NADP-specific isocitrate dehydrogenase in regulation of urea synthesis in rat hepatocytes

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The effect of inhibition of NADP-specific isocitrate dehydrogenase (EC 1.1.1.42) by DL-threo- α -methylisocitrate (3-hydroxy-1,2,3-butanetricarboxylate) on urea synthesis was studied in isolated rat hepatocytes. α -Methylisocitrate substantially inhibited the rate of urea synthesis (35–84%) with substrates requiring net reductive amination of 2-oxoglutarate to glutamate for aspartate synthesis (i.e., L-serine, D-alanine, or NH_4Cl + L-lactate). α -Methylisocitrate did not inhibit synthesis of urea from substrates not requiring reductive formation of glutamate (i.e. L-alanine, L-glutamine, L-asparagine, or NH_4Cl + L-ornithine). The rate-limiting role of NADPH in urea synthesis was correlated with the decrease in NADPH content that occurred upon addition of NH_4Cl or of α -methylisocitrate to hepatocytes incubated with lactate and pyruvate, indicating utilization of NADPH for reductive amination of 2-oxoglutarate and inhibition of NADPH generation via NADP-isocitrate dehydrogenase, respectively. Similar results were obtained with D-alanine and L-serine; however, α -methylisocitrate or NH_4Cl did not substantially decrease NADPH content when L-alanine was the substrate. Inhibitors of ornithine–2-oxo acid transaminase (L-canaline or gabaculine) decreased the uptake of ornithine by hepatocytes and inhibited the α -methylisocitrate-insensitive urea synthesis from ornithine and NH_4Cl . Canaline did not inhibit urea synthesis from lactate, ornithine, and NH_4Cl but the inhibition by α -methylisocitrate of urea formation from this combination was appreciably larger with canaline (approx. 82%) than without canaline (approx. 48%). Inhibition of urea synthesis from NH_4Cl + lactate by α -methylisocitrate was partially prevented by oleate, octanoate, or 3-hydroxybutyrate. When the NADH content of hepatocytes was increased by 3-hydroxybutyrate, the addition of NH_4Cl and/or α -methylisocitrate caused a decline in NADH (and NADPH) content, suggesting that reducing equivalents from NADH as well as from NADPH can support net reductive amination of 2-oxoglutarate when required for urea synthesis.

Glutamate dehydrogenase plays an important role in urea synthesis. The direction of nitrogen flux can proceed either toward glutamate oxidation for synthesis of carbamoyl phosphate, or toward reductive amination of 2-oxoglutarate for aspartate synthesis, depending on the substrate (Braunstein, 1957). The role of glutamate dehydrogenase activity as the predominant factor in ammoniogenesis for urea synthesis in rat liver has been questioned (McGivan *et al.*, 1973; Chappell *et al.*, 1976). It has been proposed that deamination of aspartate via the purine nucleotide cycle may be the primary pathway for NH_3 formation from those amino acids (e.g.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

L-alanine) requiring initial transamination with 2-oxoglutarate (McGivan, 1976). However, incorporation of ^{15}N into the 6-amino group of adenine nucleotides isolated from rat hepatocytes incubated with [^{15}N]alanine does not appear sufficient to account for substantial NH_3 formation by the purine nucleotide cycle (Krebs *et al.*, 1978).

Incorporation of nitrogen from NH_3 into aspartate for urea synthesis requires reductive amination of 2-oxoglutarate to form glutamate, followed by transamination with oxaloacetate to form aspartate. Identification of the major source(s) of reducing equivalents for reductive amination of 2-oxoglutarate is complex. Either NADH or NADPH can serve as a co-substrate for mammalian glutamate

dehydrogenase; the rates of reaction differ depending on the concentrations of positive and negative modulators of the enzyme (e.g. ADP, ATP, GDP and GTP). The effects of these factors on glutamate dehydrogenase activity in the cell are not clear (Goldin & Frieden, 1971). Studies by Sies and coworkers have shown that oxidation of NADPH by added *t*-butyl hydroperoxide decreased urea synthesis from NH_4Cl by 50% in perfused liver (Sies *et al.*, 1974) and in isolated hepatocytes (Sies *et al.*, 1975). They attributed this to the decline in NADPH content causing decreased reductive amination of 2-oxoglutarate, a step required to supply aspartate for urea synthesis. The oxidation of NADPH by *t*-butyl hydroperoxide occurs via the NADP-specific glutathione peroxidase–glutathione reductase system which is located in both the cytosolic and mitochondrial compartments of the liver cell. Since glutamate dehydrogenase is located exclusively in mitochondria (Hogeboom & Schneider, 1953) it became desirable to identify the mitochondrial source of reducing equivalents that could supply NADPH for reductive amination of 2-oxoglutarate. It seemed possible that a significant amount could be derived from isocitrate via NADP-specific isocitrate dehydrogenase. Earlier studies with intact rat liver mitochondria showed that the addition of α -methylisocitrate, which inhibits NADP-specific isocitrate dehydrogenase but not the NAD-linked enzyme (Plaut *et al.*, 1975), inhibited glutamate formation from added isocitrate and NH_4Cl by about 50% under aerobic or anaerobic conditions (Smith *et al.*, 1975; Plaut & Smith, 1977; Smith & Plaut, 1979). In the present investigation the effect of α -methylisocitrate on urea synthesis from NH_4Cl and from certain amino acids was studied in isolated hepatocytes to determine the importance of the flux from internally generated isocitrate through NADP-specific isocitrate dehydrogenase to glutamate and, hence, urea formation. A preliminary report of these studies has appeared (Petcu & Plaut, 1979).

Experimental

Materials

Amino acids, lithium L-lactate, DL-gabaculine and Hepes were A grade from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.). The cation exchange resin AG 50W (X12; 200–400 mesh) was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). *N*-Carbamoyl-L-glutamic acid from I.C.N.—K. & K. (Plainview, NY, U.S.A.) was found to be free of glutamic acid in several systems of thin layer chromatography. The following were purchased from the supplier indicated: bovine serum albumin (Pentex fraction V, fatty acid free) from Miles Biochemicals (Elkhart, IN, U.S.A.), Hanks balanced salt solution (free of calcium and Phenol Red) from

Grand Island Biological Co. (Grand Island, NY, U.S.A.), bovine glutamate dehydrogenase from Boehringer-Mannheim (Indianapolis, IN, U.S.A.), collagenase (from *Clostridium histolyticum*), yeast glucose 6-phosphate dehydrogenase and yeast alcohol dehydrogenase from Worthington Biochemicals (Freehold, NJ, U.S.A.), urease (type III), deoxycholate, monosodium 2-oxoglutarate, L-canadine, β -NADH and β -NADPH from Sigma Chemical Company (St. Louis, MO, U.S.A.). All reagent grade inorganic salts were from Mallinckrodt Inc. (St. Louis, MO, U.S.A.).

