The Oxidation of Glutamate by Rat-Liver Mitochondria

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1. In rat-liver mitochondria both the dehydrogenase and transaminase routes participate in glutamate oxidation. However, the rate of ammonia production by the dehydrogenase pathway progressively decreases with the time of incubation. 2. Glutamate deamination is stimulated by blocking the transaminase pathway with arsenite or malonate. On the other hand, this process is completely suppressed by succinate, malate, pyruvate and oxaloacetate. Succinate and pyruvate inhibit, whereas malate and oxaloacetate stimulate, aspartate formation. 3. Glutamate deamination increases with increasing concentrations of 2,4-dinitrophenol from 0.05 to 0.2 mM, and then becomes inhibited, together with the rate of oxygen consumption. Aspartate formation is progressively inhibited with increasing 2,4-dinitrophenol concentration from 0.05 to 0.8 mM. In the presence of 0.20 mM-2,4-dinitrophenol the rate of ammonia production is higher than in the presence of phosphate acceptors and decreases much slower and linearly with the time of incubation. 4. The addition of NAD^+ enhances glutamate deamination without affecting oxygen uptake.

Müller & Leuthardt (1950) and Krebs & Bellamy (1960) have shown the occurrence of a transaminase pathway for glutamate oxidation in liver mitochondria. This pathway consists of reaction (1), catalysed by the mitochondrial aspartate aminotransferase (de Duve, Wattiaux & Baudhuin, 1962), and reaction (2), which represents part of the citric acid cycle:

Borst & Slater (1961), Jones & Gutfreund (1961) and Borst (1962) have reported that, in intact liver mitochondria, glutamate is oxidized almost exclusively through this transaminase pathway. Papa, Saccone, Palmieri, Francavilla & Quagliariello (1963*a,b*), studying glutamate oxidation in rat-liver and -kidney mitochondria, found a significant ammonia production. This finding suggested that a dehydrogenase pathway, initiated by reaction (3), catalysed by glutamate dehydrogenase, and followed by reaction (2), may also operate during glutamate oxidation:

The experiments reported in the present paper show that both the dehydrogenase and trans-

aminase routes participate in the oxidation of glutamate by tightly coupled rat-liver mitochondria. The results obtained support the proposal (Borst, 1962; Papa *et al.* 1963*a,b,c*) that in liver mitochondria the oxidation of glutamate by glutamate dehydrogenase is controlled by the extent of reduction of the nicotinamide nucleotides. Evidence is also given that the activity of the transaminase pathway depends on the steady-state concentration of oxaloacetate.

EXPERIMENTAL

Materials. Malate dehydrogenase from pig heart, aspartate aminotransferase from pig heart and glutamate dehydrogenase from ox liver were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Hexokinase (type III) from yeast was from Sigma Chemical Co., St Louis, Mo., U.S.A. Cysteinesulphinic acid was from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

Preparation of mitochondria. Rat-liver mitochondria were isolated as described by Quagliariello, Papa, Saccone & Alifano (1964). Mitochondria from 1 g. of liver were suspended in 1 ml. of 0.25 M-sucrose. The efficiency of respiratory control and oxidative phosphorylation of mitochondria was verified on the basis of the values obtained for the ratio: rate of oxygen uptake in 'state 3'/rate of oxygen uptake in 'state 4' (polarographic measurements and procedure as described by Chance & Williams, 1956) and for the P/O ratio (manometric experiments). The values

were found to be in good agreement with those reported in the literature for tightly coupled mitochondria.

Incubation procedure. The mitochondria from 900 mg. of liver (13.5–16 mg. of protein) were incubated in 18 ml. Warburg flasks at 30°, in a final volume of 3 ml. of the standard reaction mixture containing: potassium phosphate buffer, pH 7.5 (20 mM), KCl (30 mM), MgCl₂ (5 mM), tris-HCl buffer, pH 7.4 (20 mM), and sucrose (60 mM) (derived from the mitochondrial suspension). The oxygen uptake was measured by the Warburg manometric technique. Where indicated, the respiration rate was measured polarographically with a vibrating platinum electrode. The reaction was stopped by adding to the incubation mixture 2 ml. of 0.05 M-sodium acetate buffer, pH 4.0. Ammonia and inorganic phosphate were determined in this suspension (A). For spectrophotometric determination of aspartate and α -oxoglutarate, suspension (A) was deproteinized by addition of 0.1 vol. of 35% (v/v) perchloric acid. After removal of protein, perchloric acid was removed as the potassium salt in the cold.

