Studies on the Control of 4-Aminobutyrate Metabolism in 'Synaptosomal' and Free Rat Brain Mitochondria

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1. The specific activities of 4-aminobutyrate aminotransferase (EC 2.6.1.19) and succinate semialdehyde dehydrogenase (EC 1.2.1.16) were significantly higher in brain mitochondria of non-synaptic origin (fraction M) than those derived from the lysis of synaptosomes (fraction SM₂). 2. The metabolism of 4-aminobutyrate in both 'free' (non-synaptic, fraction M) and 'synaptic' (fraction SM₂) rat brain mitochondria was studied under various conditions. 3. It is proposed that 4-aminobutyrate enters both types of brain mitochondria by a non-carrier-mediated process. 4. The rate of 4-aminobutyrate metabolism was in all cases higher in the 'free' (fraction M) brain mitochondria than in the synaptic (fraction SM₂) mitochondria, paralleling the differences in the specific activities of the 4-aminobutyrate-shunt enzymes. 5. The intramitochondrial concentration of 2-oxoglutarate appears to be an important controlling parameter in the rate of 4-aminobutyrate metabolism, since, although 2-oxoglutarate is required, high concentrations (2.5 mM) of extramitochondrial 2-oxoglutarate inhibit the formation of aspartate via the glutamate-oxaloacetate transaminase. 6. The redox state of the intramitochondrial NAD pool is also important in the control of 4-aminobutyrate metabolism; NADH exhibits competitive inhibition of 4-aminobutyrate metabolism by both mitochondrial populations with an apparent K_i of 102 μ M. 7. Increased potassium concentrations stimulate 4-aminobutyrate metabolism in the synaptic mitochondria but not in 'free' brain mitochondria. This is discussed with respect to the putative transmitter role of 4-aminobutyrate.

4-Aminobutyrate metabolism has been extensively studied at the 'whole' brain and brain 'slice' level of organization (Balazs *et al.*, 1973; Van den Berg, 1973). This has led to the concept of compartmentation of 4-aminobutyrate metabolism in the whole brain which has been related to its proposed transmitter function (see Berl *et al.*, 1975). Although Salganicoff & De Robertis (1963) established the intramitochondrial location of the 4-aminobutyrate-shunt enzymes 4-aminobutyrate aminotransferase (EC 2.6.1.19) and succinate semialdehyde dehydrogenase (EC 1.2.1.16) some time ago, studies on 4-aminobutyrate metabolism (McKhañ & Tower, 1959; Løvtrup, 1961; Sacktor & Packer, 1962; Balazs *et al.*, 1963; Bacila *et al.*, 1963) have been confusing, particularly with respect to the ability of 4-aminobutyrate to act as a substrate for mitochondrial respiration. This for the most part may be explained by the mitochondrial preparations used, which were probably either heavily contaminated with synaptosomes or metabolically inactive after long preparative procedures utilizing high-density sucrose gradients.

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With the advent of new techniques and particularly of the polymer Ficoll it is now possible to prepare relatively uncontaminated metabolically active brain mitochondria of both 'synaptic' (Lai & Clark, 1976; Lai *et al.*, 1975) and non-synaptic origin (Clark, & Nicklas, 1970).

In the present paper we have compared the control of 4-aminobutyrate metabolism in both 'synaptic' and 'free' brain mitochondria as part of a study related to the proposed transmitter role of 4-aminobutyrate (Curtis, 1975).

Materials and Methods

Chemicals

All chemicals were of the highest purity commercially available and were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. and Sigma (London) Chemical Co., London S.W.6, U.K. Enzymes were obtained from Boehringer. All solutions were prepared in double-glass-distilled water. 4-Amino[U-¹⁴C]butyric acid (specific radioactivity 232 Ci/mol), [2-¹⁴C]acetate (specific radioactivity

38 Ci/mol), [^{14}C]sucrose (specific radioactivity 9.6 Ci/mol), $^3\text{H}_2\text{O}$ (specific radioactivity 5 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks, U.K. Versilube F 50 and Bisoflex D.N.P. were donated by Jakobson, Van den Berg and Co., London W3 7RN, U.K., and B.P. Chemicals International, Saltend, Hedon, Hull, U.K., respectively.

Animals

Adult male rats (160–190 g) of the Wistar strain were used for the preparation of brain mitochondria.

