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GLUTAMATE OXIDATION IN RAT-LIVER HOMOGENATE

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SUMMARY

1. In rat-liver homogenate ammonia can be produced from adenine nucleotides by deamination of AMP when no oxidizable substrate is added. Glutamate prevents ammonia formation by lowering the AMP level and by removal of ammonia as glutamine.

2. The time course of glutamate oxidation in rat-liver homogenates is biphasic. In the first 10 min of the reaction aspartate production and deamination of glutamate are equal. In the 10–30-min period ammonia formation from glutamate declines, while the aspartate formation is correspondingly stimulated.

3. Ammonia formed by deamination of glutamate cannot be used for the synthesis of citrulline under the conditions used, due to its efficient removal as glutamine. Aspartate formed *via* the transamination pathway can be used as nitrogen donor for arginine synthesis from citrulline. This causes a stimulation of the transamination pathway.

4. The factors influencing the pathway of glutamate oxidation in rat-liver homogenate are discussed in relation to the metabolism of nitrogen *in vivo*.

INTRODUCTION

In isolated liver mitochondria, the oxidation of glutamate in the presence of phosphate and phosphate acceptor proceeds predominantly[1–4] *via* the transamination pathway first described by RATNER AND PAPPAS[5] and by MÜLLER AND LEUTHARDT[6]; in spite of the high concentration of glutamate dehydrogenase present, deamination of glutamate occurs to a very limited extent only (refs. 1, 2, 4; contrast refs. 7, 8). TAGER AND PAPA[9] and PAPA *et al.*[10] have presented evidence that glutamate dehydrogenase in rat-liver mitochondria reacts preferentially with NADP (see also ref. 11) and have concluded that the deamination of glutamate in the isolated mitochondria is inhibited because $NADP^+$ is not available. Indeed, conditions leading to a lowering of the reduction level of NADP lead to a stimulation of ammonia formation from glutamate[4,10,12].

These observations raise the question of the source of the ammonia required for citrulline synthesis. CHARLES *et al.*[13] and DE HAAN[14] have suggested that *in vivo*

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NADPH can be utilized for various synthetic purposes and that the ensuing decrease in the reduction level of NADP leads to an increased deamination of glutamate. In order to investigate this possibility, the study of the pathway of glutamate oxidation was extended to rat-liver homogenates (*cf.* ref. 7), a system in which synthetic reactions utilizing NADPH might be expected to take place. In this system, the additional reactions in the Krebs–Henseleit cycle can occur, in particular the synthesis of arginine from citrulline, for which aspartate is the specific nitrogen donor[15]. Thus, oxidation of glutamate *via* the transamination pathway could yield one of the nitrogen atoms of urea and oxidation *via* glutamate dehydrogenase the other.

In this paper, the interrelationship between the synthesis of urea and of glutamine, and the oxidation of glutamate in rat-liver homogenates is examined. In addition, observations on the formation of ammonia from the adenine nucleotides are presented. A preliminary account of this investigation has appeared[16].

METHODS

Rat-liver homogenate. The liver was removed from a decapitated rat, placed in ice-cold 0.25 M sucrose, minced, washed several times with sucrose, and homogenized in a Potter–Elvehjem homogenizer in 40–45 ml 0.25 M sucrose. The homogenate was centrifuged at 130 × *g* for 3 min. The supernatant was carefully decanted, filtered through two layers of cheese-cloth, and homogenized. The homogenate (30–35 ml) contained 10–25 mg protein/ml. In some experiments, 0.3 M mannitol was used instead of 0.25 M sucrose.

