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The Pathway of Glutamine and Glutamate Oxidation in Isolated Mitochondria from Mammalian Cells

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1. Pyruvate strongly inhibited aspartate production by mitochondria isolated from Ehrlich ascites-tumour cells, and rat kidney and liver respiring in the presence of glutamine or glutamate; the production of $^{14}CO_2$ from L-[U-^{14}C]glutamine was not inhibited though that from L-[U-^{14}C]glutamate was inhibited by more than 50%. 2. Inhibition of aspartate production during glutamine oxidation by intact Ehrlich ascites-tumour cells in the presence of glucose was not accompanied by inhibition of CO_2 production. 3. The addition of amino-oxyacetate, which almost completely suppressed aspartate production, did not inhibit the respiration of the mitochondria in the presence of glutamine, though the respiration in the presence of glutamate was inhibited. 4. Glutamate stimulated the respiration of kidney mitochondria in the presence of glutamine, but the production of aspartate was the same as that in the presence of glutamate alone. 5. The results suggest that the oxidation of glutamate produced by the activity of mitochondrial glutaminase can proceed almost completely through the glutamate dehydrogenase pathway if the transamination pathway is inhibited. This indicates that the oxidation of glutamate is not limited by a high [NADPH]/[NADP$^+$] ratio. 6. It is suggested that under physiological conditions the transamination pathway is a less favourable route for the oxidation of glutamate (produced by hydrolysis of glutamine) in Ehrlich ascites-tumour cells, and perhaps also kidney, than the glutamate dehydrogenase pathway, as the production of acetyl-CoA strongly inhibits the first mechanism. The predominance of the transamination pathway in the oxidation of glutamate by isolated mitochondria can be explained by a restricted permeability of the inner mitochondrial membrane to glutamate and by a more favourable location of glutamate–oxaloacetate transaminase compared with that of glutamate dehydrogenase.

Braunstein (1957) proposed that, before the oxidation, amino acids transaminate first with α-oxoglutarate, the glutamate formed then being oxidatively deaminated by glutamate dehydrogenase. This suggestion was supported by the fact that glutamate dehydrogenase is the only enzyme activity in different animal tissues, especially liver and kidney, that can, indirectly, catalyse the oxidative deamination of amino acids, because L-amino acid oxidases are absent or have very low activity.

Muller & Leuthardt (1950) reported, however, the occurrence of another pathway of glutamate oxidation, which was later designated the transamination pathway (Borst, 1962). In this case glutamate transaminates with oxaloacetate, with the formation of aspartate and α-oxoglutarate. The α-oxoglutarate formed is oxidized through the citric acid cycle, supplying further oxaloacetate for the transamination. This pathway was not considered to be a quantitatively important mechanism of glutamate oxidation until Krebs & Bellamy (1960) showed that aspartate production is high during glutamate oxidation. Under some conditions the amounts of glutamate removed and aspartate formed were approximately equal. These findings were confirmed by Borst & Slater (1960), Borst (1962), De Haan, Tager & Slater (1967), Papa, Tager, Francavilla, De Haan & Quagliariello (1967) and Balázs (1965). It is now generally accepted that the predominant pathway of glutamate oxidation by mitochondria isolated from different tissues is conversion into aspartate by

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glutamate–oxaloacetate transaminase. About 80–90% of the glutamate oxidized undergoes transamination and only a small percentage is deaminated by glutamate dehydrogenase. These results raised the question of the physiological role of glutamate dehydrogenase, since many tissues are very rich in this enzyme. On the basis of their results Papa, Palmieri & Quagliariello (1966) and Vinogradov (1968) suggested that the role of glutamate dehydrogenase is primarily the synthesis of glutamate rather than oxidative deamination.

In the last decade glutamate has been used extensively as a substrate for the study of respiration and oxidative phosphorylation by isolated mitochondria. Glutamine, on the other hand, has been used for this purpose in only a few cases (Hird & Marginson, 1968; Kovačević, McGivan & Chappell, 1970), despite the fact that glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) is almost exclusively a mitochondrial enzyme (Guha, 1961). The present paper describes experiments that throw more light on the problem of glutamine and glutamate oxidation. The results suggest that in some cells (Ehrlich ascites-tumour cells and perhaps kidney cells) the glutamate dehydrogenase pathway predominates in the oxidation of the glutamate that appears in mitochondria mainly by the hydrolysis of glutamine.

