Biochem. J. (1974) **142**, 359–364
Printed in Great Britain

Adaptive Changes in the Capacity of Systems used for the Synthesis of Citrulline in Rat Liver Mitochondria in Response to High- and Low-Protein Diets

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(*Received* 3 *April* 1974)

1. Citrulline synthesis was measured in mitochondria from rats fed on a standard diet, a high-protein diet, or on glucose. 2. With NH_4Cl as the nitrogen source the rate of citrulline synthesis was higher in mitochondria from rats fed on a high-protein diet than in those from rats fed on a standard diet. When rats were fed solely on glucose the rate of synthesis of citrulline from NH_4Cl was very low. 3. With glutamate as the nitrogen source the relative rates of citrulline synthesis were much lower than when NH_4Cl was present, but similar adaptive changes occurred. 4. The activity of the mitochondrial glutamate-transporting system increased two to three times on feeding rats on a high-protein diet, but the K_m for glutamate was unchanged. 5. Adaptive changes in certain intramitochondrial enzymes were also measured. 6. The results were interpreted to indicate that when an excess of substrate was present, citrulline synthesis from NH_4Cl was rate-limited by the intramitochondrial concentration of *N*-acetylglutamate, but citrulline synthesis from glutamate was rate-limited primarily by the activity of the glutamate-transporting system.

The main pathway of synthesis of urea from amino acid nitrogen in mammalian liver is thought to involve the initial formation of glutamate in the cytoplasm by transamination reactions between various amino acids and 2-oxoglutarate. Glutamate is then transported by a specific carrier system (Azzi *et al.*, 1967; Meyer *et al.*, 1972) into the mitochondrial matrix where it is deaminated by reaction with glutamate dehydrogenase. The enzymes carbamoyl phosphate synthase and ornithine transcarbamoylase are also located in the mitochondrial matrix whereas the other urea-cycle enzymes are cytoplasmic.

Isolated rat liver mitochondria synthesize citrulline from added bicarbonate, ornithine, ATP and a source of NH_3 (Charles *et al.*, 1967). However, when glutamate is the sole NH_3 source, the rate of citrulline synthesis is relatively low. The activity of the glutamate-transporting system in liver is much lower than the activities of the other substrate anion transporters, and the K_m of the system is relatively high (4mM) (Bradford & McGivan, 1973; Meyer & Vignais, 1973). Evidence has been obtained that the maximum rate of glutamate deamination in liver mitochondria may be limited by the rate of glutamate transport (McGivan *et al.*, 1973; Bradford & McGivan, 1973). It therefore seemed likely that citrulline synthesis might also be limited by the rate of glutamate transport under certain conditions.

It is known that the overall rate of urea synthesis in rat liver increases in response to a high-protein diet. The activities of the urea-cycle enzymes increase under these conditions (Schimke, 1962) as do the activities of certain aminotransferases (Krebs, 1972). In the present paper, it is shown that adaptive changes occur in the rate of citrulline synthesis by isolated mitochondria and in the activity of the glutamate-transporting system in response to high- and low-protein diets. It is concluded that under appropriate conditions the rate of citrulline synthesis by isolated mitochondria is limited by the activity of the glutamate carrier.

Experimental

Animals, diets and preparation of liver mitochondria

Rats of standard weight (200–250g) were maintained for three days either on standard rat cake (diet 41B; Oxoid Ltd., London S.E.1, U.K.) or on one of the two special diets described by Krebs (1972). The high-protein diet consisted of boiled egg white, which rats consume in quantities sufficient to maintain the normal rate of growth (Krebs, 1972). The other diet consisted of a 20% solution of glucose given instead of drinking water, and was thus protein-free. The rats were killed on the morning of the fourth day after the introduction of the appropriate diet. Liver mitochondria were prepared as described by Chappell & Hansford (1972) except that the sucrose in the isolation medium was replaced by mannitol (Charles *et al.*, 1967).

Glutamate transport

The uptake of glutamate into mitochondria was determined as described by Bradford & McGivan (1973), by using a mixture of Bromocresol Purple (1mM) and *N*-ethylmaleimide (0.15mM) to terminate the transport reaction after a predetermined time.