Chromatographic determination of amino acids. The amino acids were extracted from suspension (A) with 5% (w/v) trichloroacetic acid (final concn.). After removal of protein by centrifugation, the trichloroacetic acid was removed by extraction with diethyl ether, and 0.05 or 0.1 ml. samples of the extracts were spotted on Whatman no. 1 paper and chromatographed with phenol-water (4:1, w/v) by the one-dimensional descending technique. The chromatograms were developed for about 10 hr. at 30°, and dried at the same temperature. The amino acids were identified by dipping the paper through 0.2% ninhydrin solution in acetone in which 2 g. of pyridine was added/100 ml. immediately before use. The strips were heated at 100° for 5 min. and the spots cut out and eluted in stoppered tubes with 3 ml. of ninhydrin reagent (300 mg. of ninhydrin and 20 mg. of EDTA in 100 ml. of 95% ethanol plus 6.5 ml. of water). The tubes were heated at 50° for 45 min. and the amino acid concentration was calculated from the extinction at 578 m μ . By this method the recoveries of glutamate,

aspartate, alanine and glutamine, added to mitochondrial suspension at 0°, were 95–100%.

Spectrophotometric determination of aspartate. This was carried out by the method of Pfeleiderer (1963) on the perchloric acid extracts.

Spectrophotometric determination of α -oxoglutarate. This was carried out by the method of Bergmeyer & Bernt (1963).

Microdetermination of ammonia. Ammonia was estimated according to the microdiffusion method of Conway as reported by Salvatore, Zappia & Palmieri (1962); 0.5 μ g. of ammonia could be detected by this method.

Measurement of nicotinamide nucleotide reduction. Nicotinamide nucleotide reduction was directly monitored in the mitochondrial suspension by using the double-beam spectrophotometer (Aminco-Chance, Silver Spring, Md., U.S.A.) at a wavelength pair 340 and 374 m μ (Chance & Williams, 1956).

RESULTS

Effect of inhibitors and of incubation time on the oxidation of glutamate. The values presented in line 1 of Table 1 show that, during the oxidation of glutamate (ADP and phosphate acceptor present), the amino group of the glutamate oxidized was quantitatively converted into ammonia and into the amino group of aspartate. Aspartate accounted for 75% of the glutamate utilized, and ammonia for 24%. These values represent the respective contributions of the dehydrogenase and transaminase routes for glutamate oxidation during 12–14 min. of incubation. When arsenite, which inhibits reaction (2) at the level of oxoglutarate dehydrogenase, was added (line 2), the rate of oxygen consumption was diminished by 87%, whereas the rate of ammonia production was

Table 1. *Effect of inhibitors on the oxidation of glutamate by rat-liver mitochondria*

The reaction mixture contained the basic components with the following additions: glucose (30 mM), ADP (0.3 mM), glutamate (10 mM) and hexokinase (150 units, as defined by McDonald, 1955). The hexokinase plus substrate solution was tipped into the main compartment from the side arm after 5 min. of equilibration. The final volume was 3 ml. The reaction time was 12–14 min. The ammonia production, aspartate formation and glutamate disappearance were calculated as the differences between the amounts found after the incubation and that found in the corresponding controls stopped immediately after mixing. The mean values of endogenous ammonia, aspartate and glutamate found in the incubation mixture were respectively 0.041, 0.045 and 0.076 μ -mole/mg. of protein. On incubating mitochondria for 13 min. without the addition of substrate, glutamate disappeared at a rate of 3 μ moles/mg. of protein/min., and no significant variations of aspartate and ammonia were found. The values given in the Table are the means $\pm$ S.E.M. of ten experiments, with the ranges in parentheses.

—, Not determined.

