Factors Influencing the Activity of Ornithine Aminotransferase in Isolated Rat Liver Mitochondria

By J. D. MCGIVAN, N. M. BRADFORD and A. D. BEAVIS
Department of Biochemistry, University of Bristol Medical School,
Bristol BS8 1TD, U.K.

(Received 2 August 1976)

1. The characteristics of ornithine catabolism by the aminotransferase pathway in isolated mitochondria were determined. 2. Ornithine synthesis from glutamate and glutamate γ -semialdehyde produced by the oxidation of proline was studied. No ornithine was formed in the absence of rotenone. 3. The mechanism of ornithine transport was reinvestigated, and the existence of an ornithine⁺/H⁺ exchange system postulated. 4. The kinetics of ornithine transport, ornithine catabolism in intact mitochondria and ornithine aminotransferase activity in solubilized mitochondria were compared. It is concluded that ornithine aminotransferase activity in liver mitochondria is rate-limited by the transport of ornithine across the mitochondrial membrane, and that this enzyme is involved primarily in ornithine degradation rather than ornithine synthesis.

Ornithine aminotransferase (L-ornithine-2-oxoglutarate δ -aminotransferase, EC 2.6.1.13) catalyses the reaction:

In the rat, high activities of this enzyme occur only in the liver, kidney and small intestine (Peraino & Pitot, 1963; Herzfeld & Knox, 1968). The enzymes from all three tissues appear to be identical (Sanada *et al.*, 1970). Ornithine aminotransferase from rat liver has been purified and its properties have been extensively investigated (Peraino & Pitot, 1963; Strecker, 1965; Peraino *et al.*, 1969; Peraino, 1972; Morris *et al.*, 1974). The enzyme activity in liver and kidney undergoes very marked adaptive changes in response to variations in the metabolic state of the animal. Thus ornithine aminotransferase activity is induced by the force-feeding of amino acids (Peraino *et al.*, 1965), by administration of glucagon (Peraino & Pitot, 1964) and of oestrogen (Herzfeld & Knox, 1968), and the activity in the liver, but not in kidney, is repressed by glucose feeding (Volpe *et al.*, 1969).

The metabolic function of this enzyme has not been completely defined. In rat liver, the enzyme is located in the mitochondria (Peraino & Pitot, 1963), apparently in the matrix space (Ip *et al.*, 1974). Katanuma *et al.* (1965) have shown that in isolated liver mitochondria, added ornithine may react with a 2-oxoglutarate formed in the tricarboxylic acid cycle, with the production of glutamate. Thus ornithine aminotransferase may be involved in

ornithine catabolism. However, Herzfeld & Knox (1968) and Volpe *et al.* (1969) have suggested that ornithine aminotransferase in liver functions primarily to provide ornithine for use in the urea cycle.

It has been shown that under certain conditions the intracellular concentration of ornithine may limit the rate of urea synthesis in isolated liver cells and in perfused liver (Krebs *et al.*, 1973). The regulation of ornithine aminotransferase activity is therefore of importance, since this enzyme may mediate changes in the intracellular ornithine concentration and thus possibly in the capacity of the liver to synthesize urea. The transport of ornithine into the mitochondrial matrix has been relatively little studied. In the present paper, the restraints placed on the activity of ornithine aminotransferase by its location in the mitochondrial matrix are examined. It is concluded that ornithine aminotransferase is an enzyme primarily involved in ornithine catabolism. The rate at which the enzyme may catalyse ornithine transamination is limited by the rate of ornithine transport across the mitochondrial membrane.

Experimental

Liver mitochondria were prepared from female Wistar rats as described by Chappell & Hansford (1972). In experiments where citrulline was to be determined, the sucrose in the preparation medium was replaced by 0.3M-mannitol, since sucrose interferes with the citrulline assay. The rats were fed

on a standard laboratory diet (diet 41B; Oxoid Ltd., London S.E.1, U.K.).

Measurement of ornithine disappearance

To measure the rate of metabolism of ornithine, use was made of the fact that at neutral pH values ornithine is positively charged, whereas its major metabolic products, i.e. glutamate γ -semialdehyde, glutamate, aspartate, citrulline and 2-oxoglutarate, are either negatively charged or have no net charge. Ornithine was therefore separated from its metabolic products by ion-exchange chromatography by a method based on the procedure of Strecker (1965). Mitochondria (5–10 mg of protein/ml) were incubated in a medium containing 0.12 M-KCl, 20 mM-Tris/HCl and the appropriate concentration of [3 H]ornithine hydrochloride at pH 7.2 and 30°C. Other additions were as indicated in the text. After a given time the reaction was terminated by the addition of HClO₄ (5%, w/v, final concentration), and the denatured protein removed by centrifugation at 3000g for 2 min. The supernatant was neutralized with 3 M-KOH. A 0.2 ml sample of this solution was then applied to a column (8 cm $\times$ 0.5 cm) of the ion-exchange resin Dowex 50W (X8; K⁺ form). This column had been previously equilibrated with 10 mM-potassium morpholinopropanesulphonate at pH 7.0. The metabolic products of ornithine were eluted with 7 ml of this buffer and the residual ornithine was then eluted with 25 ml of 0.3 M-ammonium formate. The total radioactivity in each fraction was determined by liquid-scintillation counting as described below.