Reaction conditions. The reactions were carried out in round-bottom tubes (diameter, 25 mm) at 25° in a Dubnoff shaker. The basic reaction medium contained 15 mM KCl, 50 mM Tris–HCl, 2 mM EDTA, 10 mM $MgCl_2$, 16.6 mM $KHCO_3$, 5 mM P_i and 167 mM sucrose or 200 mM mannitol (derived from the homogenate) in a final volume of 3 ml. The atmosphere was O_2-CO_2 (95:5, v/v). The final pH was 7.4. The reaction mixture was gassed with O_2-CO_2 for 10 min before adding the homogenate, which had been kept at 25° for 5 min before the incubation (except in the experiment of Table I). The reaction was stopped with 0.3 ml 35% $HClO_4$. After removal of the protein by centrifugation, $HClO_4$ was removed in the cold as $KClO_4$ (see ref. 10).

Citrulline. Citrulline was measured by the method of ARCHIBALD[17], as modified by CHARLES *et al.*[12]. In experiments where citrulline was to be determined, the homogenate was prepared in 0.3 M mannitol instead of sucrose (see ref. 18). Urea was removed by pretreatment of the neutralized sample with 5 mg urease (EC 3.5.1.5) for 30 min at 25° (see ref. 17). The reaction was stopped with $HClO_4$. After removal of protein, the supernatant was used for citrulline determination.

Urea. Urea was measured by an enzymic method, developed by ROCH-RAMEL[19] and R. A. F. M. CHAMALAUN AND J. B. HOEK (unpublished observations), in which the NH_3 was liberated with urease and determined with glutamate dehydrogenase (EC 1.4.1.3). The reaction mixture (1.25 ml) contained 75 mM P_i (pH 7.4), 15 mM α-oxoglutarate, 80–160 μM NADH (free of NH_3; see ref. 4), 0.5 mg glutamate dehydrogenase (free of NH_3) and a sample of the neutralized $HClO_4$ extract containing not more than 50 nmoles urea. Any NH_3 present in the reaction mixture was removed by preincubation for 20–30 min. The reaction was started by the addition of 0.5 mg urease. The recovery of added urea was at least 85%.

Glutamine. A method for the determination of glutamine was developed analogous to that for the determination of urea, by coupling the glutaminase (EC 3.5.1.2) and glutamate dehydrogenase reactions. The reaction mixture (1.25 ml) contained 15 mM α-oxoglutarate, 80–160 μM NADH (free of NH_3), 0.5 mg glutamate dehydrogenase (free of NH_3), 75 mM P_i (pH 7.4), 30 μg aspartate transaminase (free of NH_3), 5 μg malate dehydrogenase (free of NH_3) and a sample of the neutralized $HClO_4$ extract containing not more than 100 nmoles glutamine. Any NH_3, oxaloacetate and aspartate present were removed by preincubation for about 30 min. The reaction was started by the addition of 0.1 mg glutaminase. The recovery of added glutamine was at least 90%. Oxidation of NADH, caused by NADH oxidase present in the glutaminase preparation, was corrected for by a blank containing all additions except the sample to be analysed.

Other analytical procedures. Ammonia[20], glutamate[21], aspartate[22], ATP[23], AMP[24] and ADP[24] were measured enzymically. Protein was determined by the biuret method as described by CLELAND AND SLATER[25].

Glutaminase. Glutaminase was prepared from pig kidney by the method of KLINGMAN AND HANDLER[26] with some modifications. A crude mitochondrial fraction was obtained as follows. Pig-kidney homogenate was prepared in 5 vol. 8.5 % sucrose–0.02 M potassium borate (pH 8.1) and centrifuged for 8.5 min at 900 × *g*. The supernatant was centrifuged for 15 min at 5000 × *g*. The precipitate was further treated as described by KLINGMAN AND HANDLER[26]. The purification was not continued after the second phosphate precipitation (see ref. 27). The precipitated enzyme preparation was dissolved in 0.03 M potassium borate–0.02 M P_i (pH 8.1) and dialysed against 100 vol. of the same buffer. Any precipitate present after dialysis was removed by centrifugation for 15 min at 29000 × *g*. The enzyme preparation contained about 7 mg protein/ml. Glutaminase prepared in this way does not contain any measurable lactate dehydrogenase (EC 1.1.1.27), or β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), but is contaminated with malate dehydrogenase (EC 1.1.1.37), aspartate transaminase (EC 2.6.1.1) and a low activity of NADH oxidase.

