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Glutamate Metabolism and Transport in Rat Brain Mitochondria

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1. The metabolism and transport of glutamate and glutamine in rat brain mitochondria of non-synaptic origin has been studied in various states. 2. These mitochondria exhibited glutamate uptake and swelling in iso-osmotic ammonium glutamate, both of which were inhibited by *N*-ethylmaleimide. 3. The oxidation of glutamate was inhibited by 20% by avenaciolide, but glutamine oxidation was not affected. 4. These mitochondria, when metabolizing glutamine, allowed glutamate, but very little aspartate, to efflux at considerable rates. 5. These results suggest that brain mitochondria of non-synaptic origin possess in addition to a relatively rapid glutamate–aspartate translocase, a relatively slow aspartate-independent glutamate–OH^- translocase (cf. liver mitochondria).

The existence of mitochondrial anion-translocator systems and their role in the control of cellular metabolism is now widely accepted (Chappell, 1968; Klingenberg, 1970; Meijer & Van Dam, 1974). However, mitochondria of different tissues appear to differ in their possession of certain of these translocators. A particular example of this is found in the case of the mitochondrial translocators for glutamate, at least two of which have been proposed and substantiated. These are the glutamate–OH^- translocator and the glutamate–aspartate translocator (Azzi *et al.*, 1967; Meijer & Van Dam, 1974). Whereas liver mitochondria possess both these translocators (Azzi *et al.*, 1967), heart (Chappell, 1968) and brain mitochondria (Brand & Chappell, 1974) have been reported to possess only the glutamate–aspartate translocator. The absence of the glutamate–OH^- translocator in heart mitochondria has been rationalized on the basis that heart mitochondria possess only a very low glutamate dehydrogenase activity (Klingenberg & Pette, 1962; Davis, 1968). However, such a situation does not exist in brain mitochondria, where high activities of both glutamate dehydrogenase and glutamate–oxaloacetate transaminase are present (Klingenberg & Pette, 1962; Balazs, 1965; Lai *et al.*, 1975) and glutamate may be metabolized by both oxidative deamination and transamination (Balazs, 1965; Nicklas *et al.*, 1971).

In view of the importance of glutamate in brain function and the well established compartmentation of its metabolism (Berl & Clarke, 1969; Balazs *et al.*, 1970; Van den Berg, 1973), we have studied the transport and metabolism of glutamate under a variety of conditions in mitochondria that are essentially of non-synaptic origin (Clark & Nicklas, 1970; Lai *et al.*, 1975; Lai & Clark, 1976). The experiments reported here provide suggestive evidence that both a glutamate–OH^- and a glutamate–aspartate translocator exist in these brain mitochondria.

Materials and Methods

Animals

In all cases male adult rats (150–180g) of the Wistar strain were used.

Chemicals

N-Ethylmaleimide was from BDH Chemicals Ltd., Poole, Dorset, U.K. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr. P. Heytler of E. J. Dupont De Nemours and Co., Wilmington, DE, U.S.A., and avenaciolide from Dr. W. B. Turner of I.C.I., Macclesfield, Cheshire, U.K. Glutamine and the scintillator (BBOT [2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen] was from Sigma Chemical Co., St. Louis, MO, U.S.A. ADP, NADH, NADPH, hexokinase (EC 2.7.1.1), glutamate dehydrogenase (EC 1.4.1.3), glutaminase (EC 3.5.1.2) and malate dehydrogenase (EC 1.1.1.37) were from Boehringer Corp. (London) Ltd., London W.5, U.K. Pyruvic acid was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and was twice distilled under vacuum and stored at −20°C before use. The silicone oil (Versilube F50) used in the transport studies was a gift from Jacobson Van den Berg and Co. Ltd., London, U.K. [1-^{14}C]-Glutamate (specific radioactivity 29mCi/mmol), [U-^{14}C]sucrose (specific radioactivity 9.6mCi/mmol) and ^3H_2O (specific radioactivity 5Ci/ml) were from The Radiochemical Centre, Amersham, Bucks., U.K.

All other chemicals were of the purest grade available and made up in twice-glass-distilled water.

Purification of glutamine

Before use contaminating glutamic acid (approx. 0.4%) was removed from the glutamine by passing a 200mM-glutamine solution through a 10ml column containing Dowex-1 (X8; acetate form) ion-exchange resin as described by Berl *et al.* (1962). Contaminating glutamate was measured enzymically as described by Williamson & Corkey (1969); glutamine was assayed by the method of Crompton & Chappell (1973). No glutamine was used with greater than 0.04% contaminating glutamic acid.