Preparation of brain mitochondria. The method of Clark & Nicklas (1970) was used to prepare the mitochondrial population of 'non-synaptic' origin designated M in the text. The method of Lai & Clark (1976) was used to isolate the mitochondrial population of 'synaptic' origin (fraction SM_2), the heavier 'synaptic' population (sedimenting below the 6% Ficoll/sucrose medium; see Lai & Clark, 1976). Protein was determined by the method of Lowry *et al.* (1951), with bovine plasma albumin as the standard.

Methods

Enzyme assays. 4-Aminobutyrate-2-oxoglutarate aminotransferase (EC 2.6.1.19). Despite the number of methods available for the determination of 4-aminobutyrate aminotransferase activity, none afford a rapid continuous method of measurement [for a review of the existing methods, see Baxter (1972)]. The method described in the present paper measures the glutamate formed from 4-aminobutyrate by 4-aminobutyrate aminotransferase by using commercially available glutamate dehydrogenase (EC 1.4.1.3) in glycerol in the presence of NAD^+ , and monitors spectrophotometrically the NADH produced by this coupled enzyme assay. The following concentration of reagents gave optimum 4-aminobutyrate aminotransferase activity: 100 mM-triethanolamine/HCl, pH 8.6, 6 mM-4-aminobutyrate, 2.5 mM-2-oxoglutarate (adjusted to pH 8.6 with triethanolamine base immediately before use), 1 mM- NAD^+ , 0.1% (v/v) Triton X-100 and 0.2 mg of glutamate dehydrogenase in glycerol in a final volume of 1 ml.

The routine amount of mitochondrial protein used was about 0.4 mg, although the rate of 4-aminobutyrate aminotransferase activity increased linearly with increasing protein concentration up to 1.3 mg. The reaction was started by the addition of 4-aminobutyrate, and the linear rate of NADH production was measured at 340 nm for about 3 min. As a check on the reliability of this method, a reaction mixture containing the same components except for the glutamate dehydrogenase was incubated for 30 min and the glutamate formed was measured by the method of

Frieden (1959). Rates identical with those obtained by the continuous method of 4-aminobutyrate aminotransferase measurement were obtained.

Succinate semialdehyde dehydrogenase (EC 1.2.1.16). The fluorimetric discontinuous method of measurement described by Pitts & Quick (1965) was adapted so that the rate of NADH production could be measured by a continuous spectrophotometric method. Optimal activity was achieved with the following assay mixture: 100 mM-Tris/HCl, pH 8.6, 15 mM-2-mercaptoethanol, 2.5 mM- NAD^+ , 0.1% (v/v) Triton X-100, 1 mM-succinate semialdehyde and approx. 0.2 mg of mitochondrial protein (although the assay was linear for up to 0.6 mg of enzyme protein). The reaction was started with succinate semialdehyde and the linear rate of NADH production was measured for 1–2 min at 340 nm. All enzyme assays were carried out at 25°C in a Unicam SP.800 recording spectrophotometer. Enzyme activities are expressed as the amount of NADH produced, which is a direct measure of substrate used, in nmol/min per mg of mitochondrial protein.

Succinate semialdehyde was prepared as a routine by the method of Langheld (1909*a,b*), as described by Bruce *et al.* (1971). Purification was carried out chromatographically as described by Swim & Utter (1957) by using Celite 535. Freedom from contamination by glutamate and polymeric condensation products was shown by performing high-voltage electrophoresis on the solution (Walsh, 1975). The concentrations of succinate semialdehyde solutions were determined enzymically by the method of Scott & Jakoby (1959), and the solutions were stored at –20°C.

4-Aminobutyrate shunt activity. The assay used was essentially that described for succinate semialdehyde dehydrogenase (see above), 1 mM-succinate semialdehyde being replaced by 6 mM-4-aminobutyrate and 2.5 mM-2-oxoglutarate in 0.1 M-triethanolamine/HCl buffer, pH 8.6.

The assay uses the endogenous mitochondrial 4-aminobutyrate aminotransferase to produce succinate semialdehyde, which is oxidized via the endogenous NAD^+ -linked succinate semialdehyde dehydrogenase with a concomitant production of NADH, which is measured spectrophotometrically.