Urease. Urease was obtained from Sigma Chemical Co. (type III; solution in 50% glycerol). The solution was freed of NH_3 by repeated dialysis against 250 vol. 50% glycerol. Other enzymes were obtained from Boehringer und Soehne, Mannheim. Malate dehydrogenase and aspartate transaminase were freed of NH_3 by dialysing 3 times against 300 vol. of 20 mM P_i (pH 7.4) or 10 mM α-oxoglutarate (pH 7.4), respectively.

RESULTS

In this study, experimental conditions were chosen to ensure that CO_2-fixing reactions (in particular the synthesis of citrulline) and the synthesis of arginine could occur. The conditions for the maximal synthesis of arginine from citrulline and aspartate in rat-liver homogenate (see Fig. 1) were similar to those necessary for maximal synthesis of citrulline from ornithine and NH_3 in isolated rat-liver mitochondria[13].

Fig. 1 shows the synthesis of urea from NH_3 and ornithine in rat-liver homogenate. Citrulline (3 μmoles) was added to ensure that arginine formation was not limited by the concentration of citrulline. The rate of urea formation was linear for

about 25 min and then declined. The rate of citrulline disappearance was lower than that of urea formation, showing that the former was synthesized during the incubation. The rate of synthesis of citrulline could be calculated by subtracting the amount of citrulline that disappeared from the amount of urea formed. This is plotted in Fig. 1 as Δcitrulline$_{corr}$. The rate of citrulline synthesis was much lower than that of its further reaction to urea.

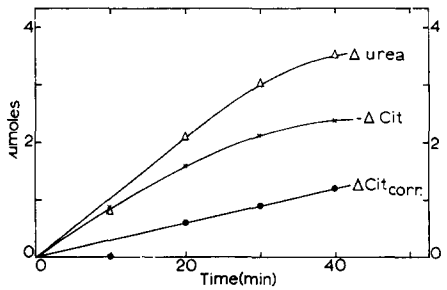

Fig. 1. Time course of urea synthesis from ammonia and ornithine in rat-liver homogenate. Experimental conditions as described under METHODS. Reaction mixture contained the basic components *plus* 15 μmoles ornithine, 30 μmoles glutamate, 30 μmoles NH_4Cl, 3 μmoles citrulline, 30 μmoles aspartate, 30 μmoles β-hydroxybutyrate, 9 μmoles ATP and 42.4 mg protein. △—△, Δurea; ×—×, —Δcitrulline; ●—●, Δcitrulline (corrected) = Δurea + Δcitrulline.

Table I shows the results of an experiment in which rat-liver homogenate was incubated with glucose, hexokinase, P_i and ADP. Without any further additions, a considerable amount of NH_3 was formed. By adding glutamate this was completely suppressed. Rotenone, which in itself slightly stimulated NH_3 production, relieved the suppression of NH_3 formation by glutamate. It is clear from the nitrogen balance that glutamate oxidation was not the only process involved.

Other experiments (not shown) suggested a relationship between the production of NH_3 in rat-liver homogenate and the presence of adenine nucleotides. As first shown by WAKABAYASI[28] and by CONWAY AND COOKE[29], quantitatively the most important way to form NH_3 from adenine nucleotides in liver is by deamination of AMP.

TABLE I

AMMONIA PRODUCTION IN RAT-LIVER HOMOGENATE

The reaction mixture (3 ml) contained 45 μmoles KCl, 6 μmoles EDTA, 15 μmoles $MgCl_2$, 150 μmoles Tris–HCl buffer, 90 μmoles glucose, 15 units (μmoles/min) hexokinase (EC 2.7.1.1), 1.5 μmoles ADP, 90 μmoles potassium phosphate buffer, 480 μmoles sucrose (derived from the homogenate) and 35.8 mg protein. Glutamate and rotenone were added as indicated. Final pH, 7.4. The reaction was carried out for 20 min at 25° in Warburg flasks (volume, 15 ml) with 0.1 ml 1 M KOH in the centre well.

