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The Pathway of Glutamate Metabolism in Rat Brain Mitochondria

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1. The pathway of glutamate metabolism in non-synaptic rat brain mitochondria was investigated by measuring glutamate, aspartate and ammonia concentrations and oxygen uptakes in mitochondria metabolizing glutamate or glutamine under various conditions. 2. Brain mitochondria metabolizing 10mM-glutamate in the absence of malate produce aspartate at 15nmol/min per mg of protein, but no detectable ammonia. If amino-oxyacetate is added, the aspartate production is decreased by 80% and ammonia production is now observed at a rate of 6.3nmol/min per mg of protein. 3. Brain mitochondria metabolizing glutamate at various concentrations (0–10mM) in the presence of 2.5mM-malate produce aspartate at rates that are almost stoicheiometric with glutamate disappearance, with no detectable ammonia production. In the presence of amino-oxyacetate, although the rate of aspartate production is decreased by 75%, ammonia production is only just detectable (0.3nmol/min per mg of protein). 4. Brain mitochondria metabolizing 10mM-glutamine and 2.5mM-malate in States 3 and 4 were studied by using glutamine as a source of intramitochondrial glutamate without the involvement of mitochondrial translocases. The ammonia production due to the oxidative deamination of glutamate produced from the glutamine was estimated as 1nmol/min per mg of protein in State 3 and 3nmol/min per mg of protein in State 4. 5. Brain mitochondria metabolizing 10mM-glutamine in the presence of 1mM-amino-oxyacetate under State-3 conditions in the presence or absence of 2.5mM-malate showed no detectable aspartate production. In both cases, however, over the first 5min, ammonia production from the oxidative deamination of glutamate was 21–27nmol/min per mg of protein, but then decreased to approx. 1–1.5nmol/min per mg. 6. It is concluded that the oxidative deamination of glutamate by glutamate dehydrogenase is not a major route of metabolism of glutamate from either exogenous or endogenous (glutamine) sources in rat brain mitochondria.

In mammalian mitochondria there are two routes by which glutamate may be metabolized, (*a*) oxidative deamination via glutamate dehydrogenase (EC 1.4.1.3) or (*b*) transamination, via glutamate–oxaloacetate transaminase (EC 2.6.1.1). In muscle (Borst, 1962) and heart (Klingenberg & Pette, 1962), where glutamate dehydrogenase activities are very low, glutamate metabolism proceeds essentially via the transaminase. However, in liver, as in brain, where both the transaminase and glutamate dehydrogenase are very active (Balázs, 1965; Lai *et al.*, 1975), the relative contributions of the two routes have been the subject of some controversy (De Haan *et al.*, 1967). Although it is now well established that in liver the primary route of oxidation of exogenous glutamate is via transamination (Papa *et al.*, 1966, 1967; De Haan *et al.*, 1967; McGivan *et al.*, 1974), the precise role of glutamate dehydrogenase in glutamate metabolism remains speculative (McGivan & Chappell, 1975). A similar situation to that in liver has been reported for brain, where some 70–90% of exogenously added glutamate is metabolized to aspartate by brain homogenates (Haslam & Krebs, 1963) or mitochondria (Balázs, 1965; Dennis *et al.*, 1976). However, it has been reported that, during the incubation of brain slices in the absence of glucose, ammonia is formed, due in considerable measure to the metabolism of endogenous glutamate by glutamate dehydrogenase (Weil-Malherbe, 1974; Benjamin & Quastel, 1974).

Glutamine may be used as a convenient source of intramitochondrial glutamate via the activity of the intramitochondrial glutaminases (Hird & Marginson, 1968; Kovacevic, 1971; Blackburn & Hird, 1972; Crompton & Chappell, 1973). Investigations using glutamine in this role with kidney mitochondria have suggested that glutamate arising from glutamine intramitochondrially may be metabolized via oxidative deamination.

The present paper reports experiments with rat brain mitochondria using glutamine as an intramitochondrial source of glutamate, and aimed at elucidating the proportion of glutamate metabolized via transamination or oxidative deamination. Further, the influence of the presence of malate, a source of oxaloacetate for transamination, has also been

investigated in certain cases. The data suggest that glutamate, whether supplied exogenously as such to the mitochondria or endogenously as glutamine, is metabolized mainly by transamination and that only a small proportion is metabolized by oxidative deamination.

Materials and Methods

Animals

Male adult rats (150–180g) of the Wistar strain were used throughout.