Mitochondrial transport studies. Incubations were performed with 1.9 ml of 100 mM- K^+ medium [100 mM-KCl, 75 mM-mannitol, 25 mM-sucrose, 5 mM-phosphate/Tris, pH 7.4, 5 mM-Tris/HCl, pH 7.4, and 50 μM -EDTA (dipotassium salt)], 2.5 mM-succinate/Tris, 2.2 μM -rotenone, 0.7 mM-sodium arsenite, 0.9 mM-amino-oxyacetate, 1.3×10^6 d.p.m. of $^3\text{H}_2\text{O}$, 3.5–5 mg of mitochondrial protein together with the appropriate concentration of substrate (200–600 d.p.m./nmol) in a final volume of 2.2 ml. The reaction mixture was incubated at 25°C for 3 min, and 2 $\times$ 200 μl samples were taken, centrifuged at 15 000 g

for 1 min through 500 μ l of silicone oil layer over 100 μ l of 1.5M-HClO₄ in an Eppendorf microcentrifuge. In this way the mitochondria were separated from the medium. The oil used for the 'free' mitochondria was Versilube F 50, and a mixture of 5 parts Versilube F 50/1 part Bisoflex was used for the synaptic mitochondria (fraction SM₂). After sampling the incubation medium, a portion of radioactive substrate was added, then allowed to equilibrate for 3 min, and the sampling procedure repeated. Addition of substrate and sampling was repeated a further three times.

Samples from the medium (top; 20 μ l) and the mitochondrial extract (bottom; 50 μ l) were counted for ¹⁴C and ³H radioactivity in 10 ml of a 2-methoxy-ethanol/toluene mixture (2:3, v/v) containing 4 g of BBOT [2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen]/litre and 80 g of naphthalene/litre in a 2425 Packard liquid-scintillation counter. [U-¹⁴C]Sucrose replaced the substrate (4-aminobutyrate) when the sucrose-permeable space was determined. Suitable quench corrections were made and the intramitochondrial volume and substrate concentrations calculated on the St. Bartholomew's Hospital Medical College Computer Facility by Dr. A. Dunlop using a program originally supplied by Dr. J. Mowbray of University College, London (see Land *et al.*, 1976).

Mitochondrial metabolic incubations. Approx. 3–4 mg of mitochondrial protein was incubated in 2 ml of 5 mM-KCl or 100 mM-KCl media containing various substrates as described in the legends. The 5 mM-K⁺ medium had the following composition: 5 mM-KCl, 225 mM-mannitol, 75 mM-sucrose, 5 mM-phosphate/Tris, pH 7.4, 5 mM-Tris/HCl, pH 7.4, and 50 μ M-EDTA (dipotassium salt), and the 100 mM-K⁺ medium was as above. The incubations were performed at 25°C, and O₂ was blown over the surface of the medium. State 3 (Chance & Williams, 1956) conditions were induced by the addition of 1 mM-ADP and maintained by means of a hexokinase trap in the medium which contained 20 mM-glucose, 5 mM-MgCl₂ and 1.5 units of hexokinase/2 ml of incubation medium.

In experiments where the mitochondria were separated from the medium, samples (the volume of which depended on the particular metabolites being assayed) were removed at time-intervals and pipetted on to 500 μ l of the appropriate oil (see above) layered over 150 μ l of 1.5M-HClO₄ contained in a microcentrifuge tube. The mitochondria were separated from the medium by centrifuging in an Eppendorf microcentrifuge at 15000g for 1 min. A portion of the top layer was removed and added to 1.5M-HClO₄ so that a final concentration of 0.2–0.4M-HClO₄ was obtained. A sample of mitochondrial extract (bottom) was also taken after the oil had been removed and both (top and bottom) samples were neutralized with 3M-K₂CO₃ in 0.5M-triethanolamine/

HCl to pH 6.5. The precipitated protein and KClO₄ were removed by centrifugation.

When separation of the mitochondria from the medium was not required, measured volumes were pipetted immediately into 1.5M-HClO₄ to give a final concentration of HClO₄ similar to that described above, and neutralization was carried out in the same way.

Fluorimetric metabolite assays. All measurements were performed at 25°C in an Eppendorf Photometer 1101M equipped with a recording adaptor 6441 and adapted for use as a fluorimeter.

Glutamate was measured fluorimetrically by the adapted spectrophotometric method of Frieden (1959). Aspartate and 2-oxoglutarate were determined by the fluorimetric assays described by Williamson & Corkey (1969).

