Stimulatory effect of arginine on acetylglutamate synthesis in isolated mitochondria of mouse and rat liver

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N-Acetyl-L-glutamate synthetase (EC 2.3.1.1) catalyses the synthesis of *N*-acetyl-L-glutamate, an allosteric activator of carbamoyl-phosphate synthetase I in the liver of ureotelic animals, and the first enzyme is activated specifically by arginine. We have proposed that arginine can stimulate acetylglutamine synthetase *in vivo* and thereby increase the mitochondrial content of acetylglutamate. The effects of arginine on acetylglutamate synthesis in isolated mitochondria were investigated in detail in the present work. (1) When rat liver mitochondria were isolated and incubated with [¹⁴C]glutamate and unlabelled acetate as substrates, acetyl[¹⁴C]glutamate synthesis in the mitochondria was more extensive in the presence than in the absence of L-arginine. There was no significant difference between the specific radioactivities of intramitochondrial [¹⁴C]glutamate in the presence and absence of arginine. (2) When rat liver mitochondria were incubated with [¹⁴C]acetate and unlabelled glutamate as substrates, arginine also stimulated acetyl[¹⁴C]glutamate synthesis in the isolated mitochondria. (3) L-Lysine or L-homoarginine, which does not activate acetylglutamate synthetase, had no effect on acetylglutamate synthesis, in the isolated mitochondria. (4) The arginine concentration giving half-maximal synthesis of acetylglutamate in isolated mitochondria was about 50 μ M, which is in the range of physiological concentrations of arginine in the liver. (5) As we previously reported [Kawamoto, Ishida, Mori & Tatibana (1982) *Eur. J. Biochem.* **123**, 637–641], the sensitivity of acetylglutamate synthetase to arginine activation undergoes marked changes after food ingestion. The extent of arginine activation of acetylglutamate synthesis in isolated mitochondria correlated well with the sensitivity of acetylglutamate synthetase extracted from the mitochondria to arginine activation. (6) These data lend further support to the idea that arginine itself activates the mitochondrial synthesis of acetylglutamate.

INTRODUCTION

Carbamoyl-phosphate synthetase I (EC 6.3.4.16) catalyses the synthesis of carbamoyl phosphate, the first intermediate in the pathway of urea synthesis in the liver of ureotelic animals, and requires, though not absolutely (Rubio *et al.*, 1983; Cohen, 1984), *N*-acetyl-L-glutamate as a catalytic cofactor (Grisolia & Cohen, 1953; Hall *et al.*, 1958). We have proposed (Shigesada & Tatibana, 1971a; Tatibana & Shigesada, 1976; Shigesada *et al.*, 1978) that the hepatic concentration of acetylglutamate is a control factor of urea biosynthesis. This proposal has been supported by findings in other laboratories (McGivan *et al.*, 1976; Saheki *et al.*, 1977; Cheung & Rajman, 1980; Hensgens *et al.*, 1980; Stewart & Walser, 1980; Lof *et al.*, 1983; for a review, see Meijer, 1979). We also showed (Shigesada & Tatibana, 1971b, 1978; Tatibana *et al.*, 1976) that the presence of acetylglutamate synthetase (EC 2.3.1.1) in mouse and rat liver mitochondria is responsible for the formation of acetylglutamate from glutamate and acetyl-CoA and that the enzyme is activated specifically by L-arginine. The enzyme from rat liver was purified to apparent homogeneity (Sonoda & Tatibana, 1983).

The stimulatory effect of arginine on [¹⁴C]glutamate incorporation into acetylglutamate was demonstrated both *in vitro*, by using isolated mitochondria (Shigesada

& Tatibana, 1971b), and *in vivo* (Shigesada *et al.*, 1978). Administration of arginine to mice *in vivo* led to an increase in hepatic acetylglutamate (Tatibana & Shigesada, 1976; Shigesada *et al.*, 1978). We proposed that the hepatic concentration of arginine, along with that of glutamate, regulates acetylglutamate synthesis and thus intramitochondrial concentrations of the compound.