Assay of intramitochondrial enzymes

The mitochondrial suspension (60–70mg of protein/ml) was diluted with an equal volume of a solution containing 0.14M-KCl, 10mM-Tris–HCl, 5mM-$MgCl_2$ and 5mM-2-mercaptoethanol. This suspension was sonicated in 4ml amounts for three successive periods of 20s with a Soniprobe (Dawe Instruments, London W.3, U.K.). The sonicate was then centrifuged at 9000*g* for 10min to sediment the unbroken mitochondria. The supernatant was then used in the assay of mitochondrial enzymes. This method allowed a reproducible extraction of enzyme activity from the mitochondria.

Ornithine–2-oxoglutarate aminotransferase (EC 2.6.1.13) was assayed by the method of Peraino & Pitot (1963). Mitochondrial sonicate (3–4mg of protein) was incubated in a medium containing 0.1M-KCl, 10mM-Tris–HCl, 20mM-ornithine hydrochloride, 10mM-2-oxoglutarate and 10mM-*o*-aminobenzaldehyde at pH7.2 and 30°C. The development of the complex formed between *o*-aminobenzaldehyde and glutamic semialdehyde was measured at 440nm.

Ornithine transcarbamoylase (EC 2.1.3.3) was assayed by incubation of the sonicate (0.1–0.2mg of protein) in 2ml of a medium containing 0.12M-KCl, 20mM-Tris–HCl, 20mM-ornithine hydrochloride and 5mM-carbamoyl phosphate at pH7.2 and 30°C. Samples (0.5ml) were deproteinized after 1, 2 and 3min by addition of $HClO_4$ (final concn. 10%, w/v) and were then assayed for citrulline.

For the assay of carbamoyl phosphate synthase (EC 2.7.2.5), mitochondrial sonicate (5–10mg) was incubated in 4ml of a medium containing 0.12M-KCl, 20mM-Tris–HCl, 15mM-$MgCl_2$, 5mM-ATP, 20mM-ornithine hydrochloride, 20mM-$KHCO_3$, 5mM-*N*-acetylglutamate and 10μg of oligomycin at pH7.2 and 30°C. Samples (1ml) were deproteinized with $HClO_4$ (final concn. 10%, w/v) after 3, 6 and 9min and assayed for citrulline. The assay depends on the rapid quantitative conversion of the carbamoyl phosphate into citrulline by the ornithine transcarbamoylase present in the sonicate. The activity of ornithine transcarbamoylase in the sonicate was always at least 20 times that of carbamoyl phosphate synthase.

Glutamate dehydrogenase (EC 1.4.1.3) was assayed in the direction of glutamate synthesis by incubation of the sonicate in a medium containing 0.12M-KCl, 10mM-Tris–HCl, 5mM-2-oxoglutarate, 0.15mM-NADH, 2mM-ADP and 1mM-EDTA at pH7.2 and 30°C. The initial rate of decrease in absorbance at 340nm on addition of 25mM-NH_4Cl was recorded. For assay in the deamination direction, the sonicate was incubated with 0.12M-KCl, 10mM-Tris–HCl, 1mM-NAD^+, 2mM-ADP and 1mM-EDTA at pH7.2 and 30°C and the initial rate of increase in absorbance at 340nm on the addition of 50mM-glutamate was recorded.

Aspartate aminotransferase (EC 2.6.1.1) was assayed enzymically (Bergmeyer & Bernt, 1963).

Citrulline was assayed by the method of Archibald (1944). Mitochondrial protein was determined by a buiret method (Gornall *et al.*, 1949).

Carbamoyl phosphate and nicotinamide nucleotides were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. *o*-Aminobenzaldehyde was purchased from Ralph N. Emmanuel Ltd., Wembley, Middx., U.K.

Results

Overall rate of citrulline synthesis

Citrulline synthesis was measured in mitochondria from rats fed on egg white, a standard diet or on glucose. Ornithine, bicarbonate and either glutamate or NH_4Cl were present in excess and extramitochondrial ATP was added. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added to permit rapid penetration of ATP, and oligomycin was present to prevent hydrolysis of ATP by the mitochondrial ATPase.* Under these conditions citrulline synthesis was rapid and the intramitochondrial nicotinamide nucleotides were oxidized allowing a maximum rate of glutamate deamination.