Control experiments showed that Dowex 50 (X8; K⁺ form) retained 94 $\pm$ 0.5% of the [3 H]ornithine added. Glutamate, 2-oxoglutarate, aspartate, citrulline and glutamate γ -semialdehyde were not retained and were completely eluted in 3–4 ml of buffer at pH 7.0. Dowex 50W (X8; H⁺ form) as supplied was unsuitable for this separation, since both citrulline and glutamate γ -semialdehyde were retained. It was also established that the concentration of endogenous ornithine in the mitochondria was low and did not significantly alter the specific radioactivity of the radioactive ornithine added.

Determination of the steady-state distribution of ornithine across the mitochondrial membrane

Mitochondria (2 mg of protein/ml) were pre-incubated with 0.5 mM-amino-oxyacetate in the appropriate medium at 20°C for 2 min; [3 H]ornithine was added together with [14 C]sucrose, which acts as a marker of the extramitochondrial space. In some experiments, 3 H₂O replaced the ornithine for determination of the total pellet volume. After a further 2 min, 2 $\times$ 1 ml samples of the incubation mixture were centrifuged for 2 min at 11000g in an Eppendorf Zentrifuge model 3200. The pellets

and supernatants were separated, acidified and assayed for radioactivity.

Measurement of the initial rate of ornithine transport by centrifugal filtration

Tubes for the Beckmann 152 microfuge were prepared as follows: 0.025 ml of 10% (v/v) HClO₄ was placed at the bottom of the tube and 0.1 ml of silicone oil was carefully layered on top of this. The silicone oil consisted of equal volumes of dinonyl phthalate and Silicone Fluid MS 550 (Hopkin and Williams, Romford, Essex, U.K.). Then 0.05 ml of a solution containing an appropriate concentration of [3 H]ornithine hydrochloride and [14 C]sucrose formed a layer above the silicone oil. Mitochondria (5 mg/ml) were suspended in a medium containing 0.12 M-KCl, 0.02 M-Tris/HCl, 2 mM-potassium phosphate, 0.5 mM-amino-oxyacetate and 1 μ g of rotenone/ml at 20°C. Portions (0.2 ml) of this suspension were added to the ornithine-containing layer in the Beckmann tubes at appropriate intervals and the tubes were then centrifuged at 9000g for 2 min. The supernatant fraction was acidified and assayed for radioactivity. The tubes were then frozen and cut at the silicone oil/HClO₄ interface with a sharp knife. The tip of each tube, containing the pellet plus HClO₄, was added to 15 ml of scintillation fluid and shaken hard before radioactivity counting. By this method samples could be centrifuged within 5 s of adding the mitochondria to the ornithine solution. Shorter incubation times could be obtained by the inclusion of an air bubble between the ornithine layer and the mitochondrial suspension; in this case mixing occurred only when the centrifuge was started. The initial rates of transport were evaluated as described previously (Bradford & McGivan, 1973).

Assay of metabolites

Glutamate (Bernt & Bergmeyer, 1965), NH₃ (Kirsten *et al.*, 1963) and aspartate (Pfleiderer, 1965) were assayed by enzymic methods. Glutamate γ -semialdehyde was assayed by measurement of the complex formed with 2-aminobenzaldehyde. A millimolar extinction coefficient of 2.71 litre $\cdot$ mmol⁻¹ $\cdot$ cm⁻¹ at 443 nm was assumed (Strecker, 1965). Citrulline was determined as described by Archibald (1944). Ornithine in the absence of other metabolites was assayed by a ninhydrin method (Chinard, 1952). Mitochondrial protein was determined by a biuret method (Gornall *et al.*, 1949). The system used for liquid-scintillation counting contained, in 1 litre: 600 ml of toluene, 400 ml of 2-methoxyethanol, 80 g of naphthalene and 6 g of 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole.

Materials

L-[G- 3 H]Ornithine hydrochloride was purchased

from NEN Chemicals G.m.b.H., Dreieichenhain, W. Germany. 2-Aminobenzaldehyde was a product of Fluka A.G. (Fluorochem Ltd., Glossop, Derbyshire, U.K.). Dowex 50W (X8) was a product of Bio-Rad Laboratories, Richmond, CA, U.S.A. Enzymes and nucleotides were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany.