However, Stewart & Walser (1980) reported that an increase in hepatic acetylglutamate was induced by administration of an amino acid mixture, regardless of whether or not the mixture contained arginine, and they claimed that the increase was not attributable to the arginine activation of acetylglutamate synthetase. Cheung & Rajman (1981) showed experimental results including the existence of significant arginase activity in isolated mitochondria as well as a very limited increase in carbamoyl phosphate synthesis with arginine over controls, and suggested that stimulation of urea synthesis by arginine by way of the activation of acetylglutamate synthetase is of limited physiological significance.

Here we report a detailed analysis of the effects of arginine on acetylglutamate synthesis in isolated mitochondria. Several lines of evidence presented here support the proposal that mitochondrial synthesis of acetylglutamate is enhanced by exogenously added arginine through a direct activating effect on mitochondrial acetylglutamate synthetase.

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MATERIALS AND METHODS

Chemicals

L-Arginine hydrochloride was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Salmine (protamine sulphate from salmon) was from Nakarai Chemicals (Kyoto, Japan), and was purified on Sephadex G-50 (fine grade) (Pharmacia Fine Chemicals, Uppsala, Sweden) before use. Malate dehydrogenase (type I) and citrate synthase were from Boehringer Mannheim (Mannheim, Germany). L-[U- 14 C]Glutamate (290 Ci/mol), [1- 14 C]acetate (sodium salt) (500 Ci/mol) and L-[guanidino- 14 C]arginine hydrochloride (60 Ci/mol) were obtained from New England Nuclear (Boston, MA, U.S.A.), Daiichi Pure Chemicals (Tokyo, Japan) and The Radiochemical Centre (Amersham, Bucks., U.K.) respectively.

Isolation of mitochondria

Liver mitochondria were isolated as described by Myers & Slater (1957) from male Wistar rats (150–200 g body wt.) or male dd/Y-strain mice (15–20 g body wt.), except that the isolation medium (pH 7.0) contained 210 mM-mannitol, 70 mM-sucrose, 0.2 mM-EGTA and 3 mM-Tris/HCl. Mitochondrial protein was determined by the method of Clarke (1976).

Incubation of mitochondria

The standard incubation medium was a slight modification of that described by Charles *et al.* (1967),

Fig. 1. Effect of arginine on acetyl[14 C]glutamate synthesis from [14 C]glutamate by rat liver mitochondria

One rat was fed on the usual laboratory chow *ad libitum*, and a single mitochondrial preparation was used in the experiments of Figs. 1 and 2. Similar results were obtained in another experiment, where a different mitochondrial preparation and a slightly different incubation system were used. Rat liver mitochondria were incubated with [14 C]glutamate and unlabelled acetate as described in the Materials and methods section for the period indicated, in the absence (O) or presence (●) of 2 mM-L-arginine. After incubation, mitochondria were precipitated and extracted. Acetyl[14 C]glutamate in the extracts was isolated and counted for radioactivity.

and contained, in a final volume of 0.25 ml (pH 7.4 at 25 °C), 15 mM-KCl, 1 mM-EGTA, 5 mM-MgCl₂, 75 mM-Tris/HCl, 10 mM-L-ornithine, 16.6 mM-KHCO₃, 3 mM-ATP, 5 mM-potassium phosphate buffer, 10 mM-NH₄Cl, 10 mM-acetate, 5 mM-L-[14 C]glutamate (10 Ci/mol) and, where indicated, 2 mM-L-arginine. The medium was preincubated at 25 °C under a gas phase of O₂/CO₂ (19:1) for 10 min. Mitochondria (about 1 mg of protein) suspended in 25 μ l of the isolation medium were added to the incubation medium and incubated, usually for 15 min. The reaction was halted by placing the tubes on ice, and 0.5 ml of the isolation medium for mitochondria was added. The diluted mixture was layered on a medium containing 0.36 M-sucrose, 3 mM-Tris/HCl and 0.1 mM-dithiothreitol, and the preparation was centrifuged at 9000 g for 1 min (Microcentrifuge, model KM-15200; Kubota Corp., Tokyo, Japan). The precipitated mitochondria were washed by this procedure. To the precipitate was added 150 μ l of 10% (w/v) trichloroacetic acid, and the mixture was homogenized and centrifuged at 9000 g for 1 min. The extract and the washings of the residue were combined and then used for the determination of the radioactivity of acetyl[14 C]glutamate and of the amount and radioactivity of glutamate in the mitochondria.

