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UTILIZATION OF GLUTAMATE FOR PHOSPHO*ENOL*PYRUVATE AND ASPARTATE SYNTHESIS IN KIDNEY CORTEX MITOCHONDRIA OF RABBIT*

JADWIGA BRYŁA and JOLANTA M. DZIK

Institute of Biochemistry, University of Warsaw, Al. Żwirki i Wigury 93, 02–089 Warsaw (Poland)

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SUMMARY

1. Utilization of glutamate for both phospho*enol*pyruvate and aspartate synthesis in kidney cortex mitochondria of the rabbit was studied under three metabolic conditions (state 3, uncoupled state and in the presence of uncoupler and oligomycin) in order to induce a high respiratory activity with an oxidized state of the nicotinamide dinucleotides but with varied intramitochondrial ATP levels.

2. The highest rates of glutamate deamination and thus of phospho*enol*pyruvate formation were observed in uncoupled mitochondria incubated without oligomycin, i.e. under conditions of low intramitochondrial ATP content. Aspartate synthesis was high in both State 3 and the uncoupled state.

3. In contrast to liver, addition of oligomycin to uncoupled kidney cortex mitochondria in order to increase the mitochondrial ATP/ADP ratio resulted in an inhibition of glutamate deamination, phospho*enol*pyruvate formation and aspartate synthesis. Atractyloside augmented an inhibitory effect of oligomycin.

4. Addition of either ATP trapping system or ADP to mitochondria incubated in uncoupled-plus-oligomycin state resulted in the abolishing of the inhibition of both phospho*enol*pyruvate and aspartate synthesis, due to a depletion of intramitochondrial ATP content.

5. In contrast to liver, leucine, which activates glutamate dehydrogenase, caused an acceleration of glutamate deamination in kidney cortex mitochondria, accompanied by an enhancement of phospho*enol*pyruvate formation. On the other hand, aspartate synthesis was not affected by leucine.

6. In the uncoupled-plus-oligomycin state, leucine abolished an inhibitory effect of ATP and/or GTP on both glutamate deamination and phospho*enol*pyruvate synthesis. However, it did not relieve the inhibition of aspartate formation.

7. In the presence of malate in addition to glutamate, oligomycin inhibited neither aspartate formation nor phospho*enol*pyruvate synthesis, since under these

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Abbreviations: *P-enol*pyruvate, phospho*enol*pyruvate; EGTA, ethylene glycol-bis(2-aminoethyl)tetraacetate; FCCP, *p*-trifluoromethoxyphenylhydrazone of carbonyl cyanide.

* Dedicated to Professor E. C. Slater on occasion of his 60th birthday.

conditions an accumulation of energy did not occur in mitochondria due to an efficient phospho*enol*pyruvate formation.

8. The data indicate that in contrast to liver, in kidney cortex mitochondria, utilizing glutamate as a substrate, the rates of both phospho*enol*pyruvate and aspartate synthesis depend greatly upon the mitochondrial energy level and the availability of both oxaloacetate and leucine.

INTRODUCTION

In previous communications from this laboratory [1–3] we have reported that in both guinea-pig and rabbit liver mitochondria the phospho*enol*pyruvate synthesis from glutamate is dependent upon the glutamate deamination. According to McGivan and Chappell [4], the main function of glutamate dehydrogenase in liver mitochondria is glutamate synthesis rather than glutamate deamination primarily because of the kinetic limitation of glutamate entry into mitochondria on the glutamate-hydroxyl translocator. Since in kidney cortex a ready deamination of glutamate occurs [5], we have used rabbit kidney cortex mitochondria for studies of the phospho*enol*pyruvate formation from glutamate.

Results reported here show that in kidney cortex mitochondria (in contrast to liver) a high intramitochondrial ATP/ADP ratio results in the inhibition of glutamate dehydrogenase activity, followed by a decrease of both phospho*enol*pyruvate and aspartate synthesis. Leucine, an activator of glutamate dehydrogenase [6, 7], abolishes the inhibitory effect of ATP and/or GTP on glutamate deamination causing a stimulation of phospho*enol*pyruvate formation. In contrast, leucine does not reverse the aspartate synthesis under these conditions.

MATERIALS AND METHODS

Mitochondrial preparation and incubation

Mitochondria were prepared from kidney cortex of white male rabbits (about 2–3 kg in weight) by a minor modification of the method of Schneider and Hogeboom [8]. The isolation medium contained 225 mM mannitol, 75 mM sucrose, 0.5 mM EGTA and 0.1 % bovine serum albumin. The final wash and suspension were made with the medium not containing bovine serum albumin.

