ACCUMULATION OF ORNITHINE AND CITRULLINE IN RAT LIVER MITOCHONDRIA IN RELATION TO CITRULLINE FORMATION

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1. Introduction

According to Gamble and Lehninger [1], transport of ornithine across the mitochondrial membrane is dependent upon respiratory energy. However, this conclusion is not consistent with the high rate of citrulline production found in uncoupled mitochondria incubated under anaerobic conditions [2,3]. Hence, studies were undertaken in order to examine the transport of both ornithine and citrulline across the mitochondrial membrane under both energized and uncoupled conditions.

Results indicate that entry of ornithine is not energy-dependent and does not require proton-carrying anions but is modulated by the extramitochondrial citrulline/arginine ratio. Accumulation of citrulline in mitochondria occurs only during its formation. Inhibition of citrulline production results in a release of this amino acid from the mitochondria in both State 4 and uncoupled conditions.

2. Materials and methods

Rat liver mitochondria were prepared as described previously [4], but the final wash and suspension were made with 0.3 M mannitol replacing sucrose.

Abbreviations: EGTA, ethylene glycol-bis(2-aminoethyl)-tetraacetate; TTFB, tetrachlortrifluoromethylbenzimidazole

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The distribution of ornithine and citrulline between the mitochondria and the medium was measured using the silicone layer technique [5]. The basic incubation medium contained 15 mM KCl, 2 mM EGTA, 5 mM $MgCl_2$, 40 mM Tris–HCl buffer, 10 mM ornithine, 30 mM $KHCO_3$, 10 mM potassium phosphate buffer, a trace amount of tritiated water, 3% dextran and 9 mM succinate (+ 1 μg/ml rotenone). The final pH was 7.4. The additions are indicated in the legends to the tables and figures.

Uptake of ornithine was measured by the use of L-[1-^{14}C]ornithine monochloride (60 mCi/mmol) from Amersham, England. When intramitochondrial levels of citrulline were determined, the incubation mixture contained a trace amount of sodium [^{14}C]-bicarbonate (60 mCi/mmol) from Amersham. After separation of mitochondria from the incubation medium, the supernatant was transferred to tubes containing 1.5 M perchloric acid; 40 μl samples were taken from both supernatant and acid extract of the pellet beneath the oil; these samples were evaporated on a boiling-water bath. The resulting acid-stable, non-volatile residue was suspended in 40 μl water and used for determination of radioactivity by liquid scintillation counting. The radioactivity of a sample taken from the incubation mixture before adding ornithine was deducted from values found in samples withdrawn at intervals from the incubation medium after adding ornithine. Extramitochondrial citrulline concentrations were also determined according to Archibald [6] with two slight modifications: the volumes of all solutions used for the assay were half those of the original method and to all the samples 50 nmol of citrulline were added in order to increase sensitivity of the

assay at low citrulline concentrations. The results obtained by radioactivity measurements are comparable with those obtained by the colorimetric assay but are more easily obtained when small quantities are to be measured.

3. Results and discussion

3.1. *Internal citrulline during citrulline formation*

Data in fig.1 show that citrulline synthesis in rat liver mitochondria is accompanied by an increase of intramitochondrial citrulline concentration. After