Additions	O ₂ uptake (μ g. atoms/min./mg. of protein)	Glutamate disappearance (μ moles/min./mg. of protein)	NH ₃ production (μ moles/min./mg. of protein)	Aspartate formation (μ moles/min./mg. of protein)
None	91.9 $\pm$ 4.6 (82.0–103.0)	30.3 $\pm$ 1.8 (24.0–39.3)	7.2 $\pm$ 0.3 (6.5–8.9)	22.7 $\pm$ 1.2 (18.6–25.9)
Arsenite (1 mM)	11.9 $\pm$ 0.8 (8.9–14.5)	11.7 $\pm$ 0.8 (8.9–13.8)	10.7 $\pm$ 0.6 (9.1–12.4)	—
Arsenite (1 mM) + cysteinesulphinate (10 mM)	30.3 $\pm$ 4.1 (22.4–45.8)	5.6 $\pm$ 0.4 (4.1–7.0)	15.1 $\pm$ 1.3 (9.8–17.8)	—
Malonate (10 mM)	41.1 $\pm$ 2.2 (35.0–50.1)	22.8 $\pm$ 1.8 (17.9–26.4)	21.1 $\pm$ 1.3 (17.9–25.4)	—

increased by 48%. The oxygen uptake appeared to be accounted for almost exclusively by reaction (3). Indeed, in the presence of this inhibitor, the oxygen uptake, ammonia production and glutamate disappearance were approximately equal. Moreover, α -oxoglutarate accumulated in amounts approximately equal to the ammonia formed. When cysteinesulphinatase, which in liver mitochondria undergoes transamination with oxoglutarate (reaction 4):

(Singer & Kearney, 1956), was added together with arsenite, the rate of ammonia production was further enhanced, whereas apparently less glutamate was oxidized. This last result may be explained by assuming that oxoglutarate, formed by reaction (3), transaminates, in the presence of arsenite, with cysteinesulphinatase, re-forming glutamate (reaction 4). The increase of the rate of oxygen uptake, compared with the experiments in the presence of arsenite alone, may be due to the further oxidation of sulphite derived from the decomposition of β -sulphinyldipyrivate (see Singer & Kearney, 1956). The addition of cysteinesulphinatase, in the absence of arsenite, had no appreciable effect on glutamate oxidation. On the addition of malonate (line 4), which inhibits succinate oxidation, the rate of oxygen uptake was diminished by 55%, whereas ammonia production was increased by 193%. The oxygen uptake/glutamate disappearance and oxygen uptake/ammonia production ratios were approximately equal to 2.

Borst & Slater (1961) and Borst (1962) have reported that, during the oxidation of glutamate by rat-liver mitochondria, glutamate was nearly

quantitatively converted into aspartate. In contrast with our experimental conditions (12–14 min. of incubation) the reaction time used by these authors was about 30 min. To study the influence of reaction time on the oxidation of glutamate, the ammonia formation and oxygen uptake were determined at different incubation times between 1 and 30 min. (Fig. 1). The rate of ammonia production gives the time-course of the rate of reaction (3). It shows a marked decrease in the

Fig. 1. Time-course of the rate of ammonia production and oxygen consumption during the oxidation of glutamate by rat-liver mitochondria. The reaction mixture contained the basic components with the following additions: glucose (30 mM), ADP (0.3 mM), glutamate (15 mM) and hexokinase (150 units). The rate of ammonia production (●) is expressed as μ moles of NH_3 formed/min./mg. of protein; the rate of oxygen uptake (○) as μ g. atoms consumed/min./mg. of protein. The rate of oxygen uptake for the first minutes (1–4) was measured polarographically in a system equivalent to that reported for manometric determinations except that the final volume was 1.5 ml. The points of the curves are mean values of five to ten measurements. The other details are those given in the text and in the legend to Table 1.

Table 2. *Effect of respiratory substrates on the oxidation of glutamate by rat-liver mitochondria*

The results refer to 3 ml. of mitochondrial suspension. The reaction mixture contained the basic components with the following additions: glucose (30 mM), ADP (0.3 mM), glutamate (15 mM) and hexokinase (150 units). The other details are those given in the legend to Table 1. The incubation time was 13 min. Expt. 1, 14.95 mg. of protein present; Expt. 2, 16.30 mg. of protein present.