Mitochondria were incubated in the reaction mixture containing: 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 40 mM Tris · HCl buffer, 10 mM potassium phosphate buffer and 10 mM glutamate. Different incubation conditions were produced by the following additions to the basic buffer: state 3, 20 mM glucose, hexokinase (2–5 units/ml) and 0.1 mM ADP; uncoupled, 0.1–0.2 μM FCCP; uncoupled plus oligomycin, 0.1–0.2 μM FCCP and oligomycin (1 μg/mg of protein). Titrations of FCCP concentration against the respiratory rate were performed with mitochondria incubated at the same protein concentration as used in the experiments in order to ascertain the correct concentration for maximal stimulation of respiration and complete loss of ATP formation by oxidative phosphorylation. In experiments carried out in either coupled or uncoupled conditions, reactions were started by addition of mitochondrial suspension to the reaction medium. In uncoupled-plus-oligomycin conditions, the

reaction was started by addition of FCCP solutions to mitochondrial suspension pre-incubated for 1 min with oligomycin. Incubations were performed in chambers maintained at 30 °C. Oxygen was blown over the surface of the stirred reaction medium.

For measurements of the total content of metabolites in the reaction medium, 1 or 2-ml aliquots were removed at 5-min intervals up to 20 min, and the protein was precipitated with 1/10 volume of 35 % (v/v) perchloric acid. The supernatant after centrifugation was neutralized with the mixture containing 5 M KOH and 0.5 M potassium acetate. $KClO_4$ was precipitated in the cold.

Intramitochondrial ATP and ADP levels were measured using the silicone layer technique [9].

Assays

Determinations of both phospho*enol*pyruvate and aspartate were done spectrophotometrically [10], while 2-oxoglutarate, ATP and ADP were measured fluorimetrically [11]. Ammonia was assayed according to Chaney and Marbach [12] following the distillation of ammonia from the sample to sulphuric acid as described by Conway [13].

Enzymes and Chemicals

Pyruvate kinase (EC 2.7.1.40) used for assays of both phospho*enol*pyruvate and ADP was isolated and purified according to Tietz and Ochoa [14]. Malate dehydrogenase (EC 1.1.1.37) and aspartate aminotransferase (EC 2.6.1.1) were obtained by the methods of Englard and Siegel [15] and Sizer and Jenkins [16], respectively. $(NH_4)_2SO_4$-free hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were purchased from Koch and Light Laboratories Ltd., Colnbrook, England, while lactate dehydrogenase (EC 1.1.1.27) was provided by Polskie Odczynniki Chemiczne, Gliwice, Poland. L-Leucine and ADP were purchased from Reanal, Hungary, while atractyloside and oligomycin were provided by Sigma Chemical Co., St. Louis, Mo., U.S.A. FCCP was the generous gift of Dr. P. Heytler. All other chemicals were of analytical grade.

RESULTS

Phosphoenolpyruvate and aspartate synthesis under various metabolic conditions

Table I shows the rates of both phospho*enol*pyruvate and aspartate synthesis in rabbit kidney cortex mitochondria under three metabolic conditions: State 3, uncoupled state and in the presence of uncoupler and oligomycin. These states were chosen in order to induce a high respiratory activity with an oxidized state of the nicotinamide dinucleotides but with varied intramitochondrial ATP levels. The rates of both phospho*enol*pyruvate and aspartate synthesis were linear up to 20 min of incubation under all conditions studied. In agreement with previous observations for liver mitochondria [1–3] in rabbit kidney cortex mitochondria, utilizing glutamate as a substrate, very little phospho*enol*pyruvate formation and a high aspartate production occurred in State 3, since under these conditions the glutamate transamination pathway [17] competes efficiently with phospho*enol*pyruvate carboxykinase for the intramitochondrial oxaloacetate. Malate production was negligible. Although the

TABLE I

FORMATION OF *P*-ENOLPYRUVATE AND ASPARTATE IN KIDNEY-CORTEX MITOCHONDRIA UNDER VARIOUS METABOLIC CONDITIONS

Aminooxyacetate and 2-oxoglutarate were added at 0.2 and 5 mM concentrations, respectively. Other details as described under Materials and Methods. Values are means±S.E. of 9 experiments, given in nmol/min per mg protein.