Results

Characteristics of ornithine catabolism in isolated mitochondria

Isolated liver mitochondria metabolize added ornithine by two different pathways. In the presence of NH_3 , bicarbonate and ATP, mitochondria synthesize carbamoyl phosphate, which reacts with ornithine to form citrulline. This latter reaction is catalysed by ornithine carbamoyl-transferase and constitutes part of the urea cycle. Ornithine can also react with 2-oxoglutarate in a reaction catalysed by ornithine aminotransferase. The glutamate γ -semialdehyde formed in this reaction may undergo isomerization to pyrroline-5-carboxylate dehydrogenase. Ornithine catabolism by this pathway in liver leads to a net loss of urea-cycle intermediates.

Table 1 shows the influence of various effectors on the rate of ornithine disappearance in the absence of added NH_3 . Under these conditions very little citrulline was formed and the disappearance of ornithine was due primarily to its catabolism by the ornithine aminotransferase pathway. The rate of ornithine disappearance was increased slightly in the presence of 2-oxoglutarate, which is the second substrate for ornithine aminotransferase. The rate

was also increased by the addition of phosphate, and the effects of phosphate and of 2-oxoglutarate were additive. Mersalyl, at concentrations which inhibit phosphate transport rather specifically, also inhibited ornithine catabolism, suggesting that the transport of endogenous or added phosphate was necessary to allow maximum rates of ornithine catabolism. The possible significance of this finding is discussed below. Ornithine catabolism was inhibited by amino-oxyacetate, a specific inhibitor of pyridoxal phosphate-dependent enzymes, and also by rotenone, which would inhibit the NAD^+ -linked oxidation of glutamate γ -semialdehyde.

The stimulation of ornithine catabolism by 2-oxoglutarate and by phosphate was further investigated. In the presence of an excess of 2-oxoglutarate, half-maximal rates of ornithine catabolism were obtained at an added phosphate concentration of 0.1 mM. Under these conditions, the concentration of endogenous phosphate outside the mitochondria was 0.15 mM. When an excess of phosphate was added, half-maximal stimulation occurred at an added 2-oxoglutarate concentration of 0.1 mM. The intramitochondrial concentration of endogenous 2-oxoglutarate was 0.2 mM.

To obtain more information about the metabolic fate of ornithine in these experiments, the formation of nitrogen-containing products of ornithine metabolism was measured (Table 2). Since ornithine contains two atoms of nitrogen per molecule, the disappearance of ornithine nitrogen was accounted for largely by the formation of glutamate, NH_3 , aspartate and citrulline. The addition of phosphate caused an increase in the disappearance of ornithine, but the overall pattern of product formation was little affected. When 2-oxoglutarate was added in excess, the ornithine nitrogen appeared almost entirely in glutamate. Approx. 2 mol of glutamate was produced per mol of ornithine metabolized. This was to be expected, since the presence of an excess of 2-oxoglutarate would inhibit further metabolism of glutamate both by glutamate dehydrogenase and by aspartate aminotransferase. The effect of adding phosphate in the presence of an excess of 2-oxoglutarate was to stimulate further both the metabolism of ornithine and the production of glutamate. Control experiments showed that neither phosphate nor mersalyl affected the activity of ornithine aminotransferase in mitochondria that had been solubilized with the detergent Triton X-100. The metabolism of ornithine in no case led to the accumulation of glutamate γ -semialdehyde. The conversion of glutamate γ -semialdehyde into glutamate was thus not rate-limiting in the catabolism of ornithine. The effect of phosphate on ornithine metabolism is consistent with a stimulation of ornithine transport if this process were rate-limiting.

Under optimal conditions for ornithine catabolism,

Table 1. *Catabolism of ornithine by liver mitochondria*
Mitochondria were incubated as described in the Experimental section with 1.5 mM-ornithine and other additions as shown. Ornithine disappearance was measured after 10 min; the rate of ornithine disappearance over this time-interval was constant. The results are means $\pm$ s.e.m. of values obtained on three separate mitochondrial preparations.

Additions	Relative rate of ornithine disappearance
None	100
Phosphate (5 mM)	140 $\pm$ 7
2-Oxoglutarate (5 mM)+malate (1 mM)	117 $\pm$ 3
2-Oxoglutarate+malate+phosphate	161 $\pm$ 6
Malate	102 $\pm$ 4
Mersalyl (20 nmol/mg of protein)	48 $\pm$ 11
Rotenone (1 μ g/ml)	24 $\pm$ 5
Amino-oxyacetate (0.5 mM)	21 $\pm$ 7

Table 2. *Products of ornithine metabolism by mitochondria*

Mitochondria were incubated with 1.5 mM-ornithine and other additions as shown. Metabolite formation was measured after 10 min. The results shown are those from a single experiment; similar results were obtained in several experiments of this type. No detectable amount of glutamate γ -semialdehyde was found in any of the samples assayed.