* Abbreviation: ATPase, adenosine triphosphatase.

Table 1. *Citrulline synthesis in liver mitochondria*

Mitochondria (15–20mg of protein) were added to a medium containing 80mM-KCl, 20mM-Tris–HCl, 10mM-ornithine hydrochloride, 5mM-potassium phosphate, 5mM-ATP, 20mM-$KHCO_3$, 0.1mM-carboxymethoxylamine, 1μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 2.5μg of oligomycin/ml and either 10mM-NH_4Cl or 20mM-glutamate at pH7.2 and 30°C. The final volume was 4ml. Samples (1ml) were deproteinized after 3, 6 and 9min and assayed for citrulline. Each value quoted represents the mean±S.E.M. of experiments performed in duplicate on at least four different mitochondrial preparations.

	Rate of citrulline synthesis (nmol/min per mg)		
Source of NH_3	Standard diet	Egg white	20% glucose
NH_4Cl	7.3±1.8	19.8±4.3	0.74±0.30
Glutamate	2.5±0.2	4.8±0.5	0.69±0.12

Table 2. *Activity of certain mitochondrial enzymes*

Enzyme activities were measured in mitochondrial sonicates as described in the Experimental section. All enzymes were assayed in the presence of excess of substrate at 30°C and pH 7.2. For each mitochondrial preparation, two separate sonicates were prepared. Each value quoted represents the mean ± S.E.M. of the results obtained from at least four separate mitochondrial preparations.

Enzyme	Activity (nmol/min per mg)		
	Standard diet	Egg white	20% glucose
Ornithine-2-oxoglutarate aminotransferase	8.5 ± 0.5	13.1 ± 1.2	0.8 ± 0.2
Carbamoyl phosphate synthase	27.8 ± 5.5	33.8 ± 6.4	21.4 ± 1.3
Ornithine transcarbamoylase	690 ± 150	1220 ± 238	697 ± 49
Aspartate aminotransferase	570 ± 100	655 ± 135	346 ± 150
Glutamate dehydrogenase (deaminating)	55 ± 10	58 ± 6	47 ± 7
Glutamate dehydrogenase (2-oxoglutarate → glutamate)	406 ± 116	410 ± 67	323 ± 80

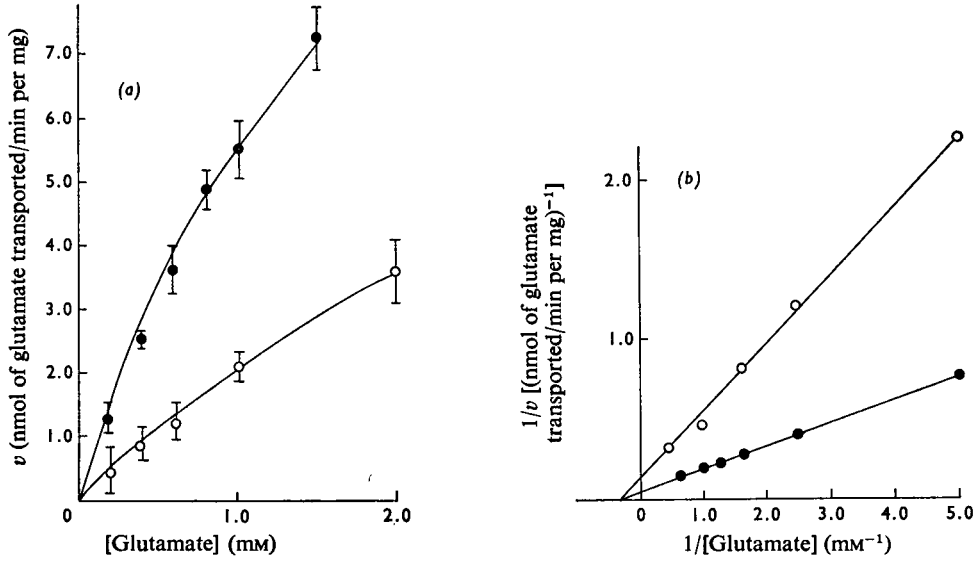

Fig. 1. *Concentration-dependence of glutamate transport*

Glutamate transport was measured at 20°C and pH 7.0. ○, Mitochondria from rats fed on a standard diet (results from Bradford & McGivan, 1973); ●, mitochondria from rats fed on egg white. (*a*) Concentration-dependence of glutamate transport and (*b*) Lineweaver–Burk plot of glutamate transport. Each point represents the mean (± S.E.M. in *a*) of determinations on at least three separate mitochondrial preparations.