MATERIALS AND METHODS

Hyperdiploid Ehrlich ascites-tumour cells were maintained by intraperitoneal transplantation in white Swiss mice. Ascites cells were harvested 9 days after inoculation. Rat kidney and liver mitochondria were obtained from male Buffalo rats (Simonsen laboratory, San Francisco, Calif., U.S.A.). The mitochondria were isolated by centrifugation after homogenization as described by Chappell & Hansford (1969). The homogenization medium contained (final concentrations) sucrose (0.25M), tris–HCl buffer (5mM) and ethanedioxybis(ethylamine)tetra-acetate (1mM), adjusted to pH7.4. Functional integrity of the isolated mitochondria was assessed before all experiments by measuring the respiratory control ratio (Chance & Williams, 1955): it was always more than 4. The incubation medium contained (final concentrations) KCl (120mM), tris–HCl buffer (10mM), P_i (K^+ salt, 10mM) and bovine serum albumin (0.1%), final pH7.4. The temperature was 30°C. O_2 consumption by isolated mitochondria was determined by using a Clark oxygen electrode (Chappell, 1964) and was automatically recorded on a Sergent recorder.

For the measurement of $^{14}CO_2$ production the mitochondria were incubated in a closed system in an air atmosphere. CO_2 was trapped in scintillation vials with 0.5ml of Hyamine during the incubation and for a further 1h after the reaction was stopped by the addition of 1ml of 20% (w/v) trichloroacetic acid by syringe. Radioactivity of CO_2 was determined by using a dioxan-based counting fluid containing naphthalene (60g), 2,5-diphenyloxazole (4g) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (0.2g), dissolved in methanol (100ml), ethylene glycol (20ml) and dioxan (to 1000ml), and a Packard Tri-Carb model 3375 scintillation counter. Radioactive aspartate, glutamate and glutamine were separated on a column (1cm × 9cm) of Dowex 1 (X8; acetate form) with 0.5M-acetic acid as the eluent (Berl, Lajtha & Waelsch, 1961). Portions (1ml) of the eluate were each mixed with 15ml of the dioxan-based counting fluid and radioactivity was determined as described above.

Intact Ehrlich ascites-tumour cells (130mg wet wt.) were incubated in 3ml (final volume) of Ca^{2+}-free Krebs–Ringer phosphate solution of the following composition: NaCl (145mM), KH_2PO_4 (1.5mM), $MgSO_4$ (1.5mM), KCl (5.8mM), Na_2HPO_4 (15mM), final pH7.4. The incubation was done in a closed system with an atmosphere of O_2. The $^{14}CO_2$ was trapped and determined as described above. Aspartate was measured enzymically. The enzymic determinations of aspartate, alanine and glutamate were done as described by Pfleiderer (1963) and Bernt & Bergmeyer (1963).

Mitochondrial protein was measured by the biuret method of Gornall, Bardawill & David (1949).

The glutamine used was a glutamate-free solution prepared by passage through an anion-exchange resin.

RESULTS

Glutamine as a substrate for respiration by isolated mitochondria. The mitochondria isolated from Ehrlich ascites-tumour cells, and rat kidney and liver respire in the presence of glutamine (Figs. 1*a*, 1*b* and 1*c*). Glutamine is a particularly good substrate for the respiration of mitochondria isolated from Ehrlich ascites-tumour cells, kidney cells and some malignant tumours (Kovačević & Morris, 1971). In these cases a high rate of O_2 uptake was achieved in the presence of low concentrations of glutamine (below 1mM). The rate of respiration of mitochondria from malignant tumours in the presence of glutamine was approximately the same as in the presence of glutamate (optimum concentrations were used), whereas with kidney mitochondria glutamine was apparently a better substrate than glutamate. The mitochondria isolated from rat liver use O_2 faster in the presence of glutamate. With liver mitochondria a very high concentration of glutamine (about 20mM) was required to achieve the maximum rate of respiration.