Enzyme assays

Acetylglutamate synthetase in the sonicated mitochondria was measured as described by Kawamoto *et al.* (1982). Malate dehydrogenase was assayed by the method of Bergmeyer *et al.* (1974). Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard.

Determination of radioisotope incorporation into acetylglutamate

Trichloroacetic acid extracts of mitochondria were treated with diethyl ether to remove the acid. Acetylglutamate from the samples was purified by ion-exchange and paper chromatography, and the radioactivity was determined as described by Shigesada & Tatibana (1971a).

Determination of specific radioactivity of glutamate

The amount of glutamate in the trichloroacetic acid extracts of mitochondria was determined with a Hitachi Amino Acid Analyser, model 835 (Hitachi, Tokyo, Japan). Fractions of glutamate from the column in the above analysis were used for determination of the radioactivity. The specific radioactivity of glutamate was calculated from these two values.

Determination of acetyl-CoA

Acetyl-CoA was assayed by the method of Decker (1974) with malate dehydrogenase and citrate synthase, after the liver mitochondria had been deproteinized with HClO₄ (final concn. 7%, v/v).

RESULTS

When rat liver mitochondria were incubated with [14 C]glutamate and unlabelled acetate as substrates, addition of 2 mM-L-arginine, a specific activator of acetylglutamate synthetase (Sonoda & Tatibana, 1983), to the medium increased, by about 4-fold, acetyl[14 C]glutamate formation for up to 15 min (Fig. 1).

Fig. 2. Effects of various arginine concentrations on mitochondrial synthesis of acetyl[¹⁴C]glutamate from [¹⁴C]glutamate

Rat liver mitochondria were incubated for 15 min in the presence of arginine at the concentrations indicated. After the incubation, the mixture was centrifuged and intramitochondrial acetyl[¹⁴C]glutamate was isolated and counted for radioactivity.

To elucidate whether the increase was due to a net increase in the rate of acetylglutamate synthesis or to changes in the specific radioactivity of [¹⁴C]glutamate, the amounts and radioactivity of intramitochondrial glutamate were measured after incubation for 5 or 15 min. There was no significant difference between the calculated specific radioactivities of glutamate in the presence (14200 and 16200 c.p.m./nmol) and absence (14400 and 15700 c.p.m./nmol) of arginine after 5 or 15 min incubation. Thus the increase by arginine of ¹⁴C incorporation into acetylglutamate from [¹⁴C]glutamate may represent the net increase in acetylglutamate synthesis.

The effect of arginine on acetylglutamate synthesis in isolated mitochondria was also examined with [¹⁴C]acetate as the labelled substrate. The mitochondria were incubated for 15 min as in Fig. 1, except that [¹⁴C]acetate (5 Ci/mol) and unlabelled glutamate were used in place of [¹⁴C]glutamate and unlabelled acetate as substrates. The radioactivities of acetyl[¹⁴C]glutamate formed in the absence and presence of 2 mM-arginine were 1030 and 2940 c.p.m. respectively. These results further support the proposal that arginine stimulates the net synthesis of acetylglutamate in the isolated mitochondria.

Effects of arginine at various concentrations (0, 0.02, 0.1, 0.5 and 2 mM) on the incorporation of [¹⁴C]glutamate into acetylglutamate in isolated mitochondria were also examined (Fig. 2). The ¹⁴C incorporation, which represents acetylglutamate formation, increased when the concentrations of arginine were increased up to 0.5 mM. The concentration giving half-maximal synthesis of acetyl[¹⁴C]glutamate was about 50 μM. When 0.02 mM-, 0.1 mM-, 0.5 mM- and 1.0 mM-[guanidino-¹⁴C]arginine was initially added to the assay system, 70, 62, 61 and 59% of the arginine was recovered, respectively, at the end of 15 min incubation, as analysed by paper electrophoresis.

The arginine concentration giving half-maximal activation of purified acetylglutamate synthetase was 5 μM (Sonoda & Tatibana, 1983), which is much lower than 50 μM. The synthetase may have a higher *K_a* value *in situ*, or arginine penetration into mitochondria may be too low or slow.