Carboxymethoxylamine was added to prevent glutamate metabolism by reactions other than glutamate dehydrogenase.

Table 1 shows the rates of citrulline synthesis obtained under these conditions. With NH_4Cl as the nitrogen source the rate of citrulline synthesis increased twofold in mitochondria from rats fed on egg white compared with mitochondria from rats fed on a standard diet. Citrulline synthesis from NH_4Cl in mitochondria from glucose-fed rats was very slow. When glutamate was the sole NH_3 source, the rates of citrulline synthesis were much lower than when NH_4Cl was present. The rate in mitochondria from rats fed on egg white was again higher than that from rats fed on a standard diet. The rate of citrulline synthesis in mitochondria from glucose-fed rats was again very low. *N*-Acetylglutamate is known to be an essential cofactor for carbamoyl phosphate synthase (Cohen & Sallach, 1961). However, in no case did the addition of 5mM-*N*-acetylglutamate cause a

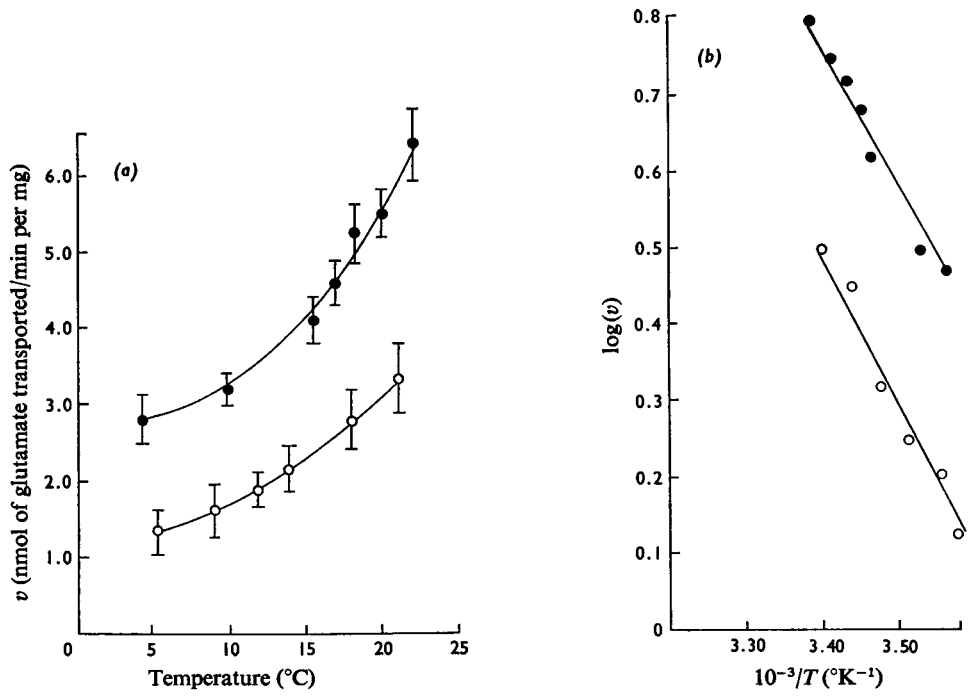

Fig. 2. *Temperature-dependence of glutamate transport*

The glutamate concentration was 1 mM and the pH was 7.0. ○, Mitochondria from rats fed on a standard diet (results from Bradford & McGivan, 1973); ●, mitochondria from rats fed on egg white. (*a*) Temperature-dependence of glutamate transport and (*b*) Arrhenius plot of glutamate transport. Each point represents the mean (±S.E.M. in *a*) of determinations on at least three separate mitochondrial preparations.

significant increase in the rate of citrulline synthesis in the experiments shown in Table 1.

