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Comparative Studies on Glutamate Metabolism in Synaptic and Non-Synaptic Rat Brain Mitochondria

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1. The apparent Michaelis constants of the glutamate dehydrogenase (EC 1.4.1.3), the glutamate–oxaloacetate transaminase (EC 2.6.1.1) and the glutaminase (EC 3.5.1.2) of rat brain mitochondria derived from non-synaptic (M) and synaptic (SM2) sources were studied. 2. The kinetics of oxygen uptake by both populations of mitochondria in the presence of a fixed concentration of malate and various concentrations of glutamate or glutamine were investigated. 3. In both mitochondrial populations, glutamate-supported respiration in the presence of 2.5mM-malate appears to be biphasic, one system (B) having an apparent K_m for glutamate of 0.25 ± 0.04mM ($n = 7$) and the other (A) of 1.64 ± 0.5mM ($n = 7$) [when corrected for low-K_m process, $K_m = 2.4 \pm 0.75$mM ($n = 7$)]. Aspartate production in these experiments followed kinetics of a single process with an apparent K_m for glutamate of 1.8–2mM, approximating to the high-K_m process. 4. Oxygen-uptake measurement with both mitochondrial populations in the presence of malate and various glutamate concentrations in which amino-oxyacetate was present showed kinetics approximating only to the low-K_m process (apparent K_m for glutamate ~0.2mM). Similar experiments in the presence of glutamate alone showed kinetics approximating only to the high-K_m process (apparent K_m for glutamate ~1–1.3mM). 5. Oxygen uptake supported by glutamine (0–3mM) and malate (2.5mM) by the free (M) mitochondrial population, however, showed single-phase kinetics with an apparent K_m for glutamine of 0.28mM. 6. Aspartate and 2-oxoglutarate accumulation was measured in 'free' non-synaptic (M) brain mitochondria oxidizing various concentrations of glutamate at a fixed malate concentration. Over a 30-fold increase in glutamate concentration, the flux through the glutamate–oxaloacetate transaminase increased 7–8-fold, whereas the flux through 2-oxoglutarate dehydrogenase increased about 2.5-fold. 7. The biphasic kinetics of glutamate-supported respiration by brain mitochondria in the presence of malate are interpreted as reflecting this change in the relative fluxes through transamination and 2-oxoglutarate metabolism.

Since the observations (Berl *et al.*, 1961) of the anomalous precursor–product relationship between glutamate and glutamine in the mammalian brain, a great deal of evidence to suggest that brain mitochondria may be heterogeneous has accumulated (Salganicoff & De Robertis, 1965; Van Kempen *et al.*, 1965; Balázs *et al.*, 1966; Neidle *et al.*, 1969; Blokhuis & Veldstra, 1970; Lai *et al.*, 1975; Reijnierse *et al.*, 1975). The proposal that this mitochondrial heterogeneity may partly explain the compartmentation of the tricarboxylate-cycle intermediates and related metabolites such as glutamate and 4-aminobutyrate has been comprehensively reviewed (Balázs & Cremer, 1973; Berl *et al.*, 1975).

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A method developed in our laboratory (Lai & Clark, 1976) for the subcellular fractionation of rat brain tissue has permitted the isolation of a mitochondrial population of synaptic origin, distinct from non-synaptic mitochondria prepared as described by Clark & Nicklas (1970). This method, besides adding further evidence for brain mitochondrial heterogeneity, offers the advantage that the derived mitochondria are relatively pure, functionally intact and metabolically active. Comparative studies (Lai & Clark, 1976) of these populations of mitochondria have demonstrated that there are considerable differences in the specific activities of certain tricarboxylate-cycle and related enzymes as well as in the metabolic capabilities of these organelles, particularly with regard to glutamate-supported respiration.

In view of the importance of glutamate and

glutamine in brain function and the well-documented compartmentation of their metabolism, a detailed study has been made of the ability of these two mitochondrial populations to metabolize both glutamate and glutamine, and this has been related to the specific activities of the enzymes involved in the metabolism of these substrates. The results are discussed with respect to the mitochondrial translocase systems which have been proposed to exist in the mammalian brain (Brand & Chappell, 1974; Dennis *et al.*, 1976).

Materials and Methods

Animals

Adult male rats (150–180g) of the Wistar strain, fed *ad libitum*, were used for all mitochondrial preparations.

Chemicals

Glutamic acid and amino-oxyacetate hemichloride were obtained from BDH Chemicals, Poole, Dorset, U.K. Glutamine and glutaminase (EC 3.5.1.2) were products of Sigma Chemical Co., St. Louis, MO, U.S.A. ADP, NAD(H), NADP(H), glutamate dehydrogenase (EC 1.4.1.3), glutamate–oxaloacetate transaminase (EC 2.6.1.1) and malate dehydrogenase (EC 1.1.1.37) were obtained from Boehringer, Lewes, Sussex, U.K. Hexokinase (EC 2.7.1.1) from the same supplier was dialysed against 3×50vol. of 50mM-potassium phosphate buffer, pH7.4, before use. Pyruvic acid, obtained from Koch–Light, Colnbrook, Bucks., U.K., was twice distilled under vacuum and stored at −20°C. All other chemicals, of the purest grade commercially available, were made up in twice-distilled water and when necessary neutralized with Tris base.