To clarify whether the stimulatory effect of arginine on mitochondrial acetylglutamate synthesis was mediated directly through the activating effect of arginine on acetylglutamate synthetase or through a non-specific effect of basic amino acids or guanidino compounds on mitochondria, the following experiments were performed. We examined the effects of L-lysine and L-homoarginine, neither of which activates acetylglutamate synthetase (Sonoda & Tatibana, 1983), or protamine (a cationic polypeptide), a potent activator of the synthetase (Sonoda & Tatibana, 1983), on acetylglutamate synthesis in isolated mitochondria. Rat liver mitochondria were incubated with [¹⁴C]glutamate for 15 min, as in Fig. 1, except that the above compounds were used instead of arginine. Lysine or homoarginine (at 0.04 or 2 mM) had no effect on the mitochondrial synthesis of acetylglutamate. Arginine (2 mM) activated acetylglutamate synthesis of the mitochondrial preparation 5.4-fold. Protamine at a concentration of 0.5 mg/ml (but not at 0.05 mg/ml) activated the acetylglutamate synthesis in the isolated mitochondria, although the extent of protamine activation was small (31 and 57% in two mitochondrial preparations). These results indicate that the stimulation of mitochondrial acetylglutamate synthesis by arginine is through a direct activating effect on mitochondrial acetylglutamate synthetase.

We reported that the sensitivity of acetylglutamate synthetase in mouse liver to arginine activation undergoes marked changes after ingestion of food (Kawamoto *et al.*, 1982) and injection of inhibitors of nucleic acid and protein synthesis (Kawamoto & Tatibana, 1983). Correlations between the extent of arginine activation of acetylglutamate synthesis in isolated mitochondria and the sensitivity to arginine activation of acetylglutamate synthetase, extracted from the mitochondria, were also examined (Table 1). When liver mitochondria from 72 h-starved mice were used, acetylglutamate synthetase activity in the sonicated homogenate was activated 4.3-fold by 1 mM-arginine, and mitochondrial acetylglutamate synthesis, as measured by [¹⁴C]glutamate incorporation, was activated 4.6-fold by 2 mM-arginine added to the medium. On the other hand, when mitochondria from fed mice were used, the extent of arginine activation of the synthetase was 6.7-fold in the sonicated homogenate, and the extent of arginine activation of mitochondrial acetylglutamate synthesis was 6.1-fold. In this particular experiment, arginine sensitivity of the synthetase from the starved mice remained fairly high, contrary to expectation. Nevertheless, the extent of arginine activation of mitochondrial acetyl[¹⁴C]glutamate synthesis was similar to the sensitivity to arginine activation of acetylglutamate synthetase prepared from the respective mitochondrial preparations. For mitochondria from liver of starved mice, the contents of intramitochondrial acetyl-CoA after the incubation were 0.405 and 0.378 nmol/mg of protein in the absence and presence of arginine respectively. For mitochondria from liver of fed mice, the values were 0.253 and 0.317 nmol/mg of protein respectively. Although acetylglutamate synthesis in the mitochondria from fed mice was less than that in mitochondria from starved mice, this is ascribable, at least in part, to the lower

Table 1. Changes in sensitivity of mitochondrial acetylglutamate synthesis to activation by arginine

Six mice were killed after starvation for 72 h (starved mice). The other six mice were allowed the usual laboratory chow for 3 h for 3 days before experiments. On day 4, they were allowed food for 3 h, and then killed at 8 h (fed mice). Mitochondria were isolated from the pooled six livers. Samples of mitochondria were suspended and sonicated, and acetylglutamate synthetase activity was measured in the presence or absence of 1 mM-L-arginine (a). The remaining portion of mitochondria was incubated with [¹⁴C]glutamate for 15 min in the presence or absence of 2 mM-L-arginine. After the incubation, the mixture was centrifuged, and intramitochondrial acetyl[¹⁴C]glutamate was isolated and counted for radioactivity (b).