Capacity of some intramitochondrial enzymes

The maximum activity of certain enzymes in sonicated mitochondria is shown in Table 2. Ornithine–2-oxoglutarate transaminase undergoes large variations in activity as a result of changes in the dietary protein intake (Peraino & Pitot, 1964; Krebs, 1972) and may be used as a reference to determine the effectiveness of dietary variations on enzyme induction and repression. Under the conditions used in the present investigation the activity increased by 50% on substituting egg white for the standard diet and decreased ten times as a result of substituting glucose for the standard diet. Carbamoyl phosphate synthase showed a smaller increase in activity in response to the egg-white diet. Ornithine transcarbamoylase activity increased twofold in response to the egg-white diet but did not decrease in response to the glucose diet. The activity of ornithine transcarbamoylase was some 20 times greater than that of carbamoyl phosphate synthase. These results are in good general agreement with the original findings of Schimke (1962).

The activities of glutamate dehydrogenase and aspartate aminotransferase showed no significant response to changes in the protein content of the diet. However, the reproducibility of extraction of enzyme activity on sonication was less good than for the other enzymes, and small adaptive changes would not be detected.

Glutamate transport

In the synthesis of citrulline from glutamate as a source of NH_3, glutamate must enter the mitochondria in exchange for OH^- via a specific transporting system. The initial rate of glutamate transport in mitochondria from rats fed on egg white and on a standard diet is shown in Fig. 1. The maximum rate of transport at 20°C increased from 9 nmol/min per mg of protein to 25 nmol/min per mg in response to the egg-white diet. The K_m (4 mM) was not significantly altered. The dependence of the initial rate of transport on temperature is shown in Fig. 2. In

Table 3. *NH_3 and aspartate formation in liver mitochondria*

Mitochondria (20–25mg of protein) were added to a medium containing 0.12M-KCl, 20mM-Tris–HCl, 10mM-glucose, 2mM-$MgCl_2$, 20mM-glutamate, 5mM-potassium phosphate, 1mM-ADP and dialysed hexokinase (0.1mg/ml) at pH7.2 and 30°C; 5mM-malonate or 5mM-malate were also present when indicated. Samples of the suspension were deproteinized after 3, 6 and 9min. The acid extracts were neutralized with K_3PO_4 and assayed for NH_3 (Kirsten *et al.*, 1963) or aspartate (Pfleiderer, 1963). Each value quoted is the mean±S.E.M. of experiments performed in triplicate on four separate mitochondrial preparations.

	Rate of NH_3 formation (nmol/min per mg)		
Substrate	Standard diet	Egg white	20% glucose
Glutamate+malonate	4.3±0.7	8.6±0.8	4.1±1.5
	Rate of aspartate formation (nmol/min per mg)		
Glutamate+malate	16.6±1.8	18.2±2.1	14.8±1.9

mitochondria from rats fed on egg white, the initial rate of transport of glutamate (1mM) at 20°C was 5.3nmol/min per mg compared with 2.8nmol/min per mg for rats fed on a standard diet. Similar experiments were performed with mitochondria from rats fed solely on glucose. In no case were the rates of transport obtained signicantly different from those presented above for rats on a standard diet.

It has previously been postulated (McGivan *et al.*, 1973; Bradford & McGivan, 1973) that the maximum rate of glutamate deamination in rat liver mitochondria is limited by the activity of the glutamate-transporting system. To obtain further evidence about this possibility, the maximum rate of NH_3 production from glutamate was measured under conditions where aspartate production was inhibited by addition of malonate. Table 3 shows that under these conditions, the rate of NH_3 production increased twofold in mitochondria from rats fed on egg white compared with those fed on a standard diet. Replacement of the standard diet by glucose had no effect on the rates of glutamate deamination obtained. These changes parallel very closely the changes observed in the activity of the glutamate-transporting system reported above. The corresponding rates of glutamate transport and glutamate deamination were also similar.

Under conditions where glutamate plus malate were present, glutamate was quantitatively converted into aspartate. In Table 3, it is shown that the maximum rates of aspartate formation were faster than the maximum rates of NH_3 formation when malonate was present, and did not vary significantly with the protein content of the diet. In this case, glutamate exchanges for aspartate on a separate transporting system (Azzi *et al.*, 1967), which has a higher activity than the glutamate/OH^- exchange system (Bradford & McGivan, 1973). There is no evidence to suggest that glutamate/aspartate exchange is rate-limiting for aspartate formation.