Additions	*ΔGlu*	*ΔNH_3*	*ΔAsp*
	(*μmoles*)		
None	−0.1	1.6	0.5
Rotenone (3 μg)	+0.1	2.0	0.0
Glutamate (10 μmoles)	−4.3	0.0	3.2
Rotenone + glutamate	−0.5	2.0	0.2

This process can occur directly by the action of AMP deaminase (EC 3.5.4.6), or indirectly by the combined action of a 5'-nucleotidase (EC 3.1.3.5) and adenosine deaminase (EC 3.5.4.4)[30]. That AMP is indeed the source of NH_3 formed in rat-liver homogenate is shown in the experiment of Figs. 2 and 3, in which 9 μmoles AMP were added. NH_3 formation was linear during the first 15 min of the incubation and was correlated with a rapid disappearance of AMP (Fig. 2). There was no correlation between the formation of NH_3 and the changes in the level of the other adenine nucleotides. The close relationship between NH_3 formation and disappearance of total adenine nucleotides shows that the latter are the only source of NH_3 under these conditions (Fig. 3).

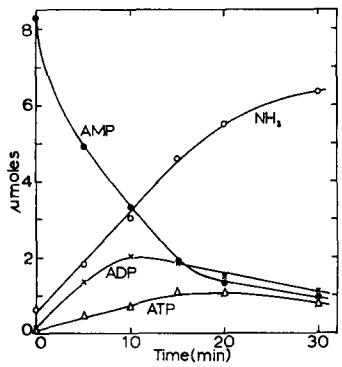

Fig. 2. Time course of AMP consumption in rat-liver homogenate. Experimental conditions as described under METHODS. The reaction mixture contained the basic components *plus* 9 μmoles AMP and 32.0 mg protein. The homogenate was prepared in mannitol.

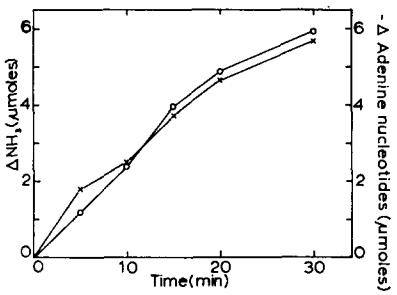

Fig. 3. Time course of ammonia formation from adenine nucleotides in rat-liver homogenate. Both curves were obtained from Fig. 2. ×—×, $-\Delta$(AMP + ADP + ATP); O—O, ΔNH_3.

Two explanations are possible for the effect of glutamate on NH_3 production from the adenine nucleotides in rat-liver homogenates (Table I). (i) The phosphorylation of ADP coupled with the oxidation of glutamate pulls the equilibrium of the adenylate kinase (EC 2.7.4.3) reaction in the direction of ADP formation, thus lowering the AMP level. This effect will be considerably more important if glucose and hexokinase are absent. (ii) The NH_3 is efficiently removed by the action of glutamine synthetase (EC 6.3.1.2), which is very active in rat liver.

Table II shows the results of a balance study of glutamate metabolism in rat-liver homogenate. Several points emerge. (i) The activity of glutamine synthetase was so high that, in the presence of glutamate, all the NH_3 produced was removed (compare ΔNH_3 and ΔGln, lines 3 and 4). (ii) In the presence of glutamate, the ATP level remained high, while in the absence of glutamate it rapidly disappeared. The level of AMP was kept so low in the presence of glutamate that its deamination did not play an important role; of the 9 μmoles ATP added only 0.5 μmole adenine nucleotides disappeared. (iii) No significant urea synthesis occurred. (iv) Of the 4.3 μmoles glutamate that disappeared in the presence of ATP (line 4), 1.8 were necessary for the synthesis of glutamine. Aspartate formation amounted to 1.9 μmoles. The total production of NH_3 is given by Δglutamine *plus* ΔNH_3, *i.e.*, 1.9 μmoles. Of this amount 0.5 μmole was derived from the adenine nucleotides; the remainder (1.4 μmoles) must

TABLE II

STOICHEIOMETRY OF GLUTAMATE METABOLISM IN RAT-LIVER HOMOGENATE

Experimental conditions as described under METHODS. Reaction mixture contained the standard components *plus* 27.6 mg protein. Glutamate and ATP were added as indicated. All values are the means of measurements of two parallel incubations. Reaction time, 20 min.

