BBA 45508

CITRULLINE SYNTHESIS IN RAT-LIVER MITOCHONDRIA

R. CHARLES, J. M. TAGER AND E. C. SLATER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Amsterdam (The Netherlands)*

(Received July 14th, 1966)

SUMMARY

1. The synthesis of citrulline from ornithine in isolated rat-liver mitochondria has been studied.

2. Maximal synthesis is obtained in the presence of ornithine, CO_2, ammonia, glutamate, ATP and inorganic phosphate (P_i). No synthesis occurs in the absence of ornithine. The optimal concentrations of glutamate, ATP and P_i have been determined.

3. Glutamate may be replaced by pyruvate or (less effectively) by other oxidizable substrates.

4. In the optimal system containing ammonia and glutamate, the principal nitrogen donor for citrulline synthesis is the added ammonia. Glutamate is a very poor nitrogen donor for citrulline synthesis in freshly prepared rat-liver mitochondria.

5. Removal for citrulline synthesis of the small amount of ammonia formed during glutamate oxidation does not lead to any further deamination of glutamate.

6. Under conditions where the deamination of glutamate is stimulated, citrulline synthesis with glutamate as nitrogen donor is also stimulated. This can be achieved by ageing or sonication of the mitochondria, by the addition of uncoupler (*plus* oligomycin), or by the addition of 2-methyl-1,4-naphthoquinone.

7. Citrulline synthesis in freshly prepared rat-liver mitochondria is independent of added *N*-acetylglutamate. After ageing or sonication the synthesis becomes *N*-acetylglutamate-dependent.

8. The ammonia derived from the amide nitrogen of glutamine by the action of mitochondrial glutaminase can be used for citrulline synthesis. The glutamate formed from glutamine is virtually exclusively oxidized *via* the transamination pathway in freshly prepared rat-liver mitochondria and plays an insignificant role as nitrogen donor for citrulline synthesis.

INTRODUCTION

Since KREBS[1] in his classic studies in 1932 established the important role of citrulline in urea synthesis in mammalian liver, many investigations have been carried out on the mechanism of citrulline synthesis. Most of the work has been done

* Formerly Laboratory of Physiological Chemistry. Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

with isolated enzymes, in particular by COHEN and co-workers (see ref. 2). Although it was discovered by COHEN AND HAYANO[3] and LEUTHARDT AND MÜLLER[4] in 1948 that citrulline synthesis takes place in the mitochondria, relatively little attention has been paid to this reaction as a mitochondrial process.

According to present ideas, the nitrogen donor for citrulline synthesis in mammals is ammonia. It is generally accepted that this ammonia can be provided by the oxidation of glutamate. However, this has been brought into question by the observations[5–10] that glutamate is virtually exclusively oxidized *via* the transamination pathway (first described by MÜLLER AND LEUTHARDT[11]) in isolated mitochondria, even those from liver[6–9] which contain high concentrations of glutamate dehydrogenase. A small amount of ammonia is formed only at the beginning of the incubation[12,13]. HIRD and co-workers[14,15], on the other hand, have suggested that the ammonia for citrulline synthesis in isolated mitochondria can be provided by glutamate oxidation if a low concentration of glutamate is maintained.

In this paper, the results of a study of the interrelationship between citrulline synthesis and glutamate oxidation in isolated rat-liver mitochondria are described. In addition, the optimal conditions for citrulline synthesis in isolated mitochondria are discussed. Some of these results have been presented in a preliminary form[16]. Several aspects of the control of glutamate oxidation in isolated rat-liver mitochondria are discussed in the accompanying papers[17,18].

METHODS

Rat-liver mitochondria

Rat-liver mitochondria were prepared by the method of HOGEBOOM[19] exactly as described by MYERS AND SLATER[20].

Reaction conditions

The standard reaction mixture used in all the experiments described in this paper (except that of Table I) contained, in a final volume of 1 ml, 15 mM KCl 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer, 10 mM ornithine, 16.6 mM $KHCO_3$, 3 mM ATP, 5 mM potassium phosphate buffer, 25 mM sucrose (derived from the mitochondrial suspension) and the additions indicated in the legends to the tables and figures. Oligomycin, antimycin and 2-methyl-1,4-naphthoquinone were added as ethanolic solutions. The same concentration of ethanol (1–2 %) was present in the controls. The final pH was 7.4.

The reaction was carried out in round-bottom tubes (diameter 25 mm) at 25° in a Dubnoff shaker. The gas phase was 95 % O_2–5 % CO_2. The standard reaction mixture without mitochondria and additions was gassed with 95 % O_2–5 % CO_2 for 10 min before starting the experiment.

The reaction was stopped by the addition of 0.1 ml 35 % $HClO_4$. After removal of the protein by centrifugation, the $HClO_4$ was removed as $KClO_4$ in the cold.

Determination of citrulline

Citrulline was measured by the method of ARCHIBALD[21] as described by OGINSKY[22], except that heating at 100° was carried out for 60 min instead of 30 min. Since the absorbance deviates from Beer's law, a standard curve was made for each

determination. Furthermore, since sucrose interferes with the colour development, the same concentration of sucrose as that present in the samples was added to the standards.

Other assays

The methods used for the determination of protein, glutamate, aspartate and ammonia are given in the accompanying paper[17].