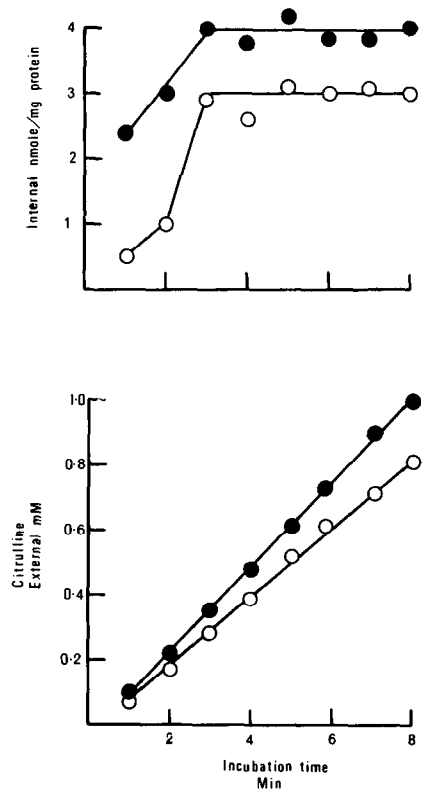

Fig.1. Accumulation and production of citrulline in rat liver mitochondria incubated in State 4 and in the presence of uncoupler + oligomycin. Mitochondria (5.4 mg/ml) were incubated in the basic medium containing a trace amount of [^{14}C]bicarbonate and 5 mM ornithine in State 4 (•) or in the presence of oligomycin (1 μg/mg protein) and 1.5 μM TTFB (○). At the indicated times, 0.25 ml samples were taken and the reaction was stopped by the rapid centrifugation through silicone oil. External and internal citrulline was determined in supernatant and pellet extracts as described in Materials and methods.

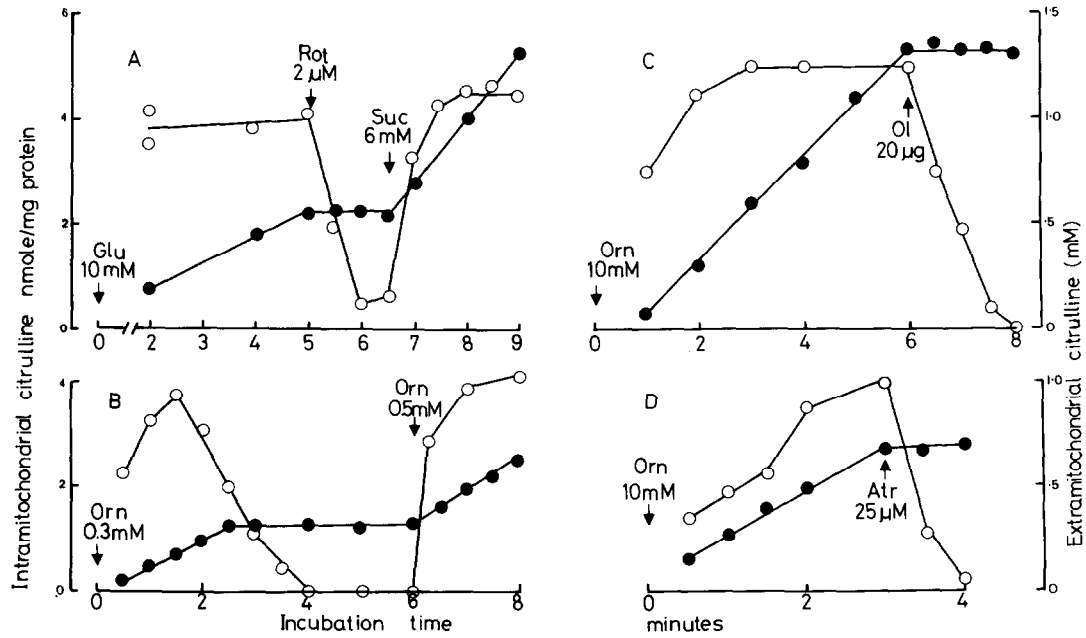

Fig.2. Legendopposite

3 min of incubation, i.e., in the presence of about 0.3 mM citrulline in the external medium, the internal citrulline level was equal to 3 and 4 nmol/mg protein respectively, in mitochondria incubated in State 4 and uncoupled conditions, and remained constant despite an increase of external citrulline concentration in course of a prolonged incubation. However, an inhibition of citrulline production either by addition of rotenone to mitochondria incubated with glutamate as respiratory substrate (fig.2A) or the lack of ornithine in the incubation medium (fig.2B), resulted in a release of citrulline from the mitochondria. The increased intramitochondrial citrulline concentration was restored on the re-initiation of citrulline synthesis by addition of succinate (fig.2A) or ornithine (fig.2B). The release of citrulline from the mitochondria was not a direct consequence of energy depletion or ornithine utilization, since this phenomenon was also observed on addition of oligomycin to mitochondria incubated in State 4 in the presence of a high ornithine concentration (fig.2C). Here, oligomycin stops synthesis of carbamyl phosphate and hence of citrulline, so the internal citrulline is no longer replenished. A release of intramitochondrial citrulline occurred also from uncoupled mitochondria (+ oligomycin) incubated with exogenous ATP as energy source for carbamyl phosphate generation when ATP entry was inhibited by adding atractylate (fig.2D).