Expt. no.	Addition	O_2 uptake (μ g. atoms)	NH_3 production (μ moles)	Aspartate formation (μ moles)
1	Without arsenite:			
	None	16.50	+1.55	4.00
	Succinate (15 mM)	18.96	−0.11	3.10
	Malate (15 mM)	17.32	−0.13	9.50
	Pyruvate (15 mM)	16.93	−0.25	3.36
	Oxaloacetate (15 mM)	17.47	−0.20	16.40
2	With arsenite (1 mM):			
	None	2.48	+2.13	—
	Succinate (15 mM)	18.70	+0.46	1.90
	Malate (15 mM)	8.50	+0.73	8.29
	Oxaloacetate (15 mM)	2.19	+2.68	16.93

first 4–5 min., followed by a further slow decrease. In contrast, the rate of oxygen consumption remained constant.

Effect of respiratory substrates on the oxidation of glutamate. In Table 2 the effects of succinate, malate, oxaloacetate and pyruvate on the oxidation of glutamate are reported. The presence of these respiratory substrates (Expt. 1) resulted in suppression of the ammonia production. The addition of succinate and pyruvate resulted in lower aspartate formation, whereas malate and oxaloacetate considerably increased it. In the presence of arsenite (Expt. 2), succinate and malate no longer completely suppressed ammonia production, and oxaloacetate increased production by 26%. In the presence of glutamate and succinate the addition of arsenite resulted in a further inhibition of aspartate formation: oxygen uptake was unaffected. In the presence of glutamate and malate, the g.atoms of oxygen consumed were approximately equal to the moles of aspartate formed. The oxygen uptake

with glutamate and oxaloacetate was markedly inhibited, but aspartate formation remained unaffected. In other experiments, the effects of increasing concentrations of succinate and oxaloacetate were studied (Fig. 2). Mitochondria were incubated in the presence of 15 mM-glutamate and various quantities of succinate or of oxaloacetate. On increasing the concentration of succinate up to 7 mM, ammonia production was progressively inhibited until it reached zero, whereas aspartate formation remained unaffected; at concentrations above 7 mM-succinate, aspartate formation was slightly inhibited. On increasing the concentration of oxaloacetate, ammonia formation was progressively inhibited (complete suppression was found at 4–5 mM-oxaloacetate), whereas aspartate formation increased linearly.

Oxidation of glutamate in the presence of 2,4-dinitrophenol. Increasing the concentration of dinitrophenol between 0.05 and 0.2 mM markedly stimulated ammonia production, which, after an optimum at 0.2 mM, became progressively inhibited at higher dinitrophenol concentrations (Fig. 3). Oxygen uptake was slightly stimulated at lower dinitrophenol concentrations, and was then inhibited at concentrations above 0.2 mM. Aspartate formation was progressively inhibited as the dinitrophenol concentration was increased from 0.05 to 0.8 mM. On repeating an experiment analogous to that illustrated in Fig. 1 with 0.2 mM-dinitrophenol, the rate of ammonia production was found to be higher than in the presence of phosphate acceptors and decreased more slowly and linearly with the time of incubation (Fig. 4).

Effect of exogenous nicotinamide nucleotides on glutamate-dehydrogenase activity. The addition of

Fig. 2. Effect of succinate or oxaloacetate concentration on ammonia and aspartate formation during the oxidation of glutamate by rat-liver mitochondria. The points of the curves refer to 3 ml. of mitochondrial suspension (15.45 mg. of protein). The reaction mixture contained the basic components with the following additions: glucose (30 mM), ADP (0.3 mM), glutamate (15 mM) and hexokinase (150 units). The other details are those given in the text and in the legend to Table 1. The incubation time was 13 min. ○, Ammonia in the presence of oxaloacetate; □, aspartate in the presence of oxaloacetate; ●, ammonia in the presence of succinate; ■, aspartate in the presence of succinate.