Special chemicals and enzymes

Special chemicals and enzymes were obtained from the following sources: ATP, NAD^+, L-citrulline and L-ornithine, Sigma Chemical Co.; L-glutamate, L-glutamine, *N*-acetylglutamate and 2,4-dinitrophenol, British Drug Houses; glutamate dehydrogenase (EC 1.4.1.2) in 50% glycerol and malate dehydrogenase (EC 1.1.1.37), Boehringer und Soehne, Mannheim. Antimycin was a gift from Kyowa Fermentation Co. and oligomycin from Upjohn Chemical Co. Aspartate transaminase (EC 2.6.1.1) was prepared as described by TAGER AND SLATER[23].

RESULTS

Optimal conditions for citrulline synthesis in isolated rat-liver mitochondria

Table I shows the effect of the composition of the reaction medium on citrulline formation in freshly prepared rat-liver mitochondria. In the presence of CO_2, NH_4Cl, ornithine and ATP, the classical substrates for citrulline synthesis, 0.5 μmole citrulline was formed in 30 min in this experiment. As would be expected, no citrulline formation occurred in the absence of ornithine. In the absence of added ammonia, the trace of citrulline that was formed may have been due to endogenous ammonia. When ATP was omitted, only 0.2 μmole citrulline was formed. Inorganic phosphate or *N*-acetylglutamate increased the amount of citrulline formed to 0.7 μmole. A con-

TABLE I

EFFECT OF COMPOSITION OF THE REACTION MIXTURE ON CITRULLINE SYNTHESIS IN FRESHLY-PREPARED RAT-LIVER MITOCHONDRIA

Experimental conditions as described under METHODS. The reaction mixture (1 ml) contained 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer, 25 mM sucrose, 16 mM $KHCO_3$ and 3.9 mg mitochondrial protein. NH_4Cl (10 mM), ATP (3 mM), ornithine (10 mM), glutamate (10 mM), potassium phosphate buffer (pH 7.4; 5 mM) and *N*-acetylglutamate (10 mM) were added where indicated. The final pH was 7.4. Reaction time, 30 min.

Additions	*ΔCitrulline (μmoles)*
NH_4Cl + ATP + ornithine	0.5
NH_4Cl + ATP	0
NH_4Cl + ATP + ornithine + *N*-acetylglutamate	0.7
ATP + ornithine	<0.1
NH_4Cl + ATP + ornithine + glutamate	2.1
NH_4Cl + ornithine	0.2
NH_4Cl + ATP + ornithine + P_i	0.7
NH_4Cl + ornithine + glutamate	1.3
NH_4Cl + ornithine + glutamate + P_i	2.2
NH_4Cl + ATP + ornithine + glutamate + P_i	4.3

siderable stimulation of citrulline synthesis was obtained by adding glutamate or P_i, and the largest amount of citrulline (4.3 μmoles) was formed in the complete reaction mixture containing NH_4Cl, ornithine, ATP, glutamate and P_i.

Table II shows that the stimulatory effect of glutamate could be duplicated by pyruvate or, less effectively, by β-hydroxybutyrate or malate or (not shown in the table) α-oxoglutarate, succinate, fumarate and isocitrate. This suggests that the stimulation by glutamate is due at least in part to its functioning as an oxidizable

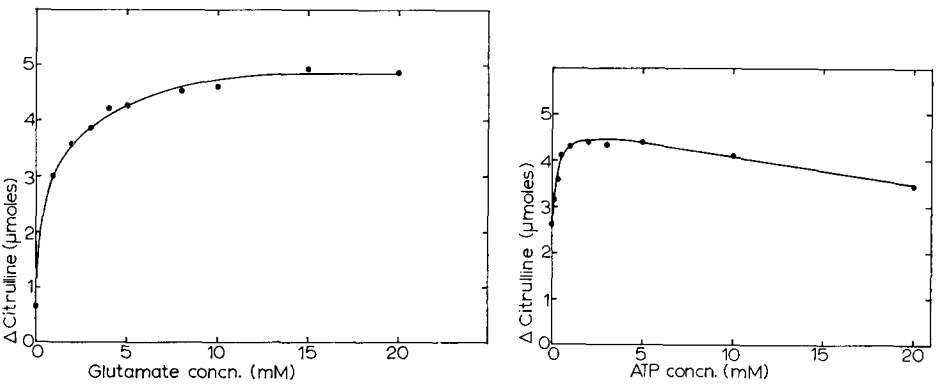

Fig. 1. Effect of glutamate concentration on citrulline formation in rat-liver mitochondria. Experimental conditions as described under METHODS. Reaction mixture contained the basic components *plus* 10 mM NH_4Cl and 3.1 mg mitochondrial protein. Reaction time, 30 min.

Fig. 2. Effect of ATP concentration on citrulline formation in rat-liver mitochondria. Experimental conditions as described under METHODS. Reaction mixture contained the basic components (except that the ATP concentration was varied) *plus* 10 mM NH_4Cl, 10 mM glutamate and 2.8 mg mitochondrial protein. Reaction time, 30 min.