Mitochondrial experiments

Mitochondria from rat brain were prepared by the method of Clark & Nicklas (1970). Evidence has been presented elsewhere (Lai *et al.*, 1975) suggesting that mitochondria prepared by this method form a population distinct from those derived from synaptosomes. For that reason they will be referred to as mitochondria of 'non-synaptic' origin.

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Glutamate–oxaloacetate transaminase (EC 2.6.1.1)

This enzyme was measured by the method of Gutfreund *et al.* (1961) in the direction of aspartate formation. The reaction mixture (final volume 3ml) contained 40mM-L-glutamate, 1mM-oxaloacetate, 100mM-H_3PO_4 adjusted to pH7.4 with Tris, 0.1mM-NADPH, 50mM-NH_4Cl, 0.17% Triton X-100 and 11.25 units of glutamate dehydrogenase (EC 1.4.1.2). In the direction of glutamate formation the assay was as described by Boyd (1961). The reaction mixture (final volume 3ml) contained 100mM-H_3PO_4/Tris, pH7.4, 16mM-L-aspartate, 8mM-2-oxoglutarate, 0.25mM-NADH, 0.17% Triton X-100 and 22 units of malate dehydrogenase.

Swelling experiments

Absorbance changes associated with mitochondrial swelling were monitored at 640nm on an Aminco DW2 spectrophotometer in the split-beam mode.

Mitochondrial incubation

The standard incubation medium contained 100mM-KCl, 75mM-mannitol, 25mM-sucrose, 5mM-H_3PO_4/Tris, pH7.4, 20mM-Tris/HCl, pH7.4, 50μM-EDTA adjusted to pH7.4. O_2 uptake was measured polarographically by using a Clark-type electrode as described previously (Clark & Land, 1974). In the case of mitochondrial incubations in which metabolites were subsequently assayed, incubations were conducted at 25°C with O_2 blown over the surface to provide aeration. In these cases state 3 was induced by the presence of a hexokinase trap (20mM-glucose, 5mM-$MgCl_2$, 1mM-ADP and 0.1 unit of hexokinase/mg of mitochondrial protein). Samples (0.5ml) of the incubation medium were removed for analysis at timed intervals, pipetted into a micro-centrifuge tube (1.5ml) containing 500μl of silicone oil (Versilube F50) layered on top of 150μl of 14% (v/v) $HClO_4$ and rapidly centrifuged (15000g for 1min) in a Quickfit micro-centrifuge. After centrifugation a measured volume (400μl) of the upper layer was immediately removed and added to 200μl of 14% $HClO_4$. The region above the oil was then washed, the oil sucked off and the pellet dispersed in the lower layer to release internal metabolites. After re-centrifuging, a 100μl sample was removed and added to 500μl of 1M-Tris/HCl, pH7.4. The samples from both above and below the oil were then neutralized to pH6.5±0.2 with 3M-K_2CO_3/0.5M-triethanolamine and centrifuged to remove precipitated perchlorate and protein.

Metabolite assays

Glutamate and aspartate were determined in the neutralized incubation extracts by fluorimetric enzyme assays as described by Williamson & Corkey (1969). When *N*-ethylmaleimide was present in the extracts 5mM-dithiothreitol was included in the assay system, and similarly, when amino-oxyacetic acid was present, 5mM-acetaldehyde was added (see Meijer & Van Dam, 1974). In incubations including high concentrations (in the mM range) of 2-oxoglutarate and ammonium acetate, glutamate could not be measured by the methods of Williamson & Corkey (1969). Even after NH_3 removal by vacuum desiccation of the extract, adjusted to pH10, residual 2-oxoglutarate prevented glutamate estimation, and 5mM-H_2O_2 was included in all assays to remove the 2-oxoglutarate. Glutamate recoveries after vacuum desiccation indicated a 2% loss.

Mitochondrial loading (transport) experiments

A sample of the mitochondrial preparation (200μl; 6–10mg of mitochondrial protein) was added to 2ml of an incubation medium of the following final composition: 100mM-KCl; 12.5mM-succinate/Tris, pH7.4; 4μM-rotenone; 1mM-amino-oxyacetic acid; 13×10^3 d.p.m. ^3H_2O; the appropriate initial concentration of substrate (specific radioactivity approx. 3×10^5 d.p.m./μmol). The reaction mixture was incubated at 25°C for 3min, at which time two 200μl samples were taken and carefully layered on to 500μl of Versilube F50 oil above 100μl of 1.5M-$HClO_4$ in a micro-centrifuge tube and then centrifuged at 15000g for 1.5min. After