Production of aspartate during the oxidation of glutamine and the effect of pyruvate. A large amount of aspartate was produced (Table 1) during respiration of isolated mitochondria in the presence of glutamine. The addition of pyruvate greatly decreased the production of aspartate by mitochondria respiring in the presence of glutamine or glutamate. However, the addition of pyruvate to a suspension of tumour-cell mitochondria respiring in the presence of glutamine markedly stimulated O_2 uptake, with a very satisfactory respiratory control ratio (Fig. 1*a*). Measurement of $^{14}CO_2$ production

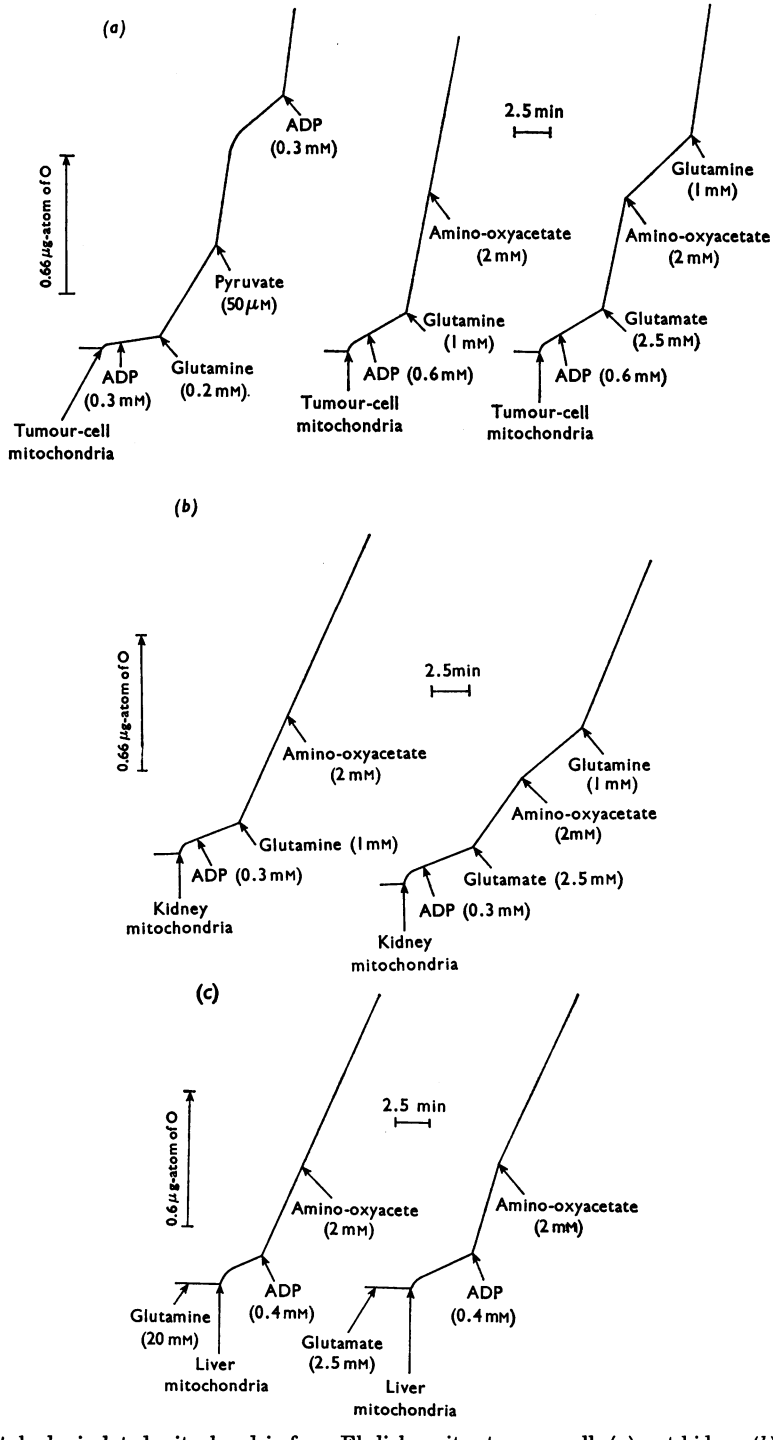

Fig. 1. O_2 uptake by isolated mitochondria from Ehrlich ascites-tumour cells (*a*), rat kidney (*b*) and rat liver (*c*) in the presence of glutamine and glutamate, and the effect of pyruvate and amino-oxyacetate. The incubation medium was as described in the Materials and Methods section. The amount of mitochondria was approx. 5 mg of protein; the final volume was 5 ml.

by the mitochondria respiring in the presence of L-[U-^{14}C]glutamine or L-[U-^{14}C]glutamate showed that the addition of pyruvate did not inhibit the oxidation of glutamine, but that the oxidation of glutamate was inhibited by about 50% (Table 1).

Effect of amino-oxyacetate on the respiration of isolated mitochondria in the presence of glutamine or glutamate. Amino-oxyacetate is a powerful inhibitor of transaminases (Wallach, 1961). Thus, if glutamine oxidation goes mainly through the transamination pathway, the addition of amino-oxyacetate should inhibit O_2 uptake in the presence of this substrate. In fact, no inhibition was observed with glutamine as substrate (Figs. 1*a*, 1*b* and 1*c*). However, amino-oxyacetate did inhibit mitochondrial respiration in the presence of glutamate, and this inhibition was more pronounced with the tumour-cell and kidney mitochondria. The addition of glutamine after amino-oxyacetate stimulated the respiration. Measurement of [^{14}C]aspartate production from labelled glutamine by the tumour-cell and liver mitochondria revealed that amino-oxyacetate almost completely inhibited the transamination pathway (Table 2).