Preparation of rat brain mitochondria

Non-synaptic brain mitochondria (M fraction), distinct from the mitochondria derived from synaptosomes (Lai & Clark, 1976), were prepared essentially by the method of Clark & Nicklas (1970). Synaptic mitochondria (SM2 fraction) were prepared by the procedure of Lai & Clark (1976).

Enzyme assays

Enzyme activity determinations were carried out at 25°C in Unicam SP.800 or SP.1800 recording spectrophotometers. Incubations were performed in the presence of sufficient (0.16%) Triton X-100 to release all activity, and rates in the presence of excess substrate and cofactor concentrations were linearly proportional to the amount of mitochondrial protein.

NAD^+– and $NADP^+$–glutamate dehydrogenase were measured as described by Lai & Clark (1976).

Glutamate–oxaloacetate transaminase activity in the directions of either aspartate or glutamate formation was assayed by methods reported by Dennis *et al.* (1976).

Glutaminase activities were determined by a method modified from that of Kvamme *et al.* (1965). NH_3 production from phosphate-independent glutaminase was assayed in a reaction mixture which contained (final concns.): 15mM-Hepes,* pH8.8, 0.4mM-EDTA (potassium salt), 0.4mM-NADH, 15mM-2-oxoglutarate, 19mM-glutamine, 0.015% (v/v) Triton 100, 9 units of glutamate dehydrogenase in 50% (v/v) glycerol and approx. 0.2mg of mitochondrial protein. Phosphate-dependent glutaminase activity was determined in a similar assay system but at pH7.4 and supplemented with 19mM-potassium phosphate buffer, pH7.4.

Mitochondrial incubations

The standard incubation medium contained (final concns.): 100mM-KCl, 75mM-mannitol, 25mM-sucrose, 5mM-phosphate/Tris, pH7.4, 20mM-Tris/HCl, pH7.4, and 50μM-EDTA, adjusted to pH7.4.

Oxygen-uptake experiments were performed with a Clark-type oxygen electrode as described previously (Clark & Nicklas, 1970).

Mitochondrial incubations, from which samples were removed for metabolite determinations, were conducted at 25°C with agitation and aeration. State-3 conditions (Chance & Williams, 1956) were achieved by the presence of a hexokinase trap (20mM-glucose, 5mM-$MgCl_2$, 1mM-ADP and dialysed hexokinase at approx. 7 units/mg of mitochondrial protein). Samples were removed from the incubations and pipetted into 1.5M-$HClO_4$. Samples were adjusted to pH6.0–6.5 with 3M-K_2CO_3 in 0.5M-triethanolamine. Precipitated $KClO_4$ and protein were then removed by centrifugation at 15000*g* for 3min in a Quickfit micro-centrifuge.

Metabolite essays

Glutamate, aspartate and 2-oxoglutarate were determined by either fluorimetric or spectrophotometric assays as described by Williamson & Corkey (1969). Glutamine was assayed by the method of Crompton & Chappell (1973).

Purification of glutamine

Contaminating glutamic acid (~0.4%) was removed from a 200mM-glutamine solution by passage through a column (10ml) of Dowex-1 (X8; acetate form) as described by Berl *et al.* (1962). Glutamine used was <0.05% contaminated with glutamic acid.

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Protein determinations

Protein was determined by either the method of Lowry *et al.* (1951) or the biuret method of Gornall *et al.* (1949), with bovine plasma albumin as standard. Close agreement of mitochondrial-protein values determined by both methods was found (J. M. Walsh, personal communication).

Results

Table 1 outlines the rates of oxidation of glutamate+malate and glutamine+malate by synaptic and non-synaptic mitochondria under State-3 conditions. Additionally, the rates of production of aspartate and, for glutamine oxidation, of glutamate are included. Clearly, whereas the 'free' (i.e. non-synaptic) mitochondria have the ability to oxidize glutamate and malate at twice the rate of the synaptic mitochondria, there is relatively little difference between the rates of oxidation of glutamine and malate by the two mitochondrial populations. Comparison of these results with those in Table 2, which includes the Michaelis parameters of the enzymes involved in glutamate and glutamine metabolism, shows that the differences in the oxidation of glutamate are paralleled by the difference in the $V_{max.}$ of the glutamate–oxaloacetate transaminase in the two mitochondrial populations. Specific activities of both glutamate dehydrogenase and glutamate–oxaloacetate transaminase have been reported extensively in 'free' brain mitochondria (Balázs, 1965; Salganicoff & De Robertis, 1965; Balázs *et al.*, 1966; Patel & Tilghman, 1973). However, previous studies involving comparison of specific activities of mitochondrial enzymes from synaptic and non-synaptic sources have been hampered by the difficulty of assessing actual mitochondrial protein in the preparations. In the present study it is shown (Table 2), in agreement with the results of most workers (Salganicoff & De Robertis, 1965; Van Kempen *et al.*, 1965; Balázs *et al.*, 1966; Fonnum, 1968; Van den Berg, 1973), that the specific activity of the glutamate–oxaloacetate transaminase is significantly higher ($P<0.001$) in the non-synaptic mitochondrial preparation, whereas, contrary to previous reports, the glutamate dehydrogenase specific activity is significantly higher ($P<0.001$) in the synaptic mitochondria (see Lai *et al.*, 1975; Lai & Clark, 1976).