DL-*threo*- α -Methylisocitric lactone was prepared by the method of Plaut *et al.* (1975). Before use, it was hydrolysed to the free acid by heating in 1.7M-NaOH at 100°C, followed by cooling and neutralization with 3M-HCl to pH 7.4.

Isolation of hepatocytes

Male Sprague–Dawley rats (180–200 g) were fed on Purina chow for at least one week and were starved for 48 h before the isolation of cells. Hepatocytes were isolated by a modification of the method of Berry & Friend (1969). The isolated liver was first perfused via a cannula in the vena cava with 200 ml of medium containing Hanks balanced salt solution supplemented with 10 mM-MgSO₄, 1 mg of bovine serum albumin/ml, 0.5 mM-EGTA, 25 mM-Hepes (sodium salt) at pH 7.4, and 0.2 mM-L-methionine (to slow glutathione loss) (Viña *et al.*, 1978) at a rate of 20 ml/min at 35°C. This was followed by perfusion at the same rate with 150 ml of the above medium without EGTA, but containing 5 mM-CaCl₂ and collagenase (90 units/ml). The liver was then gently minced in 20 ml of the collagenase-containing medium, the resulting suspension was passed sequentially through a 253 μm and a 64 μm screen nylon filter at 4°C, the filtrate was brought to 40 ml with cold wash medium (which had the same composition as the initial perfusion medium, except that EGTA was omitted) and centrifuged at about 60 g in a swinging bucket rotor for 1 min. The supernatant fluid was discarded and the pellet was washed three times at 4°C by suspension in 25 ml each time of the cold wash medium, followed by centrifugation. The final volume of the cell suspension was adjusted to 40 ml.

Cell viability was determined by the Trypan Blue exclusion test (Seglen, 1976). Measurement of the cells by this method after isolation and before incubation indicated an average of 96% viability initially; an average of 85% viability was found after 2 h of incubation. Under our conditions, the cell count showed consistent agreement with measurements of the protein content by the method of Gornall *et al.* (1949) (1.05 mg of protein/10⁶ cells) and the DNA content as determined by the method of Burton (1956) (8.2 μg of DNA/10⁶ cells). Rates of

urea (or citrulline) synthesis are expressed as nmol of urea formed/min per mg of protein.

Incubation of cells and determination of urea

Cells were incubated in 250ml polypropylene or polycarbonate Erlenmeyer flasks fitted with a gassing manifold in a rotary shaker water bath (100rev./min) at 35°C. Media contained 20ml of Krebs-Henseleit (1932) bicarbonate buffer supplemented with 11.8mM-MgSO₄, 25mM-Hepes (sodium salt) at pH 7.4, 1mg of bovine serum albumin/ml, 2mM-L-ornithine, substrates and inhibitors as specified in the text, and hepatocytes at 0.95–1.4mg of protein/ml of incubation medium (0.9×10^6 – 1.3×10^6 cells/ml). Before the addition of cells, the medium was equilibrated with humidified O₂/CO₂ (19:1, v/v) at 0.625 litre/min per flask for 1h at 35°C. The reaction was started by the addition of cell suspension and the gas atmosphere was maintained throughout the incubation period. Portions (1.0ml) of incubation medium were removed at zero time and at the incubation times indicated and were added to chilled centrifuge tubes containing 0.10ml of 3M-HClO₄, kept on ice for 10min and centrifuged.

The sum of urea and citrulline contents was determined in the deproteinized supernatant fluids by the method of Archibald (1945). To distinguish between the contents of urea and citrulline, urea was determined separately by a urease assay as follows. The deproteinized fluid was neutralized with 3M-KOH, and KClO₄ was removed by centrifugation in the cold. To remove NH₄⁺, the supernatant fluid (0.5ml) was diluted to 2.0ml with water and passed through a column (0.5cm × 4cm) of AG 50W (X12; H⁺ form) followed by 5.0ml of water. A portion of the effluent (1.0ml) was adjusted to pH 6.0 and incubated with 5U of urease for 20min at 25°C. The NH₃ formed was liberated by diffusion in a Conway cell and was determined by the method of Chaney & Marbach (1962).

Ornithine was determined by the ninhydrin method of Shimura & Vogel (1966). Coupled enzymic assays were used for the determination of aspartate, glutamate, or glutamine (Williamson & Corkey, 1969).

Determination of nicotinamide nucleotides in hepatocytes

The concentrations of NADH and NADPH were determined in samples deproteinized with ethanolic (11%, w/v) KOH followed by neutralization with triethanolamine hydrochloride; NAD⁺ and NADP⁺ were assayed in samples deproteinized with HClO₄ followed by neutralization with KOH. The fluorimetric enzymatic assays of Estabrook *et al.* (1967) and of Williamson & Corkey (1969) were used. NADH and NADPH were determined differentially

by measurement of the decline of reduced nicotinamide nucleotide fluorescence after addition of acetaldehyde and alcohol dehydrogenase followed by 2-oxoglutarate, NH₄Cl and glutamate dehydrogenase. NAD⁺ and NADP⁺ were estimated by measuring the increase in nicotinamide nucleotide fluorescence after addition of ethanol, hydrazine and alcohol dehydrogenase, or of glucose 6-phosphate and glucose 6-phosphate dehydrogenase, respectively.

Analysis of data

The rates of net urea formation/min per mg of protein obtained at various incubation times for each medium were averaged. The standard deviations from the mean were calculated for velocities in the absence (v_0) and presence (v_i) of α -methylisocitrate. Thus, S.D. (v_i) and S.D. (v_0) are the standard deviations of n determinations of v_i and v_0 , respectively. When the data were plotted as v_i/v_0 versus the reciprocals of α -methylisocitrate concentrations (see the Results section) the error bars at each α -methylisocitrate concentration refer to error in v_i/v_0 [$\varepsilon(v_i/v_0)$]. Therefore:

$$\varepsilon(v_i/v_0) = \text{abs.} \left[\frac{\text{S.D.} (v_i)}{v_i} - \frac{\text{S.D.} (v_0)}{v_0} \right]$$

The statistical variations for the v_i/v_0 values at infinite α -methylisocitrate concentration were estimated graphically from the average of the minimal and maximal values of the extrapolated intercepts of lines drawn at minimum and maximum slopes through the error bars of the data points. Means and variations of these results are represented as percentage inhibitions in Table 1 and Table 3.

The nicotinamide nucleotide values (Table 2) and rates of urea formation in the absence of α -methylisocitrate (v_0) (Table 1 and Table 3) were the means of a number of experiments $\pm$ the error determined at the $P \leq 0.05$ level of significance from the pooled data.