Additions	Metabolite formation (nmol per mg of protein in 10 min)				
	Ornithine disappearance	Glutamate	Aspartate	NH ₃	Citrulline
None	23.1	17.8	7.4	5.5	6.5
Phosphate (5 mM)	28.2	26.6	4.9	8.6	7.7
2-Oxoglutarate (5 mM)+malate (1 mM)	27.2	58.6	0	0	3.1
2-Oxoglutarate+malate+phosphate	29.7	60.6	0	0	3.8

Fig. 1. *Ornithine catabolism as a function of ornithine concentration*

The incubation was started by the addition of mitochondria (10 mg of protein/ml final concentration) to a medium containing 0.12 M-KCl, 20 mM-Tris/HCl, 1 mM-malate, 1 mM-2-oxoglutarate, 5 mM-phosphate and various concentrations of [³H]-ornithine at pH 7.2 and 30°C. The reaction was terminated after 10 min, and ornithine disappearance was assayed as described in the Experimental section.

i.e. in the presence of an excess of 2-oxoglutarate and phosphate, the rate of ornithine disappearance as a function of ornithine concentration is shown in Fig. 1. At 30°C the maximum rate was 6.2 ± 0.26 nmol/min per mg of protein, with an apparent K_m for ornithine of 1.25 ± 0.13 mM. These results may be compared with the kinetic constants of ornithine aminotransferase in mitochondria which had been solubilized with Triton X-100 (Fig. 2). The maximum rate of ornithine transamination in solubilized mitochondria (25 nmol/min per mg) was four times the rate at which ornithine was metabolized in intact mitochondria at the same temperature (Fig. 1). The apparent K_m for ornithine of ornithine aminotransferase in solubilized mitochondria (4 mM; Fig. 2a) was also considerably

higher than that for ornithine catabolism in intact mitochondria. In contrast, the apparent K_m for 2-oxoglutarate of ornithine aminotransferase in solubilized mitochondria (0.15 mM; Fig. 2b) was closely similar to the concentration of 2-oxoglutarate which produced half-maximal stimulation of ornithine catabolism in intact mitochondria.

The above results taken together indicate that ornithine is readily catabolized by the ornithine aminotransferase pathway in isolated mitochondria. The results are also consistent with the postulate that the rate-limiting reaction in ornithine catabolism is the transport of ornithine across the mitochondrial membrane. The characteristics and mechanism of ornithine transport are considered below.

Competition for ornithine between the ornithine aminotransferase and ornithine carbamoyltransferase reactions

One of the chief metabolic functions of the liver is the synthesis of urea from NH₃. It may be questioned whether the degradation of ornithine by the ornithine aminotransferase pathway can be of metabolic significance in relation to the rate at which ornithine reacts with carbamoyl phosphate to produce citrulline in the urea cycle. An experiment was performed to test whether the ornithine aminotransferase pathway operates to a significant extent when mitochondria are synthesizing citrulline. Ornithine was added to mitochondria in the presence of an excess of bicarbonate and respiratory substrate; NH₃ was produced continuously in the incubation by the inclusion of 5 mM-adenosine plus a limiting amount of adenosine deaminase. Under these conditions, ornithine could undergo both transamination and reaction with carbamoyl phosphate, but the rate of carbamoyl phosphate synthesis was limited by the rate of NH₃ production. The concentration of adenosine deaminase was varied to give rates of citrulline production of between 0 and 10 nmol/min per mg of protein. The rate of ornithine disappearance always exceeded that of citrulline formation under these conditions. Further,

Fig. 2. *Ornithine aminotransferase activity in solubilized mitochondria*

The enzyme was assayed essentially as described by Stfecker (1965). Mitochondria were incubated at pH 7.2 and 30°C in 3 ml of a medium containing 0.12 M-KCl, 20 mM-Tris/HCl, 0.3% (w/v) Triton X-100 and 2 mg of 2-aminobenzaldehyde together with appropriate concentrations of 2-oxoglutarate and ornithine. The increase in A_{443} was followed continuously with a recording spectrophotometer. (a) +8 mM-2-oxoglutarate; (b) +20 mM-ornithine.

the rate of ornithine metabolism by the amino-transferase pathway (taken as the difference between the rate of ornithine disappearance and that of citrulline formation) was independent of the rate of citrulline synthesis at the concentrations of ornithine used (2-3 mM). It was not possible to repeat this experiment at ornithine concentrations below 1 mM, owing to technical difficulties in metabolite determination under such conditions.