Additions	$-\Delta Glu$	ΔAsp	ΔNH_3	ΔGln	$\Delta Urea$	ΔATP	ΔADP	ΔAMP
	(*μmoles*)							
None	0.1	0.2	0.2	0.0	0.1	0.0	−0.2	−0.2
Glutamate (15 μmoles)	3.0	1.6	−0.1	0.6	0.2	0.0	−0.1	−0.2
ATP (9 μmoles)	0.1	0.1	1.5	0.0	0.0	−8.6	2.8	2.6
Glutamate + ATP	4.3	1.9	0.1	1.8	0.1	−2.9	1.9	0.5

have come from glutamate. Thus the products formed from glutamate amounted to 5.1 μmoles.

Total NH_3 production = $\Delta NH_3 + \Delta Gln = 1.9$ (A)

NH_3 derived from glutamate = (A) − Δadenine nucleotides = 1.4 (B)

Products formed from glutamate = Δaspartate + ΔGln + (B) = 5.1 (C)

Table III shows the results of an analogous balance study in which the effect of an active urea synthesis on the metabolism of glutamate (and *vice versa*) was examined. Ornithine and citrulline were added to the reaction mixture. The first two lines illustrate the same points as in Table II. The AMP level remained so low that no net deamination occurred. Glutamine synthesis was very active. No urea or citrulline was synthesized. Even when ornithine was present, there was no citrulline synthesis (lines 3 and 4). The most likely explanation for this is that glutamine synthesis kept the concentration of NH_3 too low to allow citrulline synthesis to proceed at an appreciable rate. The synthesis of arginine from citrulline, by removing aspartate, caused a stimulation of the total aspartate production (= Δaspartate *plus* Δurea) from 2.4 to 3.5 μmoles.

Fig. 4A shows the time course of glutamate metabolism. The level of citrulline and urea did not change significantly during the reaction (not shown). The NH_3

TABLE III

EFFECT OF ORNITHINE AND CITRULLINE ON GLUTAMATE METABOLISM IN RAT-LIVER HOMOGENATE

Experimental conditions as described under METHODS. Reaction mixture contained the standard components *plus* 15 μmoles glutamate and 34.8 mg protein. ATP (9 μmoles), ornithine (15 μmoles) and citrulline (3 μmoles) were added as indicated. Reaction time, 20 min.

Additions	$-Glu$	ΔAsp	ΔNH_3	ΔGln	$\Delta Urea$	ΔCit	ΔATP	ΔADP	ΔAMP
	(*μmoles*)								
None	4.5	1.8	−0.5	1.6	0.2	0.0	0.5	0.0	−0.1
ATP	6.4	2.4	−0.5	2.8	0.0	0.0	0.6	0.3	−0.1
Ornithine + citrulline	4.2	1.1	−0.4	1.3	2.0	−1.6	0.5	0.0	−0.1
ATP + ornithine + citrulline	6.1	1.4	−0.4	2.4	2.1	−2.2	−1.9	1.4	0.2

present in the system at zero time disappeared almost completely in the first 10 min. Fig. 4B shows that during the course of the experiment the amount of ATP remained high (9 μmoles) and that of AMP very low; the total amount of adenine nucleotides did not change. The changes in the levels of ATP and ADP in the 20–30-min period could be correlated with a decrease in glutamine synthesis (see Fig. 4A). The curves in Fig. 5 were obtained from Fig. 4A by the following corrections. Glutamate disappearance was corrected for the amount required for glutamine synthesis ($-\Delta Glu_{corr.} = -\Delta Glu + \Delta Gln$). The NH_3 used for glutamine synthesis was derived in part from that present in the reaction mixture at the start of the experiment and in part from the oxidative deamination of glutamate. The latter could thus be estimated by applying a suitable correction to the total amount of glutamine formed. ΔAspartate was plotted without any corrections. The sum of the products formed from glutamate is given by the curve aspartate *plus* glutamine.