Results

The formation of urea with time was studied with a number of substrates in the absence and presence of varying concentrations of α -methylisocitrate. Urea formation from NH₄Cl and L-lactate is shown in Fig. 1. Urea accumulation was linear with time (all values are corrected for urea content at zero time); the addition of up to 1000 μ M- α -methylisocitrate progressively inhibited urea production. A linear relationship between product formation and time was also observed with other substrates tested. The average rates of urea formation at different time points were replotted as a function of the ratios (v_i/v_0) of velocity in the presence (v_i) and absence

Fig. 1. Urea synthesis at various α -methylisocitrate concentrations as a function of incubation time

The conditions of incubation of hepatocytes were as described in the Experimental section. The incubation media contained 5.4 mM- NH_4Cl , 6.0 mM-L-lactate, and 2.0 mM-L-ornithine. The concentrations of α -methylisocitrate are: $\circ$, zero; $\square$, 200 μM ; $\triangle$, 333 μM ; $\bullet$, 500 μM ; $\blacksquare$, 1000 μM . The lines were fitted to the data by the method of least squares.

(v_0) of α -methylisocitrate (Fig. 2). The inhibition of NADP-specific isocitrate dehydrogenase by α -methylisocitrate is competitive with respect to isocitrate (Plaut *et al.*, 1975) and a plot of v_i (or v_i/v_0) versus the reciprocal of inhibitor ($1/I$) usually would not be linear. However, these plots approach linearity when the ratio K_m/K_i is large [K_m for isocitrate/ K_i for α -methylisocitrate is 140–185 for NADP-specific isocitrate dehydrogenase (Plaut *et al.*, 1975)] and the concentration of inhibitor is relatively large (the concentration of α -methylisocitrate added here was 200–1000 μM) compared with K_i (0.1 μM for α -methylisocitrate in the presence of Mg^{2+}). As derived previously (Smith & Plaut, 1979), under such conditions the equation for competitive inhibition can be approximated by:

$$v_0/v_i = K_m[I]/K_iA$$

Deviations from linearity of the plots could occur if different concentrations of α -methylisocitrate (I) caused substantial changes in mitochondrial isocitrate (A) concentrations in hepatocytes. However, when isocitrate was generated internally from pyruvate and malate with isolated liver mitochondria, and α -methylisocitrate was at a matrix concentration leading to 99.99% inhibition of NADP-isocitrate dehydrogenase, only a small

Fig. 2. Effect of α -methylisocitrate on synthesis of urea from ammonia

The conditions of incubation were as in Table 1. The incubation media contained 2.0 mM-L-ornithine, 5.4 mM- NH_4Cl with (●) 50 mM-glucose or (○) 6.0 mM-L-lactate. Relative velocity = v_i/v_0 , where v_i and v_0 are the velocities in the presence and absence respectively of the inhibitor.

change of D-isocitrate content (40–60 μM) was found in the total mitochondrial space (Smith & Plaut, 1979). When v_i/v_0 is plotted against $1/[I]$, the intercept at $1/[I] = 0$ occurs at the relative rate of urea synthesis (v_i/v_0) where NADP-specific isocitrate dehydrogenase should be completely inhibited. Thus, when urea synthesis is completely dependent on NADP-specific isocitrate dehydrogenase activity, $v_i/v_0 = 0$, and $v_i/v_0 = 1$ when urea formation is not affected by α -methylisocitrate. The utility of the method of estimation is demonstrated by the linearity of plots of $1/[I]$ versus v_i/v_0 whenever α -methylisocitrate inhibited urea synthesis (Fig. 2 and Fig. 3). Values for percentage inhibition of urea synthesis at infinite α -methylisocitrate concentration $\{[1 - (v_i/v_0)] \times 100\}$ for several substrates are reported in Tables 1 and 3.

NH_4Cl as substrate

Urea synthesis from NH_4Cl (5.4 mM) in the presence of 2 mM-L-ornithine was examined with L-lactate (6 mM) or D-glucose (50 mM) as the energy source. In the absence of α -methylisocitrate, the rates of urea formation from NH_4Cl were about the same with L-lactate or glucose (Table 1). α -Methylisocitrate inhibited urea production from both (Fig. 2), but the inhibition was somewhat larger with L-lactate (49%) than with glucose (38%) (Table 1).

Combinations of NH_4Cl with lactate and pyru-

Fig. 3. Effect of α -methylisocitrate on synthesis of urea from L-alanine and L-serine

The conditions of incubation were as in Fig. 2. The incubation media contained 2.0 mM-L-ornithine and (●) 50 mM-L-alanine or (○) 50 mM-L-serine + 5.4 mM-NH $_4$ Cl. Relative velocity was calculated as in Fig. 2. Values of v_0 were 5.8 ± 0.9 and 8.8 ± 0.7 nmol/min per mg of protein for L-alanine and L-serine, respectively.

vate as used previously by other investigators (Krebs *et al.*, 1976) did not give a larger yield of urea than NH $_4$ Cl + L-lactate alone under our conditions, and the extent of inhibition by α -methylisocitrate was the same with all of these substrates (cf. Table 3). However, the further addition of substrates that increase the cellular reduction state decreased the inhibition of urea production by α -methylisocitrate from NH $_4$ Cl + L-lactate. At infinite α -methylisocitrate concentration the inhibition of urea formation from NH $_4$ Cl + lactate alone was about 49%. The inhibition decreased to about 35, 25, and 24% upon the further addition of oleate (1 mM), octanoate (2 mM), or DL-3-hydroxybutyrate (20 mM), respectively (Table 1). These substances did not cause a substantial increase in rate of urea synthesis in the absence of α -methylisocitrate (Table 1) (cf. Krebs *et al.*, 1976; Meijer *et al.*, 1975) and hence the decreased inhibition by α -methylisocitrate cannot be attributed to this factor. The effect of 3-hydroxybutyrate especially would indicate that this decrease of α -methylisocitrate inhibition is due to increase concentrations of reduced nicotinamide nucleotides, since the only oxidation product is acetoacetate, which cannot be metabolized further

Table 1. Effect of α -methylisocitrate on the synthesis of urea from various substrates

The conditions of incubation are described in the Experimental section; all incubation mixtures contained 2 mM-L-ornithine. The numbers in parentheses refer to the numbers of determinations. Values are means $\pm$ errors as described in the Experimental section. Inhibition at infinite α -methylisocitrate concentration was calculated as $[1 - (v_i/v_0)] \times 100$