sampling the incubation mixture the appropriate quantity of additional substrate was added to the remaining incubation mixture and this was allowed to equilibrate for 2min, at which time two further 200μl samples were taken and treated as before. This procedure was repeated a further three times. Samples from the top and bottom of the micro-centrifuge tube were counted for ^{14}C and ^{3}H radioactivity in a Packard 2425 scintillation counter in 10ml of 2-methoxyethanol/toluene (2:3, v/v) containing 4g of BBOT and 80g of naphthalene/l. In all experiments the sucrose impermeable space was determined by using [U-^{14}C]sucrose in place of substrate, in both the presence and absence of inhibitors. Intramitochondrial substrate contents and volume measurements were computed by using a computer program kindly supplied by Dr. J. Mowbray of the Department of Biochemistry, University College London, London, U.K., on the University College London I.B.M. 360 machine.

Results

Enzyme activities

Table 1 indicates the observed maximum velocities of a number of enzymes concerned with glutamate metabolism in the brain mitochondria used in these experiments. They indicate that, unlike rat heart (Klingenberg & Pette, 1962; Davis, 1968) and muscle mitochondria (Borst, 1962), considerable quantities of glutamate dehydrogenase are present, although in the direction of glutamate utilization the ratio of glutamate–oxaloacetate transaminase to glutamate dehydrogenase is of the order of 16:1. This is in accord with Balazs's (1965) proposal that transamination was the main (70–90%) pathway of glutamate utilization in brain, and preliminary experiments in this laboratory, which have indicated that very little NH_3 as compared with aspartate, is produced from glutamate by these mitochondria (S. C. Dennis, unpublished work). It is also worth noting the mitochondrial localization of the glutaminases (cf. Katanuma *et al.*, 1967) in view of experiments reported below in which glutamine is used to generate intramitochondrial glutamate.

Oxygen uptake experiments

It may be seen from Table 2 that brain mitochondria are capable of oxidizing either exogenously supplied glutamate or endogenous glutamate supplied from the hydrolysis of glutamine by glutaminase. In the presence of 20μM-avenaciolide [an inhibitor of the liver mitochondrial glutamate–OH^- translocase (McGivan & Chappell, 1970; Meyer & Vignais, 1973)] the glutamate and malate oxidation was decreased by almost 20%, whereas glutamine oxidation was unaffected. However, the addition of 1mM-amino-oxyacetate (a transaminase inhibitor) inhibited both glutamate and glutamine oxidation by almost two-thirds. Further, it is apparent that the O_2 uptake due to exogenously supplied glutamate alone is completely inhibited in the presence of both avenaciolide and amino-oxyacetate, the remaining uninhibited respiration being of the same order as that due to malate alone. However, glutamine respiration in the presence of both inhibitors is only inhibited to the extent previously seen for amino-oxyacetate alone.

Table 1. *Enzyme activities associated with glutamate metabolism in non-synaptic rat brain mitochondria*

Glutamate–oxaloacetate transaminase activity was measured as described in the Materials and Methods section. The specific activity is expressed as the means±S.D.; the numbers of experiments are in parentheses. In each experiment the enzyme activity is the mean of at least three separate determinations.

Enzyme and reaction	Observed $V_{max.}$ (nmol/min per mg of protein)	Reference
Glutamate–oxaloacetate transaminase		
(1) Glutamate+oxaloacetate → 2-oxoglutarate+aspartate	804±77 (4)	This paper
(2) Aspartate+2-oxoglutarate → glutamate+oxaloacetate	1752±83 (4)	This paper
Glutamate dehydrogenase		
(1) Glutamate+NAD^+ → 2-oxoglutarate+NH_3+NADH	50	J. C. K. Lai & J. B. Clark (unpublished work)
(2) 2-Oxoglutarate+NH_3+NADH → glutamate+NAD^+	578±44 (9)	Lai *et al.* (1975)
(3) 2-Oxoglutarate+NH_3+NADPH → glutamate+$NADP^+$	492±39 (4)	Lai *et al.* (1975)
4-Aminobutyrate transaminase	38± 3 (7)	Lai *et al.* (1975)
Glutaminase		
(1) Phosphate dependent	338	J. C. K. Lai & J. B. Clark (unpublished work)
(2) Phosphate independent	70	J. C. K. Lai & J. B. Clark (unpublished work)

Table 2. *Oxygen uptake studies*

O_2 uptake was measured polarographically at 25°C in the standard incubation medium, as outlined in the Materials and Methods section, with approx. 1.5mg of mitochondrial protein/ml. State 3 was induced by the addition of 250μM-ADP for glutamate and malate experiments, but 1mM-ADP for glutamine experiments. The respiratory control ratio is defined as the ratio of the respiratory rate in state 3 to that in state 4 (Chance & Williams, 1956). Avenaciolide was added as an ethanolic solution to give a final concentration of 20μM and amino-oxyacetate as the aqueous Tris salt to give a final concentration of 1mM. The glutamate state 3 respiration rates are the means of at least two determinations. The glutamine state 3 respiration rates were measured when linearity had been established, which was at least 5min after the addition of the ADP. All respiration rates are expressed as ng-atoms of O/min per mg of mitochondrial protein.