Consideration of the other kinetic parameters of the enzymes investigated (Table 2) shows that they do not differer markedly between the two brain mitochondrial preparations. Indeed, within experimental limitations, the values of the Michaelis parameters agree reasonably well with those from the enzymes from bovine liver (Fahien & Strmecki, 1969). Two exceptions are the K_m for 2-oxoglutarate of the NAD(P)–glutamate dehydrogenase, which is 4–6-fold higher for the brain enzyme than the value of 0.25mM for the bovine liver enzyme, and the K_i for aspartate of the glutamate–oxaloacetate transaminase, which was much higher (>5mM) (Henson & Cleland, 1964) for the liver enzyme than the brain enzyme. Effective control of the brain mitochondrial glutamate–oxaloacetate transaminase activity by aspartate might explain the lack of intramitochondrial aspartate accumulation in glutamate-loaded brain mitochondria (Dennis *et al.*, 1976) as compared with heart and liver mitochondria (La Noue *et al.*, 1974*a*,*b*). The K_m for glutamine of the brain glutaminases (Table 2) of about 1mM is of the same order as that for the pig kidney glutaminases (Crompton & Chappell, 1973), but considerably lower than those of the rat kidney (Katunuma *et al.*, 1967), which were 40 and 4mM respectively for the phosphate-dependent and -independent glutaminases. Also, Fig. 1 shows that the glutamate dehydrogenase from both brain mitochondrial populations is, in common with that of most

Table 1. *Comparison of glutamate and glutamine metabolism in synaptic and non-synaptic rat brain mitochondria*
Mitochondria were incubated in State-3 conditions in 100mM-K^+ medium in the presence of substrates at 25°C (see the Materials and Methods section). Oxygen uptake and metabolite production was assayed as outlined in the Materials and Methods section. Results are the means of at least two experiments ± S.D. Oxygen uptakes are expressed as ng-atoms/min per mg of protein and aspartate/glutamate production as nmol/min per mg of protein.

	Mitochondrial activities		
Substrates present	Non-synaptic	Synaptic	
10mM-Glutamate+2.5mM-malate	158 ± 11 ($n=6$)	78 ± 16 ($n=6$)	Oxygen uptake
	46 ± 10 ($n=6$)	29 ± 6 ($n=6$)	Aspartate production
10mM-Glutamine+2.5mM-malate	55.5 ± 2 ($n=3$)	43 ± 3 ($n=3$)	Oxygen uptake
	8.1:8.9	5.5:6.5	Aspartate production
	43.5:40.6	34.0:38.0	Glutamate production

Table 2. *Kinetic parameters of the glutamate dehydrogenase, glutamate–oxaloacetate transaminase and glutaminase of non-synaptic (M) and synaptic (SM2) rat brain mitochondria*

Enzyme activities at 25°C were measured as outlined in the Materials and Methods section. $V_{max.}$ values are measured in nmol/min per mg of mitochondrial protein and apparent K_m (or K_i) values in mM. These data were derived from double-reciprocal (Lineweaver–Burk) plots by inspection, and values in parentheses are the calculated K_i values for the observed competitive inhibition by the product with respect to the corresponding amino acid substrate.

		Non-synaptic mitochondria		Synaptic mitochondria	
Enzyme	Substrate	$V_{max.}$	K_m (K_i) (mM)	$V_{max.}$	K_m (K_i) (mM)
NADH–glutamate dehydrogenase	NADH	607	0.04	1016	0.04
	2-Oxoglutarate		1.0		1.1
	NH_4^+		18.2		21
NAD^+–glutamate dehydrogenase	NAD^+	53	0.9	100	1.1
	Glutamate	55	7.1		3.5
	2-Oxoglutarate		(0.2)		(0.25)
NADPH–glutamate dehydrogenase	NADPH	586	0.1	762	0.14
	2-Oxoglutarate		1.5		1.0
	NH_4^+		16.7		12.5
$NADP^+$–glutamate dehydrogenase	$NADP^+$		1.25		1.1
	Glutamate	36	3.7	90	2.2
	2-Oxoglutarate		(0.26)		(0.20)
Glutamate–oxaloacetate transaminase					
→Glutamate (forward reaction)	Aspartate	1752	0.67	1216	0.87
	Glutamate		(13.2)		(12.3)
	2-Oxoglutarate		1.45		1.1
→Aspartate (reverse reaction)	Glutamate	1870	22	1473	23
	Aspartate		(0.55)		(0.9)
	Oxaloacetate		0.05		0.05
Glutaminase					
Phosphate-independent	Glutamine	220	3.5	182	1.0
Phosphate-dependent	Glutamine	338	1.0	267	1.5

other tissues (Goldin & Frieden, 1971), activated by ADP. The relationship between ADP concentration and enzyme activation is sigmoidal, suggesting a co-operative interaction (Koshland, 1970). In both mitochondrial populations plots of the NAD–glutamate dehydrogenase activity against substrate concentration showed more marked sigmoidicity than those with the NADP–glutamate dehydrogenase.

