory effect comes largely from replenishment of these metabolites. Further evidence supporting this idea is the fact that the stimulatory effect of lactate is hardly apparent in cells prepared from non-starved rats, which have a potential source of lactate/pyruvate in stored liver glycogen. Lactate concentrations in rat liver have been reported to range from 1.5 to 2.6μmol/g fresh wt. in fed rats and to be near 1μmol/g in starved rats (Williamson & Brosnan, 1974). The K values (i.e. the concentrations of lactate giving half-maximal stimulation) reported here are of the same order of magnitude as the physiological concentrations of lactate in liver. The lactate present *in vivo* and in perfused liver may be sufficient already to display the major part of its stimulatory effect. Further rises in lactate concentration may have relatively small stimulatory effects that are difficult to distinguish from variation between animals and experimental error.

If the major role of lactate in stimulating urea synthesis lies in providing C_4 intermediates (i.e. aspartate), one should expect the primary control of the urea cycle to lie between citrulline and arginine. Thus the formation of citrulline should not be a rate-limiting factor. The fact that citrulline accumulates in the isolated cell system when ornithine is abundant implies that citrulline formation, and hence carbamoyl phosphate formation, are not limiting. The citrulline accumulation and the low concentrations of succeeding intermediates (argininosuccinate and arginine) (Ratner, 1975; Buttery & Rowsell, 1974) suggest a limitation in the rate of formation of argininosuccinate. Schimke (1962) has shown argininosuccinate synthetase to have the lowest activity of the urea-cycle enzymes. Though the activities of the urea-cycle enzymes depend on the long-term content of dietary protein, with a 15%-casein diet argininosuccinate synthetase had an activity of 2–3μmol/min per g wet wt. of liver. The commercial diet fed to our rats was somewhat higher in protein content than a 15%-casein diet. Our estimate of 1.8μmol of urea/min per g of liver produced by the isolated cells from ammonia in the presence of 10mM-ornithine and 10mM-lactate is very near the maximum possible rate, according to Schimke's (1962) values. Therefore, under our best conditions, argininosuccinate synthetase activity may be limiting the rate of urea production in our system. The fact that flux through the pathway is increased when lactate is present, however, indicates that the limited capacity of this enzyme is not responsible for the lower rate in the control flasks.

A more likely explanation for the low control rate of urea formation and for the accumulation of citrulline is a limitation on the availability of aspartate in the cytosol to condense with citrulline. Other investigators have suggested the importance of cytosolic aspartate in urea synthesis (Stubbs & Krebs, 1975). Enzymes for the formation of aspartate from oxaloacetate exist in both cytosol and mitochondria, but regardless of the compartment in which transamination takes place, it is in the mitochondria that C_4 intermediates are synthesized from the various glucogenic substrates. This creates the need for transport systems between mitochondria and cytosol, which may be regulatory in pathways that involve both

compartments, such as urea formation and gluconeogenesis. Other researchers have suggested that when lactate is provided as a substrate for gluconeogenesis, aspartate is the major form of transfer of oxaloacetate from mitochondria, whereas when pyruvate is provided, oxaloacetate is transferred mainly as malate, which contains reducing equivalents needed for continued production of glucose (Krebs *et al.*, 1967; Rognstad & Katz, 1970). A greater efflux of aspartate should be stimulatory to urea production, and our data show that lactate stimulates urea production to a greater extent than does pyruvate.

One would expect that when the limitation at the argininosuccinate synthetase step is diminished or removed (e.g. by providing more aspartate), the concentration of accumulated citrulline would decrease. But, curiously, when urea formation increases, the rate of citrulline accumulation does not decrease (compare control and lactate treatments, Table 3). The greater rate of formation of citrulline (equal to the urea formed plus the citrulline accumulated) when lactate is present implies that the capacities of carbamoyl phosphate synthase and ornithine transcarbamoylase are not limiting in the control, but the unchanging rate of citrulline accumulation suggests the existence of some form of negative feedback on one or other, or both, of these enzymes. Carbamoyl phosphate synthase would be the more likely step for feedback regulation, since ornithine transcarbamoylase is reversible.