Chemicals

Glutamate dehydrogenase (EC 1.4.1.3), glutamate–oxaloacetate transaminase (EC 2.6.1.1), malate dehydrogenase (EC 1.1.1.37) and hexokinase (EC 2.7.1.1) were from Boehringer Corp. (London) Ltd., London W.5, U.K. Glutamine, purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., was purified from contaminating glutamic acid (0.4%) before use. A 200mM-glutamine solution was passed through a 10ml column containing Dowex 1 (X8; acetate form) anion-exchange resin, as described by Berl *et al.* (1962). Glutamine, assayed by the method of Crompton & Chappell (1973), was less than 0.04% contaminated with glutamic acid after this procedure.

All other chemicals were of the purest grade commercially available and made up in double-glass-distilled water. Substrates for mitochondrial experiments were adjusted to pH6.5–6.8 with 2M-Tris.

Preparation of mitochondria

Rat brain mitochondria of non-synaptic origin were prepared by the method of Clark & Nicklas (1970). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Mitochondrial incubations

The basic incubation medium contained 100mM-KCl, 75mM-mannitol, 25mM-sucrose, 5mM-phosphate/Tris, pH7.4, 20mM-Tris/HCl, pH7.4, and 50μM-EDTA adjusted to pH7.4. O_2-uptake measurements were performed polarographically with a Clark-type oxygen electrode as described previously (Clark & Land, 1974). Incubations for subsequent assay of metabolites were carried out with shaking at 25°C with O_2 blown over the surface to ensure adequate aeration. State 3 (Chance & Williams, 1956) was induced by the presence of a hexokinase trap (20mM-glucose, 5mM-$MgCl_2$, 1mM-ADP and 1 unit of hexokinase/mg of mitochondrial protein). Samples for metabolic assays were removed at timed intervals and added to $HClO_4$ to give a final concentration of 0.2–0.4M. Neutralization was effected by titration with 3M-K_2CO_3 in 0.5M-triethanolamine and the precipitated perchlorate and protein were removed by centrifugation at 15000*g* for 3min. Samples for ammonia* analysis were neutralized with 3M-K_2CO_3 without triethanolamine, which interfered with the ammonia-electrode response.

Metabolite assays

Glutamate and aspartate were determined in the neutralized $HClO_4$ incubation extracts by fluorimetric or spectrophotometric enzyme assays as described by Williamson & Corkey (1969). When amino-oxyacetate was present in the extracts, 5mM-acetaldehyde was included in the aspartate assay system (see Meijer & Van Dam, 1974).

Ammonia production was determined by two methods. Spectrophotometrically detectable NH_4^+ was determined in a 1 ml assay system of the following composition: 50mM-triethanolamine/HCl, pH7.0, 5mM-2-oxoglutarate, 1.5mM-NADH, extract, and 24 units of glutamate dehydrogenase in glycerol. For measurements of lower NH_4^+ concentrations (<50μM), it was necessary to use a method involving an automated continuous-flow system coupled to an ammonia-selective electrode as described by Park & Fenton (1973).

Results

Exogenous glutamate oxidation

Fig. 1 and Table 1 present the results of experiments designed to assess the proportion of glutamate metabolized by the transamination or oxidative deamination routes by rat brain mitochondria. The almost stoicheiometric accumulation of aspartate associated with glutamate disappearance (Fig. 1) indicates that, for a wide range of glutamate concentrations, oxidative deamination of added glutamate is very limited (cf. Hird & Marginson, 1964). This is further substantiated by the data of Table 1, in which glutamate dehydrogenase activity has been assessed by measuring NH_4^+ production. From these results it appears that unless the transamination route of glutamate metabolism is suppressed by adding amino-oxyacetate (Hopper & Segal, 1964), the oxidative deamination of glutamate did not proceed at a measurable rate. Further, even when amino-oxyacetate was present, the inclusion of malate in the incubation mixture had a marked effect on the rate at which ammonia was produced from glutamate

* Either as NH_4^+ or as NH_3, since the ammonia electrode utilizes the $NH_4^+ \rightleftharpoons NH_3 + H^+$ equilibrium.