(a)			
Mice	Acetylglutamate synthetase activity in disrupted mitochondria (nmol/h per mg of protein)		Activation by arginine (fold)
	No arginine	+ arginine	
Starved	1.35	5.78	4.3
Fed	0.88	5.92	6.7

(b)			
Mice	$10^{-3} \times$ Acetyl[¹⁴ C]glutamate synthesized by intact mitochondria (c.p.m./mg of protein)		Activation by arginine (fold)
	No arginine	+ arginine	
Starved	2.11	9.85	4.6
Fed	1.56	9.58	6.1

content of acetyl-CoA in the mitochondria from fed mice. The specific radioactivity of intramitochondrial glutamate showed only slight variation for each incubation. In mitochondria from liver of starved mice, the specific radioactivities of intramitochondrial glutamate were 1.27×10^4 and 1.37×10^4 c.p.m./nmol in the absence and presence of arginine respectively. In mitochondria from liver of fed mice, the values were 1.53×10^4 and 1.36×10^4 c.p.m./nmol in the absence and presence of arginine respectively. From the two values of [¹⁴C]glutamate incorporation into mitochondrial acetylglutamate and the specific radioactivities of intramitochondrial glutamate, the amounts of acetylglutamate synthesized during the incubation by mitochondria from the liver of starved mice were estimated to be 0.166 and 0.719 nmol/mg of protein in the absence and presence of arginine, respectively, giving a value of 4.3 for the arginine activation ratio. For mitochondria from liver of fed mice, the values were 0.102 and 0.704 nmol/mg of protein in absence and presence of arginine respectively, giving a value of 6.9 for the arginine activation ratio. There was a good correlation between the extent of arginine activation of mitochondrial acetylglutamate synthesis and the sensitivity to arginine activation of acetylglutamate synthetase. The results presented in Table 1 and the estimation made here are compatible with the notion that the effect of arginine, added exogenously to increase mitochondrial acetylglutamate synthesis, is mediated directly by the activating effect of the amino acid on the enzyme.

DISCUSSION

Arginine stimulated acetyl[¹⁴C]glutamate synthesis from [¹⁴C]glutamate and unlabelled acetate in isolated

mitochondria (Figs. 1 and 2). The specific radioactivity of glutamate in mitochondria incubated with or without arginine was much the same, thereby suggesting that the stimulation is due to the net increase in acetylglutamate synthesis. Moreover, the specific radioactivity of intramitochondrial glutamate (14000–16000 c.p.m./nmol) was nearly equal to that of added glutamate (10 Ci/mol = 22000 d.p.m./nmol). This is not unexpected, since the rate of glutamate transport across the mitochondrial membrane seems to be rather high, as was reported by Williamson *et al.* (1980) and Wanders *et al.* (1983). Hence it appears unlikely that arginine acts by activating [¹⁴C]glutamate permeation into mitochondria and increases ¹⁴C incorporation into acetylglutamate without significant effect on net synthesis of the compound. This was further supported by findings with [¹⁴C]acetate and unlabelled glutamate as substrates.

The purified acetylglutamate synthetase is activated specifically by arginine, but lysine and homoarginine, like other structural analogues of arginine, have no activating effect on the synthetase (Sonoda & Tatibana, 1983). Here, we demonstrated that the analogues had no effect on mitochondrial synthesis of acetylglutamate. On the other hand, protamine, which is an activator of the synthetase (Sonoda & Tatibana, 1983), activated acetylglutamate synthesis in isolated mitochondria, albeit to a small extent only. Therefore the stimulation of mitochondrial acetylglutamate synthesis by arginine is ascribed to the direct effect of arginine on acetylglutamate synthetase. Additional support for this view was obtained with different mitochondrial preparations, the acetylglutamate synthetase of which had different sensitivities to arginine activation. Acetylglutamate synthesis in mitochondria with acetylglutamate synthetase having a higher sensitivity to arginine activation responded more extensively to exogenous arginine than did mitochondria with synthetase

of a lower sensitivity (Table 1). The correlation was quite obvious. It is also unlikely that ornithine, a product of arginine degradation, is an actual activator of acetylglutamate synthesis in isolated mitochondria, because addition of 2 mM-arginine to the medium increased acetylglutamate synthesis in isolated mitochondria regardless of whether the medium contained 10 mM-ornithine (the present paper) or not (Shigesada & Tatibana, 1971b).