Experiments on whole mitochondria

Fig. 2 shows the results of experiments in which brain mitochondrial respiration was measured in the presence of increasing concentrations of glutamate (up to 10mM) but a fixed concentration of malate (2.5mM) under State-3 conditions. The data are plotted as double-reciprocal plots, and with both the 'free' and synaptic mitochondrial populations it is apparent that two processes are involved. When the extrapolated Michaelis parameters are derived (see Table 3) one process (A) has a relatively high apparent K_m for glutamate, 1.3mM for the free mitochondria (M) and 1.8mM for the synaptic mitochondria (SM2), and the other process (B) has a low K_m for glutamate, 0.26 and 0.3mM for the 'free' and synaptic mitochondria respectively. However, if the same experiment is performed in the presence of amino-oxyacetate, an inhibitor which suppresses glutamate transamination (Hopper & Seegal, 1964). then the diminished oxygen uptakes when plotted as in Fig. 2 can be extrapolated to an apparent K_m value approaching that of the low-K_m process (B) [K_m for glutamate 0.19mM for free mitochondria (M) and 0.21mM for synaptic mitochondria (SM2); see Table 3]. This suggests that the high-K_m process of glutamate oxidation which is eliminated by amino-oxyacetate is associated with glutamate transamination. Further, when experiments are carried out under conditions similar to those in Fig. 2, but in the absence of amino-oxyacetate, and aspartate production is measured as a function of increasing glutamate concentrations (Fig. 3), no inflexion is seen in the double-reciprocal plots, and the extrapolated apparent K_m values for glutamate are consistent with those of the high-K_m process (A) as seen in the respiration experiments [apparent K_m for glutamate for free mitochondria

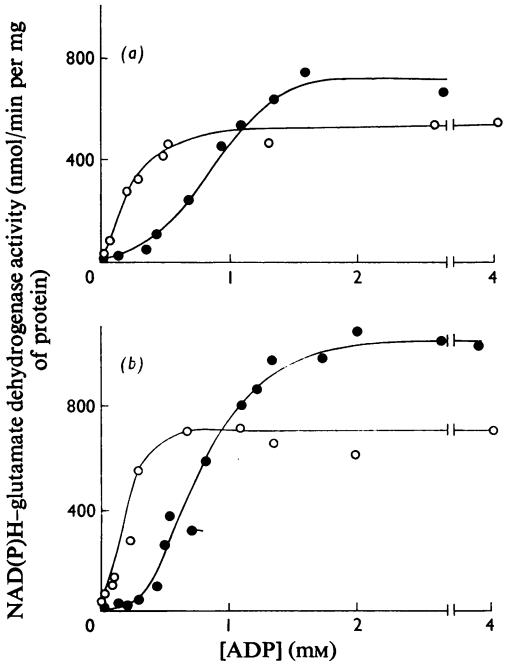

Fig. 1. *Effects of ADP on NAD(P)H-glutamate dehydrogenase activities in (a) free (M) and (b) synaptic (SM2) rat brain mitochondria*

Glutamate dehydrogenase activity (●, with NADH; ○, with NADPH), in nmol/min per mg of protein, at 25°C was measured as outlined in the Materials and Methods section.

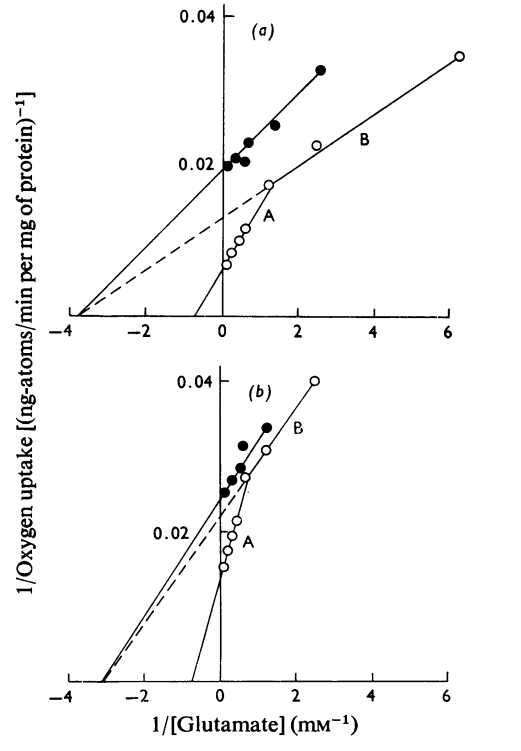

Fig. 2. *Glutamate+malate supported respiration in (a) free (M) and (b) synaptic (SM2) rat brain mitochondria in the presence and absence of amino-oxyacetate*