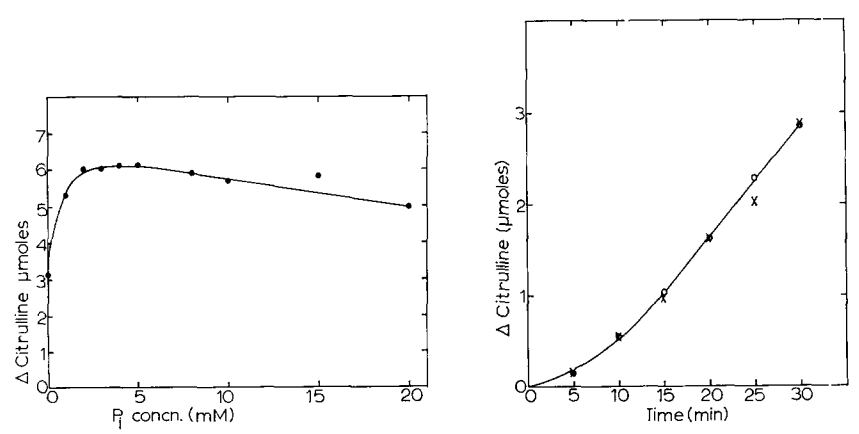

Fig. 3. Effect of the concentration of P_i on citrulline formation in rat-liver mitochondria. Experimental conditions as described under METHODS. Reaction mixture contained the basic components (except that the concentration of P_i was varied) *plus* 10 mM NH_4Cl, 10 mM glutamate and 3.8 mg mitochondrial protein. Reaction time, 30 min.

Fig. 4. Time course of citrulline formation in rat-liver mitochondria. Experimental conditions as described under METHODS. Reaction mixture contained the basic components *plus* 10 mM NH_4Cl, 10 mM glutamate and 4.1 mg mitochondrial protein. O, control; ×, with *N*-acetylglutamate.

substrate and thereby furnishing ATP. It is clear, however, that glutamate must have another function, since maximal stimulation of citrulline synthesis is only obtained if rather high concentrations (10 mM) of glutamate are added (Fig. 1).

Added ATP stimulates citrulline synthesis in intact mitochondria even in the presence of glutamate (Table I). However, this stimulation by ATP is not due to its utilization as energy donor for citrulline synthesis, as will be shown in a forthcoming paper (see also Table VIII). Fig. 2 shows that maximal citrulline synthesis is obtained at an ATP concentration of 1–5 mM. At higher concentrations, citrulline synthesis declines slightly.

P_i stimulates citrulline formation (Table I), and Fig. 3 shows that maximal synthesis occurs at a P_i concentration of approx. 2–5 mM.

Citrulline synthesis with isolated enzymes is *N*-acetylglutamate dependent[24,25]. There are reports that *N*-acetylglutamate also stimulates citrulline synthesis in mitochondria[26–29]. However, we have found little, if any, effect of *N*-acetylglutamate on citrulline synthesis with freshly-prepared rat-liver mitochondria in the presence of oxidizable substrate (Table II and Fig. 4). This will be discussed later.

The time course of citrulline formation is shown in Fig. 4. A definite lag occurs,

TABLE II

THE EFFECT OF OXIDIZABLE SUBSTRATES AND *N*-ACETYLGLUTAMATE ON CITRULLINE FORMATION IN RAT-LIVER MITOCHONDRIA

Experimental conditions as described under METHODS. Reaction mixture contained the basic components *plus* 10 mM NH_4Cl and 3.9 mg mitochondrial protein. Reaction time, 30 min.

Additions	*ΔCitrulline (μmoles)*
None	0.5
Glutamate (10 mM)	4.1
β-Hydroxybutyrate (10 mM)	2.8
Pyruvate (10 mM)	4.1
Malate (10 mM)	1.8
N-Acetylglutamate (10 mM)	0.7
Glutamate + *N*-acetylglutamate	4.3
β-Hydroxybutyrate + *N*-acetylglutamate	2.8
Pyruvate + *N*-acetylglutamate	4.0
Malate + *N*-acetylglutamate	2.3

TABLE III

NITROGEN DONORS FOR CITRULLINE SYNTHESIS IN RAT-LIVER MITOCHONDRIA

Experimental conditions and reaction mixture as described under METHODS. Reaction time, 30 min.

Additions	*ΔCitrulline (nmoles/min per mg protein)*		*Number of measurements*
	Mean	*Range*	
Glutamate (10 mM)	1.9	1.6–2.6	5
NH_4Cl (10 mM)	4.4	1.2–10	11
Glutamate + NH_4Cl	25	13–35	8

the reason for which is unknown. The lag occurs both in the absence and in the presence of *N*-acetylglutamate.

Nitrogen donors for citrulline synthesis

In Table III, the results of a number of experiments on citrulline synthesis from NH_4Cl and glutamate in rat-liver mitochondria are shown. Neither ammonia nor glutamate alone supported an active synthesis of citrulline. Only in the presence of both substrates was an extensive synthesis observed. The balance studies reported in Table IV show that the nitrogen donor for the extra citrulline synthesis in the presence of ammonia *plus* glutamate is the added ammonia and not glutamate (*cf.* ref. 30). In both experiments of Table IV glutamate was almost quantitatively converted to aspartate, and the citrulline formation was approximately equal to the disappearance of ammonia. The results indicate that glutamate is not a good source of ammonia for citrulline synthesis in isolated rat-liver mitochondria and are in agreement with the observations[6–9] that in these mitochondria glutamate is almost quantitatively converted to aspartate.