Substrate	Metabolite measured	State 3	FCCP	FCCP+oligomycin
Glutamate	*P-enol*pyruvate	2.4±0.2	4.7±0.6	1.4±0.4
	Aspartate	20.0±0.7	20.5±1.5	4.5±0.3
Glutamate+ aminooxyacetate	*P-enol*pyruvate	6.7±0.4	7.0±0.6	3.3±0.3
2-Oxoglutarate	*P-enol*pyruvate	11.2±0.5	10.8±1.0	11.8±0.8

uncoupler is known to switch the glutamate transamination to dehydrogenation [17] under the conditions of our experiment, the rates of aspartate formation were not decreased below those observed in State 3. However, in uncoupled mitochondria, phospho*enol*pyruvate production was about 2-fold higher than in State 3, probably due to a higher intramitochondrial oxaloacetate concentration available for phospho*enol*pyruvate carboxykinase since in the uncoupled state the NADH/NAD^+ ratio is lower than in State 3. Addition of aminooxyacetate, an inhibitor of transamination [18], resulted in about a 3-fold stimulation of phospho*enol*pyruvate production in State 3. The rate of phospho*enol*pyruvate formation in uncoupled mitochondria incubated in the presence of aminooxyacetate was only slightly higher than in State 3.

In contrast to liver mitochondria [1–3], addition of oligomycin to uncoupled kidney cortex mitochondria incubated with glutamate resulted in a marked decrease of both phospho*enol*pyruvate and aspartate synthesis. Inhibition of phospho*enol*pyruvate formation was not due to an inhibition of flux through 2-oxoglutarate dehydrogenase by the state of phosphorylation of the nucleotide systems [19], since oligomycin did not inhibit the phospho*enol*pyruvate production when 2-oxoglutarate was used as the source of both oxaloacetate and energy. The diminished aspartate production was not due to an inhibition of aspartate efflux from the mitochondria [20], since the intramitochondrial aspartate levels were not increased in the presence of oligomycin (not shown). The complete inhibition of aspartate formation on addition of aminooxyacetate was accompanied by a partial restoration of phospho*enol*pyruvate formation.

Reversal of phosphoenolpyruvate and aspartate synthesis by either the ATP trapping system or ADP in uncoupled-plus-oligomycin state

In uncoupled mitochondria incubated with glutamate, energy is generated only via the substrate level phosphorylation. Thus, the addition of oligomycin could increase the ratio of intramitochondrial GTP/GDP and ATP/ADP and inhibit the glutamate dehydrogenase activity providing less 2-oxoglutarate and hence oxaloacetate for both phospho*enol*pyruvate production as well as aspartate formation. In order to check this possibility, both the ATP levels and the ammonia generation were estimated. As is shown in Table II, on addition of oligomycin to uncoupled mito-

TABLE II

EFFECT OF ATP TRAPPING SYSTEM ON *P-ENOL*PYRUVATE AND ASPARTATE SYNTHESIS, AMMONIA GENERATION, ATP LEVELS AND OXYGEN UPTAKE IN MITOCHONDRIA INCUBATED IN BOTH UNCOUPLED AND UNCOUPLED PLUS OLIGOMYCIN CONDITIONS

Samples with hexokinase (about 5 units/ml) contained also 20 mM glucose and 0.1 mM ADP. Other details as described under Materials and Methods. Values shown are means±S.E. of 7 experiments.

State	Hexokinase	(nmol/min per mg protein)			ATP levels (nmol/mg protein)	O_2 uptake (ngatoms/min per mg protein)
		*P-enol*pyruvate	Ammonia	Aspartate		
Uncoupled	−	4.8±0.5	7.2±1.7	17.8±4.4	2.7±1.5	72.1±6.6
	+	5.2±0.8	7.9±1.5	18.1±3.5	–	84.2±8.7
Uncoupled-plus-oligomycin	−	1.3±0.3	2.6±0.8	5.1±1.7	5.5±1.7	27.8±2.9
	+	4.7±0.4	6.2±1.1	18.0±3.4	–	75.9±6.3

chondria, the 3 to 4-fold decrease of both phospho*enol*pyruvate and aspartate synthesis was accompanied by a marked inhibition of both oxygen uptake and ammonia generation (to about 1/3) as well as by an approx. 2-fold elevation of ATP in the incubation medium. Addition of an ATP trapping system (hexokinase, glucose and ADP) to cause depletion of intramitochondrial ATP resulted in a marked stimulation of all processes studied.