Fig. 3. Rate of ammonia production (●), aspartate formation (■) and oxygen uptake (○) during the oxidation of glutamate by rat-liver mitochondria in the presence of various concentrations of 2,4-dinitrophenol. The reaction mixture contained the basic components with the addition of glutamate (15 mM). The other details are those given in the text and in the legends to Table 1 and Fig. 1. The points of the curves are mean values of five measurements. The incubation time was 13 min.

NAD⁺ resulted in a marked stimulation of glutamate deamination in tightly coupled rat-liver mitochondria, but it had no effect on the oxygen uptake supported by glutamate and other NAD-linked respiratory substrates (α -oxoglutarate, β -

hydroxybutyrate). The stimulatory effect was dependent on the concentration of added NAD⁺. NADP⁺ had no significant effect either on oxygen uptake or on ammonia production (Table 3).

Reduction of nicotinamide nucleotides with glutamate. On the addition of glutamate and phosphate acceptor to mitochondria preincubated for 5 min., a rapid reduction of nicotinamide nucleotides occurred, followed by a progressive one until anaerobiosis was reached. The extent of nicotinamide nucleotide reduction was greatly enhanced by the addition of an excess of succinate (Fig. 5*b* compared with Fig. 5*a*) or of an excess of malate (Fig. 5*c* compared with Fig. 5*a*) to liver mitochondria oxidizing glutamate.

DISCUSSION

The results presented show that, in liver mitochondria, glutamate is oxidized by the two pathways described in the introduction. However, the rate of glutamate deamination is very slow in spite of the high concentration of glutamate dehydrogenase present (Jones & Gutfreund, 1961; Chappell, 1964). The sharp decline of the rate of reaction (3) in the first 4–5 min., followed by a slower progressive decrease, could at first sight be ascribed to an accumulation of the reaction products. However, it cannot be due to α -oxoglutarate, since direct analysis showed that this compound did not accumulate (see also Borst, 1962). Further, the small amounts of ammonia produced can only partly account for this decrease in deamination. In fact the higher rate of ammonia production found in the presence of 0.2 mM-dinitrophenol (Fig. 4) does not show the initial rapid decrease observed with phosphate acceptor. The inhibition of glutamate deamination may be due to the reduced nicotinamide nucleotides. This is directly supported by the time-course of nicotinamide nucleotide reduction in the presence of glutamate and phosphate acceptor (Fig. 5). It is conceivable that the continued slow reduction of nicotinamide nucleotides, observed in state 3 in the presence of glutamate, and the consequent sharp decline of

Fig. 4. Time-course of the rate of ammonia production during the oxidation of glutamate by rat-liver mitochondria in the presence of phosphate acceptors (●) or 2,4-dinitrophenol (▲). The reaction mixture contained the basic components with the following additions: glutamate (15 mM), glucose (30 mM), ADP (0.3 mM) and hexokinase (150 units) as phosphate acceptors, or 2,4-dinitrophenol (0.2 mM). The points of the graphs are mean values of five to ten measurements. The other details are those given in the text and in the legends to Table 1 and Fig. 1.

Table 3. *Effect of nicotinamide nucleotides on the oxidation of glutamate by rat-liver mitochondria*

The results refer to 3 ml. of mitochondrial suspension (11.33 mg. of protein). The reaction mixture contained the basic components with the following additions: glucose (30 mM), ADP (0.3 mM), glutamate (10 mM) and hexokinase (150 units). The other details are those given in the legend to Table 1. The incubation time was 15 min.

Addition	O ₂ uptake (μ g. atoms)	NH ₃ production (μ moles)	Aspartate formation (μ moles)
None	13.53	1.20	3.43
NAD (1 mM)	13.30	2.09	3.28
NAD (5 mM)	13.00	3.15	3.30
NADP (1 mM)	13.39	1.35	3.50