In the accompanying paper[17] it is shown that the small amount of ammonia that is found after incubating rat-liver mitochondria with glutamate in State 3 is formed during the first few minutes (*cf.* refs. 12, 13). The effect of removal of this ammonia for citrulline synthesis on the deamination of glutamate was studied. In

TABLE IV

STOICHEIOMETRY OF CITRULLINE SYNTHESIS IN RAT-LIVER MITOCHONDRIA

Experimental conditions as described under METHODS. Reaction mixture contained the basic components *plus* 10 mM glutamate, 10 mM NH_4Cl and 5.4 mg (Expt. 1) or 4.0 mg (Expt. 2) mitochondrial protein. Reaction time, 30 min.

Expt. No.	*ΔCitrulline (μmoles)*	*$-\Delta NH_3$ (μmoles)*	*$-\Delta$Glutamate (μmoles)*	*ΔAspartate (μmoles)*
1	4.7	4.6	2.0	1.7
2	2.5	2.3	0.9	0.9

TABLE V

EFFECT OF AMMONIA ON CITRULLINE FORMATION FROM GLUTAMATE IN RAT-LIVER MITOCHONDRIA

Experimental conditions as described under METHODS. Reaction mixture contained basic components *plus* 10 mM glutamate and 5.0 mg mitochondrial protein. Reaction time, 30 min.

NH_4Cl added (μmole)	*ΔCitrulline (μmole)*	*$-\Delta NH_3$ (μmole)*	*ΔAspartate (μmoles)*
0	0.35	0	0.90
	0.38	0	0.92
0.20	0.60	0.20	0.94
	0.55	0.23	0.97
0.50	0.87	0.47	1.00
	—	0.50	1.02

the experiment of Table V, 0.37 μmole citrulline was formed when glutamate was the sole nitrogen donor. When a small amount of ammonia was added, this was quantitatively converted to citrulline. Glutamate oxidation in coupled mitochondria is dependent on the availability of phosphate acceptor; the amount of glutamate oxidized in an experiment of this type will therefore depend on the amount of citrulline synthesized, since the latter process makes ADP available[31]. The enhanced citrulline synthesis found when ammonia was added was accompanied by an enhanced glutamate oxidation (see Column 4 of Table V). Nevertheless the contribution of glutamate as nitrogen donor for citrulline synthesis remained constant. It may be concluded that removal of the small amount of ammonia that is formed during the oxidation of glutamate by isolated mitochondria does not lead to any increase in the deamination of glutamate.

Factors influencing citrulline synthesis with glutamate as nitrogen donor

The deamination of glutamate can be stimulated by impairing the structural integrity of the mitochondria[12,17], by the addition of uncouplers[12,13,17], or by the addition of 2-methyl-1,4-naphthoquinone[18]. Accordingly, experiments were performed to see if the enhanced ammonia production that occurs under these conditions could lead to an increase in citrulline synthesis with glutamate as nitrogen donor.

Effect of structural integrity of the mitochondria. Table VI shows the effect of ageing of mitochondria on citrulline synthesis from glutamate. In the incubations with aged mitochondria, oligomycin was added to inhibit the high ATPase in these particles, extra ATP was present since respiratory-chain phosphorylation is inhibited by oligomycin, and NAD^+ was added to replace the nucleotide lost from the mitochondria during ageing. In the aged mitochondria, citrulline synthesis was completely dependent on added *N*-acetylglutamate. When *N*-acetylglutamate alone was added to the aged mitochondria, 0.7 μmole citrulline was synthesized; this can be ascribed to ammonia liberated from the mitochondria during ageing and during the incubation. Glutamate was actively deaminated by the aged mitochondria (Line 5 of Table VI)

TABLE VI

CITRULLINE SYNTHESIS IN FRESH AND IN AGED RAT-LIVER MITOCHONDRIA

Experimental conditions and reaction mixture as described under METHODS. In the case of aged mitochondria 10 μg oligomycin, 0.45 mM NAD^+ and 4 mM ATP were added to the reaction mixture. The mitochondria were aged by incubating the mitochondrial suspension for 3 h at 25°. Each tube contained 5.3 mg mitochondrial protein. Reaction time, 30 min.

Mitochondria	*Additions*	*ΔCitrulline (μmoles)*	*ΔNH_3 (μmoles)*
Fresh	None	0.1	0
	Glutamate (10 mM)	0.5	0
	NH_4Cl (10 mM)	0.3	—
Aged	None	0	0.2
	Glutamate	0	3.6
	NH_4Cl	0	—
	N-Acetylglutamate (10 mM)	0.7	−0.5
	Glutamate + *N*-acetylglutamate	2.1	0.3
	NH_4Cl + *N*-acetylglutamate	2.7	—

and in the presence of *N*-acetylglutamate, the ammonia that was formed could be used for citrulline synthesis. After correcting for the control in which only *N*-acetylglutamate was present, a value of 1.4 μmoles is obtained for citrulline synthesis with glutamate as nitrogen donor. This represents a pronounced stimulation compared with the corrected value of 0.4 μmole obtained with fresh mitochondria.