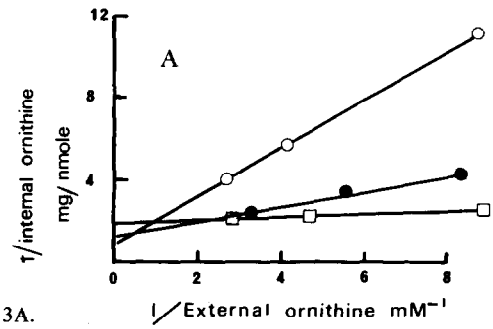

Fig.3A.

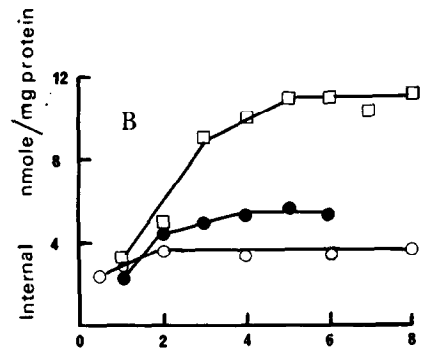

Fig.3B. Ordinate: Citrulline

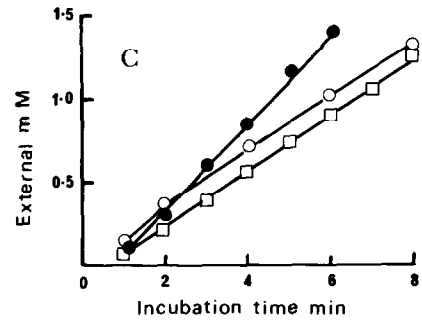

Fig.3C. Ordinate: Citrulline

Fig.3. Effect of pH on the ornithine and citrulline accumulation in rat liver mitochondria. The system for experiment 1 (section A) consisted of mitochondria (4.8 mg/ml) incubated for 2 min in the basic medium. Oligomycin (1 μg/mg protein), 5 mM glucose, 0.1 mM ADP and 2 units of hexokinase were added in order to prevent ATP production and hence carbamyl phosphate synthesis. In experiment 2 (sections B and C), mitochondria (6.2 mg/ml) were incubated in State 4 in the presence of a trace amount of [^{14}C]bicarbonate. At indicated times, 0.25 ml samples were taken and spun down through the silicone oil in order to determine both external and internal citrulline concentrations following the procedure described in Materials and methods. (•) pH 7.4, (○) pH 6.5, (□) pH 7.9.

Fig.2. Release of citrulline from mitochondria after inhibition of citrulline production. Mitochondria (4.8 mg/ml) were incubated in the basic medium containing a trace amount of [^{14}C]bicarbonate in either State 4 (experiments A–C) or in the presence of 1.5 μM TTFB and 1 μg/mg protein of oligomycin (experiment D). In experiments A, 10 mM ornithine was added before addition of glutamate. In experiments B and C, 9 mM succinate (1 μg/ml rotenone) was present as a respiratory substrate. In experiment D, 5 mM ATP was used as an energy source for carbamyl phosphate formation. Other additions were made where indicated. *Abbreviations*: Glu, glutamate; Rot, rotenone; Suc, succinate; Orn, ornithine; Ol, oligomycin; Atr, atractylate. (○) and (•) correspond to internal and external levels of citrulline, respectively.