Accumulation of 2-oxoglutarate varied considerably under different metabolic conditions, being about 2-fold higher in State 3, uncoupled mitochondria or uncoupled mitochondria incubated with oligomycin and ATP trapping system than in the uncoupled plus oligomycin state (Fig. 1). A marked inhibition of 2-oxoglutarate accumulation on the addition of oligomycin to uncoupled mitochondria could be due to a decline of 2-oxoglutarate formation in the reaction catalyzed by the glutamate dehydrogenase.

The data presented in Fig. 2 show that exogenous ADP (about 0.5 mM), like the ATP trapping system, resulted also in an enhancement of both phospho*enol*-

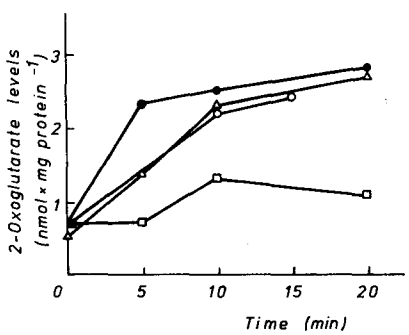

Fig. 1. Accumulation of 2-oxoglutarate under various conditions. State 3 (△), uncoupled state (○), uncoupled-plus-oligomycin state (□) and uncoupled-plus-oligomycin state in the presence of ATP trapping system (●).

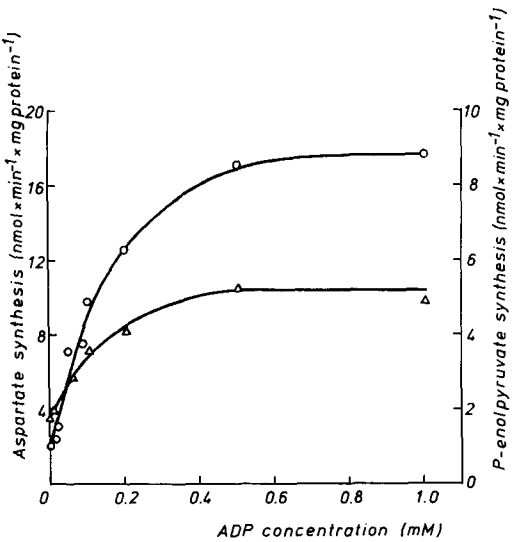

Fig. 2. Effect of exogenous ADP on both *P-enol*pyruvate and aspartate synthesis in kidney cortex mitochondria incubated with glutamate in uncoupled plus oligomycin state. The rates of *P-enol*-pyruvate formation (△) and aspartate production (○) in uncoupled mitochondria (without oligomycin added) were 4.8 and 16.9 nmol/min per mg of protein, respectively.

pyruvate and aspartate synthesis. This could be due to a stimulation of ATP-ADP exchange on the nucleotide translocator, causing a decrease of intramitochondrial ATP/ADP ratio and probably GTP/GDP ratio.

Effect of leucine

Leucine (2.5 mM), an activator of glutamate dehydrogenase [6, 7], increased glutamate deamination by about 2-fold in uncoupled mitochondria as measured by the ammonia production (Table III). It did not change the rate of phospho*enol*-pyruvate synthesis from either malate or 2-oxoglutarate in State 3 (not shown), indicating that its stimulatory effect on phospho*enol*pyruvate formation in the presence

TABLE III

EFFECT OF LEUCINE ON PHOSPHO*ENOL*PYRUVATE FORMATION, ASPARTATE SYNTHESIS, AMMONIA GENERATION AND ATP LEVELS IN MITOCHONDRIA INCUBATED WITH GLUTAMATE IN BOTH UNCOUPLED AND UNCOUPLED PLUS OLIGOMYCIN STATES

L-Leucine was added at 2.5 mM concentration. Values shown are means±S.E. of 4 experiments.