Three points emerge. (i) The oxidation of glutamate and the formation of products occurred at the same rate for 30 min except for a lag in the corrected glutamate consumption in the first few min. This lag was probably responsible for the fact that in the balance studies a surplus of nitrogen was measured. Apparently a small amount of glutamate was synthesized in this period; this will be discussed later. (ii) During the first 10 min the formation of aspartate and NH_3 proceeded at the same rate (1.4 μmoles/10 min). After this period, the rate of NH_3 formation decreased and aspartate production was correspondingly stimulated (Fig. 5). (iii) The

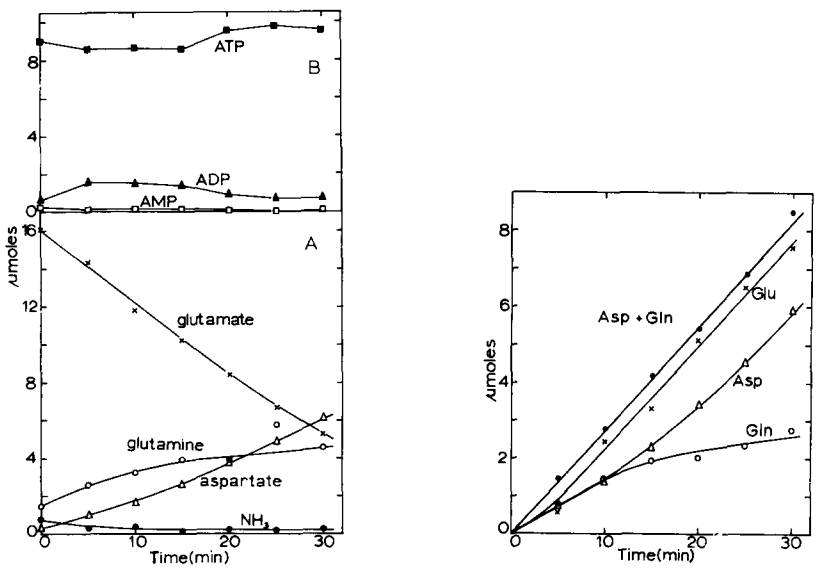

Fig. 4. Time course of glutamate metabolism and level of adenine nucleotides in rat-liver homogenate. Experimental conditions as described under METHODS. The reaction mixture contained the basic components *plus* 15 μmoles glutamate, 9 μmoles ATP and 36.0 mg protein. The homogenate was prepared in mannitol. A. Glutamate metabolism. B. Level of adenine nucleotides.

Fig. 5. Time course of glutamate oxidation in rat-liver homogenate. The curves were calculated from Fig. 4. ×—×, $-\Delta$glutamate (corrected) = $-\Delta$glutamate + Δglutamine; △—△, Δaspartate; ○—○, Δglutamine (corrected) = Δglutamine + ΔNH_3; ●—●, Δaspartate + Δglutamine (corrected).

rate of NH_3 formation in the 15–30-min period was not zero (as in isolated mitochondria), but proceeded at about one third of the initial rate (Fig. 5).

DISCUSSION

The experiment of Fig. 1 demonstrates that, under the conditions used, both steps of the Krebs–Henseleit cycle can occur simultaneously. Although many authors have stated that the rate-limiting step in the urea cycle is the synthesis of argininosuccinate, under our experimental conditions citrulline synthesis was found to be limiting. Possible explanations are: (i) A rather high glutamate concentration is necessary for an optimal citrulline synthesis in rat liver[13,31]. The 10 mM NH_4Cl added to the reaction mixture in our experiments would lower the glutamate concentration due to glutamine synthesis. (ii) The recovery of mitochondria in the homogenate may be low, so that the activity of the citrulline-synthesizing system (mitochondrial) is low with respect to that for arginine synthesis (soluble fraction). (iii) Intracellular permeability factors may play a role in the regulation of urea synthesis.