Production of aspartate and carbon dioxide during the oxidation of glutamine in intact Ehrlich ascites-tumour cells in the presence of glucose. Intact tumour cells oxidize glutamine rapidly and a large amount of aspartate is produced. In the presence of glucose there is a marked inhibition of aspartate production, though $^{14}CO_2$ production from labelled glutamine was not inhibited except in the first 30min (Fig. 2). This inhibition is caused by the Crabtree effect, which operates until all the glucose is used up. The same results (aspartate and CO_2 production) were obtained with pyruvate instead of glucose.

Stimulatory effect of aspartate on O_2 consumption by kidney mitochondria respiring in the presence of pyruvate. The addition of aspartate to kidney mitochondria that had been preincubated with ADP and P_i and were respiring in the presence of pyruvate markedly stimulated O_2 consumption (Fig. 3). The same effect was observed with the tumour-cell mitochondria, but it was almost absent with rat liver mitochondria.

Stimulatory effect of glutamate on the respiration of kidney mitochondria in the presence of glutamine. Kovačević *et al.* (1970) showed that the addition of glutamate stimulated O_2 uptake by kidney mitochondria respiring in the presence of glutamine. The same effect was observed with mitochondria isolated from the tumour cells and liver. No satisfactory explanation for this phenomenon can be given, as the activity of mitochondrial glutaminase (production of glutamate) is not the rate-limiting step. In connexion with this problem I measured the rate of O_2 uptake and the production of aspartate in the presence of glutamine, glutamate or both substrates together. Whereas the rate of the respiration of the mitochondria in the presence of both substrates together was approximately equal to the sum of the rates of respiration in the presence of glutamine and glutamate separately, the production of aspartate was approximately equal to the amount found during the respiration of the mitochondria in the presence of glutamate alone (Table 3). Further, the production of aspartate during the oxidation of glutamine was

Table 1. *Production of aspartate, alanine, glutamate and carbon dioxide by mitochondria from Ehrlich ascites-tumour cells and rat liver in the presence of glutamine or glutamate with and without the addition of pyruvate*