Fig. 5. Kinetics of the reduction of nicotinamide nucleotides during the oxidation of glutamate by rat-liver mitochondria. Mitochondrial suspension (0.15 ml.) was added to 2.85 ml. of reaction mixture. All the basic components were at the final concentrations given in the Experimental section. (a) After 5 min. of preincubation, 30 μ moles of glutamate, 0.9 μ mole of ADP and 150 units of hexokinase were added at point A. (b) As for (a) plus 30 μ moles of succinate at point A. (c) As for (a) plus 30 μ moles of malate at point A. Upward deflexion of the trace indicates increased extinction at 340 m μ measured with respect to that at 374 m μ . At point B anaerobiosis was reached.

glutamate deamination is due to the functioning of the transaminase pathway. Indeed, the addition of inhibitors of the transaminase pathway, such as arsenite or malonate, or of the transamination to aspartate, such as dinitrophenol (Tager & de Haan, 1964), resulted in a stimulation of ammonia production. On the other hand, ammonia production was

completely inhibited when the citric acid-cycle dehydrogenases, involved in the transaminase pathway, were maximally stimulated by the addition of the corresponding substrates (Expt. 1 in Table 2). Spectrophotometric monitoring shows that there is an enhancement of extent of reduction of nicotinamide nucleotides when succinate or malate was added together with glutamate (Fig. 5b and Fig. 5c). The enhancement of the reduction of nicotinamide nucleotides on the addition of higher concentrations of succinate (Chance & Hollunger, 1957; Wu & Tsou, 1955) inhibits not only ammonia production but also aspartate formation at the level of malate dehydrogenase. Malate, in the presence of glutamate, which removes oxaloacetate, is actively oxidized; the enhancement of the reduction of nicotinamide nucleotides is due to an insufficiently rapid rate of electron transport by the phosphorylating respiratory chain. The inhibitory effect of oxaloacetate (Table 2 and Fig. 2) appears to be connected with the oxidation of α -oxoglutarate and of its products; in fact, arsenite eliminates this effect. Finally, after the addition of pyruvate, the condensing enzyme competes with aspartate aminotransferase for oxaloacetate, thus inhibiting aspartate formation. The increased activity of the citric acid cycle inhibits, in turn, reaction (3). The reported results provide substantial evidence that the oxidation of glutamate by glutamate dehydrogenase is controlled by the extent of reduction of nicotinamide nucleotides. A similar proposal has been suggested by Borst (1962) and Papa *et al.* (1963a,b,c). It is known that glutamate dehydrogenase reacts with both NAD and NADP. Klingenberg & Slenczka (1959) have suggested that in liver mitochondria glutamate dehydrogenase reacts preferentially with NADP. It is conceivable that, in liver mitochondria, the oxidation of glutamate is initiated by reaction (3). As incubation proceeds, reactions (2) and (1) assume more importance with a consequent higher and widely distributed reduction of NAD, followed by the reduction of NADP through the energy-dependent transhydrogenase reaction (Danielson & Ernster, 1963). Indeed, Klingenberg & Slenczka (1959) and Estabrook, Gonze & Nissley (1963) have reported a high NADPH/NADP⁺ ratio in 'state 3'. The enhancement of the NADH/NAD⁺ ratio, and more especially of the NADPH/NADP⁺ ratio, could inhibit glutamate deamination either by affecting the equilibrium of reaction (3) or by an allosteric effect (Frieden, 1963; Yielding, Tomkins & Trundle, 1964) on glutamate dehydrogenase. Added NAD⁺ can possibly also affect glutamate deamination by these mechanisms. However, this explanation would require the assumption that external NAD⁺ may either freely reach mitochondrial glutamate dehydrogenase, or accept re-

ducing equivalents from nicotinamide nucleotides in the glutamate dehydrogenase compartment via transhydrogenase.

For the transaminase route, the results reported in Table 2 (Expt. 1) suggest that, owing to the high activity of aspartate aminotransferase in liver mitochondria (Table 2 and Fig. 2), the rate-limiting step is the formation of oxaloacetate by reaction (2). Chappell (1964) has suggested that in liver mitochondria the slowest step of reaction (2) is the conversion of α -oxoglutarate into succinate. Considering the relatively high endogenous concentration of glutamate compared with oxaloacetate in liver mitochondria (Bellamy, 1962), it may be suggested that aspartate formation in liver mitochondria is controlled by the steady-state concentration of oxaloacetate.

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