Completely analogous results were obtained when the structural integrity of the mitochondria was impaired by exposing them to ultrasonic vibrations (Table VII).

Effect of 2,4-dinitrophenol. In the experiment shown in Table VIII, 0.2 μmole citrulline was formed in 30 min with glutamate as nitrogen donor. This synthesis was completely inhibited by 0.3 mM 2,4-dinitrophenol (*cf.* SIEKEVITZ AND POTTER[31]), due to the dinitrophenol-induced ATPase. The deamination of glutamate was greatly stimulated by the uncoupler (*cf.* refs. 12, 13, 17). When in addition to dinitrophenol oligomycin was added to inhibit the ATPase, a 4.5-fold stimulation of citrulline synthesis was obtained in comparison with the control containing only glutamate. The nitrogen balance shows that citrulline production proceeded at the expense of

TABLE VII

CITRULLINE SYNTHESIS IN FRESH AND IN SONICATED RAT-LIVER MITOCHONDRIA

Experimental conditions and reaction mixture as described under METHODS. In the case of sonicated mitochondria, 10 μg oligomycin, 0.45 mM NAD^+ and 4 mM ATP were added to the reaction mixture. The mitochondria were sonicated for 30 sec at 20 kHz. Each tube contained 5.2 mg mitochondrial protein. Reaction time, 30 min.

Mitochondria	*Additions*	*ΔCitrulline* (*μmoles*)	ΔNH_3 (*μmoles*)
Fresh	None	0.3	0
	Glutamate (10 mM)	0.4	0
	NH_4Cl (10 mM)	1.2	—
Sonicated	None	0	0.4
	Glutamate	0	2.1
	NH_4Cl	0	—
	N-Acetylglutamate (10 mM)	0.5	0
	Glutamate + *N*-acetylglutamate	1.7	0.7
	NH_4Cl + *N*-acetylglutamate	2.4	—

TABLE VIII

EFFECT OF 2,4-DINITROPHENOL AND OLIGOMYCIN ON THE FORMATION OF CITRULLINE IN RAT-LIVER MITOCHONDRIA

Experimental conditions and reaction mixture as described under METHODS. Each tube contained 4.0 mg mitochondrial protein. Reaction time, 30 min.

Additions	*ΔCitrulline* (*μmole*)	ΔNH_3 (*μmoles*)	$-\Delta$*Glutamate* (*μmoles*)	*ΔAspartate* (*μmoles*)
Glutamate (10 mM)	0.2	0	0.7	0.5
Glutamate + 2,4-dinitrophenol (0.3 mM)	0	2.3	3.7	1.5
Glutamate + oligomycin (10 μg)	0	0.1	0	0.2
Glutamate + 2,4-dinitrophenol + oligomycin	0.9	1.0	2.9	1.6

ammonia formation. The inhibition of citrulline synthesis by oligomycin in the absence of dinitrophenol will be discussed in a forthcoming paper.

Effect of 2-methyl-1,4-naphthoquinone. In the accompanying paper[18], it is shown that the deamination of glutamate by isolated rat-liver mitochondria can be stimulated by the addition of 2-methyl-1,4-naphthoquinone. Table IX shows that the addition of 2-methyl-1,4-naphthoquinone to the reaction mixture also gives a pronounced stimulation of citrulline synthesis with glutamate as the nitrogen donor. This increase in citrulline synthesis makes more ADP available and thus also stimulates the oxidation of glutamate to aspartate*.

TABLE IX

EFFECT OF 2-METHYL-1,4-NAPHTHOQUINONE ON THE FORMATION OF CITRULLINE IN RAT-LIVER MITOCHONDRIA

Experimental conditions and reaction mixture as described under METHODS. Each tube contained 5.4 mg mitochondrial protein. Reaction time, 30 min.

Additions	*ΔCitrulline* (*μmole*)	*ΔNH_3* (*μmole*)	*−ΔGlutamate* (*μmoles*)	*ΔAspartate* (*μmoles*)
Glutamate (10 mM)	0.3	0.1	0.9	1.0
Glutamate + 2-methyl-1,4-naphthoquinone (10 μM)	0.9	0.1	3.1	2.4

TABLE X

STOICHEIOMETRY OF CITRULLINE FORMATION FROM GLUTAMINE IN RAT-LIVER MITOCHONDRIA

Experimental conditions and reaction mixture as described under METHODS. The reaction mixture contained 10 mM glutamine and 5.2 mg mitochondrial protein. This experiment was carried out in collaboration with Mr. H. F. TABAK.

Reaction time (*min*)	*ΔCitrulline* (*μmoles*)	*ΔNH_3* (*μmoles*)	*ΔGlutamate* (*μmoles*)	*ΔAspartate* (*μmoles*)
20	2.3	1.8	3.1	0.8
30	4.0	1.8	4.2	1.3

Glutamine as nitrogen donor for citrulline formation

HIRD *et al.*[14,15] have shown that under their experimental conditions, the deamination pathway of glutamate oxidation is favoured by low concentrations of glutamate. However, as suggested in the accompanying paper[17], this is the case only with mitochondria whose structural integrity has been impaired. By using glutamine rather than glutamate as respiratory substrate, the steady-state concentration of glutamate can be kept low. However, even under these conditions, at least 90% of the glutamate that is oxidized is recovered as aspartate and only 10% or less as ammonia[17].