Substrate	Additions	Respiration rate (state 3) (ng-atoms of O/min per mg of protein)	Respiratory control ratio
2.5mM-Malate	—	25	—
10mM-Glutamate plus 2.5mM-malate	—	148	4.1
	Avenaciolide	120	2.9
	Amino-oxyacetate	61	3.0
	Avenaciolide plus amino-oxyacetate	31	—
10mM-Glutamine	—	62	—
	Avenaciolide	63	—
	Amino-oxyacetate	23	—
	Avenaciolide plus amino-oxyacetate	24	—
5mM-Pyruvate plus 2.5mM-malate	—	180	6.5

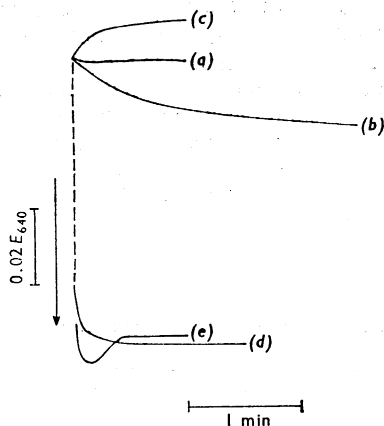

Fig. 1. *Swelling of brain mitochondria in iso-osmotic solutions*

Mitochondria (approx. 0.3mg/ml) were suspended in various iso-osmotic solutions of permeant anions containing 5mM-Tris/HCl, pH 7.4, and 7μg of antimycin/mg of mitochondrial protein at 25°C. The change in E_{640} with reference to a control sample containing 250mM-sucrose in place of the permeant anion was recorded. The following iso-osmotic solutions were used: (*a*) 125mM-ammonium aspartate; (*b*) 125mM-ammonium glutamate; (*c*) 125mM-ammonium glutamate and 0.5mM-*N*-ethylmaleimide; (*d*) 125mM-ammonium acetate; (*e*) 125mM-ammonium acetate and 0.5mM-*N*-ethylmaleimide. The arrow indicates the direction of swelling.

Swelling experiments

The swelling of mitochondria in iso-osmotic solutions of NH_4 salts of permeant and non-permeant anions has been used as a method of establishing the existence of mitochondrial translocases (Chappell, 1968). Fig. 1 shows the results of such experiments, and it is apparent that no change in light-scattering occurs in the presence of 125mM-ammonium aspartate (Fig. 1*a*). However, a typical swelling pattern was seen in the presence of ammonium acetate (Fig. 1*d*), which was not affected by *N*-ethylmaleimide (Fig. 1*e*), and a small, but significant, swelling in the presence of ammonium glutamate (Fig. 1*b*), which was *N*-ethylmaleimide sensitive (Fig. 1*c*). This suggests that these brain mitochondria possess a glutamate–OH^- translocase.

Uptake experiments

Further support for this suggestion was obtained by studying the *N*-ethylmaleimide sensitivity of glutamate uptake by brain mitochondria (Fig. 2). Although *N*-ethylmaleimide has complex inhibitory properties (Meijer & Van Dam, 1974) it has been shown to be an inhibitor of the glutamate–OH^- translocase in liver mitochondria (Meijer *et al.*, 1972), but not of the glutamate–aspartate translocase. In Fig. 2 the metabolism of glutamate has been inhibited by 5μM-rotenone and 1mM-amino-oxyacetate, and the uptake of [^{14}C]glutamate monitored. It can be seen from Fig. 2 that there is a marked uptake

of glutamate by these mitochondria, which is inhibited by *N*-ethylmaleimide even in the presence of uncoupler. This has also been confirmed in experiments that indicated that glutamate oxidation was inhibited by *N*-ethylmaleimide in uncoupled mitochondria (S. C. Dennis & J. B. Clark, unpublished work).