Discussion

The results presented in the present paper provide some information about the nature of the rate-limiting reactions in mitochondrial citrulline synthesis under various conditions.

When mitochondria from glucose-fed rats were incubated with an excess of NH_4Cl, bicarbonate, ornithine and ATP, the rate of citrulline synthesis was extremely low (Table 1). However, the maximum activities of the enzymes carbamoyl phosphate synthase and ornithine transcarbamoylase were not greatly different from those in mitochondria from rats on a standard diet (Table 2). Preliminary experiments (N. M. Bradford, unpublished work) indicated that the rate of ornithine transport in mitochondria from glucose-fed rats was not significantly different from that in mitochondria from control rats and was considerably higher than the maximum rate of carbamoyl phosphate synthase. Hence the very low rate of citrulline synthesis in mitochondria from glucose-fed rats did not appear to be due to a lack of enzyme capacity or of activity of the ornithine-transporting system.

The NH_3-dependent carbamoyl phosphate synthase in liver is absolutely dependent on the presence of *N*-acetylglutamate (Cohen & Sallach, 1961). *N*-Acetylglutamate is synthesized in liver mitochondria, and the liver content of this compound increases on feeding a high-protein diet and decreases when dietary protein content is low (Tatibana & Shigesada, 1971, 1972). The K_a for the activation of carbamoyl phosphate synthetase by *N*-acetylglutamate in frog liver is 1mM (Fahien & Cohen, 1964) and the intramitochondrial concentration of *N*-acetylglutamate is estimated to be 0.1mM–0.2mM (Tatibana & Shigesada, 1972). From these considerations and the results presented in the present paper, it appears that in glucose-fed rats, the rate of citrulline synthesis from excess of NH_4Cl is limited by the presumably low intramitochondrial content of *N*-acetylglutamate. Addition of *N*-acetylglutamate to mitochondria from glucose-fed rats did not increase the rate of citrulline synthesis. However, it is likely that this compound does not penetrate the mitochondrial membrane (see also Charles *et al.*, 1967). For rats fed on egg white, the rate of citrulline synthesis approached the maximum capacity of carbamoyl phosphate synthase (Table 2) and

sufficient *N*-acetylglutamate to saturate this enzyme was presumably present.

The rates of citrulline synthesis from glutamate were much lower than the corresponding rates of synthesis from NH_4Cl as substrate (Table 1). The conditions of this experiment were chosen so that the rate of glutamate deamination was the maximum that could be achieved. The K_m for glutamate transport is relatively high (4mM) as is the K_m of glutamate dehydrogenase for glutamate (Bradford & McGivan, 1973). The $V_{max.}$ of glutamate transport is much lower than that of glutamate dehydrogenase (Fig. 1 and Table 2). The synthesis of citrulline from NH_3 has a relatively low K_m (0.2mM) for NH_3 (McGivan *et al.*, 1973). The rate of citrulline synthesis from glutamate increased twofold in response to the egg-white diet (Table 1) and a parallel increase in the rate of glutamate transport also occurred (Fig. 1). The absolute rates of citrulline synthesis and glutamate transport were similar. It therefore appears that when glutamate is the NH_3 source, the rate of citrulline synthesis is limited by the rate of glutamate transport across the mitochondrial membrane. In the case of glucose-fed rats the rates of citrulline synthesis from both NH_4Cl and glutamate were very low, and were presumably limited by the intramitochondrial content of *N*-acetylglutamate.

Factors controlling the rate of urea synthesis in the liver cell have been discussed in detail by Krebs *et al.* (1973). The activity of argininosuccinate synthetase is lower than the activity of the other urea-cycle enzymes. However, the citrulline concentration in liver is normally very low, suggesting that citrulline synthesis may limit the rate of urea synthesis *in vivo*. For this reason, a knowledge of factors influencing the rate of citrulline synthesis in isolated mitochondria may prove to be of importance in considering the control of urea synthesis in liver.

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

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