Results

The mean observed specific activities of the 4-aminobutyrate aminotransferase and succinate semialdehyde dehydrogenase from 'free' (fraction M) and synaptic (fraction SM₂) rat brain mitochondria together with the Michaelis parameters are shown in Table 1. In both cases the 'free' mitochondria showed significantly higher specific activities, although only slight differences in the *K_m* values for substrates were apparent between the two mitochondrial populations. For the 4-aminobutyrate aminotransferase, 2-oxoglutarate showed substrate inhibition at concentrations above 4 mM in both mitochondrial populations (results not shown; for 'free' mitochondrial enzyme *K_i* for 2-oxoglutarate was 4.7 mM). However, for the succinate semialdehyde dehydrogenase no substrate inhibition by succinate semialdehyde was observed in either mitochondrial population up to concentrations of 2 mM, in contrast with the reports of other workers (Albers & Koval, 1961; Embree & Albers, 1964; Kammeraat & Veldstra, 1968). The distribution and specific activities of the 4-aminobutyrate-shunt enzymes agree well with the values obtained by Salganicoff & De Robertis (1965) when suitable temperature corrections are applied. The specific activities of the 4-aminobutyrate aminotransferase are, however, considerably higher (two to three times) than those reported by Buu & Van Gelder (1974) and may reflect the different mitochondrial preparative procedure used by these authors. It is also worth noting that the 4-aminobutyrate aminotransferase from both mitochondrial populations showed a broad pH profile with an optimum activity at pH 8.6–8.8 (cf. Waksman *et al.*, 1968).

Transport studies

Initially, experiments were carried out to establish how 4-aminobutyrate was transported across the mitochondrial membrane. Brand & Chappell (1974)

Table 1. Kinetic characteristics of 4-aminobutyrate-shunt enzymes in brain mitochondrial populations

V_{observed} is the mean optimal specific activity $\pm$ S.D. of n experiments. At least two enzyme concentrations were measured in each experiment. The Michaelis parameters (V_{max} , K_m) were obtained by extrapolation of Lineweaver–Burk plots, the assay methods described under 'Methods' being used. The V_{observed} values for fraction M versus fraction SM₂ are significantly different ($P < 0.001$).

	Mitochondrial populations	
	M fraction	SM ₂ fraction
4-Aminobutyrate aminotransferase		
V_{observed} (nmol/min per mg of protein)	38 ± 3 ($n = 7$)	26 ± 5 ($n = 4$)
K_m for 4-aminobutyrate (mM)	1.25	0.8
K_m for 2-oxoglutarate (mM)	0.5	0.24
V_{max} (nmol/min per mg of protein)	44	34.5
Succinate semialdehyde dehydrogenase		
V_{observed} (nmol/min per mg of protein)	57 ± 0.6 ($n = 7$)	29 ± 5 ($n = 7$)
K_m for NAD ⁺ (10^{-5} M)	9.4	17
K_m for succinate semialdehyde (10^{-5} M)	1.5	1.4
V_{max} (nmol/min per mg of protein)	64.5	30.3

Fig. 1. 4-Amino[¹⁴C]butyrate uptake by brain mitochondria

Both 'free' (○) and synaptic (□) brain mitochondria (3–5 mg of protein) were incubated in 1.9 ml of the 100 mM-K⁺ medium (see under 'Methods') together with various concentrations of 4-aminobutyrate. Samples of the incubation mixture were centrifuged and counted for radioactivity as outlined under 'Methods'. Internal spaces of the mitochondria and internal mitochondrial substrate concentrations were calculated as described by Land *et al.* (1976). Each point represents the mean of two experiments. The average internal space for 'free' mitochondria (fraction M) was $1.49 \pm 0.4 \mu\text{L}/\text{mg}$ of protein and for synaptic mitochondria (fraction SM₂) $1.58 \pm 0.6 \mu\text{L}/\text{mg}$ of protein.

have previously suggested, on the basis of swelling experiments, that this occurs by passive diffusion and is not 'carrier'-mediated. Fig. 1 shows the results of experiments in which both 'free' and 'synaptic' mitochondria were incubated with radioactively labelled 4-aminobutyrate in conditions where metabolism of 4-aminobutyrate was inhibited. The mitochondria

were separated from the medium, and the internal 4-aminobutyrate was measured. The results indicate that the uptake of 4-aminobutyrate showed non-saturable kinetics up to concentrations of 18 mM; experiments with radioactively labelled acetate showed similar results. These observations support the suggestion of Brand & Chappell (1974) that 4-aminobutyrate enters mitochondria by passive diffusion. The entry of 4-aminobutyrate into these brain mitochondria was further confirmed by following the intramitochondrial reduction of NAD(P)⁺ fluorimetrically in the presence of 2-oxoglutarate and malate (Walsh, 1975).