Additions	Velocity (v_0) in absence of α -methylisocitrate (nmol urea/min per mg of protein)	Inhibition at infinite α -methylisocitrate concentration (%)
5.4 mM-NH $_4$ Cl + 6 mM-L-lactate (20)	14.9 ± 0.8	49 ± 2
5.4 mM-NH $_4$ Cl, 6 mM-L-lactate + 20 mM-N-carbamoylglutamate (5)	28.7 ± 2.6	47 ± 3
5.4 mM-NH $_4$ Cl, 6 mM-L-lactate + 1 mM-oleate (11)	$15.8 \pm 1.3^*$	35 ± 1
5.4 mM-NH $_4$ Cl, 6 mM-L-lactate + 2 mM-octanoate (11)	15.7 ± 1.1	25 ± 1
5.4 mM-NH $_4$ Cl, 6 mM-L-lactate + 20 mM-DL-3-hydroxybutyrate (12)	17.9 ± 1.4	24 ± 2
5.4 mM-NH $_4$ Cl, 6 mM-L-lactate, 20 mM-DL-3-hydroxybutyrate + 20 mM-methylmalonate (12)	15.0 ± 1.3	46 ± 4
5.4 mM-NH $_4$ Cl + 50 mM-D-glucose (15)	$17.6 \pm 2.6^*$	38 ± 3
50 mM-L-Serine (15)	5.0 ± 0.3	35 ± 4
50 mM-D-Alanine + 5.4 mM-NH $_4$ Cl (15)	$2.4 \pm 0.4^*$	47 ± 5
50 mM-L-Alanine (20)	5.8 ± 0.9	0 ± 4
50 mM-L-Alanine + 5.4 mM-NH $_4$ Cl (10)	16.2 ± 1.2	2 ± 8
50 mM-L-Alanine + 20 mM-N-carbamoylglutamate (10)	7.3 ± 0.3	5 ± 7
40 mM-L-Asparagine (10)	5.4 ± 0.3	6 ± 8
40 mM-L-Asparagine + 6 mM-L-lactate (5)	5.8 ± 0.7	3 ± 5
40 mM-L-Asparagine + 5.4 mM-NH $_4$ Cl (5)	12.8 ± 2.6	0 ± 3
40 mM-L-Glutamine (10)	14.4 ± 1.1	0 ± 11

* Citrulline was also determined in these experiments. The accumulation of citrulline was 2–4% of that reported here for urea.

by liver mitochondria. Addition of methylmalonate, an inhibitor of 3-hydroxybutyrate dehydrogenase (Tan *et al.*, 1975), to hepatocytes completely reversed the effect of added 3-hydroxybutyrate, restoring the inhibition of urea formation by α -methylisocitrate to the level observed when NH_4Cl and L-lactate only were the substrates (Table 1).

Effect of α -methylisocitrate on urea formation from serine and alanine

Urea synthesis from L-serine or D-alanine was inhibited significantly by α -methylisocitrate (Table 1). In contrast, there was essentially no inhibition by α -methylisocitrate when L-alanine was the substrate (Table 1). This is particularly apparent in Fig. 3, where the effects of varying concentration of α -methylisocitrate on urea production from L-serine and L-alanine are compared. With all of these substrates the rates of urea formation without α -methylisocitrate were lower than with NH_4Cl + L-lactate (Table 1). It seemed possible that the intracellular supply of energy-yielding intermediates with these amino acids was rate-limiting. However, the further addition of lactate or pyruvate did not increase urea production or change the extent of inhibition by α -methylisocitrate.

With L-alanine as the substrate the addition of NH_4Cl increased the rate of urea formation to the

Fig. 4. Stimulation of urea synthesis by *N*-carbamoyl-L-glutamate in hepatocytes

The conditions of incubation are described in the Experimental section. The incubation mixtures contained 2.0 mM-L-ornithine, 5.4 mM- NH_4Cl and 6.0 mM-L-lactate, and the concentrations of *N*-carbamoyl-L-glutamate as indicated. The results are plotted as a function of the reciprocals of *N*-carbamoyl-L-glutamate concentration and of $(v - v_0)$, where v and v_0 are the velocities (nmol urea/min per mg of protein) in the presence (v) and absence (v_0) of *N*-carbamoyl-L-glutamate. K_m was calculated from a program for a rectangular hyperbola that does not intersect the ordinate at zero (Cleland, 1967).

level found with NH_4Cl + L-lactate. However, as with L-alanine alone, essentially no inhibition by α -methylisocitrate was obtained with L-alanine + NH_4Cl (Table 1). Thus, increased rate of urea production *per se* could not be correlated with the effect of α -methylisocitrate. These results suggested that, when L-alanine was the substrate, the intracellular concentration of NH_4^+ was rate-limiting for urea synthesis. This was supported by the finding that *N*-carbamoyl-L-glutamate (20 mM) enhanced the rate of urea synthesis from L-alanine, alone in hepatocytes by about 26% without increasing the susceptibility to α -methylisocitrate inhibition (Table 1). *N*-Carbamoyl-L-glutamate, a structural analogue of *N*-acetyl-L-glutamate, is a positive modulator of carbamoyl phosphate synthetase (Grisolia & Cohen, 1953). *N*-Carbamoyl-L-glutamate is less susceptible to enzymic hydrolysis than the natural allosteric effector, and it has been found to decrease NH_3 toxicity in rats, apparently by increasing urea synthesis at the level of carbamoyl phosphate synthetase (Kim *et al.*, 1972). The effectiveness of *N*-carbamoyl-L-glutamate in increasing urea synthesis in hepatocytes could also be shown with NH_4Cl (5.4 mM) and L-lactate (6 mM) as substrates (Fig. 4). $K_{m \text{ app.}}$ for *N*-carbamoyl-L-glutamate was estimated to be about 3.9 mM in this experiment, which compares with $K_{m \text{ app.}}$ of 1.6 mM for a soluble carbamoyl phosphate synthetase from rat liver determined at pH 7.4 with 50 mM- NH_4Cl and 100 mM- KHCO_3 (Kennedy & Grisolia, 1965). However, the percentage inhibition by α -methylisocitrate of urea synthesis from NH_4Cl + L-lactate in hepatocytes was the same in the absence and presence of *N*-carbamoyl-L-glutamate (20 mM) (Table 1).

Urea synthesis from L-asparagine and L-glutamine

α -Methylisocitrate did not inhibit urea formation from either glutamine or asparagine in isolated hepatocytes (Fig. 5 and Table 1). The rate of urea synthesis from glutamine was 2–3-fold larger than from asparagine under our incubation conditions. This compares with about equal rates of urea formation from these substrates under the conditions used by Krebs *et al.* (1976). The rate of urea synthesis from asparagine was not stimulated by the addition of lactate, but the addition of NH_4Cl more than doubled the rate of urea formation with asparagine. Supplementation with either lactate or NH_4Cl did not alter the lack of α -methylisocitrate inhibition of urea synthesis from asparagine (Table 1).