Table 1. *Ammonia production from rat brain mitochondria oxidizing glutamate*

All incubations were performed with rat brain mitochondria (approx. 1 mg of protein/ml) under State-3 conditions (Chance & Williams, 1956) in the 100 mM-K^+ medium at 25°C. Flux rates were linear with time, and changes in the rates of metabolite production or oxygen consumption were coincident with the addition of amino-oxyacetate. O_2-consumption rates were determined polarographically in parallel incubations. The data are expressed as means ± S.D. for at least three experiments. Rates of ammonia production below 0.2 nmol/min per mg of protein are indicated as 'N.D.' (not detectable).

Conditions	Ammonia production (mol/min per mg of mitochondrial protein)	Aspartate production (mol/min per mg of mitochondrial protein)	O_2 uptake (ng-atoms/min per mg of mitochondrial protein)
10 mM-Glutamate	N.D.	15±1	79±6
+2.5 mM-amino-oxyacetate	6.3±2.0	3.3±2.0	26±3
10 mM-Glutamate			
+2.5 mM-malate	N.D.	57±3	164±2
+2.5 mM-amino-oxyacetate	0.3±0.1	15±2	67±3

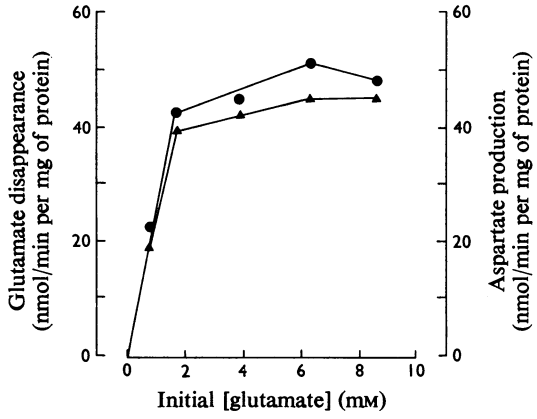

Fig. 1. *Effect of glutamate concentration on glutamate metabolism by rat brain mitochondria*

Rat brain mitochondria were incubated with 2.5 mM-malate and the indicated initial concentrations of glutamate in State 3 (Chance & Williams, 1956) in the 100 mM-K^+ medium (see the Materials and Methods section). Samples (0.5 ml) were removed at 0, 10 and 20 min, and glutamate and aspartate determined as indicated in the Materials and Methods section. Linear progress curves were observed. Mitochondrial protein concentrations were varied from 0.65 to 5 mg of protein/ml to obtain a measurable decrease in glutamate concentration. From these results the rates of glutamate disappearance (●) and aspartate production (▲) were calculated and plotted as a function of the initial glutamate concentration.

(6.3 nmol/min per mg of protein in the absence of malate; 0.3 nmol/min per mg in its presence). If the mitochondria were preincubated with 2.5 mM-amino-oxyacetate rather than being pulsed with it as in the data of Table 1, the aspartate production became undetectable and ammonia production was increased to 2.3 nmol/min per mg of protein. A further increase in ammonia production to 3.3 nmol/min per mg was obtained by preincubating the mitochondria in the presence of both 2.5 mM-amino-oxyacetate and uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 0.35 nmol/mg of protein) or vitamin K-3 (23 nmol/mg of protein) (S. C. Dennis & J. B. Clark, unpublished work).

Endogenous glutamate oxidation

Glutamate arising inside the mitochondria from the hydrolysis of glutamine by the glutaminases can either efflux via the glutamate–OH^- translocator and then re-enter on the glutamate–aspartate translocator to be metabolized, via transamination, to aspartate (see Dennis *et al.*, 1976) or it can be oxidatively deaminated directly by glutamate dehydrogenase. If glutamate dehydrogenase has any involvement in the oxidation of glutamate produced endogenously from glutamine, then a measure of this flux can be obtained by subtracting from the ammonia production the glutamate and aspartate formed. The remaining ammonia then represents the combined product of both glutamate dehydrogenase activity and glutaminase activity, unaccounted for by aspartate or glutamate accumulation. Therefore, assuming that no other systems are producing or fixing ammonia, one-half of this excess ammonia should be an estimate of the oxidative deamination of glutamate, i.e.

$$\text{Glutamate dehydrogenase flux} = \frac{\Delta NH_3 - (\Delta Glu + \Delta Asp)}{2}$$

where ΔNH_3 = rate of ammonia production, ΔGlu = rate of glutamate formed and ΔAsp = rate of aspartate formed.