Metabolic studies

The studies so far are suggestive that brain mitochondria possess a glutamate–OH^- translocase in addition to that translocase already described, namely glutamate–aspartate translocase (Brand & Chappell, 1974). The studies are, however, concerned with glutamate uptake. The following experiments were carried out to demonstrate the reverse, namely the efflux of glutamate from brain mitochondria. Figs. 3 and 4 show the results of experiments in which brain mitochondria were incubated in the presence of glutamine and malate and both the extra- and intramitochondrial glutamate and aspartate produced were measured. Fig. 3 shows that glutamate was rapidly transported out of the mitochondria at a rate of 42nmol/min per mg of protein compared with a rate of 8nmol/min per mg of protein for the aspartate efflux. The presence of 1mM-amino-oxyacetate resulted in a negligible efflux of intramitochondrial aspartate together with a proportionate increase in the rate of glutamate efflux to 52nmol/min per mg of protein. The presence or absence of amino-oxyacetate did not alter the intramitochondrial glutamate or aspartate concentrations, which remained essentially constant over the incubation period.

Fig. 4 illustrates glutamate and aspartate production by mitochondria oxidizing glutamine and malate in state 3, state 4 (Chance & Williams, 1956) and in the presence of uncoupler. As in Fig. 3, the intramitochondrial glutamate and aspartate concentrations remained essentially constant during the course of the incubation. However, glutamate efflux from the mitochondria appears to be very dependent on the energy state of the mitochondria: 53, 42 and 18nmol/min per mg of protein in the uncoupled state, state 3 and state 4 respectively. Aspartate efflux is only significant in state 3 and even then shows an initial lag over the first 5min. Further, in state 3 the decrease in glutamate efflux as compared with the uncoupled state was compensated for by an equivalent increase in the aspartate efflux, rendering the total glutamate and aspartate efflux in both state 3 and the uncoupled state the same. The glutamate efflux observed in these experiments is complicated by the possibility that having come out of the mitochondria, the glutamate may then re-enter the mitochondria via the glutamate–aspartate translocase in a stoicheiometric exchange for aspartate. The apparent lag in aspartate efflux observed in state 3 (Fig. 4) suggests that this may be occurring, and the

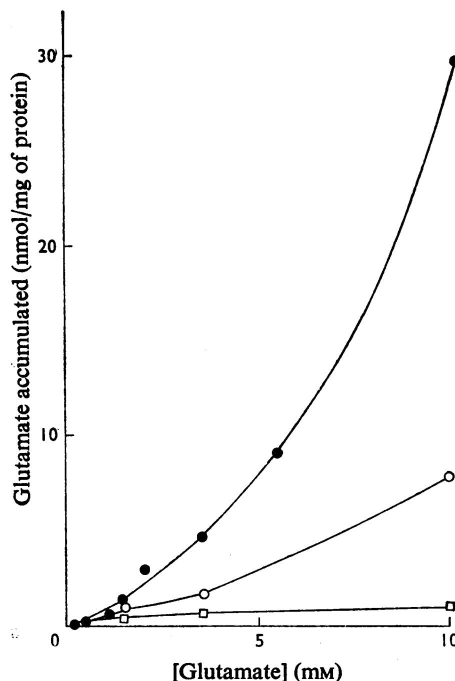

Fig. 2. *N-Ethylmaleimide-sensitive glutamate uptake by rat brain mitochondria*

Mitochondria (approx. 6–10mg of protein) were incubated with L-[U-^{14}C]glutamate of known specific radioactivity and $^{3}H_2O$ in a medium containing rotenone and amino-oxyacetate exactly as specified in the Materials and Methods section. Samples of the incubation mixture were taken and the mitochondria separated from the incubation medium by centrifugation as specified in the Materials and Methods section. Further additions of substrate were then made and further samples taken. Samples of the extramitochondrial medium and the intramitochondrial contents were then counted for ^{14}C and ^{3}H radioactivity. The internal mitochondrial matrical content of [^{14}C]glutamate was then computed, as outlined in the Materials and Methods section. The matrical space ranged from 0.6 to 1.7μl/mg of mitochondrial protein with a mean of 1.25μl/mg of mitochondrial protein, and the sucrose-accessible space ranged from 5.1 to 6.1μl/mg of mitochondrial protein with a mean of 5.6μl/mg of mitochondrial protein. The values of glutamate accumulated in the absence of inhibitor represent the mean of four values obtained from two separate experiments. Although the mitochondrial matrical glutamate contents varied for the same applied glutamate concentration in all cases, the S.D. observed was less than 7% of the observed content. (The maximal deviation was observed at the highest applied glutamate concentration, 10mM, where the content was 29.8 ± 2.1nmol/mg of mitochondrial protein.) The results are expressed as nmol of glutamate accumulated/mg of mitochondrial protein. (●) Control; (○) in the presence of 0.5mM-*N*-ethylmaleimide plus 0.05nmol of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone/mg of protein; (□) in the presence of 0.5mM-*N*-ethylmaleimide.