Effect of α -methylisocitrate and NH_4Cl on NADH/NAD⁺ and NADPH/NADP⁺ ratios

The inhibition by α -methylisocitrate on urea synthesis from certain substrates in isolated hepa-

Fig. 5. Urea synthesis from L-glutamine or L-asparagine with various concentrations of α -methylisocitrate. The conditions of incubation were as in Fig. 3. The incubation media contained 2.0 mM-L-ornithine and (○) 40 mM-L-asparagine or (●) 40 mM-L-glutamine, and α -methylisocitrate as indicated. Relative velocity was calculated as in Fig. 2.

tocytes (Figs. 1–3) and previous studies of the effect of α -methylisocitrate on glutamate formation from isocitrate and NH_4Cl by isolated rat liver mitochondria (Smith & Plaut, 1979) suggested that this compound, by inhibiting NADP-specific isocitrate dehydrogenase, decreased the supply of reduced nicotinamide nucleotides necessary for reductive amination of 2-oxoglutarate.

The effect of α -methylisocitrate and of NH_4Cl on nicotinamide nucleotide contents was studied in a medium supplemented with 2 mM-L-ornithine, 5 mM-L-lactate and 1 mM-pyruvate. Comparison of the nicotinamide nucleotide contents of hepatocytes at the beginning and the end of the incubations showed no significant loss of total NAD and NADP attributable to incubation with any of the materials added (Table 2). The hepatocyte contents for total NAD at the beginning and the end of incubation were 879 ± 9 and 868 ± 7 nmol/g wet wt. of cells, respectively; the corresponding values for total NADP were 295 ± 13 and 286 ± 18 nmol/g wet wt. of cells respectively after 30 min incubations. These values compare with a total nicotinamide nucleotide content of freeze-clamped liver from fed rats of 660 nmol/g wet wt. for NAD and 377 nmol/g wet wt. for NADP (Bücher & Sies, 1976). When compared with hepatocytes incubated with lactate, pyruvate and ornithine, the further addition of NH_4Cl caused essentially no change in the NADH/NAD $^+$ ratio, but resulted in a marked fall (about

30%) of NADPH content (Table 2). These observations on the effects of NH_4Cl are in accord with those of Sies *et al.* (1975). The addition of α -methylisocitrate to the incubation mixture resulted in no significant change in the cell NADH/NAD $^+$ ratio (0.11) but caused a substantial decline in NADPH concentration when compared with the incubated control (about 45%); NADPH contents decreased even further (about 62%) when the hepatocytes were incubated with a combination of NH_4Cl and α -methylisocitrate (Table 2).

The addition of 3-hydroxybutyrate decreased the inhibition by α -methylisocitrate of urea synthesis from NH_4Cl and lactate (Table 1). This effect could be due to increasing the supply of reduced nicotinamide nucleotides for reductive amination of 2-oxoglutarate by way of the 3-hydroxybutyrate dehydrogenase reaction; the latter is not inhibited by α -methylisocitrate (Smith & Plaut, 1979). The increased hepatocyte content of NADH + NADPH upon incubation with 3-hydroxybutyrate (675 nmol/g wet wt.) compared with the incubated control (339 nmol/g wet wt.) is consistent with this possibility; however, the increased reduced nicotinamide nucleotide content resulting from 3-hydroxybutyrate addition was entirely due to the rise in NADH (Table 2). The addition of methylmalonate, which inhibits NAD-specific 3-hydroxybutyrate dehydrogenase and counteracts the effect of 3-hydroxybutyrate on α -methylisocitrate inhibition of urea synthesis (Table 1), lowered NADH to about the same level as without added 3-hydroxybutyrate but did not affect the concentration of NADPH (Table 2). In the presence of 3-hydroxybutyrate, the additions of NH_4Cl , α -methylisocitrate, and NH_4Cl + α -methylisocitrate lowered the NADPH content by about 29%, 38%, and 48% respectively (compared with the control containing 3-hydroxybutyrate). The decreases in NADPH contents when α -methylisocitrate or α -methylisocitrate + NH_4Cl was added were somewhat smaller with than without 3-hydroxybutyrate (Table 2). Whereas no changes in NADH content could be detected when NH_4Cl and α -methylisocitrate were added without 3-hydroxybutyrate, these additions resulted in significant decreases of NADH content (20–40%) in the presence of 3-hydroxybutyrate (Table 2).

Contribution of ornithine to urea synthesis

In studies of urea synthesis by hepatocytes, ornithine is usually added to incubation mixtures since it enhances urea synthesis from nitrogenous compounds (see Krebs *et al.*, 1976; Briggs & Freedland, 1977). The addition of ornithine also increased urea synthesis under our incubation conditions. For example, the rate of urea formation from NH_4Cl + lactate (expt. A) or NH_4Cl , lactate and pyruvate (expt. B) was twice as large in the

Table 2. *Effect of NH_4Cl and α -methylisocitrate on the nicotinamide nucleotide content of hepatocytes*

Hepatocytes (3.9–6.0 mg of protein/ml) were incubated for 30 min under the conditions described in Table 1, except that in the experiments of series A all flasks contained 2 mM-L-ornithine, 5 mM-L-lactate and 1 mM-pyruvate. In series B, lactate and pyruvate were omitted when L-alanine (50 mM), L-serine (50 mM) or D-alanine (50 mM) were used as the substrates. If specified, 1000 μM - α -methylisocitrate, 5.4 mM- NH_4Cl , 20 mM-DL-3-hydroxybutyrate, or 10 mM-methylmalonate were added. Nicotinamide nucleotides were determined at the beginning and at the end of the incubation period as described in the Experimental section. The number of experiments with a given substrate combination is indicated in parentheses; an average number of four determinations were done per experiment. The variations from the mean are for $P < 0.05$. Nucleotides were determined at the start of incubation with each substrate; the averages of zero-time values are given for each series of experiments.