* The main reason for this increased oxidation of glutamate to aspartate is that in the presence of 2-methyl-1,4-naphthoquinone the high-energy intermediates of oxidative phosphorylation are utilized for the energy-linked reduction of $NAD(P)^+$, which is regenerated by the oxidation of NAD(P)H *via* NAD(P)H dehydrogenase (EC 1.6.99.2), as has been shown by WU AND SHANG[50] and confirmed by E. J. DE HAAN (unpublished observations).

The results of an experiment in which glutamine was tested as a substrate for citrulline synthesis are shown in Table X. The amount of citrulline formed after 20 and 30 min was 2.3 and 4.0 μmoles, respectively. Calculations based on the data of Table X show that 89 and 90%, respectively, of the glutamate that was oxidized was converted to aspartate. Threfore, the principal nitrogen donor for the synthesis of citrulline was the amide group of glutamine.

DISCUSSION

Requirements for citrulline synthesis

Citrulline synthesis by isolated enzymes takes place in two steps according to the following reactions:

$$NH_4^+ + HCO_3^- + 2\ ATP \xrightarrow[Mg^{2+}]{N\text{-acetylglutamate}} \text{carbamylphosphate} + 2\ ADP + P_i \qquad (1)$$

$$\text{Carbamylphosphate} + \text{ornithine} \rightleftharpoons \text{citrulline} + P_i \qquad (2)$$

We have found that ornithine and ammonia are required for citrulline synthesis in rat-liver mitochondria (*cf.* refs. 3, 26, 29) but *N*-acetylglutamate has little or no effect on the reaction. With solubilized enzymes there is an absolute requirement for *N*-acetylglutamate[24,25]. Three possible explanations come to mind:

(i) The mitochondrial process is essentially independent of *N*-acetylglutamate. This is unlikely, since an absolute requirement for *N*-acetylglutamate could be demonstrated in our experiments when the mitochondrial integrity was impaired (Tables VI and VII).

(ii) *N*-Acetylglutamate is present in sufficient quantities or is synthesized (*cf.* ref. 32) rapidly enough within the mitochondria to allow citrulline synthesis to proceed at a maximal rate. This seems much more likely. Several investigators (refs. 27, 28) have suggested that the reason for the requirement for high concentrations of glutamate for maximal citrulline synthesis (*cf.* Fig. 1) is that glutamate is necessary for the formation of some derivative, perhaps *N*-acetylglutamate. In the optimal system containing glutamate, we observe a time lag in citrulline formation (Fig. 4). An attractive possibility is that this lag is due to an enhancement of citrulline formation by newly synthesized *N*-acetylglutamate. However, it is difficult to vizualize how *N*-acetylglutamate would be synthesized from substrates like malate.

(iii) Intact mitochondria are impermeable to added *N*-acetylglutamate.

The rate of citrulline synthesis in rat-liver mitochondria is low unless oxidizable substrate is added (Tables I, II and III). Of the substrates tested, the most effective in promoting citrulline synthesis were glutamate and pyruvate. The primary function of oxidizable substrate is to provide ATP. As will be shown in a forthcoming paper, citrulline synthesis in freshly prepared rat-liver mitochondria is completely dependent on ATP generated during oxidative phosphorylation. Although exogenous ATP must be present for maximal synthesis to occur, it apparently has some other function than that of acting as a source of energy, since in the optimal system containing oxidizable substrate and ATP, citrulline synthesis is completely oligomycin-sensitive and atractyloside-insensitive*. P_i stimulates citrulline formation in the optimal system containing oxidizable substrate, since it is required for the synthesis of ATP.

* R. CHARLES AND H. F. TABAK, unpublished observations.

The reason for the different degrees of stimulation of citrulline synthesis by different oxidizable substrates is obscure. If the only function of oxidizable substrate were to provide ATP, one might expect that maximal stimulation would be obtained with a relatively low concentration of glutamate, since each molecule of glutamate oxidized to aspartate can provide a maximum of 9 molecules of ATP. Furthermore, the K_m for glutamate in rat-liver mitochondria in State 3 is low (*cf.* ref. 17). Yet rather high concentrations of glutamate are required for maximal stimulation of citrulline synthesis. This suggests that glutamate has a second function. As discussed above, this may be to serve as a substrate for *N*-acetylglutamate synthesis.

Glutamine as nitrogen donor for citrulline synthesis

BRAUNSTEIN[33] has pointed out that glutamine may be an important ammonia donor for citrulline synthesis. Because of its ability to penetrate cell membranes readily, glutamine is particularly suitable to act as a carrier for ammonia and to prevent the accumulation of this toxic product in the body.

Our results indicate that it is the amide group of glutamine that is the principal nitrogen donor for citrulline formation. Many investigators[29,30,34,35] have suggested the existence in mammals of a special enzyme that catalyzes the synthesis of carbamylphosphate or citrulline from glutamine without the intermediary formation of ammonia. Evidence for the existence of such an enzyme in *Escherichia coli* [36–39], *Lactobacillus arabinosus*[40], *Agaricus bisporus*[41] and green-pea seedlings[42] has been reported. In our studies, we have obtained no evidence for the existence of a special glutamine-dependent citrulline synthesis different from the system utilizing ammonia (H. F. TABAK AND R. CHARLES, unpublished observations).