In our experiment, mitochondrial synthesis of acetylglutamate was activated by arginine at concentrations over 20 μ M (about 20 nmol/g) (Fig. 2), such being in the average range of physiological concentrations, 20–170 nmol/g wet wt. in normal liver of mice (Tatibana & Shigesada, 1976). For arginine to activate acetylglutamate synthetase *in vivo*, it must be transported into mitochondria at an appropriate rate. Although this has not been established experimentally *in vitro*, and permeation of arginine into liver mitochondria is low (Gamble & Lehninger, 1973), data presented by Cheung & Raijman (1981) suggested that arginine does permeate the mitochondria. In light of the small K_a value (5 μ M) of acetylglutamate synthetase for arginine (Sonoda & Tatibana, 1983), it is likely that a small amount of arginine penetrating the mitochondria is sufficient to stimulate acetylglutamate synthetase activity, though the mitochondria do appear to have a distinct mitochondrial arginase activity (Tatibana *et al.*, 1976; Cheung & Raijman, 1981), but with a high K_m value (10 mM) for arginine (Soberón & Palacios, 1976). The other possibility is that the arginine site for activation of the synthetase is exposed to the outer surface of mitochondria and that arginine, present in the cytosol, activates the synthetase. The findings that protamine (salmine), a cationic polypeptide (M_r approx. 8000), activated acetylglutamate synthesis in the isolated mitochondria, and that approx. 40% of the activity of acetylglutamate synthetase was recovered in the particulate fraction (100000 g pellets) of sonicated mitochondria, together with only 6% of the activity of malate dehydrogenase (a marker enzyme of the mitochondrial matrix) in the same fraction (S. Kawamoto, unpublished work), may favour the above view.

From the data in Fig. 1, the rates of acetylglutamate synthesis, with and without arginine, in isolated mitochondria are estimated to be about 1.0 and 0.3 nmol/mg of protein, respectively, during the incubation for 5 min. Therefore the increase in acetylglutamate caused by arginine in isolated mitochondria during the incubation is estimated to be 0.7 nmol/mg of protein. On the other hand, the liver mitochondria isolated from fed (60% casein) rats contained acetylglutamate at a concentration of 3.05 nmol/mg of protein (Morita *et al.*, 1982). If the acetylglutamate concentration in mitochondria from rats fed *ad libitum* is assumed to be 2–3 nmol/mg of protein, the arginine-related increase in acetylglutamate during the incubation corresponds to 20–35% over the controls. The increase is expected to enhance the citrulline synthesis in the isolated mitochondria at most by 20–35%, since a close relationship between the ureogenic capacity and acetylglutamate concentration was demonstrated (Tatibana & Shigesada, 1976; Shigesada *et al.*, 1978; Meijer & Van Woerkom, 1978). Thus the limited increase in carbamoyl phosphate synthesis with arginine over controls, as reported by Cheung & Raijman (1981), is reasonable. Their statement is not in accord with our proposal that the hepatic concentration of

arginine regulates that of acetylglutamate. The stimulatory effect of arginine on mitochondrial synthesis of acetylglutamate (Figs. 1 and 2; Table 1) will increase the mitochondrial concentration of acetylglutamate *in vivo*, and is expected subsequently to stimulate citrulline synthesis. An increase in intramitochondrial concentration of acetylglutamate, which needs a net accumulation of acetylglutamate in mitochondria, may take place soon after acetylglutamate synthesis is stimulated by arginine.

Stewart & Walser (1980) found that the hepatic concentration of arginine rose only slightly after the administration of arginine-free amino acid mixture, but rose remarkably when arginine was included. They reported that the hepatic arginine content was about 50–100 nmol/g wet wt. in animals given the arginine-free mixture. From our data in Fig. 2, it seems likely that this arginine concentration was high enough to enhance the mitochondrial acetylglutamate synthesis. This may explain to some extent why the omission of arginine from the administered mixture did not alter the increase in acetylglutamate (Stewart & Walser, 1980).

The data of Lof *et al.* (1983), plus the findings previously presented (for a review, see Meijer, 1979), support the proposal that acetylglutamate participates in the control of urea biosynthesis through mediation of the hepatic concentration. The present study, in addition to our previous data (Shigesada & Tatibana, 1971a, 1978; Tatibana & Shigesada, 1976), provides further evidence for the stimulatory effect of arginine on acetylglutamate synthesis in liver mitochondria. Thus arginine, which we consider a representative of exogenous amino acids, may be one factor related to the control of urea synthesis *in situ*.

During the preparation of this paper, Freedland *et al.* (1984) reported the uptake of arginine by isolated rat liver mitochondria.

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