Series	Additions	Nicotinamide nucleotide content (nmol/g wet wt. of cells)			
		NADH	NADH + NAD ⁺	NADPH	NADPH + NADP ⁺
A. Zero time		94 $\pm$ 3	879 $\pm$ 9	279 $\pm$ 8	295 $\pm$ 13
	None (13)	87 $\pm$ 7	874 $\pm$ 8	252 $\pm$ 5	284 $\pm$ 10
	α -Methylisocitrate (8)	86 $\pm$ 9	876 $\pm$ 25	139 $\pm$ 20	284 $\pm$ 17
	NH_4Cl (8)	86 $\pm$ 7	869 $\pm$ 3	177 $\pm$ 17	273 $\pm$ 6
	α -Methylisocitrate + NH_4Cl (13)	93 $\pm$ 8	861 $\pm$ 10	97 $\pm$ 8	295 $\pm$ 17
	3-Hydroxybutyrate (11)	397 $\pm$ 19	863 $\pm$ 23	279 $\pm$ 27	276 $\pm$ 20
	3-Hydroxybutyrate + α -methylisocitrate (10)	284 $\pm$ 9	865 $\pm$ 18	174 $\pm$ 13	272 $\pm$ 5
	3-Hydroxybutyrate + NH_4Cl (11)	316 $\pm$ 31	857 $\pm$ 19	198 $\pm$ 5	313 $\pm$ 31
	3-Hydroxybutyrate, α -methylisocitrate + NH_4Cl (10)	240 $\pm$ 23	879 $\pm$ 23	144 $\pm$ 15	262 $\pm$ 11
	3-Hydroxybutyrate + methylmalonate (2)	113 $\pm$ 4	866 $\pm$ 6	256 $\pm$ 17	311 $\pm$ 0
	3-Hydroxybutyrate, methylmalonate + NH_4Cl (1)	104		165	
B. Zero time		94 $\pm$ 4		279 $\pm$ 8	
	L-Alanine (2)	92 $\pm$ 8		251 $\pm$ 18	
	L-Alanine + NH_4Cl (2)	85 $\pm$ 11		220 $\pm$ 12	
	L-Alanine + α -methylisocitrate (2)	85 $\pm$ 6		219 $\pm$ 14	
	D-Alanine (2)	88 $\pm$ 8		202 $\pm$ 15	
	D-Alanine + α -methylisocitrate (2)	87 $\pm$ 7		141 $\pm$ 14	
	L-Serine	89 $\pm$ 7		201 $\pm$ 12	
	L-Serine + α -methylisocitrate	85 $\pm$ 9		145 $\pm$ 20	

presence as in the absence of ornithine (Table 3). However, the amount of ornithine added (2 mM) might not only raise the intracellular steady state concentration to support optimal citrulline synthesis, but might also serve as a carbon, energy and nitrogen source for urea synthesis.

The contribution of ornithine to the latter processes appears significant, since the rate of urea synthesis from NH_4Cl + ornithine was still about one-third of that with a combination of NH_4Cl , ornithine and lactate (Table 3). These results are in accord with those of Briggs & Freedland (1977) for urea production from these substrates by hepatocytes from starved rats. Urea formation from ornithine + NH_4Cl was not inhibited appreciably by α -methylisocitrate (Table 3). The initial steps of ornithine metabolism involve its transamination to glutamic semialdehyde and dehydration to Δ^1 -pyrroline-5-carboxylate; the latter is oxidized to glutamate (Strecker, 1960). The addition of L-canaline (50 μM), a potent inhibitor of ornithine-2-oxo acid transaminase (Katunuma *et al.*, 1965), resulted in a marked decrease of urea synthesis from

NH_4Cl + ornithine (76% inhibition), and the activity approached that obtained without added ornithine (see NH_4Cl + L-canaline, Table 3). The inhibition of urea formation by canaline under the present conditions appears to occur specifically at the level of degradative metabolism of ornithine, since urea synthesis from NH_4Cl + lactate or from NH_4Cl , lactate and ornithine was not affected by canaline. However, in the presence of canaline, the inhibition by α -methylisocitrate of urea synthesis from NH_4Cl , lactate and ornithine was significantly larger (81–84%) than without canaline (47–49%); a similar increase of α -methylisocitrate inhibition in the presence of canaline was also observed when only NH_4Cl and lactate (or NH_4Cl , lactate and pyruvate) were the added substrates (Table 3).

Gabaculine, a suicide inhibitor of ornithine-2-oxo acid transaminase (Jung & Seiler, 1978), gave results identical with those obtained with canaline. Thus, DL-gabaculine (50 μM) inhibited urea synthesis from NH_4Cl + ornithine by 83%; it did not affect urea formation from NH_4Cl , lactate and ornithine, but with this substrate combination the

Table 3. *Contribution of ornithine to urea synthesis*

The conditions of incubation were the same as in Table 1 except that only those substrates were present that are specified in this table. The concentrations of substances were as follows: NH_4Cl (5.4 mM), L-ornithine (2.0 mM), and L-canaline (50 μM). In expt. A, L-lactate (6.0 mM) was added where indicated, and in expt. B, L-lactate (5.0 mM) and pyruvate (1.0 mM) were present when specified. Each substrate combination represents a single flask sampled after 0, 60, 75, 90, and 120 min of incubation; determinations for urea were in duplicate. Inhibition at infinite α -methylisocitrate concentration was calculated as $[1 - (v_i/v_0)] \times 100$. Abbreviation: n.d., not determined. Values are means $\pm$ errors as described in the Experimental section.

Additions	Expt.	Velocity (v_0) in absence of α -methylisocitrate (nmol/min per mg of protein)	Inhibition at infinite α -methylisocitrate concentration (%)
NH_4Cl , lactate (pyruvate) + ornithine	A	14.9 ± 0.8	49 ± 2
	B	15.3 ± 0.9	47 ± 5
NH_4Cl , ornithine, lactate (pyruvate) + canaline	A	13.2 ± 0.7	81 ± 7
	B	14.6 ± 0.8	84 ± 5
NH_4Cl + ornithine	A	5.0 ± 0.3	10 ± 8
	B	5.9 ± 0.6	15 ± 6
NH_4Cl , ornithine + canaline	A	$1.2 \pm 0.2^*$	n.d.
NH_4Cl + lactate (pyruvate)	A	7.4 ± 0.8	53 ± 3
	B	7.2 ± 0.7	65 ± 7
NH_4Cl , lactate (pyruvate) + canaline	A	8.0 ± 0.6	66 ± 6
	B	7.6 ± 0.7	74 ± 8
NH_4Cl + canaline	A	$1.1 \pm 0.7^*$	n.d.

* Product formation declined with time. The rate was estimated from urea accumulation during the initial 45-min incubation period.

Table 4. *Effects of inhibitors on ornithine and NH_3 uptake and product formation*

The conditions of incubation were as in Table 3, except that the flask contents were sampled at zero time and after 30 min of incubation. The concentrations of hepatocytes were 0.95–1.05 mg of protein/ml. The averages $\pm$ s.d. of five determinations are reported.