The intramitochondrial citrulline content was increased at pH 7.9 and decreased at pH 6.5 (fig.3B), although the rates of its production were similar (fig.3C). Although K_m for ornithine of ornithine carbamyltransferase decreases with increasing pH [7], the rate of citrulline production was lower at both pH 6.5 and 7.9 than at pH 7.4 (fig.3C). The latter observation is in agreement with Rijman [7].

3.2. *Internal ornithine*

[^{14}C]Ornithine was used to find the distribution of this amino acid between medium and mitochondria under conditions ensuring no conversion to citrulline. The net ornithine uptakes, corrected for the non-specific carry down, were plotted reciprocally against the applied concentrations (fig.3A), showed that saturation uptakes were only 1.25, 0.83 and 0.53 nmol/mg protein at pH 6.5, 7.4 and 7.9 respectively.

The data presented in table 1 show that the intramitochondrial ornithine accumulation was similar in both respiring and uncoupled non-respiring mitochondria (experiments 1 and 5, respectively). Moreover, the ornithine uptake was almost unaffected by addition of either phosphate or bicarbonate (experiment 4). These data differ from those reported by Gamble and Lehninger [1] who however, used a medium based on mannitol plus sucrose rather than on Tris–chloride. The ratios between internal ornithine content (nmol/mg protein) and its external concentration (mM) included in table 1 are between 2 and 3, which corresponds to one value

Table 1
Uptake of [^{14}C]ornithine by rat liver mitochondria

Expt	System	Ornithine uptake (nmol/mg protein)
1	Control	0.25
	No succinate	0.26
2	Control	0.27
	+ 1 mM citrulline	0.64
	+ 1 mM arginine	0.11
3	Control	0.24
	+ 0.1 mM citrulline	0.51
	+ 0.1 mM citrulline + 0.1 mM arginine	0.42
	+ 0.1 mM citrulline + 0.2 mM arginine	0.32
	+ 0.1 mM citrulline + 1 mM arginine	0.26
4	No phosphate, no bicarbonate	0.23
	+ 5 mM phosphate	0.28
	+ 10 mM bicarbonate	0.28
5	Uncoupler + oligomycin	0.29
	+ 1 mM citrulline	0.70

The system for experiments 1–4 consisted of mitochondria (5 mg/ml) incubated at 30°C in the basic medium in the presence of oligomycin (1 μg/mg protein), 10 mM glucose, 0.1 mM ADP and 2 units of hexokinase in order to trap ATP and prevent citrulline formation. 0.1 mM [^{14}C]ornithine was added after 1 min preincubation. In the control of experiment 4, phosphate and bicarbonate were omitted from the basic incubation mixture. In experiment 5, succinate was omitted from the medium and mitochondria were preincubated for 1 min in the presence of 20 μM rotenone and oligomycin followed by addition of 1.5 μm TTFB. Time of incubation was 3 min. Accumulation of ornithine was not increased after a longer time of incubation.

in Gamble and Lehninger's table 2, namely their experiment 4. Their other values in the same table are as much as 10-fold higher, for which we have no explanation. Trials of citrulline formation in two differently based media showed that the rates in mannitol were very low compared with those in the Tris mixture. Addition of 1 mM citrulline increased the ornithine accumulation (experiment 2) although the saturation capacity of the mitochondria for ornithine was unchanged under these conditions (not shown). Citrulline at 0.1 mM concentration, which is the concentration only slightly higher than that found in rat liver [8] and human plasma [9], also increased ornithine accumulation (experiment 3). Since citrulline appears to pass in and out of cells unhindered [10] and its concentration in liver is similar to that in plasma, it is likely that it could increase intramitochondrial ornithine in vivo. In contrast to the effect of citrulline, arginine added at concentrations similar to those found in rat liver [8] and human plasma [9] significantly decreased ornithine accumulation (experiment 2). The inhibitory effect of arginine was diminished in the presence of 0.1 mM citrulline (experiment 3).