Source of the ammonia for citrulline synthesis

The nitrogen donor for citrulline synthesis in isolated mitochondria is ammonia, as it is in isolated enzyme systems (Table I; see also refs. 3, 26, 43). The question arises of the source of this ammonia. It is generally considered that a major part of the ammonia for citrulline synthesis is provided by transamination of amino acids with α-oxoglutarate to form glutamate, followed by the oxidative deamination of glutamate (see, *e.g.* refs. 44, 45).

Our studies have shown that in isolated rat-liver mitochondria very little citrulline synthesis occurs when glutamate is the only nitrogen donor. This is in accord with the observations (refs. 12, 13, 17) that very little ammonia is formed during the oxidation of glutamate by isolated rat-liver mitochondria. The small amounts of ammonia that are formed during the first few minutes of glutamate oxidation can be quantitatively converted to citrulline (Table V). Removal of this ammonia does not lead to any further deamination of glutamate, indicating that the decrease in deamination of glutamate is not caused by an accumulation of ammonia (see also ref. 17).

PAPA *et al.*[18] have shown that in rat-liver mitochondria glutamate dehydrogenase reacts preferentially with NADP, as originally postulated by KLINGENBERG AND SLENCZKA[46]. This finding lends support to the suggestion of BORST[7,8] and of QUAGLIARIELLO AND PAPA[13] that the oxidative deamination of glutamate is limited by the oxidoreduction state of NADP. Because of the presence of the energy-linked transhydrogenase in mitochondria, NADP is kept almost completely reduced. This

high degree of reduction of NADP may be the reason why very little ammonia is formed from glutamate in isolated mitochondria (see ref. 18).

It seems probable that the oxidative deamination of glutamate in the intact cell, as in the isolated mitochondrion, is controlled by the oxidoreduction state of NADP. However, it is known that in the intact cell NADPH is utilized for various synthetic purposes[47,48]. Thus it is possible that the $NADP^+$ that becomes available thereby can be used for the oxidative deamination of glutamate. This coupling of synthetic reactions utilizing NADPH with oxidative deamination of glutamate by $NADP^+$ may be the mechanism by which ammonia is provided for citrulline synthesis *in vivo*.

The experiments with 2-methyl-1,4-naphthoquinone described in this and the accompanying paper[18] may be considered as a model for the oxidation of intramitochondrial NADPH *in vivo*. When 2-methyl-1,4-naphthoquinone was added to mitochondria oxidizing glutamate in State 3, NADP became oxidized and, concomitantly, ammonia production was stimulated; NAD was already completely oxidized. As could be expected 2-methyl-1,4-naphthoquinone stimulated citrulline synthesis (Table IX). Thus when NADPH can be oxidized, ammonia production from glutamate can occur, and glutamate can therefore serve as nitrogen donor for citrulline synthesis.

H. A. KREBS (personal communication) has found that in the intact liver the glutamate:α-oxoglutarate–NH_3 couple is in equilibrium with the β-hydroxybutyrate:acetoacetate couple. If our hypothesis that glutamate dehydrogenase *in vivo* reacts preferentially with NADP is correct, this finding suggests that *in vivo* the removal of intramitochondrial NADPH for synthetic purposes occurs very efficiently and that the transfer of reducing equivalents from NADH to $NADP^+$ *via* the energy-linked transhydrogenase results in a rapid equilibration between the β-hydroxybutyrate and the glutamate dehydrogenase couples. *In vivo* therefore, the deamination of glutamate may proceed rapidly enough to provide the ammonia required for citrulline synthesis. A delicate balance must exist between the deamination and transamination pathways of glutamate oxidation; glutamate must also provide the aspartate required for urea synthesis[49] and other processes.

ACKNOWLEDGEMENTS

The authors are indebted to Mr. E. J. DE HAAN and Mr. H. F. TABAK for many helpful discussions and to Miss B. KELDER for her excellent technical assistance.

REFERENCES

1 H. A. KREBS AND K. HENSELEIT, *Z. Physiol. Chem.*, 210 (1932) 33.
2 P. P. COHEN AND H. J. SALLACH, in D.M. GREENBERG, *Metabolic Pathways*, Vol. 2, Academic Press, New York, 1961, p. 1.
3 P. P. COHEN AND M. HAVANO, *J. Biol. Chem.*, 172 (1948) 405.
4 F. LEUTHARDT AND A. F. MÜLLER, *Experientia*, 4 (1948) 478.
5 H. A. KREBS AND D. BELLAMY, *Biochem. J.*, 75 (1960) 523.
6 P. BORST AND E. C. SLATER, *Biochim. Biophys. Acta*, 41 (1960) 170.
7 P. BORST, *Een biochemisch onderzoek over mitochondriën geïsoleerd uit een ascitescel tumor*, M.D. Thesis, Jacob van Campen, Amsterdam, 1961.
8 P. BORST, *Biochim. Biophys. Acta*, 57 (1962) 256.
9 J. B. CHAPPELL AND G. D. GREVILLE, *Nature*, 190 (1961) 502.
10 E. A. JONES AND H. GUTFREUND, *Biochem. J.*, 79 (1961) 608.