Formation of ornithine by the ornithine amino-transferase reaction

It is in principle possible that the ornithine amino-transferase reaction may operate in the reverse direction in intact mitochondria, with the formation of ornithine from glutamate plus glutamate γ -semialdehyde. Glutamate γ -semialdehyde may arise in liver mitochondria from the oxidation of proline to pyrroline-5-carboxylate, followed by the isomerization of the latter compound. The characteristics of ornithine formation from proline are shown in Fig. 3. No ornithine was formed unless both glutamate and rotenone were present. These

results are entirely consistent with those in Table 2 and may be interpreted as follows. Pyrroline-5-carboxylate formed by the flavoprotein-linked oxidation of proline is rapidly oxidized to glutamate and is thus not available for isomerization to glutamate γ -semialdehyde and subsequent transamination to form ornithine. Rotenone inhibits the NAD⁺-linked oxidation of pyrroline-5-carboxylate, and this compound now forms glutamate γ -semialdehyde, which transaminates with added glutamate to produce ornithine. It appears that when NAD⁺-linked oxidations are not inhibited, little or no synthesis of ornithine from proline can occur, at least in isolated mitochondria.

Mechanism of ornithine transport

The pK of the δ -amino group of ornithine is 10.8, so that at neutral pH values this molecule carries one net positive charge. In principle ornithine may be transported across the mitochondrial membrane in exchange for a cation or together with an anion; alternatively ornithine may be transported electrogenically without compensating charge

Fig. 3. Formation of ornithine from proline

The reaction was started by the addition of mitochondria (5 mg/ml) to an oxygen-saturated medium which contained 0.12M-KCl, 20mM-Tris/HCl, 10mM-proline, 10mM-carbamoyl phosphate and various concentrations of glutamate at pH7.2 and 30°C. The reaction was terminated after 5 min and samples were assayed for citrulline. The rate of citrulline formation was linear with time over this period. The ornithine formed reacted with the added carbamoyl phosphate to form citrulline in a reaction catalysed by the very active mitochondrial enzyme ornithine carbamoyl-transferase. Ornithine production was taken as the rate of citrulline synthesis in this system. ○, No further additions; ●, +rotenone (1 µg/ml final concentration).

Fig. 4. Apparent steady-state distribution of ornithine across the inner mitochondrial membrane as a function of ornithine concentration

The incubation medium contained 2mM-phosphate, 3mM-succinate, 1 µg of oligomycin/ml, 1 µg of rotenone/ml, 10mM-sucrose, 10mM-Tris/morpholinopropanesulphonate and [¹⁴C]sucrose together with [³H]ornithine at pH7.0. The osmolarity of the medium was adjusted to 250mosm with choline chloride. ●, No further additions; ○, +0.5mM-amino-oxyacetate; ▲, +0.5mM-amino-oxyacetate+10mM-L-lysine.

Table 3. Steady-state distribution of ornithine across the mitochondrial inner membrane at pH7.0

The incubation medium contained 2mM-phosphate, 3mM-succinate, 1 µg of oligomycin/ml, 1 µg of rotenone/ml, 10mM-sucrose, 10mM-lysine, 0.5mM-amino-oxyacetate, [³H]ornithine, [¹⁴C]sucrose and 10mM-Tris/morpholinopropanesulphonate together with choline chloride to make the final osmolarity 250mosm. The matrix concentration of ornithine was determined as described in the Experimental section.

Concn. of ornithine in medium (mM)	Concn. of ornithine in matrix space (mM)
0.5	0.45
0.75	0.75
1.50	1.65
3.20	4.00
6.4	6.0
10.0	10.2

movement. According to Gamble & Lehninger (1973), ornithine transport is mediated by a specific transporting system which transports ornithine electrogenically. This conclusion is, however, of uncertain validity since no account was taken of ornithine metabolism by ornithine aminotransferase, and ornithine transport was not clearly differentiated from ornithine binding to the membrane in this investigation. It was therefore decided to re-assess the mechanism of ornithine transport.

The steady-state distribution of [³H]ornithine across the mitochondrial membrane as a function of ornithine concentration is shown in Fig. 4. An apparent accumulation of ornithine occurred, particularly at low external ornithine concentrations. The addition of amino-oxyacetate, which inhibited ornithine aminotransferase, largely abolished this accumulation, suggesting that the apparent accumulation was in fact due to some metabolism of the radioactive ornithine by the aminotransferase reaction. The addition of amino-oxyacetate together with 10mM-L-lysine further decreased the apparent uptake of ornithine at low concentrations. In this case, it is probable that that part of the total [³H]ornithine 'uptake' which was abolished by lysine represented the binding of ornithine to the outer surface of the negatively charged mitochondrial inner membrane rather than ornithine transport. Lysine does not penetrate the inner membrane (Gamble & Lehninger, 1973). It may be concluded that studies of the steady-state distribution of labelled ornithine are valid as a measure of ornithine transport only when amino-oxyacetate is present to inhibit transamination and when lysine is added to inhibit binding.