Oxygen uptakes were measured in 100mM-K^+ medium (see the Materials and Methods section) at 28°C by using free (*a*, 0.7mg of protein) or synaptic (*b*, 0.9mg of protein) brain mitochondria. In the final incubation (1ml), 2.5mM-malate and 2.5mM-ADP to maintain continuous State-3 conditions were present. Various concentrations of glutamate were added to initiate the reaction. Amino-oxyacetate (2.5mM), where present (●), was added during the course of State-3 respiration and the decreased rate of oxygen consumption, when linear, was measured. State-3 oxidation rates in the presence of malate (2.5mM) alone have been subtracted from the data; these values were 22 and 28ng-atoms of oxygen/min per mg of protein for free (M) and synaptic (SM2) mitochondria respectively. The data are plotted as double-reciprocal plots and the derived $V_{max.}$ and apparent K_m values are tabulated in Table 3. A and B refer to the high- and low-K_m processes described in the text.

(M) was 1.8mM and for synaptic mitochondria (SM2) 2mM in the experiments of Fig. 3; see Table 3]. Fig. 4 shows results of oxygen-uptake studies conducted under similar conditions to those in Figs. 2 and 3 but in either the presence or the absence of a fixed concentration of malate. Again, as in Fig. 2, in the presence of malate an inflexion is apparent in the double-reciprocal plot, with the apparent K_m values of the processes approximating to those in the high- and low-K_m processes (A, B) seen previously (see Fig. 2 and Table 3). However, in the absence of malate, when the glutamate concentration was varied, only the system with a high K_m for glutamate (A) was apparent (see Fig. 4 and Table 3). The data of Figs. 2, 3 and 4 and Table 3 suggest that the process which has a high apparent K_m for glutamate (A) is associated in some way with the transamination route of glutamate metabolism, since this process is no longer observable in the presence of amino-oxyacetate. However, experiments similar to those reported in Fig. 4 with substrates other than glutamate, e.g. pyruvate or 2-oxoglutarate (variable) + malate (fixed), do not show biphasic kinetics (S. C. Dennis & J. B. Clark, unpublished work). In fact the apparent K_m values for these substrates are in close agreement with those for pyruvate and 2-oxoglutarate of the corresponding isolated enzyme complexes [pyruvate dehydrogenase, 90μM (Land & Clark, 1973); 2-oxo-

Table 3. *Summary of kinetic parameters of glutamate oxidation by free (M) and synaptic (SM2) brain mitochondria*
Free (M) and synaptic (SM2) mitochondria were prepared as outlined in the Materials and Methods section. The apparent K_m value (mM) and $V_{max.}$ rates (ng-atoms of oxygen/min per mg of protein for oxygen uptakes or nmol/min per mg of protein for aspartate production) have been derived from linear-regression analysis of the double-reciprocal Lineweaver–Burk plots of the corresponding experimental data (appropriate Figure noted) on a Munroe 1860 programmable calculator. A and B are the high- and low-K_m processes described in the text.

Experimental conditions		Free mitochondria (M)				Synaptic mitochondria (SM2)			
Process ...		A		B		A		B	
Substrate	Parameter measured	K_m	$V_{max.}$	K_m	$V_{max.}$	K_m	$V_{max.}$	K_m	$V_{max.}$
Glutamate+malate	O_2 uptake (Fig. 2)	1.3	153	0.26	75	1.8	76	0.3	44
Glutamate+malate+amino-oxyacetate	O_2 uptake (Fig. 2)	—	—	0.19	49	—	—	0.21	41
Glutamate+malate	Aspartate production (Fig. 3)	1.8	72	—	—	2.0	51	—	—
Glutamate+malate	O_2 uptake (Fig. 4)	1.1	187	0.26	101	1.0	118	0.28	76
Glutamate	O_2 uptake (Fig. 4)	1.0	83	—	—	1.3	99	—	—