With this approach, the data in Fig. 2 show that, without inhibition of transamination, glutamate dehydrogenase activity is low in brain mitochondria oxidizing glutamine and malate. In State 3, associated with a respiration of 60 ng-atoms of O/min per

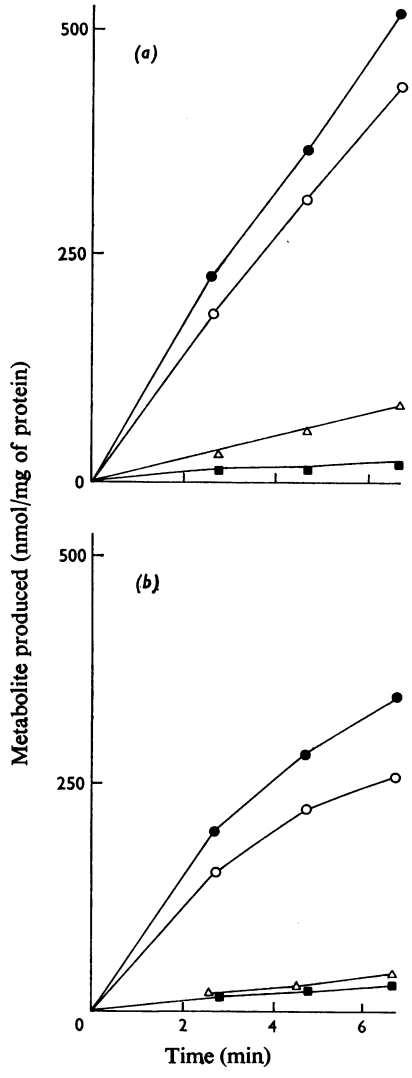

Fig. 2. *Glutamine and malate oxidation by rat brain mitochondria in States* 3 (*a*) *and* 4 (*b*)

Rat brain mitochondria (1.9mg of protein/ml) were incubated in State 3 or 4 (Chance & Williams, 1956) in 100mM-K^+ medium (see the Materials and Methods section) in the presence of 10mM-glutamine and 2.5mM-malate. Samples (0.4ml) were removed at 30s, 3, 5 and 7min for the determination of ammonia (●), glutamate (○) and aspartate (△) (see the Materials and Methods section). ■ Represents the flux through glutamate dehydrogenase and has been calculated from the expression $\frac{1}{2}[\Delta NH_3-(\Delta Glu+\Delta Asp)]$, where ΔNH_3 is the rate of total ammonia production (●), ΔGlu is the rate of glutamate production (○) and ΔAsp is the rate of aspartate production (△). O_2 consumption during the experiment (results not shown) was 60ng-atoms of O/min per mg of protein in State 3 and 21ng-atoms of O/min per mg of protein in State 4.

mg of protein, ammonia, glutamate and aspartate accumulated at mean rates of approx. 78, 66 and 10nmol/min per mg of protein respectively. Introduction of these flux values into the above equation provided an estimate of the rate of oxidative deamination of glutamate under these conditions of approx. 1nmol/min per mg of protein. In State 4 (Chance & Williams, 1956), when the rate of O_2 consumption supported by glutamine and malate was 21ng-atoms of O/min per mg of protein, the glutamate dehydrogenase flux was estimated in a similar way from equivalent mean rates of appearance of ammonia, glutamate and aspartate (36, 24 and 4nmol/min per mg of protein respectively) to be approx. 3nmol/min per mg of protein. This is somewhat faster than in State 3, and may be due to the lowered transamination rate consequent on the highly reduced state of the mitochondrial NAD.

The effect of the presence of amino-oxyacetate on State-3 glutamine metabolism by mitochondria in the presence and absence of malate is shown in Fig. 3. Under these conditions, the transamination of glutamate to aspartate was undetectable. However, for both incubations, calculations (see the legend to Fig. 3) revealed an initial rapid rate of oxidative deamination of glutamate over the first 4–6min. This initial rate of glutamate dehydrogenase activity was of the order of 21–27nmol/min per mg of protein and was not dependent on the presence of malate. After this initial period, ammonia production from oxidative deamination markedly diminished to a rate of the order of 1–1.5nmol/min per mg of protein. However, after this initial period (4–6min) the rates of total ammonia and glutamate production remained relatively constant, at 92 and 90nmol/min per mg of protein respectively in the presence of malate, and 90 and 87nmol/min per mg of protein in absence. Further, O_2 consumption in the presence of glutamine remained linear with time for the duration of these experiments (Fig. 3), at 28 and 21ng-atoms of O/min per mg of protein in the presence and absence of malate respectively. The presence of malate and the transamination of glutamate complicate any attempt to estimate flux calculations from the glutamine-supported O_2 uptake. However, in the absence of malate and transamination (Fig. 3), it is perhaps of significance that the rate of O_2 consumption (21ng-atoms of O/min per mg of protein) is of the same order as the initial burst of ammonia production from oxidative deamination of glutamate (21–27nmol/min per mg of protein). The 2-oxoglutarate that accumulates as a result of this might inhibit further oxidative deamination and, at the same time, act as a substrate for the tricarboxylic acid cycle, leading to the continued O_2 uptake. However, the observation that the rapid rate of oxidative deamination (Fig. 3) did not persist even when transamination was inhibited suggests that, in