In the presence of amino-oxyacetate plus lysine, ornithine appeared to equilibrate across the membrane at concentrations up to 10mM (Table 3). That this uptake of radioactivity represented the

Fig. 5. *Ornithine uptake as a function of matrix volume*
The incubation medium was that described in the legend to Fig. 4, including amino-oxyacetate and lysine. The final osmolarity was adjusted in the range 70–500mosm with choline chloride, and ornithine uptake and the matrix volume were determined as described in the Experimental section. The external concentration of ornithine was 1 mM.

Table 4. *Influence of a trans-membrane pH gradient on ornithine distribution*

Mitochondria were incubated in the medium described in the legend to Table 3 with the further additions of 10mM-KCl and 1 μg of valinomycin/ml at pH7.0. Succinate and phosphate were added as the Tris salts. (A), No uncoupler present; matrix alkaline with respect to medium; (B), +1 μM-carbonyl cyanide phenylhydrazone; matrix acid with respect to medium. This technique of establishing trans-membrane pH gradients is described in detail by Nicholls (1974).

Concn. of ornithine in medium (mM)	Concn. of ornithine in matrix (mM)	
	(A) Matrix alkaline	(B) Matrix acid
0.1	0.075	0.145
1.0	0.82	1.34
10.0	6.30	11.30

transport of ornithine rather than ornithine binding was verified by measuring the uptake of radioactive ornithine as a function of the matrix volume when the matrix volume was altered by changing the osmolarity of the suspension medium (Fig. 5).

The linear relationship between ornithine uptake and matrix volume indicates clearly that transport was the parameter measured in such experiments. These results also suggest that ornithine does not exchange for any metabolite which is present at low concentrations in the matrix space.

If ornithine were transported electrogenically, as has been proposed (Gamble & Lehninger, 1973), the steady-state distribution should be affected by the electrical potential across the mitochondrial membrane. However, in experiments similar to those in Table 3 it was found that neither succinate respiration (which establishes a membrane potential) nor the addition of an uncoupling agent (which abolishes such a potential) had any significant effect on ornithine distribution in the steady state.

Ornithine distribution was found to vary as a function of the difference in pH between the matrix space and the external medium. Such a pH difference may be established by the addition of succinate plus valinomycin to mitochondria. In this case the intramitochondrial pH becomes more alkaline than the medium. The further addition of an uncoupling agent causes the intramitochondrial pH to become more acid than that of the medium. The magnitude of the transmembrane pH difference may be varied by varying the K⁺ concentration of the external medium. This method of establishing a pH gradient has been characterized in detail elsewhere (Nicholls, 1974).

Table 4 shows that when the intramitochondrial pH was more alkaline than that of the medium the steady-state concentration of ornithine was higher in the medium than in the matrix space. Conversely, the relative acidification of the matrix led to an accumulation of ornithine. Further evidence that ornithine distributed according to the pH gradient was obtained by comparing the distribution of ornithine with that of methylamine when the pH gradient was varied (Fig. 6). Methylamine is commonly used as an indicator of the pH gradient across the membrane (Nicholls, 1974). The linear relationship between the distribution of ornithine and that of methylamine indicates that ornithine must cross the membrane in an electro-neutral manner in a process which is pH-dependent. Ornithine transport may therefore be regarded as an exchange of ornithine⁺ for H⁺ or as the transport of the form of the ornithine molecule which has no net charge. At present these two possibilities cannot be differentiated experimentally. The results presented above are not consistent with the electrogenic transport of the ornithine⁺ cation.

Initial rate of ornithine transport

Ornithine transport was measured by the method of centrifugal filtration (see Klingenberg, 1970).

Fig. 6. Distribution of ornithine across the mitochondrial membrane in relation to the distribution of methylamine

Mitochondria were incubated in a basic medium which contained 2mM-phosphate, 3mM-succinate, 1 μ g of rotenone/ml, 1 μ g of oligomycin/ml, 0.5mM-amino-oxyacetate, 10mM-lysine and 1 μ g of valinomycin/ml, together with 10mM-Tris/morpholinopropane-sulphonate at pH7.0; 1mM-[3 H]ornithine and 50 μ M-[14 C]methylamine were added and their distributions determined as described in the Experimental section. The pH gradient was varied by adding KCl to the medium at concentrations in the range 0–10mM in order to create a pH gradient with the matrix alkaline with respect to the medium; a pH gradient in the opposite direction was established by varying the external KCl concentration between 0 and 60mM with the further addition of 1 μ M-carbonyl cyanide phenylhydrazine. The osmolarity was kept at 250mosM throughout with choline chloride, and phosphate and succinate were present as the Tris salts. See Nicholls (1974) for a full explanation of this technique.

Fig. 7. Kinetics of ornithine transport

Transport was measured at pH7.2 and 20°C as described in the Experimental section. Rates were evaluated from uptake of ornithine after 5, 10, 15, 20, 30 and 60s.