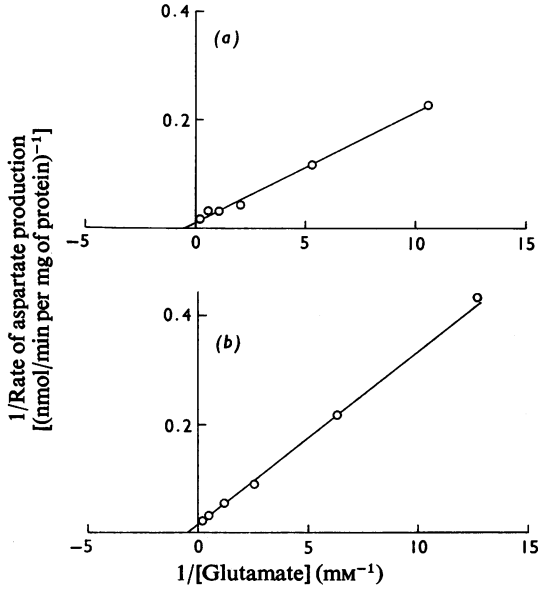

Fig. 3. *Aspartate production by (a) free (M) or (b) synaptic (SM2) brain mitochondria in the presence of 2.5 mM-malate at various glutamate concentrations*
Incubations (2ml final vol.) under State-3 conditions (including a hexokinase trap; see the Materials and Methods section) were performed at 25°C in the 100mM-K^+ medium with mitochondria in the presence of a fixed concentration of malate (2.5mM) and various (0.1–5mM) glutamate concentrations. The incubations were initiated by the addition of either free (M) (*a*, 1.7mg of protein) or synaptic (SM2) (*b*, 1.2mg of protein) mitochondria, and samples removed for fluorimetric analysis of aspartate (see the Materials and Methods section or legend to Table 4). Data derived from these experiments are shown in Table 3.

glutarate dehydrogenase, 1.4mM (S. C. Dennis & J. B. Clark, unpublished work)]. Further, experiments with glutamine, which diffuses into the mitochondrion (and after hydrolysis by glutaminase acts as an intramitochondrial source of glutamate), also indicate single-phase kinetics (Fig. 5) with an apparent K_m for glutamine of 0.28mM, which is of the same order as that for the low-K_m process (B) for glutamate oxidation (see Table 3). Thus the presence of biphasic kinetics of oxygen consumption appears to be a function peculiar to the oxidation of glutamate which is added externally to the mitochondria in the presence of malate. Some insight into the relative fluxes of transamination and 2-oxoglutarate metabolism in these experiments is found in Table 4, where the rates of production of aspartate and 2-oxoglutarate have been measured in State-3 conditions at a fixed malate concentration at various glutamate concentrations with 'free' rat brain mitochondria. As the added glutamate concentration increases, so do the rates of production of both aspartate and 2-oxoglutarate. If it is assumed that relatively little glutamate is metabolized via the glutamate dehydrogenase under these conditions [NH_3 production as measured by the NH_3 electrode is not detectable (S. C. Dennis, unpublished work; see Dennis *et al.*, 1976)], then the difference between the rates of accumulation of aspartate and 2-oxoglutarate may be taken as a relative measure of the flux throught the 2-oxoglutarate dehydrogenase. If the aspartate production is similarly taken as a relative measure of the glutamate–oxaloacetate transaminase flux, then it is clear that, as the glutamate concentration increases by some 30-fold, the transaminase flux increases by almost eightfold, compared with the 2-oxoglutarate dehydrogenase flux, which increases by less than threefold. This suggests that the 2-oxoglutarate produced by transamination is sufficient to saturate

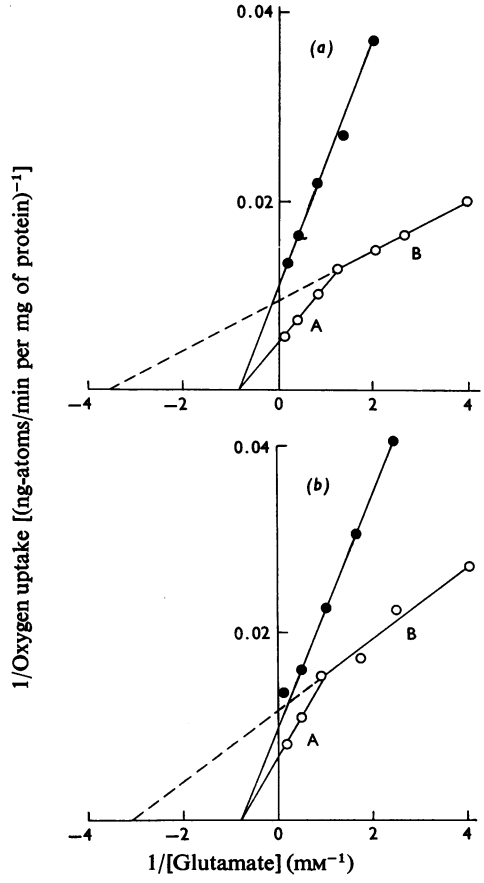

Fig. 4. *Oxygen uptakes by (a) free (M) or (b) synaptic (SM2) brain mitochondria in the presence or absence of malate at various glutamate concentrations*

Incubation conditions were similar to those of Fig. 2 except that 2.5mM-malate was either present (○) or absent (●). Oxygen uptakes in the presence of malate alone were 32 and 16ng-atoms of O/min per mg of protein for free (M) and synaptic (SM2) mitochondria respectively and have been subtracted from the glutamate- and malate-oxidation rates. A and B refer to the high- and low-K_m processes described in the text.

the 2-oxoglutarate dehydrogenase at relatively low glutamate concentrations. Further increases in the rate of transamination at the higher glutamate concentrations will be dependent on the availability of oxaloacetate from malate and on the export of any excess of 2-oxoglutarate from the mitochondria via the 2-oxoglutarate translocase (mitochondrial transport system) to prevent product-inhibition of the glutamate–oxaloacetate transaminase.