11 A. F. MÜLLER AND F. LEUTHARDT, *Helv. Chim. Acta*, 33 (1950) 268.
12 J. M. TAGER AND E. J. DE HAAN, in E. QUAGLIARIELLO, *Atti dell Seminario di Studi Biologici*, Vol. 1, Cressati, Bari, 1964, p. 307.
13 E. QUAGLIARIELLO AND S. PAPA, in E. QUAGLIARIELLO, *Atti del Seminario di Studi Biologici*, Vol. 1, Cressati, Bari, 1964, p. 351.
14 F. J. R. HIRD AND M. A. MARGINSON, *Nature*, 201 (1964) 1224.
15 F. J. R. HIRD AND D. J. MORTON, *Biochim. Biophys. Acta*, 85 (1964) 353.
16 R. CHARLES, H. F. TABAK, E. J. DE HAAN, J. M. TAGER AND E. C. SLATER, *Abstr. 3rd Meeting Fed. Europ. Biochem. Soc., Warsaw, 1966*, p. 150.
17 E. J. DE HAAN, J. M. TAGER AND E. C. SLATER, *Biochim. Biophys. Acta*, 131 (1967) 1.
18 S. PAPA, J. M. TAGER, A. FRANCAVILLA, E. J. DE HAAN AND E. QUAGLIARIELLO, *Biochim. Biophys. Acta*, 131 (1967) 14.
19 G. H. HOGEBOOM, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p.16.
20 D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558.
21 R. M. ARCHIBALD, *J. Biol. Chem.*, 156 (1944) 121.
22 E. L. OGINSKY, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 642.
23 J. M. TAGER AND E. C. SLATER, *Biochim. Biophys. Acta*, 77 (1963) 227.
24 S. GRISOLIA AND P. P. COHEN, *J. Biol. Chem.*, 204 (1953) 753.
25 M. GRASSL AND S. J. BACH, *Biochim. Biophys. Acta*, 42 (1960) 154.
26 P. P. COHEN AND S. GRISOLIA, *J. Biol. Chem.*, 182 (1950) 747.
27 J. CARAVACA AND S. GRISOLIA, *J. Biol. Chem.*, 235 (1960) 684.
28 R. CHARBONNEAU AND L. BERLINGUET, *Can. J. Biochem.*, 42 (1964) 1325.
29 F. LEUTHARDT, A. F. MÜLLER AND H. NIELSEN, *Helv. Chim. Acta*, 32 (1949) 744.
30 A. F. MÜLLER AND F. LEUTHARDT, *Helv. Chim. Acta*, 33 (1950) 262.
31 P. SIEKEVITZ AND V. R. POTTER, *J. Biol. Chem.*, 201 (1953) 1.
32 L. M. HALL, R. L. METZENBERG AND P. P. COHEN, *J. Biol. Chem.*, 230 (1958) 1013.
33 A. E. BRAUNSTEIN, in F. F. NORD, *Advances in Enzymology*, Vol. 19, Interscience, New York, 1957, p. 371.
34 H. KAMIN AND P. HANDLER, *J. Biol. Chem.*, 188 (1951) 193.
35 W. T. BURK AND L. L. MILLER, *Federation Proc.*, 15 (1956) 227.
36 A. PIÉRARD AND J. M. WIAME, *Biochem. Biophys. Res. Commun.*, 15 (1964) 76.
37 S. M. KALMAN, P. H. DUFFIELD AND T. BRZOZOWSKI, *Biochem. Biophys. Res. Commun.*, 18 (1965) 530.
38 S. M. KALMAN, P. H. DUFFIELD AND T. BRZOZOWSKI, *J. Biol. Chem.*, 241 (1966) 1871.
39 P. M. ANDERSON AND A. MEISTER, *Biochemistry*, 4 (1965) 2803.
40 R. L. ORY, D. W. HOOD AND C. M. LYMAN, *J. Biol. Chem.*, 207 (1954) 267.
41 B. LEVENBERG, *J. Biol. Chem.*, 237 (1962) 2590.
42 K. KLECZKOWSKY, *Acta Biochim. Polon.*, 12 (1965) 243.
43 S. GRISOLIA AND P. P. COHEN, *J. Biol. Chem.*, 191 (1951) 189.
44 A. E. BRAUNSTEIN, in M. L. ANSON AND J. T. EDSALL, *Advances in Protein Chemistry*, Vol. 3, Academic Press, New York, 1947, p. 24.
45 P. KARLSON, *Kurzes Lehrbuch der Biochemie für Mediziner und Naturwissenschaftler*, Thieme Verlag, Stuttgart, 1966, p. 136.
46 M. KLINGENBERG AND W. SLENCZKA, *Biochem. Z.*, 331 (1959) 486.
47 H. A. KREBS, *Bull. Johns Hopkins Hosp.*, 95 (1954) 19.
48 H. A. KREBS, *Bull. Johns Hopkins Hosp.*, 95 (1954) 34.
49 S. RATNER, in F. F. NORD, *Advances in Enzymology*, Vol. 15, Interscience, New York, 1954, p. 319.
50 C. H. WU AND H. Y. CHENG, *Acta Biochim. Biophys. Sin.*, 4 (1964) 462 (*Chem. Abstracts*, 62 (1965) 6705).