Fig. 7 shows the initial rate of ornithine transport as a function of ornithine concentration at 20°C in the presence of phosphate. The maximum rate

was approx. 8.3 nmol/min per mg, with an apparent K_m for ornithine at a concentration of 1.0mM. The rate of ornithine transport did not vary significantly with pH between 6.8 and 7.4 (results not shown). The kinetic parameters for ornithine transport (Fig. 7) and the overall process of ornithine catabolism (Fig. 1) are similar, a finding which is again consistent with the postulate that the activity of ornithine aminotransferase in the direction of ornithine degradation is limited by the rate of ornithine transport.

The activity of ornithine aminotransferase in mitochondria from rats fed for 3 days on 20% glucose is decreased some 10-fold compared with the controls (McGivan *et al.*, 1974). However, the rate of ornithine transport in mitochondria from glucose-fed rats was not significantly different from that in the controls although the rate of ornithine catabolism by such mitochondria was very much diminished (results not shown).

Discussion

Restrictions on the activity of ornithine aminotransferase as a result of its location in the mitochondrial matrix

Previous investigations of ornithine aminotransferase have largely ignored the metabolic consequences of the location of this enzyme in the mitochondrial matrix. In intact mitochondria, the activity of this enzyme is influenced by two major factors: the rate of transport of ornithine across the mitochondrial membrane and the presence in the matrix space of an active enzyme catalysing the oxidation of pyrroline-5-carboxylate to glutamate.

Ornithine aminotransferase in intact mitochondria catalyses the first reaction in the catabolism of ornithine to glutamate and oxidation products of glutamate. The results presented in this paper lead to the conclusion that the catabolism of ornithine by this pathway is rate-limited by the transport of ornithine into the matrix space. This conclusion is derived from the following lines of evidence. Phosphate increased the rate of ornithine catabolism, but had little effect on the pattern of product formation and did not affect the activity of ornithine aminotransferase directly. Since the further metabolism of glutamate γ -semialdehyde was clearly not rate-limiting (Table 2), it is probable that phosphate increased the rate-limiting transport of ornithine. More direct evidence is provided by measurements of the rate of ornithine transport. The kinetic constants of ornithine transport are similar to those of ornithine catabolism, whereas both the K_m and V_{max} of ornithine aminotransferase in solubilized mitochondria are considerably higher than the corresponding values for ornithine catabolism. It may be noted that the transport of

2-oxoglutarate is relatively fast (Palmieri *et al.*, 1972) and cannot be limiting for ornithine transamination.

The second consequence of the intramitochondrial location of ornithine aminotransferase is its effective inability to operate in the direction of ornithine formation. When pyrroline-5-carboxylate is formed by the oxidation of proline, it may undergo isomerization to glutamate γ -semialdehyde and subsequent transamination with glutamate to produce ornithine. However, the rapid oxidation of pyrroline-5-carboxylate in the mitochondria ensures that very little glutamate γ -semialdehyde can accumulate and therefore little formation of ornithine by the transamination reaction can occur (Fig. 3). If these results on isolated mitochondria apply also to the intact tissue, it follows that ornithine aminotransferase in liver does not catalyse the net formation of ornithine, and that the suggestion that this enzyme functions primarily to increase the cellular concentration of urea-cycle intermediates (Herzfeld & Knox, 1968; Volpe *et al.*, 1969) is incorrect.

Ornithine transport in liver mitochondria

The results presented above are consistent with the postulate that ornithine⁺ crosses the mitochondrial membrane in exchange for H⁺. The specificity of ornithine transport (Gamble & Lehninger, 1973) and the saturation kinetics of this process (Fig. 7) indicate that ornithine is transported on a specific carrier system similar to those that have been identified for other substrate ions. It appears that the activity of this ornithine⁺/H⁺ exchange system is considerably lower than the activities of the systems which catalyse the transport of adenine nucleotides, phosphate and tricarboxylate-cycle intermediates (see, e.g., Bradford & McGivan, 1973). Whether the ornithine⁺/H⁺ exchange system is involved in the mitochondrial synthesis of citrulline in the urea cycle remains to be determined.

The effects of P_i and of mersalyl on ornithine catabolism may be explained as effects primarily on ornithine transport. The exchange of ornithine for H⁺ would be facilitated by the presence of P_i which

Scheme 1. Interrelationship between the transport of ornithine and of phosphate

exchanges for the excess of OH^- ions produced in the matrix (Scheme 1). The inhibition of the movement of endogenous P_i on the addition of mersalyl prevents the movement of such OH^- ions; in this case, ornithine transport leads to an alkalization of the matrix space.

Interaction of ornithine aminotransferase with the urea cycle

In liver, the concentrations of urea-cycle intermediates may be increased by the influx of dietary arginine from the blood, followed by hydrolysis of this compound to give ornithine. The major metabolic function of ornithine aminotransferase is to remove excess of ornithine from the urea cycle, allowing the maintenance of a relatively constant intracellular ornithine concentration. The capability of this enzyme to catabolize ornithine in isolated mitochondria has been established in the present investigation.