The incubation medium for the tumour-cell mitochondria was as described in the Materials and Methods section, with the following additions: glutamine (5mM), glutamate (2.5mM), ADP (5mM), pyruvate (2.5mM, where indicated) and the mitochondria (8.3mg of protein); the final volume was 2ml. The incubation time was 10min. In the experiments where $^{14}CO_2$ was measured L-[U-^{14}C]glutamine (5mM; 1.5μCi/mmol) or L-[U-^{14}C]-glutamate (2.5mM; 36μCi/mmol) was used. The incubation medium for the liver mitochondria was as described in the Materials and Methods section, with the following additions: glutamine (10mM), pyruvate (10mM, where indicated), ADP (2.5mM) and mitochondria (6mg of protein); the final volume was 1ml. The incubation time was 10min. The results given are averages of those obtained with three samples.

Source of mitochondria	Substrate	Metabolite production (μmol/sample)			$^{14}CO_2$ production (c.p.m./sample)
		Aspartate	Glutamate	Alanine	
Ehrlich ascites-tumour cells	Glutamine	2.00	0.57	0	1360
	Glutamine+pyruvate	0.39	0.70	0	1411
	Glutamate	1.37	—	—	546
	Glutamate+pyruvate	0.24	—	—	264
Rat liver	Glutamine	0.80	—	—	—
	Glutamine+pyruvate	0.16	—	—	—

Table 2. *Production of labelled aspartate and glutamate by, and disappearance of glutamine from, mitochondria from Ehrlich ascites-tumour cells and rat liver in the presence of* L-[U-^{14}C]*glutamine with and without amino-oxyacetate*

The incubation medium for the tumour-cell mitochondria was as described in the Materials and Methods section, with the following additions: L-[U-^{14}C]glutamine (5mM), ADP (5mM) and mitochondria (3.6mg of protein); the final volume was 1ml. Amino-oxyacetate (2mM) was added where indicated. The incubation time was 10min. The incubation medium for the liver mitochondria was as described in the Materials and Methods section, with the following additions: L-[U-^{14}C]glutamine (10mM), ADP (2.5mM) and mitochondria (6mg of protein); the final volume was 1ml. Amino-oxyacetate (2mM) was added where indicated. The incubation time was 10min. The results given are averages of those obtained with three samples.

Source of mitochondria	Amino-oxyacetate	Aspartate production (c.p.m./sample)	Glutamate production (c.p.m./sample)	Glutamine consumption (c.p.m./sample)
Ehrlich ascites-tumour cells	−	8550	22200	40600
	+	1300	31000	44000
Rat liver	−	2370	5840	16000
	+	160	9700	20000

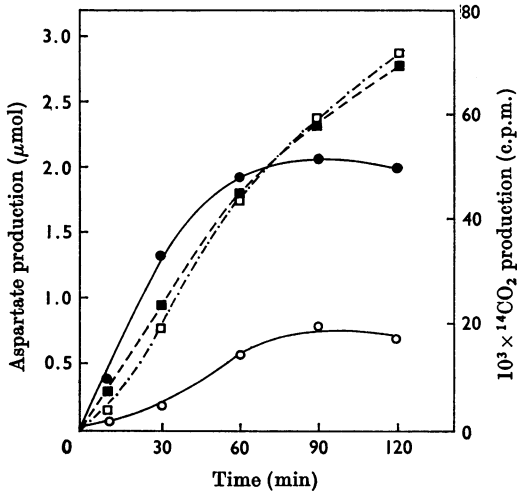

Fig. 2. Production of aspartate and $^{14}CO_2$ during the oxidation of glutamine (2mM) by intact Ehrlich ascites-tumour cells in the presence and the absence of glucose (2mM). In the experiments where $^{14}CO_2$ was measured L-[U-^{14}C]glutamine (2mM; 0.5μCi/4μmol) was used. The incubation medium was otherwise as described in the Materials and Methods section. ●, Aspartate (control); ○, aspartate (glucose present); ■, CO_2 (control); □, CO_2 (glucose present).

50% lower than during the oxidation of glutamate, though the rate of respiration of the mitochondria was faster in the first case.