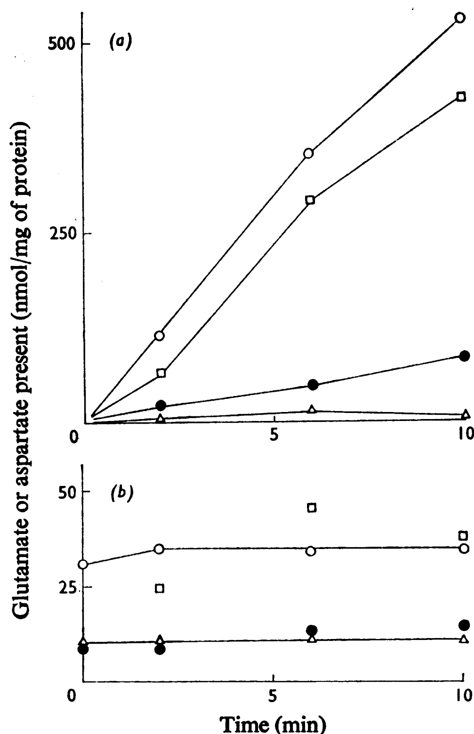

Fig. 3. *Effect of amino-oxyacetate on the state 3 oxidation of glutamine plus malate by rat brain mitochondria*

Mitochondria (approx. 5mg of protein) were incubated at 25°C in 2ml of the 100mM-KCl medium (see under 'Mitochondrial incubation') in state 3 (induced by the presence of 10mM-glucose, 5mM-$MgCl_2$, 1mM-ADP and 0.5 unit of hexokinase/ml) in the presence of 10mM-glutamine and 2.5mM-malate. Amino-oxyacetate, when present, was at a final concentration of 1mM. Samples (0.5ml) were withdrawn at timed intervals, and the mitochondria and extramitochondrial incubation medium were separated by centrifugation through a silicone-oil layer as described in the Materials and Methods section. Zero-time values were obtained in triplicate by the addition of 5mg of mitochondrial protein to 2ml of the state 4 medium (as the state 3 medium less $MgCl_2$, ADP and hexokinase) containing 10mM-glutamine and 2.5mM-malate, followed by the immediate withdrawal of 3× 0.5ml samples, which were centrifuged as described in the Materials and Methods section. The glutamate and aspartate contents of the extramitochondrial space and the intramitochondrial space (matrix) were assayed fluorimetrically as described in the Materials and Methods section. The mitochondrial metabolite contents have been corrected for any metabolite adherence during centrifugation by using a mean sucrose-accessible space of 5.6μl/mg of mitochondrial protein, obtained from the loading experiments (see legend to Fig. 2). The results are expressed as nmol of metabolite/mg of mitochondrial protein in the extramitochondrial space (*a*) or in the matrical space (*b*), in the presence (○, glutamate; △, aspartate) or absence (□, glutamate; ● aspartate) of amino-oxyacetate.

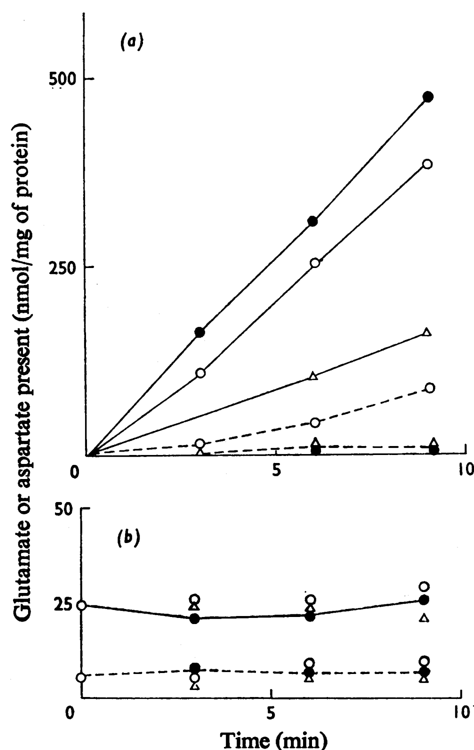

Fig. 4. *Effect of the respiratory state on the metabolism of glutamine and malate by rat brain mitochondria*

Rat brain mitochondria (approx. 5mg of protein) were incubated at 25°C in 2ml of the 100mM-KCl medium (see the Materials and Methods section) in the presence of 10mM-glutamine and 2.5mM-malate. State 3 (○) and 4 (△) conditions were produced by using or omitting a hexokinase trap (see legend to Fig. 3). The uncoupled state was induced by using state 4 conditions plus 0.1 nmol of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone/ mg of protein (●). All other conditions and manipulations were as described in the legend to Fig. 3. The results are expressed as nmol of metabolite/mg of mitochondrial protein in the extramitochondrial space (*a*) or in the matrical space (*b*). Glutamate, ——; aspartate, ----.