If the results obtained with isolated mitochondria are applicable also to the intact cell, it follows that the maximum rate at which ornithine can be removed from the urea cycle is dependent on the rate of ornithine transport into the matrix. The control of ornithine transport then becomes of considerable metabolic importance. In the present study, no control of this process by any factors other than the concentration of ornithine could be demonstrated. It is possible that the transport of ornithine is affected by some naturally occurring metabolite in the liver cytoplasm, which is lost on isolating the mitochondria. The inhibition of ornithine aminotransferase by branched-chain amino acids may also be of significance in the regulation of ornithine metabolism (Matsuzawa, 1974). It is clear that further studies are required to elucidate the mechanisms which control the intracellular ornithine concentration in liver. When the dietary protein concentration is low, the most important mechanism for the conservation of urea-cycle intermediates appears to be the repression of ornithine aminotransferase activity, which decreases the rate of ornithine catabolism, at least in isolated mitochondria, to a very low value.

It is common in the study of amino acid metabolism in liver to add an excess of ornithine to perfused liver or isolated liver cells in order to increase the concentration of urea-cycle intermediates and hence to increase the rate of synthesis of urea from amino acid nitrogen. The results of such experiments should be interpreted with care, since the present investigation has shown that ornithine at millimolar concentrations is quite rapidly catabolized by mitochondria, and may itself provide NH_3

for urea synthesis at rates comparable with the rates of deamination of the amino acids under investigation.

This investigation was supported by a Project Grant from the Medical Research Council. We thank Mrs. L. M. Clark for able technical assistance.

References

- Archibald, R. M. (1944) *J. Biol. Chem.* **156**, 121–142
- Bernt, E. & Bergmeyer, H. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H., ed.), 2nd edn., pp. 384–388, Academic Press, London
- Bradford, N. M. & McGivan, J. D. (1973) *Biochem. J.* **134**, 1023–1029
- Chappell, J. B. & Hansford, R. G. (1972) in *Subcellular Components: Preparation and Fractionation* (Birnie, G. D., ed.), 2nd edn., pp. 77–91, Butterworth, London
- Chinard, F. P. (1952) *J. Biol. Chem.* **199**, 91–95
- Gamble, J. G. & Lehninger, A. L. (1973) *J. Biol. Chem.* **248**, 610–618
- Gornall, H. E., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Herzfeld, A. & Knox, W. E. (1968) *J. Biol. Chem.* **243**, 3327–3332
- Ip, M. M., Chee, P. Y. & Swick, R. W. (1974) *Biochim. Biophys. Acta* **354**, 29–38
- Katanuma, N., Okada, M., Matsuzawa, T. & Otsuka, Y. (1965) *J. Biochem. (Tokyo)* **57**, 445–449
- Kirsten, E., Gerez, C. & Kirsten, R. (1963) *Biochem. Z.* **337**, 312–319
- Klingenberg, M. (1970) *Essays Biochem.* **6**, 119–159
- Krebs, H. A., Hems, R. & Lund, P. (1973) *Adv. Enzyme Regul.* **11**, 361–377
- Matsuzawa, T. (1974) *J. Biochem. (Tokyo)* **75**, 610–619
- McGivan, J. D., Bradford, N. M. & Chappell, J. B. (1974) *Biochem. J.* **142**, 359–364
- Morris, J. E., Peraino, C. & Strayer, D. (1974) *Proc. Soc. Exp. Biol. Med.* **147**, 706–709
- Nicholls, D. G. (1974) *Eur. J. Biochem.* **50**, 305–315
- Palmieri, F., Quagliariello, E. & Klingenberg, M. (1972) *Eur. J. Biochem.* **29**, 408–416
- Peraino, C. (1972) *Biochim. Biophys. Acta* **289**, 117–127
- Peraino, C. & Pitot, H. C. (1963) *Biochim. Biophys. Acta* **73**, 222–231
- Peraino, C. & Pitot, H. C. (1964) *J. Biol. Chem.* **239**, 4308–4313
- Peraino, C., Blake, R. L. & Pitot, H. C. (1965) *J. Biol. Chem.* **240**, 3039–3043
- Peraino, C., Bunville, L. G. & Tahmisian, T. N. (1969) *J. Biol. Chem.* **244**, 2241–2249
- Pfleiderer, G. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H., ed.), pp. 381–383, 2nd edn., Academic Press, London
- Sanada, Y., Suemori, I. & Katanuma, N. (1970) *Biochim. Biophys. Acta* **220**, 42–50
- Strecker, H. J. (1965) *J. Biol. Chem.* **240**, 1225–1230
- Volpe, P., Sawamura, R. & Strecker, H. J. (1969) *J. Biol. Chem.* **244**, 719–726