DISCUSSION

The presence of a highly active glutaminase in the mitochondria isolated from the tissues studied enables them to respire in the presence of glutamine. Besides glutaminase, glutamine–oxaloacetate transaminase and glutamine–pyruvate transaminase might be responsible for the beginning of the pathway of glutamine oxidation. If this were so, glutamate would not be the product of the enzymic reaction. However, parallel measurement of the activity of glutaminase in intact mitochondria isolated from different tissues and estimation of the rate of respiration of the mitochondria in the presence of glutamine revealed that there is a very good correlation between these two phenomena (Kovačević & Morris, 1971). Even in the presence of pyruvate there was no production of alanine during the oxidation of glutamine, indicating that glutamine–pyruvate transaminase activity is absent. The experiments with amino-oxyacetate showed that almost complete suppression of aspartate production was not accompanied by inhibition of mitochondrial respiration in the presence of glutamine. In addition, the mainly extramitochondrial location of glutamine transaminases greatly decreases the possibility that these enzymes are responsible for the extensive oxidative metabolism of glutamine.

It is known that the concentration of glutamine in blood is much higher than that of glutamate (Moore & Stein, 1954; Drewes & McKee, 1967) and that the outer cell membrane is poorly permeable to glutamate (Herscovics & Johnstone, 1964; Hems, Stubbs & Krebs, 1968) whereas glutamine is transported rapidly (Herscovics & Johnstone, 1964; Charles, Tager & Slater, 1967). These facts suggest that under physiological conditions glutamine might be a more important substrate than glutamate for the respiratory activity of mitochondria. This seems particularly so with the tumour-cell and kidney mitochondria, which respire rapidly in the presence of low concentrations of glutamine.

Aspartate production during the oxidation of glutamine by isolated mitochondria is strongly inhibited in the presence of pyruvate, though there is no inhibitory effect of pyruvate on the production of $^{14}CO_2$ from labelled glutamine. The same results were obtained in experiments with intact Ehrlich ascites-tumour cells (glucose present), indicating that the oxidation of glutamine may proceed almost completely through the glutamate dehydrogenase pathway. This is supported by the experiments with amino-oxyacetate, in which a 85–95% inhibition of the transaminase pathway did not have any inhibitory effect on the rate of O_2 uptake by the mitochondria respiring in the presence of glutamine.

With glutamate as substrate for mitochondrial respiration instead of glutamine, the addition of pyruvate again strongly inhibited aspartate formation, and in this case about 50% of the $^{14}CO_2$ production from labelled glutamate was also inhibited. Further, the addition of amino-oxyacetate inhibited O_2 uptake by mitochondria respiring in the presence of glutamate. The inhibition was particularly marked with the tumour-cell and kidney mitochondria. To explain the difference found between the oxidation of glutamine and that of glutamate in the presence of pyruvate or amino-oxyacetate I have assumed that glutamate dehydrogenase and aspartate transaminase do not have the same mitochondrial location. It is generally accepted that glutamate dehydrogenase is located in the mitochondrial matrix. It is believed that aspartate transaminase has the same location in rat liver mitochondria; however, the present experiments (Fig. 3) have shown that the addition of aspartate, which is a non-penetrant anion in the absence of glutamate (Chappell, 1968; Klingenberg, 1970), markedly stimulated O_2 uptake by the tumour-cell and kidney mitochondria respiring in the presence of pyruvate. The same effect was found after preincubation of the mitochondria in the presence of ADP and P_i to remove endogenous glutamate. These findings suggest that aspartate does not have to penetrate the inner mitochondrial membrane to reach the aspartate transaminase. Also, the inner mitochondrial membrane is less permeable to glutamate than to glutamine. This is so especially in mitochondria from kidney, Ehrlich ascites-tumour cells and some other tumours (fast-growing Morris hepatomas and MK 3 tumour). These mitochondria do not swell in an iso-osmotic solution of ammonium glutamate, but they do swell in an iso-osmotic solution of glutamine (Kovačević *et al.* 1970; Kovačević & Morris, 1971). Hence it may be assumed that, because of the restricted permeability of the inner mitochondrial membrane to glutamate and the