Chappell et al. [11] suggested that ornithine and citrulline are transported across the mitochondrial membrane on a single carrier by strict antiport. We have tested the consequence of exposing citrulline-loaded mitochondria to ornithine. This was possible by centrifuging them through a layer of [^{14}C]-ornithine-containing medium. The results, presented in fig.4, indicate that an increasing citrulline content in the mitochondria coupled with a flux of citrulline into the medium led to a low steady amount of ornithine being taken up during the brief exposure to the [^{14}C]ornithine solution, i.e., the influx was constant. When all the ornithine in the incubation mixture had been utilized, internal citrulline fell and remained at a negligible level, but the [^{14}C]-ornithine uptake by the mitochondria during the brief exposure was increased. This shows that the ornithine influx into mitochondria not exporting citrulline is greater than when they are exporting it. The fact that the internal ornithine does not rise between 0 and 2 min, despite the production of citrulline, implies that internal citrulline prevents the increased accumulation of ornithine induced by external citrulline. When internal citrulline has emerged the ornithine uptake increases.

The data described in this paper indicate that ornithine and citrulline are not coupled by a strict antiport. This follows from the observations that (i) intramitochondrial ornithine accumulation is similar in absence and in presence of intramitochondrial citrulline (compare values in table 1 with those obtained between 0 and 2 min in fig.4) and (ii)

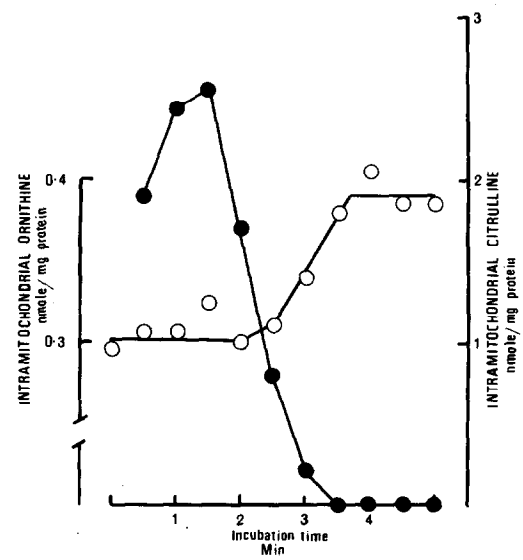

Fig.4. Relation between ornithine and citrulline accumulation. The internal citrulline was generated by incubation of mitochondria (5.4 mg/ml) in the dextran-free basic medium containing 0.2 mM ornithine. At various times, 0.15 ml samples were taken and layered onto the contents of tubes containing (in order from the bottom): 50 μl 1.5 M perchloric acid, 25 μl silicone oil, 150 μl ammonia- and bicarbonate-free basic medium containing 0.05 mM [^{14}C]-ornithine and 3% dextran, 100 μl medium without [^{14}C]-ornithine but with 1.5% dextran. The latter served as 'insulation' prior to the commencement of centrifugation. After centrifugation, samples from both the fluid above the oil and the pellet extract beneath the oil were taken for ^{14}C and ^{3}H assays. A parallel direct separation was made of the sample supplemented with [^{14}C]aspartate in order to relate the $^{3}H_2O$ and ^{14}C activities in the carry down. It was assumed that the same ratio would hold for the $^{3}H_2O$ and [^{14}C]ornithine carried outside the mitochondria into the oil. In order to determine the internal citrulline concentration, direct separations in parallel were made of samples taken at corresponding times from the basic medium containing 0.2 mM ornithine and a trace amount of [^{14}C]bicarbonate. (○) and (●) correspond to internal concentrations of ornithine and citrulline, respectively.

elevation of the external concentration of citrulline increases the mitochondrial capacity for ornithine (table 1: experiments 2, 3 and 5). The movements of the two amino acids do not appear to be related but the interactions between the external citrulline and arginine on the ornithine accumulation points to modification of the affinity of the binding sites. Trials were made of lysine, urea and guanidine at 1 mM, but these did not influence the ornithine accumulation. It appears that citrulline is only to be found accumulated while it is being produced.

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