inhibition of aspartate efflux (Fig. 4) seen in the uncoupled state suggests that the glutamate–aspartate translocase is energy-dependent, as in other tissues (La Noue *et al.*, 1974*a*). Fig. 5 shows experiments in which glutamate has been generated intramitochondrially by an alternative system, namely by the synthesis of glutamate from 2-oxoglutarate and ammonium acetate in the presence of pyruvate and malate. This leads to an efflux of glutamate from the mitochondria at a rate of approx. 1 nmol/min per mg of protein, although intramitochondrial glutamate remains constant. The presence of 1mM-amino-

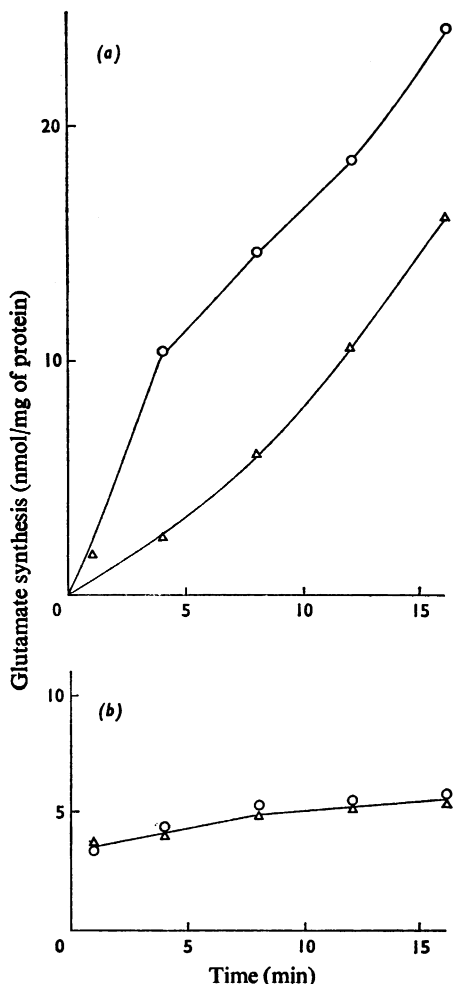

Fig. 5. *Effect of amino-oxyacetate on brain glutamate synthesis by brain mitochondria oxidizing pyruvate and malate*

Rat brain mitochondria (approx. 4.5mg of protein) were incubated at 25°C in 4ml of the 100mM-KCl medium (see the Materials and Methods section) together with 5mM-pyruvate, 2.5mM-malate, 5mM-2-oxoglutarate and 10mM-ammonium acetate. Samples (0.5ml) of the incubation mixture were removed at timed intervals and the mitochondria separated from the extramitochondrial medium by centrifugation as described in the Materials and Methods section. Glutamate was measured in both the extramitochondrial (*a*) and matrix (*b*) spaces by enzymic techniques with certain adaptations to overcome the problems of high concentrations of 2-oxoglutarate and NH_4^+ present (see the Materials and Methods section). The results are expressed as nmol of glutamate/mg of mitochondrial protein and have been corrected for any glutamate adherence (see legend to Fig. 3). Amino-oxyacetate (1mM) present (△) or absent (○).

oxyacetate, although causing an initial lag period, does not inhibit in any significant fashion the ultimate rate of glutamate efflux. The absence of any effect of amino-oxyacetate suggests also that transaminase enzymes, e.g. alanine aminotransferase are not involved in this glutamate efflux.

Discussion

Interest in the transport and metabolism of glutamate and glutamine in the brain has increased since the discovery of the putative transmitter role of glutamate (Krnjevic & Phillis, 1963), and more particularly in the wake of the proposal of Benjamin & Quastel (1972), that glutamate is deactivated at the nerve ending by conversion into glutamine. Brand & Chappell (1974) proposed that rat brain mitochondria of non-synaptic origin possess only a glutamate–aspartate translocase and in this respect were similar to heart mitochondria (La Noue *et al.*, 1973). They also proposed that glutamate efflux from these brain mitochondria was carried out via a pyruvate–alanine shuttle involving a cytosolic alanine aminotransferase.