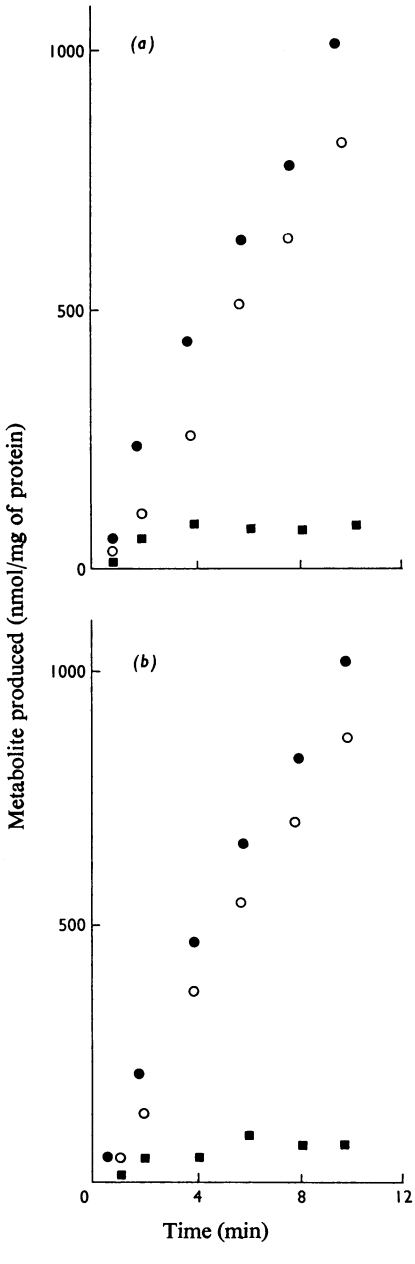

Fig. 3. *Effect of malate on glutamine metabolism by rat brain mitochondria in the presence of amino-oxyacetate*
Mitochondria (0.6mg of protein/ml) were incubated in the 100mM-K^+ medium (see the Materials and Methods section) in State 3 in the presence of 10mM-glutamine, 1mM-amino-oxyacetate and 2.5mM-malate (*b*) or no malate (*a*). Samples (0.5ml) were removed at 1, 2, 3, 4, 6, 8 and 10min, and ammonia and glutamate measured (see the Materials and Methods section). Aspartate appearance was not detectable. ■ Represents the flux through glutamate dehydrogenase = $\frac{1}{2}(\Delta NH_3 - \Delta Glu)$, where ΔNH_3 is the rate of ammonia production (●) and ΔGlu is the rate of glutamate production (○), and the rate of aspartate production was negligible. O_2 consumptions (results not shown) in these experiments were 28ng-atoms of O/min per mg of protein in the presence of malate, and 21ng-atoms of O/min per mg of protein in its absence.

brain mitochondria, sustained glutamate dehydrogenase activity is probably not a major metabolic route for even endogenous (glutamine-derived) glutamate.