Additions	Changes in metabolite concentrations (ng-atoms of N/30 min per ml of medium)							
	Ornithine	NH_3	Urea	Citrulline	Glutamate	Glutamine	Aspartate	Alanine
Expt. A								
L-Ornithine (2033 ± 4)	-800 ± 95	$+320 \pm 49$	290 ± 57	96 ± 16	25 ± 9	2 ± 5	17 ± 5	60 ± 9
+ NH_4Cl (5485 ± 7)*								
As above + 50 μM -L-canaline	-110 ± 30	-140 ± 0	70 ± 11	60 ± 15	19 ± 3	4 ± 5	14 ± 6	48 ± 5
Expt. B								
L-Ornithine (2033 ± 9), NH_4Cl (5477 ± 10) + 6 mM-L-lactate*	-470 ± 60	-660 ± 26	980 ± 19	24 ± 12	26 ± 3	44 ± 0	6 ± 0	32 ± 5
As above + 50 μM -L-canaline	-130 ± 60	-970 ± 27	940 ± 59	18 ± 13	23 ± 3	44 ± 0	4 ± 0	18 ± 4
As above + 1 mM- α -methylisocitrate	-2000 ± 66	$+1430 \pm 30$	560 ± 59	3 ± 9	9 ± 6	22 ± 4	2 ± 2	12 ± 3
As above + 50 μM -L-canaline + 1 mM- α -methylisocitrate	-120 ± 27	-80 ± 0	190 ± 14	-24 ± 13	3 ± 4	6 ± 0	1 ± 2	8 ± 2

* The concentrations (nmol/ml) of ornithine and NH_4Cl shown in parentheses were determined at zero time.

inhibition by α -methylisocitrate was larger with gabaculine (85%) than without gabaculine (50%) (results not shown).

The effect of canaline and α -methylisocitrate on nitrogenous metabolites was examined in hepatocytes incubated with ornithine and NH_4Cl in the absence and presence of lactate. Changes of the

concentrations of ornithine, NH_3 and urea were quantitatively most significant and, in combination with alterations in concentrations of other metabolites, substrate consumption and product formation accounted for the balance of nitrogenous compounds within experimental errors (Table 4). With ornithine and NH_4Cl , the further addition of

canaline decreased ornithine uptake and urea synthesis (Table 4, expt. A). With NH_4Cl , ornithine and lactate as substrates (Table 4, expt. B), ornithine and NH_3 uptake were appreciable. With this substrate combination, canaline had little effect on urea synthesis (Table 3 and Table 4), but canaline addition almost completely prevented ornithine uptake and increased NH_3 utilization (Table 4, expt. B). The decrease in urea formation by α -methylisocitrate was paralleled by increased ornithine uptake and NH_3 release. The addition of both α -methylisocitrate and canaline resulted in the most pronounced inhibition of urea synthesis and utilization of substrates (Table 4, expt. B).

Discussion

α -Methylisocitrate inhibited urea formation from NH_4Cl + L-lactate, NH_4Cl + glucose, L-serine, and D-alanine but not from L-alanine, L-asparagine and L-glutamine in hepatocytes (Table 1). These results suggest that, with substrates that require reductive amination of 2-oxoglutarate to glutamate for aspartate synthesis, mitochondrial NADP-specific isocitrate dehydrogenase becomes rate limiting for urea synthesis when the dehydrogenase is inhibited by increasing concentrations of α -methylisocitrate. With L-serine or D-alanine as substrates, the initial steps in metabolism involve extramitochondrial formation of pyruvate and NH_3 . In livers from adult rats, metabolism of serine occurs predominantly via cytosolic serine dehydratase, whereas transamination of serine is relatively minor (Snell, 1976). Oxidation of D-alanine to NH_3 and pyruvate is probably catalysed exclusively by D-amino acid oxidase located in peroxisomes. In subsequent reactions in mitochondria, pyruvate is carboxylated to oxaloacetate, and the generation of aspartate from oxaloacetate by transamination is dependent on maintenance of the glutamate concentration by reductive amination of 2-oxoglutarate. The latter is dependent on the availability of reducing equivalents in mitochondria, and inhibition of NADP-specific isocitrate dehydrogenase by α -methylisocitrate could limit glutamate formation if NADPH supplied a significant portion of the reduced nicotinamide nucleotides for the glutamate dehydrogenase reaction. This is supported by the finding that α -methylisocitrate decreased urea synthesis (Table 1) and the content of NADPH (Table 2) when L-serine, D-alanine, or NH_4Cl + lactate were the substrates.

NH_3 is also produced in the deamination of glutamine or asparagine. However, net reductive amination of 2-oxoglutarate for aspartate synthesis is not a requirement for urea synthesis with these substrates, and urea formation was not inhibited by α -methylisocitrate (Table 1). Thus, glutamate and NH_3 produced by hydrolysis of glutamine can be

utilized for formation of aspartate by transamination of oxaloacetate and for synthesis of carbamoyl phosphate, respectively. Aspartate and NH_3 , liberated by hydrolysis of asparagine would be directly available for reactions entering the urea cycle at argininosuccinate synthetase and carbamoyl phosphate synthetase, respectively. The further metabolism of the carbon skeleton from either glutamate or aspartate would lead to the generation of ATP needed for urea synthesis and gluconeogenesis.

Glutamate would be formed in the initial transamination reaction required for utilization of L-alanine, eliminating the need for reductive amination of 2-oxoglutarate and consistent with the lack of inhibition of urea formation by α -methylisocitrate. This is also in accord with the finding that the NADPH content was not lowered when L-alanine was the substrate and that the further addition of NH_4Cl or α -methylisocitrate resulted in, at best, a minor (approx. 13%) decrease in NADPH content when compared with the control (Table 2). In contrast, D-alanine lowered the NADPH content by about 20% and the further addition of α -methylisocitrate was accompanied by a substantial fall (about 44%) in NADPH content when compared with the control incubated with lactate and pyruvate (Table 2). Indeed, with L-alanine as substrate, oxidative deamination of glutamate must occur to provide NH_3 for carbamoyl phosphate synthesis. The present results suggest that the steady state concentration of NH_3 was rate limiting when L-alanine was the substrate, since the addition of NH_4Cl or of N-carbamoyl-L-glutamate, a positive modulator of carbamoyl phosphate synthetase, enhanced urea synthesis without affecting the failure to inhibit urea formation with α -methylisocitrate (Table 1).

Sies *et al.* (1974) found that the addition of t-butyl hydroperoxide depressed NADPH levels and urea synthesis in isolated perfused liver. NADP-specific isocitrate dehydrogenase is an important source of NADPH in isolated mitochondria, since α -methylisocitrate substantially decreases the formation of glutamate from added NH_4Cl and isocitrate in intact coupled rat liver mitochondria (Smith & Plaut, 1979). Furthermore, the addition of α -methylisocitrate markedly decreased the NADPH/NADP⁺ ratio but had little effect on the NADH/NAD⁺ ratio of the isolated mitochondria; the latter ratio tended to be very low even in State 4. The present results suggest that whenever net reductive amination of 2-oxoglutarate to glutamate is required for urea synthesis in isolated hepatocytes, mitochondrial NADP-specific isocitrate dehydrogenase is an important source of NADPH.