Carbamoyl phosphate synthase has been shown to have an allosteric cofactor, *N*-acetylglutamate (Cohen & Sallach, 1961), the concentration of which has been found in mitochondria to be near or below its activation constant for the enzyme (Shigesada & Tatibana, 1971; McGivan *et al.*, 1976). The concentration of *N*-acetylglutamate, which has been shown to change in various dietary conditions (Shigesada & Tatibana, 1971; McGivan *et al.*, 1976), could therefore alter carbamoyl phosphate synthase activity, and hence carbamoyl phosphate and citrulline formation. Conceivably, some of the glucogenic substances tested in our experiments could cause increased synthesis of *N*-acetylglutamate and thus stimulate carbamoyl phosphate synthase, and this might account for the greater total citrulline formation observed in some cases in the presence of these substances as compared with the control. However, since the final citrulline concentration is not notably different after the addition of lactate, and since citrulline does accumulate over time, again it appears that citrulline formation and hence carbamoyl phosphate formation are not rate-limiting.

From the present work and that of others, it appears that there are two primary limitations on the production of urea from ammonia in isolated liver cells. These can be overcome by the addition of urea-cycle intermediates such as ornithine and by addition of certain glycolytic or tricarboxylic acid-cycle intermediates, probably needed for the synthesis of aspartate. But to understand the regulation of urea production, many questions remain to be answered, including those concerning why various C_3 and C_4 intermediates display different stimulatory effects on urea and citrulline production.

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References

Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520

Briggs, S. & Freedland, R. A. (1975) *Physiologist* **18**, 152

Buttery, P. J. & Rowsell, E. V. (1974) *Comp. Biochem. Physiol. B* **47**, 473–483

Cohen, P. P. & Sallach, H. J. (1961) in *Metabolic Pathways* (Greenberg, D., ed.), vol. 2, pp. 1–66, Academic Press, New York

Foster, L. B. & Hochholzer, J. M. (1971) *Clin. Chem.* **17**, 921–925

Freedland, R. A. & Sodikoff, C. H. (1962) *Proc. Soc. Exp. Biol. Med.* **109**, 349–396

Greenstein, J. P., Winitz, M., Gullino, P., Birnbaum, S. M. & Otey, M. C. (1956) *Arch. Biochem. Biophys.* **64**, 342–354

Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292

Kramer, J. W. & Freedland, R. A. (1972) *Proc. Soc. Exp. Biol. Med.* **141**, 833–835

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Krebs, H. A., Gascoyne, T. & Notton, B. M. (1967) *Biochem. J.* **102**, 275–282

Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) *Proc. Alfred Benzon Symp. 6th* 549–564

McGivan, J. D., Bradford, N. M. & Mendes-Mourão, J. (1976) *Biochem. J.* **154**, 415–421

Meijer, A. J., Gimpel, J. A., Deleeuw, G. A., Tager, J. M. & Williamson, J. R. (1975) *J. Biol. Chem.* **250**, 7728–7738

Ontko, J. A. (1972) *J. Biol. Chem.* **247**, 1788–1800

Ratner, S. (1954) *Adv. Enzymol. Relat. Subj. Biochem.* **15**, 319–387

Ratner, S. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol.* **39**, 1–90

Ratner, S. (1975) *Anal. Biochem.* **63**, 141–155

Rognstad, R. & Katz, J. (1970) *Biochem. J.* **116**, 483–491

Ross, B. D., Hems, R. & Krebs, H. A. (1967) *Biochem. J.* **102**, 942–951

Saheki, T. & Katunuma, N. (1975) *J. Biochem. (Tokyo)* **77**, 659–669

Schimke, R. T. (1962) *J. Biol. Chem.* **237**, 459–468

Shigesada, K. & Tatibana, M. (1971) *J. Biol. Chem.* **246**, 5588–5595

Stubbs, M. & Krebs, H. A. (1975) *Biochem. J.* **150**, 41–45

Williamson, D. H. & Brosnan, J. T. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. A., ed.), vol. 4, pp. 2266–2302, Academic Press, New York, San Francisco and London