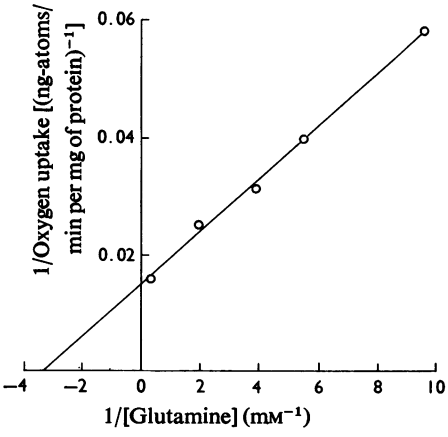

Fig. 5. *Oxygen uptake by free (M) brain mitochondria at various glutamine concentrations in the presence of a fixed concentration of malate*

Oxygen-uptake experiments were performed essentially as described in Fig. 2 except that glutamine was the substrate present in various concentrations. Oxygen uptake in the presence of malate alone (23ng-atoms/min per mg of protein) was deducted from the data.

Discussion

The observations and results in the present paper are consistent with the thesis that oxidative deamination by glutamate dehydrogenase in brain is relatively limited and that the main route of glutamate metabolism is by transamination (Balázs, 1965; Dennis *et al.*, 1976). This is a situation which also occurs in liver (De Haan *et al.*, 1967; McGivan *et al.*, 1974). Two main theories have been advanced for this in liver, which would also be appropriate in brain: (*a*) that the glutamate dehydrogenase is preferentially linked to NADP and that this coenzyme is maintained highly reduced by an energy-linked transhydrogenase (Ernster *et al.*, 1969; Hoek *et al.*, 1974); (*b*) that the rate of glutamate transport into the mitochondria in an aspartate-independent fashion, i.e. via the glutamate–OH^- translocase, is very slow (Brand & Chappell, 1974; Dennis *et al.*, 1976). That the glutamate–OH^- translocase in brain is relatively low in activity has been shown (Dennis *et al.*, 1976). This, however, does not explain the relatively low oxygen uptake seen with glutamine as a substrate (Table 1; Fig. 5), which is not itself subject to the restraints of a mitochondrial translocase and acts as an intramitochondrial source of glutamate. Thus the other possibility, i.e. that the glutamate dehydrogenase is restricted in its activity in the direction of glutamate oxidation, seems also to be true in brain. This could be for a number of reasons, but notably because of the highly reduced state (86%) of the NADP in brain

Table 4. *2-Oxoglutarate accumulation by 'free' (M) brain mitochondria oxidizing different concentrations of glutamate in the presence of a fixed malate concentration*

Incubations (final vol. 1 ml) were conducted at 25°C in the 100mM-K^+ medium in State-3 conditions (see the Materials and Methods section) in the presence of approx. 1.5mg of mitochondrial protein, 2.5mM-malate and various concentrations of glutamate (0.35–9.4mM). Samples (0.25ml) were removed at 0, 3, 6 and 9min into 1.5M-$HClO_4$, then neutralized with 3M-K_2CO_3/0.5M-triethanolamine to pH6.8, centrifuged at 15000*g* for 3min, and the supernatants assayed enzymically for aspartate and 2-oxoglutarate. From these measurements, rates of aspartate and 2-oxoglutarate production in nmol/min per mg of protein were calculated. 2-Oxoglutarate dehydrogenase flux was calculated as the difference between aspartate production and 2-oxoglutarate accumulation, assuming a stoicheiometric production of 2-oxoglutarate and aspartate from the transamination of glutamate with oxaloacetate and negligible 2-oxoglutarate production from the oxidative deamination of glutamate by glutamate dehydrogenase.

	Flux values (nmol/min per mg of protein)				
[Glutamate] (mM) ...	0.35	0.47	0.94	2.35	9.4
Aspartate production (x)	4.2	5.7	9.0	16.7	31.3
2-Oxoglutarate accumulation (y)	1.0	2.1	5.2	11.0	23.0
2-Oxoglutarate dehydrogenase flux ($x-y$)	3.2	3.6	3.8	5.7	8.3
2-$\frac{\text{Oxoglutarate dehydrogenase flux}}{\text{Glutamate–oxaloacetate transaminase flux}}$ $[(x-y)/x]$	0.76	0.63	0.42	0.34	0.26

mitochondria in State 3 oxidizing glutamate and malate (Nicklas *et al.*, 1971). If the brain glutamate dehydrogenase is preferentially linked to NADP, as has been suggested for the liver enzyme (Ernster *et al.*, 1969; Hoek *et al.*, 1974), then this state of NADP reduction would markedly decrease the capacity for any glutamate oxidation via the glutamate dehydrogenase. The metabolism of glutamate via transamination is also subject to some restraints when occurring in the intact mitochondria, notably the activity of the glutamate–aspartate translocase. This is because the glutamate–oxaloacetate transaminase will be inhibited by accumulation of relatively low concentrations of aspartate (K_i 0.5–0.9mM; Table 2), so that, in the absence of aspartate efflux by the means of the translocase (mitochondrial transport system), glutamate metabolism via transamination will be very limited.