4-Aminobutyrate metabolism

This was assessed by measuring the glutamate formed by the transamination of 4-aminobutyrate to succinate semialdehyde under conditions where further metabolism of these products was inhibited by the presence of rotenone and arsenite. Initially, conditions were established under which 4-aminobutyrate was optimally metabolized. Fig. 2(a) shows that with non-synaptic mitochondria (fraction M) 4-aminobutyrate metabolism in the presence of 2-oxoglutarate but in the absence of malate appears to be non-linear over the first 15 min. However, in the presence of 1 mM-malate the rate is both increased to 6.1 nmol/min per mg of protein and made linear over the time-period measured. With synaptic mitochondria (fraction SM₂; Fig. 2b) the rate of 4-aminobutyrate metabolism remains linear over 30 min in the presence or absence of malate, but the rate is approximately half that observed with the 'free' mitochondria, 3.5 nmol/min per mg of protein in the presence of 1 mM-malate. A similar stimulation of 4-aminobutyrate metabolism was observed with both mitochondrial populations if malonate replaced

Fig. 2. Glutamate produced from 4-aminobutyrate by 'free' (M) and synaptic (SM₂) mitochondria

Approx. 2mg of mitochondrial protein (a, 'free' mitochondria; b, synaptic mitochondria) was incubated in 2ml of the 100mM-K⁺ medium (see under 'Methods') containing 2.5 μ M-rotenone and 0.75 mM-sodium arsenite, 5 mM-4-aminobutyrate and 2.5 mM-2-oxoglutarate. All experimental procedures were carried out as indicated under 'Methods'. (a) ●, Glutamate formation (total=extra- plus intra-mitochondrial) by 'free' mitochondria in the above conditions; ○, glutamate formation (total) by 'free' mitochondria as above plus 1 mM-malate. (b) ▲, Glutamate formation (extramitochondrial only) by synaptic mitochondria in basic conditions; △, glutamate formation (extramitochondrial only) by synaptic mitochondria as above plus 1 mM-malate. For the synaptic mitochondria, intramitochondrial glutamate was undetectable.

malate in the incubation medium (J. M. Walsh & J. B. Clark, unpublished work). The intramitochondrial glutamate concentrations under conditions of maximal 4-aminobutyrate metabolism (i.e. plus malate) remained essentially constant, 8–9 nmol/mg of protein in the 'free' (fraction M) mitochondria and 2 nmol/mg of protein in the synaptic mitochondria (at the limits of the level of determination). Fig. 3 indicates the rates of glutamate accumulation from 4-aminobutyrate by 'free' mitochondria (Fig. 3a) and 'synaptic' mitochondria (Fig. 3b) in the presence and absence of rotenone. The rate of glutamate accumulation is lower in the presence of rotenone than in its absence possibly because the high NADH/NAD⁺ ratio causes an inhibition of the succinate semialdehyde dehydrogenase, thus pushing the 4-aminobutyrate aminotransferase equilibrium in the direction of 4-aminobutyrate. Under these conditions the rate of glutamate accumulation may be taken as a measure of the flux through the 4-aminobutyrate aminotransferase–succinate semialdehyde dehydrogenase shunt, since succinate metabolism is inhibited by the malonate, 2-oxoglutarate metabolism by arsenite and glutamate dehydro-