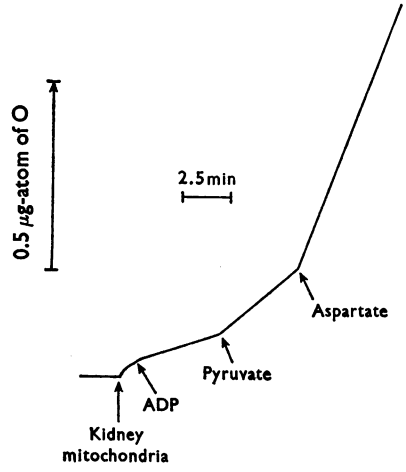

Fig. 3. Stimulation by the addition of aspartate (2.5mM) of O_2 uptake by kidney mitochondria (3.5mg of protein) respiring in the presence of pyruvate (0.5mM). The incubation medium was otherwise as described in the Materials and Methods section.

Table 3. *Oxygen uptake and production of aspartate by rat kidney mitochondria in the presence of glutamine, glutamate or both substrates together*

For the measurement of O_2 uptake with the oxygen electrode the incubation medium was as described in the Materials and Methods section, with the following additions: glutamine (2mM) or glutamate (2.5mM) or both, ADP (0.3mM) and mitochondria (2.3mg of protein); the final volume was 5ml. For the experiments where aspartate production was measured the incubation medium was as described in the Material and Methods section, with the following additions: glutamine (4mM) or glutamate (2.5mM) or both, ADP (2.5mM) and mitochondria (3mg of protein); the final volume was 1ml. The incubation time was 10min. The results given are averages of those obtained with three samples.

Substrate	O_2 uptake (ng-atom of O/min per mg of protein)	Aspartate production (μmol/sample)
Glutamine	24.0	0.192
Glutamate	18.1	0.390
Glutamine+glutamate	46.0	0.375

location of glutamate dehydrogenase in the matrix, the inhibition of glutamate–oxaloacetate transaminase by the addition of pyruvate or amino-oxyacetate resulted in an inhibition of mitochondrial respiration and CO_2 production in the presence of this substrate.

It has been suggested (Papa *et al.* 1966; De Haan *et al.* 1967) that the most important factor controlling the activity of glutamate dehydrogenase during glutamate oxidation by isolated mitochondria is the oxidoreduction state of NADP. A high [NADPH]/[$NADP^+$] ratio inhibits the deamination of glutamate by glutamate dehydrogenase. The present experimental results suggest that under physiological conditions the transamination pathway does not predominate in the oxidation of glutamate in intact Ehrlich ascites-tumour cells, as the rapid formation of acetyl-CoA from pyruvate probably keeps the steady-state oxaloacetate concentration too low for substantial utilization by the glutamate–oxaloacetate transaminase. The same may be expected for the kidney, as the glutamate dehydrogenase pathway would ensure a greater production of ammonia. However, in the liver the oxidation of glutamine or glutamate would probably involve a large production of aspartate, which is required for urea synthesis. That a highly reduced state of the intramitochondrial NADP system is probably not the cause of the inhibition of glutamate oxidation through the glutamate dehydrogenase pathway is indicated by the experiments in which the oxidation of glutamine was not inhibited in the presence of pyruvate or amino-oxyacetate despite a strong inhibition of the transamination pathway. The main reasons why the transamination pathway is predominant when isolated mitochondria respire in the presence of glutamate may be a restricted permeability of the inner mitochondrial membrane to glutamate and a more accessible location of the glutamate–oxaloacetate transaminase relative to that of glutamate dehydrogenase. When glutamine is a substrate for respiration the permeability of the mitochondrial membrane is not the rate-limiting step, as glutamate is produced inside the mitochondria.

The stimulatory effect of glutamate on O_2 uptake by kidney mitochondria respiring in the presence of glutamine can also be explained on the basis of the above considerations. External addition of glutamate saturates the transamination pathway, whereas glutamate produced by the activity of mitochondrial glutaminase saturates the glutamate dehydrogenase pathway. This would result in a stimulation of O_2 consumption, whereas the production of aspartate would correspond to its production in the presence of glutamate alone.

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