The kinetics of oxygen uptake by brain mitochondria (synaptic or free) in the presence of increasing amounts of glutamate but a fixed malate concentration (Figs. 2 and 4) are consistent with there being two oxygen-requiring processes, one (B) with an apparent K_m for glutamate in the region of 0.25± 0.04mM (7 experiments) and the other (A) with an apparent K_m of 1.64±0.5mM (7 experiments). The high-K_m process, when corrected for the low-K_m process by the technique of Walker (1963), yields a recalculated K_m of 2.4±0.75mM (7 experiments). There are, however, a number of problems associated with determining the identity of the systems to which these K_m values may be assigned. First, there is the complexity of the oxygen consumption itself, which is a combination of oxygen uptake from (*a*) the glutamate dehydrogenase step (relatively small), (*b*) the metabolism of 2-oxoglutarate to oxaloacetate, (*c*) the oxidation of the added malate to oxaloacetate. Secondly, there is the disparity in the stoicheiometries of oxygen consumption required for the production of 1 mol of oxaloacetate by procedures (*b*) and (*c*) above, i.e. three atoms of O per mol of oxaloacetate produced for (*b*) but only one atom of O per mol of oxaloacetate for (*c*). Thirdly, there is the added complication that the relative fluxes of oxaloacetate produced by these two routes, and hence the oxygen consumption, do not bear a constant relationship to each other as a function of increase in glutamate concentration (Table 4). In fact the further metabolism of 2-oxoglutarate to provide oxaloacetate becomes saturated at relatively low glutamate concentrations, the continuing transamination of glutamate being permitted by the availability of oxaloacetate from malate which enters the mitochondrial matrix in stoicheiometric exchange for the 2-oxoglutarate produced in this process. In addition, the aspartate produced by transamination will be transported out of the mitochondrion on the glutamate–aspartate translocase, thus preventing any inhibition of the transaminase and also providing a further glutamate molecule in exchange for transamination. It seems reasonable to propose that the high-K_m process observed in Figs. 2, 3 and 4, which is abolished in the presence of amino-oxyacetate, is associated with transamination, since when transamination is measured by aspartate production (Fig. 3) a similar K_m is found. The same process would also be expected for oxidation of glutamate alone (Fig. 4), since, although a small amount of glutamate might be metabolized via the glutamate–OH^- translocase and glutamate dehydrogenase reaction (Dennis *et al.*, 1976), as soon as any oxaloacetate became available transamination would take place and the glutamate–aspartate translocase would become activated. The process of transamination of

externally added glutamate would of course involve both the glutamate–aspartate translocase and the transaminase enzyme complex itself. This latter, in the isolated state, has been shown to have a K_m for glutamate of 22–23mM (Table 2), an order of magnitude higher than the K_m for glutamate of 2.4mM recorded for the oxygen-uptake experiments. This suggests that the likely identity of the system which has an apparent K_m for glutamate of 2.4mM in these experiments is the glutamate–aspartate translocase. This would represent the K_m for external glutamate for this translocase in brain mitochondria and compares favourably with the value of 6mM reported for heart mitochondria (Williamson *et al.*, 1973) and 1–2mM for liver mitochondria (J. R. Williamson, personal communication). The successful identification of the low-K_m process seen in these experiments is more complex. The fact that it remains in the presence of amino-oxyacetate suggests that it is associated with glutamate metabolism by means other than transamination, e.g. transport of external glutamate by the glutamate–OH^- translocase and its subsequent metabolism by glutamate dehydrogenase. Preliminary experiments in which NH_3 production from glutamate in the presence of amino-oxyacetate was estimated indicate an increased NH_3 production over that found in the absence of amino-oxyacetate (S. C. Dennis & J. B. Clark, unpublished work). Also, the kinetics of glutamine oxidation (Fig. 5) indicate a process with a low K_m for glutamine present in this system. It has been previously shown (Dennis *et al.*, 1976) that brain mitochondria oxidizing glutamine export glutamate, possibly via the glutamate–OH^- translocase, to the surrounding medium and that this glutamate may then be taken up again on the glutamate–aspartate translocase and metabolized by transamination. As glutamine is thought to diffuse into brain mitochondria (Brand & Chappell, 1974; Dennis *et al.*, 1976), glutamine oxidation will reflect the kinetics of both the intramitochondrial glutaminase (Table 2) and also possibly those of the glutamate–OH^- translocase working in reverse, i.e. glutamate out of the mitochondria, as well as the kinetics of transamination. In a system as complex as that involving glutamine oxidation it is impossible to assign the low apparent K_m for glutamine to any particular stage in this system. However, taken in conjunction with the experiments on glutamate oxidation, it is plausible that one possible but not unique explanation of the low-K_m process is that it is associated with the glutamate–OH^- translocase.

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