Discussion

That the major route of glutamate metabolism in mammalian brain is transamination rather than oxidative deamination has been well documented (Balázs, 1965; Dennis *et al.*, 1976, 1977). The same is also true of the heart (Davis, 1968), but in this case the reason may be the very low activity of the enzyme glutamate dehydrogenase. However, in brain (Lai *et al.*, 1975; Dennis *et al.*, 1976, 1977), as in liver, a considerable activity of this enzyme is present. The question arises therefore in brain, as in liver (see Papa *et al.*, 1966, 1967; De Haan *et al.*, 1967; Hoek *et al.*, 1974), as to why the potential activity of the glutamate dehydrogenase is not more fully utilized in the metabolism of glutamate. Three main proposals to explain this have emanated from work mainly on liver mitochondria. Firstly the equilibrium of the glutamate dehydrogenase reaction has been demonstrated to be strongly in favour of reductive amination (Engel & Dalziel, 1967). The equilibrium constant, however, is not such as to preclude the reverse reaction (Krebs, 1973). Secondly it has been suggested that the liver glutamate dehydrogenase preferentially acts with NADP(H) (Papa *et al.*, 1966, 1967; De Haan *et al.*, 1967) with the energy-linked transhydrogenase acting as an intermediary between the NAD and NADP pools. However, evidence (Hoek *et al.*, 1974) has suggested that the liver glutamate dehydrogenase is in equilibrium with a NAD pool that is linked non-energetically with the NADP pool. The third proposal (McGivan & Chappell, 1975) suggests that the inward transport of glutamate available for glutamate dehydrogenase activity is rate-limiting. There appear to be two mechanisms of glutamate entry into liver and brain mitochondria, the glutamate–aspartate translocase (Azzi *et al.*, 1967; Brand & Chappell, 1974; Dennis *et al.*, 1976), which is relatively active, and the glutamate–OH^- translocase (Meijer & Van Dam, 1974; Dennis *et al.*, 1976), which is relatively slow. Glutamate entering on the glutamate–aspartate translocase is necessarily transaminated for the obligatory coupling of aspartate efflux with glutamate

entry. As a consequence, glutamate to be oxidatively deaminated must enter the mitochondrion via the glutamate–OH^- translocase.

The object of the experiments reported here was to test the third hypothesis by comparing glutamate metabolism with that of glutamine, by using the latter to generate intramitochondrial glutamate via the action of the intramitochondrial glutaminases (Katanuma *et al.*, 1967; Dennis *et al.*, 1977). Thus glutamine would provide intramitochondrial glutamate without involving the glutamate–OH^- translocase, or indeed any translocase, since in brain mitochondria all the evidence suggests that the glutamine simply diffuses in (Brand & Chappell, 1974; Dennis *et al.*, 1976). The data in the present paper (Figs. 2, 3 and Table 1) suggest that even when transamination is inhibited, the metabolism of glutamate derived from glutamine via oxidative deamination is still very limited (6.3 nmol/min per mg of protein), in both rate and extent (see Fig. 3). Although swelling experiments have indicated that glutamate entry is very slow via the glutamate–OH^- translocase (Minn *et al.*, 1975; Dennis *et al.*, 1976), time courses of [^{14}C]glutamate uptake by rat brain mitochondria under similar conditions to those in Fig. 3 (i.e. in the presence of amino-oxyacetate and a concentration of glutamate similar to that of glutamine) indicate a glutamate uptake of approx. 25 nmol/min per mg of protein, which is four times the maximum rate of oxidative deamination in the presence of glutamine (6.3 nmol/min per mg of protein; Table 1). These data, coupled with the low rates of oxidative deamination occurring with glutamate derived from glutamine inside the mitochondria, suggest that the constraint on the forward reaction of glutamate dehydrogenase in these experimental conditions may be due to some parameter(s) other than the glutamate–OH^- translocase.

A possible explanation may be the proposal by Balázs (1965), that a competition for available NAD^+ may exist between glutamate dehydrogenase and 2-oxoglutarate dehydrogenase, which favours 2-oxoglutarate oxidation, since the apparent K_m for NAD^+ of 2-oxoglutarate dehydrogenase is very low (4.5 μM; Sanadi, 1963) compared with that for glutamate dehydrogenase (0.9 mM; Dennis *et al.*, 1977). In fact in kidney mitochondria the inhibition by 2-oxoglutarate of oxidative deamination of glutamate was overcome by the addition of arsenite (Preuss & Weiss, 1971), which inhibits 2-oxoglutarate dehydrogenase activity. This suggests that it is the actual 2-oxoglutarate metabolism with its NAD turnover that is responsible for the inhibition of glutamate deamination rather than simple 2-oxoglutarate accumulation feeding back on the glutamate dehydrogenase. The data of Table 1, in which it is shown that glutamate dehydrogenase activity in the direction of oxidative deamination only occurs to a significant extent in the absence of malate and when transamination had been inhibited, i.e. when little 2-oxoglutarate is available for further metabolism by 2-oxoglutarate dehydrogenase, would support this hypothesis.

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