However, the swelling data (Fig. 1) suggest that these mitochondria possess a glutamate–OH^- translocase, which is inhibited by *N*-ethylmaleimide (Fig. 2). Further, the O_2 uptake data (Table 2) indicate that some 23% of the O_2 consumption due to glutamate alone may be inhibited by avenaciolide, a glutamate analogue that specifically inhibits the glutamate–OH^- translocase in liver (Meyer & Vignais, 1973) and kidney (Kovacevic, 1975) mitochondria. Amino-oxyacetate, however, inhibits more than 70% of the glutamate-induced respiration (Table 2). Since glutamate entering the mitochondria on the glutamate–aspartate translocase must obligatorily be transaminated, these values suggest that about 70% of the externally added glutamate is transaminated, the rest, the entry of which is avenaciolide-sensitive, being metabolized via glutamate dehydrogenase. This supports the original suggestion by Balazs (1965) of the relative importance of oxidative deamination and transamination of glutamate in brain. The oxidation of glutamate generated intramitochondrially from glutamine appears, however, insensitive to avenaciolide, suggesting that this inhibitor acts at the level of glutamate transport across the mitochondrial membrane even in the presence of amino-oxyacetate (Table 2). In fact the glutamine oxidation rates reported in Table 2 are those measured after at least 5 min in state 3 (i.e. plus ADP), as before this period glutamine oxidation exhibits a gradually increasing rate, which becomes linear after approx. 5min (S. C. Dennis, J. M. Land & J. B. Clark, unpublished work). In the presence of amino-oxyacetate (Table 2), not only is the O_2 uptake inhibited by almost two-thirds, but the initial non-linearity of

glutamine oxidation is also removed (S. C. Dennis, J. M. Land & J. B. Clark, unpublished work). A likely explanation of this is that initially intramitochondrially generated glutamate cannot be transaminated to any extent, because any intramitochondrial aspartate so formed cannot be effluxed on the glutamate–aspartate translocase until some external glutamate is available for translocation. This will not be the case until some intramitochondrial glutamate has effluxed from the mitochondria, e.g. on the glutamate–OH^- translocase, and is available for re-entry; hence the lag phase in glutamine oxidation, which is removed when the transamination route is made unavailable by amino-oxyacetate (Table 2; Fig. 3).

Other possible mechanisms for the glutamate efflux from these mitochondria, other than the glutamate–OH^- translocase, are the pyruvate–alanine shuttle proposed by Brand & Chappell (1974) and the glutamine–glutamate translocase proposed to exist in kidney mitochondria (Crompton & Chappell, 1973).

The pyruvate–alanine shuttle seems unlikely, since both in the glutamine experiments (Figs. 3 and 4) and in those where glutamate is synthesized (Fig. 5) the addition of amino-oxyacetate does not inhibit the glutamate efflux. The glutamine–glutamate translocase, the existence of which is controversial (see Brosnan, 1975; Kovacevic, 1975), also seems improbable for the following reasons. In the experiments of Fig. 5, glutamate is synthesized intramitochondrially and effluxes in the absence of glutamine. Although the rate of efflux is slow compared with the glutamine experiments (Figs. 3 and 4), this is probably a reflection of the synthetic system rather than the translocase itself. In the experiments involving glutamine metabolism (Table 2; Figs. 3 and 4), if the glutamine–glutamate translocase were operative, a net O_2 uptake would only occur if the glutamate, having effluxed, was then transported back in again, e.g. by the glutamate–aspartate translocase, which in turn should efflux an amount of aspartate stoicheiometric with the glutamate (glutamine). It is clear from Table 2 that a net O_2 uptake does occur in the presence of glutamine and from Figs. 3 and 4 that aspartate does not efflux stoicheiometrically with respect to glutamate. Unless, therefore, aspartate is re-entering the mitochondria and masking the stoicheiometry, the glutamine–glutamate translocase does not explain these results. The swelling data suggest that these mitochondria are not permeable to aspartate (Fig. 1) and no re-oxidation of intramitochondrial NAD(P)H by added aspartate can be achieved in glutamate-loaded brain mitochondria unless they are uncoupled (S. C. Dennis & J. B. Clark, unpublished work; Brand & Chappell, 1974).

The data reported here suggest that brain mitochondria of non-synaptic origin possess both a glutamate–aspartate and a glutamate–OH^- translocase. In agreement with Brand & Chappell (1974), there is no evidence to support the existence of a glutamine–glutamate translocase in these mitochondria. However, the relative rates at which each of the glutamate translocases allow glutamate to enter the mitochondrion may be very different. These rates will depend on a variety of kinetic parameters, including the affinities of translocases for both external and internal glutamate and aspartate (La Noue *et al.*, 1974*b*). The swelling data (Fig. 1) indicate that, although glutamate may enter independently of aspartate, it does so only at a very slow rate. This may afford some explanation for the relative proportions of externally added glutamate that are metabolized via transamination or oxidative deamination.

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