State	Leucine	(nmol/min per mg protein)			ATP (nmol/mg protein)
		*P-enol*pyruvate	Ammonia	Aspartate	
Uncoupled	−	5.5±0.9	6.1±0.7	17.8±2.0	3.8±1.2
	+	7.6±1.4	12.5±1.5	19.2±2.4	4.2±1.1
Uncoupled-plus-oligomycin	−	1.8±0.8	2.3±0.8	4.6±1.6	7.0±0.5
	+	5.9±1.3	6.6±1.0	6.4±1.1	7.3±1.4

TABLE IV

EFFECT OF LEUCINE AND ATRACTYLOSIDE ON PHOSPHO*ENOL*PYRUVATE FORMATION, ASPARTATE SYNTHESIS AND MITOCHONDRIAL ATP AND ADP LEVELS IN UNCOUPLED MITOCHONDRIA INCUBATED WITHOUT OR WITH OLIGOMYCIN

Atractyloside and L-leucine were added at 25 μM and 2.5 mM concentrations, respectively. Intramitochondrial ATP and ADP levels were measured using the silicone layer technique. Assays of adenine nucleotides were performed in duplicate.

State	Additions	(nmol/min per mg protein)		(nmol/mg protein)		ATP/ADP
		*P-enol*pyruvate	Aspartate	ATP	ADP	
Uncoupled	None	5.0	20.0	0.8	2.6	0.3
	Leucine	6.4	17.6	–	–	–
	Atractyloside	4.2	19.2	1.3	1.8	0.7
	Atractyloside plus leucine	6.0	20.1	1.5	1.6	0.9
Uncoupled-plus-oligomycin	None	1.8	5.6	1.6	1.5	1.1
	Leucine	6.3	7.2	–	–	–
	Atractyloside	0.9	2.4	2.3	1.1	2.1
	Atractyloside plus leucine	3.9	4.3	2.1	1.2	1.8

of glutamate as substrate is not due to an increase of phospho*enol*pyruvate carboxykinase activity but due to an stimulation of glutamate deamination. Leucine reversed an inhibitory effect of oligomycin on both ammonia generation and phospho*enol*pyruvate formation in the presence of glutamate as substrate. The stimulatory effect of leucine was not accompanied by a decrease of ATP concentration in the mitochondrial suspension, suggesting that this amino acid could abolish an inhibitory action of ATP and/or GTP on the glutamate dehydrogenase. Surprisingly, leucine did not relieve the inhibition of aspartate production in uncoupled mitochondria incubated with oligomycin.

In order to find an interrelationship between glutamate dehydrogenase activity and adenine nucleotide levels, intramitochondrial ATP and ADP concentrations were measured in the presence and absence of atractyloside, an inhibitor of adenine nucleotide translocator. As shown in Table IV, atractyloside did not diminish the rates of either phospho*enol*pyruvate or aspartate synthesis in uncoupled mitochondria incubated with or without leucine, probably because of uncoupler-stimulated ATPase maintaining a low ATP/ADP ratio and probably a low GTP/GDP ratio. Although according to Gmaj et al. [21] kidney cortex mitochondria of rats contain an oligomycin-insensitive ATPase: addition of oligomycin to kidney cortex mitochondria of rabbits resulted in a 3-fold increase of intramitochondrial ATP/ADP ratio, followed by a much more pronounced decrease of both phospho*enol*pyruvate and aspartate synthesis than that observed in the absence of atractyloside. Leucine abolished an inhibitory effect of ATP and/or GTP on glutamate dehydrogenase activity as concluded from an increase of phospho*enol*pyruvate synthesis in the presence of high intramitochondrial ATP/ADP ratio. On the other hand, leucine did not relieve the inhibition of aspartate production in uncoupled mitochondria incubated with oligomycin and atractyloside.

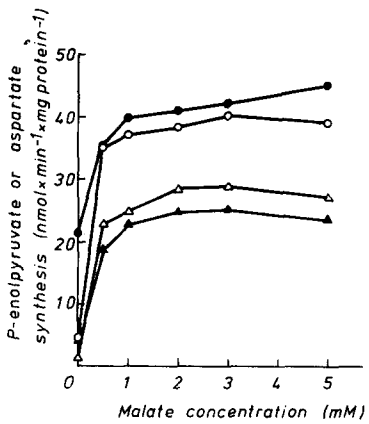

Fig. 3. Effect of increasing concentrations of malate on *P-enol*pyruvate and aspartate synthesis in kidney cortex mitochondria incubated under both uncoupled and uncoupled-plus-oligomycin conditions. ▲ and △ correspond to *P-enol*pyruvate formation in uncoupled and uncoupled plus oligomycin states, respectively, while ● and ○ correspond to aspartate formation in uncoupled and uncoupled plus oligomycin states, respectively.