Maximal inhibition of urea synthesis was approx. 50% at extrapolated values of infinite α -methyl-

isocitrate concentration with NH_4Cl , lactate and ornithine (Table 1). This might suggest (a) that reducing equivalents for reductive amination of 2-oxoglutarate were generated through pathways in addition to the NADP-specific isocitrate dehydrogenase reaction and/or (b) that ornithine (added under the incubation conditions shown in Tables 1 and 2) was converted at a significant rate to glutamate, bypassing formation of glutamate by the glutamate dehydrogenase reaction.

NADH could supply the reducing equivalents for reductive amination of 2-oxoglutarate as a direct cosubstrate for glutamate dehydrogenase, or after transhydrogenation ($\text{NADH} \rightarrow \text{NADP}^+$) to form NADPH. When hepatocytes were incubated with pyruvate, lactate, and ornithine, the addition of NH_4Cl , α -methylisocitrate, or both resulted in a marked decline in NADPH content, but no corresponding significant change in NADH content was observed (Table 2). These results do not exclude the contribution of NADH, since the levels of NADH were very low and at the limits of accurate detection. Participation of NADH was suggested by the observation that substrates that generate mitochondrial NADH (oleate, octanoate or 3-hydroxybutyrate) decreased inhibition of urea synthesis from NH_4Cl and lactate by α -methylisocitrate (Table 1). The addition of 3-hydroxybutyrate increased the NADH content of hepatocytes 4–5-fold; the further addition of NH_4Cl (and of NH_4Cl + α -methylisocitrate) significantly decreased the NADH (and NADPH) content (Table 2). These results would be consistent with increased utilization of reducing equivalents from NADH for reductive amination of 2-oxoglutarate when the NADH/NAD $^+$ ratio was increased, decreasing the dependence on NADPH from NADP-specific isocitrate dehydrogenase. It is uncertain whether the reducing equivalents from NADH are utilized directly at the glutamate dehydrogenase step, or preferentially, after transhydrogenation to form NADPH. Sies *et al.* (1975) obtained inhibition of urea synthesis in hepatocytes with the transhydrogenase inhibitor rhein at a concentration that appeared not to interfere with urea formation due to depression of mitochondrial electron transport processes at NADH dehydrogenase (Kean *et al.*, 1971). Our results are not inconsistent with the occurrence of transhydrogenation ($\text{NADH} \rightarrow \text{NADP}^+$) in hepatocytes but they do not exclude direct reaction of NADH at glutamate dehydrogenase. For example, when NADP-specific isocitrate dehydrogenase was inhibited by α -methylisocitrate, the level of NADPH decreased less (compared to the incubated control containing lactate, pyruvate and ornithine) when the NADH content was raised with 3-hydroxybutyrate ($174 \pm 13 \text{ nmol/g wet wt.}$) than in the absence of 3-hydroxybutyrate ($139 \pm 20 \text{ nmol/g wet wt.}$) (Table

2, series A); when reductive amination was stimulated by the further addition of NH_4Cl , the level of NADPH remained significantly higher with 3-hydroxybutyrate ($144 \pm 15 \text{ nmol/g wet wt.}$) than without 3-hydroxybutyrate ($97 \pm 8 \text{ nmol/g wet wt.}$). Nevertheless, even the latter results may merely reflect competition between NADH and NADPH reacting directly at glutamate dehydrogenase, rather than transhydrogenation ($\text{NADH} \rightarrow \text{NADP}^+$) followed by an obligatory utilization of NADPH for reductive amination of 2-oxoglutarate.

L-Ornithine (2 mM) was added routinely to maintain optimal steady state levels for its catalytic role in urea synthesis (Tables 1 and 2). However, if there were substantial degradation of ornithine by the multistep reaction:

net reductive amination of 2-oxoglutarate would not be required to maintain the nitrogen flux for urea synthesis. Utilization of ornithine for urea synthesis by hepatocytes from starved rats was reported previously by Meijer *et al.* (1975) under conditions where ornithine was the only anaplerotic precursor added. As shown in Table 3, urea synthesis from NH_4Cl and ornithine proceeded at about one-third the rate of that with NH_4Cl , ornithine and lactate. When transamination of ornithine to glutamic semialdehyde was inhibited with L-canaline (Katunuma *et al.*, 1965), urea synthesis from NH_4Cl and ornithine was inhibited about 75% and ornithine uptake declined (Table 4, expt. A), indicating that products of degradation of ornithine could support urea formation. Urea synthesis from NH_4Cl + ornithine was not inhibited by α -methylisocitrate (Table 3), suggesting that NADPH generated from NADP-specific isocitrate dehydrogenase was not required for reductive amination of 2-oxoglutarate in this case. Canaline did not inhibit urea synthesis from NH_4Cl , ornithine and lactate; however, the uptake of substrates shifted from ornithine to NH_3 (Table 4, expt. B). On the other hand, with this substrate combination the inhibition of urea synthesis by α -methylisocitrate was accompanied by increased utilization of ornithine, NH_3 release and a decline in accumulation of citrulline, glutamate, glutamine and alanine. The lowering of NADPH content by inhibition of NADP-specific isocitrate dehydrogenase by α -methylisocitrate caused an increase in NAD-specific isocitrate dehydrogenase activity in isolated liver mitochondria (Smith & Plaut, 1979). The increased availability of 2-oxoglutarate from NAD-specific isocitrate dehydrogenase and the displacement of the glutamate dehydrogenase reaction toward oxidative deamination, in response to the decrease of the NADPH/NAD $^+$ ratio by α -methylisocitrate (Table 2), may be responsible for

the increased utilization of ornithine at the 2-oxo acid-ornithine transaminase step in hepatocytes. It remains to be established whether these are also the sole mechanisms causing the large release of NH_3 and lowered content of glutamate upon addition of α -methylisocitrate (Table 4, expt. B). The marked increase in inhibition of urea synthesis by the combination of canaline and α -methylisocitrate (Table 3) was caused by the pronounced decrease in uptake of both ornithine and NH_3 (Table 4, expt. B). These results suggest that the degradation of ornithine may lead to underestimation of the contribution of the NADP-specific isocitrate dehydrogenase flux to urea synthesis with substrates requiring reductive amination of 2-oxoglutarate (e.g., NH_4Cl + lactate, L-serine, or D-alanine in Table 1).

When model systems were restricted to utilization of NH_4Cl and lactate by inhibiting the α -methylisocitrate-insensitive degradation of ornithine, the quantitative contribution of flux through mitochondrial NADP-specific isocitrate dehydrogenase to urea synthesis was more than 80% (Table 3). NADH, formed in the oxidative degradation of lactate (or from oxidation of endogenous fatty acids), may contribute the remaining reducing equivalents required for urea synthesis.

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