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

By J. D. McGIVAN, NORAH M. BRADFORD and J. B. CHAPPELL
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(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 NH₄Cl 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 NH₄Cl was very low. 3. With glutamate as the nitrogen source the relative rates of citrulline synthesis were much lower than when NH₄Cl 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 Km 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 NH₄Cl 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 NH₃ (Charles et al., 1967). However, when glutamate is the sole NH₃ 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 Km 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 (1 mm) and N-ethylmaleimide (0.15 mm) to terminate the transport reaction after a predetermined time.

Assay of intramitochondrial enzymes

The mitochondrial suspension (60–70 mg of protein/ml) was diluted with an equal volume of a solution containing 0.14 M-KCl, 10 mM-Tris·HCl, 5 mM-MgCl₂ and 5 mM-2-mercaptoethanol. This suspension was sonicated in 4 ml amounts for three successive periods of 20 s with a Soniprobe (Dawe Instruments, London W.3, U.K.). The sonicate was then centrifuged at 9000 g for 10 min 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 (3–4 mg of protein) was incubated in a medium containing 0.1 M-KCl, 10 mM-Tris·HCl, 20 mM-ornithine hydrochloride, 10 mM-2-oxoglutarate and 10 mM-o-aminobenzaldehyde at pH 7.2 and 30°C. The development of the complex formed between o-aminobenzaldehyde and glutamic semialdehyde was measured at 440 nm.

Ornithine transcarbamoylase (EC 2.1.3.3) was assayed by incubation of the sonicate (0.1–0.2 mg of protein) in 2 ml of a medium containing 0.12 M-KCl, 20 mM-Tris·HCl, 20 mM-ornithine hydrochloride and 5 mM-carbamoyl phosphate at pH 7.2 and 30°C. Samples (0.5 ml) were deproteinized after 1, 2 and 3 min by addition of HClO₄ (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 (5–10 mg) was incubated in 4 ml of a medium containing 0.12 M-KCl, 20 mM-Tris·HCl, 15 mM-MgCl₂, 5 mM-ATP, 20 mM-ornithine hydrochloride, 20 mM-KH₂CO₃, 5 mM-N-acetylglutamate and 10 μg of oligomycin at pH 7.2 and 30°C. Samples (1 ml) were deproteinized with HClO₄ (final concn. 10%, w/v) after 3, 6 and 9 min 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 0.12 M-KCl, 10 mM-Tris·HCl, 20 mM-2-oxoglutarate, 0.15 mM-NADH, 2 mM-ADP and 1 mM-EDTA at pH 7.2 and 30°C. The initial rate of decrease in absorbance at 340 nm on addition of 25 mM-NH₄Cl was recorded. For assay in the deamination direction, the sonicate was incubated with 0.12 M-KCl, 10 mM-Tris·HCl, 1 mM-NAD⁺, 2 mM-ADP and 1 mM-EDTA at pH 7.2 and 30°C and the initial rate of increase in absorbance at 340 nm on the addition of 50 mM-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 biuret method (Gornall et al., 1949).

Carbamoyl phosphate and nicotinamide nucleotides were purchased from C. F. Boehringer und Sohne 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 NH₄Cl were present in excess and extra-mitochondrial 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

<table>
<thead>
<tr>
<th>Source of NH₃</th>
<th>Standard diet</th>
<th>Egg white</th>
<th>20% glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>7.3 ± 1.8</td>
<td>19.8 ± 4.3</td>
<td>0.74 ± 0.30</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2.5 ± 0.5</td>
<td>4.8 ± 0.5</td>
<td>0.69 ± 0.12</td>
</tr>
</tbody>
</table>
EFFECT OF DIET ON MITOCHONDRIAL CITRULLINE SYNTHESIS

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.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Standard diet</th>
<th>Egg white</th>
<th>20% glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ornithine–2-oxoglutarate aminotransferase</td>
<td>8.5 ± 0.5</td>
<td>13.1 ± 1.2</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Carbamoyl phosphate synthase</td>
<td>27.8 ± 5.5</td>
<td>33.8 ± 6.4</td>
<td>21.4 ± 1.3</td>
</tr>
<tr>
<td>Ornithine transcarbamoylase</td>
<td>690 ± 150</td>
<td>1220 ± 238</td>
<td>697 ± 49</td>
</tr>
<tr>
<td>Aspartateaminotransferase</td>
<td>570 ± 100</td>
<td>655 ± 135</td>
<td>346 ± 150</td>
</tr>
<tr>
<td>Glutamate dehydrogenase (deaminating)</td>
<td>55 ± 10</td>
<td>58 ± 6</td>
<td>47 ± 7</td>
</tr>
<tr>
<td>Glutamate dehydrogenase (2-oxoglutarate → glutamate)</td>
<td>406 ± 116</td>
<td>410 ± 67</td>
<td>323 ± 80</td>
</tr>
</tbody>
</table>

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 NH4Cl 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 NH4Cl in mitochondria from glucose-fed rats was very slow. When glutamate was the sole NH3 source, the rates of citrulline synthesis were much lower than when NH4Cl 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 5 mM-N-acetylglutamate cause a
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 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 NH₃, 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.
Table 3. NH₃ and aspartate formation in liver mitochondria

Mitochondria (20–25 mg of protein) were added to a medium containing 0.12 M-KCl, 20 mM-Tris-HCl, 10 mM-glucose, 2 mM-MgCl₂, 20 mM-glutamate, 5 mM-potassium phosphate, 1 mM-ADP and dialysed hexokinase (0.1 mg/ml) at pH 7.2 and 30°C; 5 mM-malate or 5 mM-malate were also present when indicated. Samples of the suspension were deproteinized after 3, 6 and 9 min. The acid extracts were neutralized with K₂PO₄ and assayed for NH₃ (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.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of NH₃ formation (nmol/min per mg)</th>
<th>Rate of aspartate formation (nmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard diet</td>
<td>Egg white</td>
</tr>
<tr>
<td>Glutamate+malonate</td>
<td>4.3±0.7</td>
<td>8.6±0.8</td>
</tr>
</tbody>
</table>

mitochondria from rats fed on egg white, the initial rate of transport of glutamate (1 mm) at 20°C was 5.3 nmol/min per mg compared with 2.8 nmol/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 significantly 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 NH₃ 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 NH₃ production increased two-fold 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 NH₃ 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 NH₄Cl, 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 NH₃-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 Kᵣ for the activation of carbamoyl phosphate synthetase by N-acetylglutamate in frog liver is 1 mm (Fabien & Cohen, 1964) and the intramitochondrial concentration of N-acetylglutamate is estimated to be 0.1 mm–0.2 mm (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 NH₄Cl 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 NH₄Cl 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 (4 mM) 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 NH₃ has a relatively low $K_m$ (0.2 mM) for NH₃ (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 NH₃ 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 NH₄Cl 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.

References

Archibald, R. M. (1944) J. Biol. Chem. 156, 121–142