Effect of malate

As is shown in Fig. 3, when malate at concentrations exceeding 1–2 mM was present in the reaction medium, addition of oligomycin to the uncoupled mitochondria incubated with glutamate did not result in a decrease of either phospho*enol*pyruvate or aspartate synthesis. Since in the presence of malate, intramitochondrial ATP concentration did not exceed 0.7 nmol/mg of protein in both uncoupled and uncoupled-plus-oligomycin states, it seems that an increased generation of oxaloacetate due to malate oxidation could cause a marked stimulation of phospho*enol*pyruvate formation and prevent an accumulation of both GTP and ATP, inhibitors of glutamate dehydrogenase [22].

DISCUSSION

Data presented in this paper seem to indicate that the formation of both phospho*enol*pyruvate and aspartate in kidney cortex mitochondria of rabbit utilizing glutamate as the substrate is strongly dependent upon the intramitochondrial energy level as well as the availability of oxaloacetate and leucine. There is an evidence that in kidney glutamate is transported only via the glutamine-glutamate and glutamate-aspartate exchange [23]. In our hands however, a high rate of phospho*enol*pyruvate production in the presence of aminooxyacetate (cf. Table I) suggests that in the kidney cortex mitochondria of rabbit the glutamate-hydroxyl antiporter is also operative.

It is known that (i) glutamate dehydrogenase can form a complex with aspartate aminotransferase [24, 25] and (ii) the latter enzyme does not prevent GTP from both inducing a conformational change in glutamate dehydrogenase [26] and inhibiting reactions catalyzed by the aminotransferase · glutamate dehydrogenase complex [24, 27]. In kidney cortex mitochondria incubated with glutamate in uncoupled plus oligomycin state energy is generated only via the substrate level phosphorylation. Thus, an increase of mitochondrial GTP/GDP ratio accompanied by an elevation of

ATP/ADP ratio (cf. Table IV) could result in an inhibition of the aspartate aminotransferase · glutamate dehydrogenase complex reactions and hence a decrease of oxaloacetate generation followed by markedly diminished rates of both phosphoenolpyruvate and aspartate synthesis.

A depletion of intramitochondrial ATP due to the addition of either ATP trapping system or ADP could result in a stimulation of reactions catalyzed by the glutamate dehydrogenase · aspartate aminotransferase complex, resulting in an increase of both phospho*enol*pyruvate formation as well as aspartate production. Stimulation of glutamate dehydrogenase activity in the deamination direction by ADP in kidney cortex mitochondria of rabbit (cf. Table II and Fig. 2) is opposite to an inhibitory effect of ADP on the oxidative deamination of glutamate in rat liver mitochondria [28]. However, ADP is known to enhance the glutamate dehydrogenase reaction in direction of glutamate synthesis in rat liver mitochondria [28]. Similarly, an activation by leucine of glutamate dehydrogenase reaction in the direction of glutamate deamination in kidney cortex mitochondria of rabbit (cf. Table III) is in contrast to rat liver mitochondria. According to McGivan et al. [29], a stimulation of glutamate dehydrogenase activity in the deamination direction occurs only in disrupted but not in intact mitochondria. An activatory effect of both ADP and leucine on the glutamate dehydrogenase activity in the direction of glutamate synthesis in liver on one hand and in the direction of glutamate deamination in kidney on the other, could be of physiological significance, since in liver the main function of glutamate dehydrogenase seems to be the glutamate synthesis [4], while in kidney, this enzyme could play an important role in ammoniagenesis, especially under conditions of metabolic acidosis [30].

In the presence of elevated ATP and/or GTP levels, as observed in kidney cortex mitochondria of rabbit incubated in uncoupled-plus-oligomycin state, leucine activated only the glutamate dehydrogenase reaction, causing an increased oxaloacetate generation and phospho*enol*pyruvate formation while the reaction of aspartate synthesis remained inhibited. Since in kidney the glutamate dehydrogenase reaction is of central importance in ammonia production and in disposal of glutamine or glutamate carbon [31], the reversal by leucine of the inhibitory effect of ATP and/or GTP on the glutamate deamination could be of particular importance in metabolic acidosis preventing (i) an increased ammoniagenesis to maintain the acid-base homeostasis and (ii) a high phospho*enol*pyruvate production for gluconeogenesis. Studies on the regulation of glutamate metabolism under conditions of metabolic acidosis are now being undertaken.

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