Added glutamate affects NH_3 production from adenine nucleotides (see Table I) in three different ways: (i) The source of this NH_3 is AMP, formed from the added ADP. During the oxidation of glutamate the AMP level is kept low (Fig. 4B) and would not be expected to give rise to a significant NH_3 production (compare Fig. 2). (ii) Glutamate is oxidized partly by glutamate dehydrogenase, thus giving rise to NH_3. (iii) Due to the very active glutamine synthetase, any NH_3 is immediately bound as glutamine.

Assuming that also in rat-liver homogenate glutamate dehydrogenase reacts preferentially with NADP (*cf.* ref. 10), a stimulated rate of deamination should be correlated with a change in the reactions involving NADP. It might be supposed that this would lead to a reduction level of NADP lower than that usually found in isolated mitochondria under similar conditions. But since glutamate dehydrogenase is present in high concentration in the mitochondrion, a rapid turnover of NADPH might also occur, so that the change in the oxidoreduction state of NADP might not be large. Factors that might influence the reduction level of NADP, and thus be responsible for the high rate of deamination of glutamate in rat-liver homogenate in the first 10 min of the reaction (Fig. 5), are the following: (i) The presence of CO_2. In isolated rat-liver mitochondria bicarbonate lowers the reduction level of NADP[32], and under these conditions the deamination of glutamate is stimulated (R. CHARLES, unpublished observations), perhaps due to the reductive carboxylation of α-oxoglutarate to isocitrate, a reaction that utilizes NADPH[33–35]. Also, inhibition of succinate dehydrogenase by CO_2 (see ref. 36) may lead to a reduction in the flow of electrons to NAD[37] (and NADP). (ii) The rate of the reactions of the transamination pathway. Inhibition of the oxidative reactions of this pathway would lead to a reduction of the flow of electrons to NAD and hence to NADP. Indeed, a lag in aspartate formation was observed, and this may be due to removal of α-oxoglutarate by transamination with other amino acids. This could also explain the surplus of nitrogen in the balance studies. Possibly it takes a rather long time to build up a sufficient steady-state level of oxaloacetate and its precursors. However, other reactions leading to the formation of oxaloacetate and precursors may play a role in the stimulation of aspartate formation after 10 min, *e.g.*, the reductive carboxylation of endogenous

pyruvate by "malic" enzyme (EC 1.1.1.40) or the synthesis of oxaloacetate from pyruvate by pyruvate carboxylase (EC 6.4.1.1).

The removal of one of the compounds involved in the glutamate dehydrogenase reaction, *in casu* NH_3, will cause a stimulation of the deamination by pulling the equilibrium to the side of NH_3 formation. Until a new equilibrium state is reached, a rapid deamination of glutamate will be possible. Indeed, even in isolated mitochondria the removal of NH_3 for citrulline synthesis can cause a very low level of NH_3, which is correlated with a stimulated deamination of glutamate[38].

Although citrulline synthesis can occur with added NH_4Cl (Fig. 1), the NH_3 formed from glutamate by deamination can not be used for the synthesis of citrulline in rat-liver homogenate, due to its removal as glutamine (Table III). Hydrolysis of glutamine by intramitochondrial glutaminase does not occur in this system; this reaction requires a rather high glutamine concentration and is activated by NH_3 (ref. 38). The aspartate formed from glutamate can be used for the synthesis of arginine. Thus both nitrogen donors for urea synthesis can be derived from glutamate. The direct source of the NH_3 for citrulline synthesis *in vivo* probably is glutamine, which is broken down intramitochondrially by glutaminase. The mutual influences of the processes of glutamate oxidation, glutamine synthesis and hydrolysis, and urea synthesis open the possibilities for complex regulatory mechanisms in the metabolism of nitrogen in the cell.

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