genase by the high NADH/NAD⁺ ratio induced by rotenone. However, there seems to be very limited oxidation of 2-oxoglutarate under the conditions of these experiments (Fig. 3), since there was no detectable change in glutamate produced when arsenite was omitted from the incubation ['free' mitochondria (fraction M) under the conditions of Fig. 3 accumulated glutamate at the rate of 5.75 nmol/min per mg of protein and in the absence of arsenite at 5.7 nmol/min per mg of protein; likewise synaptic mitochondria (fraction SM₂) under the conditions of Fig. 3 accumulated glutamate at the rate of 1.12 nmol/min per mg of protein and in the absence of arsenite at 1.09 nmol/min per mg of protein (J. M. Walsh & J. B. Clark, unpublished work)]. It is possible, however, that the high NADH concentration induced by rotenone might inhibit the succinate semialdehyde dehydrogenase activity which in turn will lead to an inhibited 4-aminobutyrate aminotransferase–succinate semialdehyde dehydrogenase shunt. The effect of increasing the NADH/NAD⁺ ratio on the activity of the shunt pathway is shown in Fig. 4 for the 'free' mitochondria. Increased NADH concentrations inhibited the 4-aminobutyrate-metabolizing system competi-

Fig. 3. Effect of rotenone on 4-aminobutyrate metabolism

Mitochondrial protein (3–4 mg) was incubated in 2 ml of the 100 mM- K^+ medium. The rate of glutamate production (extra-mitochondrial + intramitochondrial) is plotted for each mitochondrial population. (a) ○, Glutamate production from 'free' mitochondria in the presence of 5 mM-4-aminobutyrate, 2.5 mM-2-oxoglutarate and 10 mM-malonate; ●, as above, but additionally 2.5 μM-rotenone and 0.75 mM-arsenite were present. (b) Δ, Glutamate production from synaptic mitochondria in the presence of 5 mM-4-aminobutyrate, 2.5 mM-2-oxoglutarate and 10 mM-malonate; ▲, as above, but additionally 2.5 μM-rotenone and 0.75 mM-arsenite were present.

Fig. 4. Effect of NADH on the 4-aminobutyrate aminotransferase-succinate semialdehyde dehydrogenase shunt in 'free' brain mitochondria

The enzyme activity was assayed as described under 'Methods', and is expressed as nmol of NADH produced/min per mg of mitochondrial protein. The V_{max} of this linked system was 20.4 nmol/min per mg of protein and the K_m for NAD $^+$ was 22 μM. The initial rate of activity over the first minute was measured. At maximum enzyme activity this increased the NADH concentration in the cuvette by a maximum of 11% over that originally added; no correction has been made for this. ○, no added NADH; ■, 150 μM-NADH; Δ, 200 μM-NADH.

tively with an 'apparent' K_i for NADH of 102 μM. Similar results were obtained for synaptic mitochondria (fraction SM $_2$).

The effect of the state of energization of both mitochondrial populations on the rate of 4-aminobutyrate metabolism is shown in Fig. 5, in which the mitochondria were incubated under State 3 (+ADP) and State 4 (−ADP) conditions. Little difference was observed in the glutamate accumulation of both populations under State 4 conditions, but the State 3 rate of glutamate accumulation by the 'free' mitochondria (fraction M) was almost 50% higher at 2.7 nmol/min per mg of protein than for the synaptic mitochondria (fraction SM $_2$) at 1.9 nmol/min per mg of protein. It is noteworthy that glutamate accumulation is lower under State 3 conditions than under State 4 conditions. This apparently lower rate may be explained by an increased metabolism of the glutamate accumulated under these conditions. From the measurements of aspartate production (Fig. 5) under these conditions it seems unlikely that the decreased glutamate accumulation from 4-aminobutyrate in State 3 conditions may be explained by an increased glutamate metabolism via transamination. Very little difference in the production of extramito-

Fig. 5. Effect of State 3 and State 4 on 4-aminobutyrate metabolism by free and synaptic mitochondria

Mitochondria were prepared as described in the Materials and Methods section. (a) 'Free' (M) mitochondria; (b) synaptic (SM₂) mitochondria. Approx. 4mg of mitochondrial protein was incubated in 2ml of the 100mM-K⁺ medium (see under 'Methods') in the presence of 5mM-4-aminobutyrate, 2.5mM-2-oxoglutarate and 1mM-malate under State 3 and State 4 conditions (Chance & Williams, 1956). The mitochondria were separated from the medium and metabolites assayed as described in the Materials and Methods section; State 3 was initiated with 1mM-ADP and maintained by a hexokinase trap. ●, Extramitochondrial glutamate concentration (State 4); □, extramitochondrial glutamate concentration (State 3); △, extramitochondrial aspartate concentration (State 4); ■, extramitochondrial aspartate concentration (State 3).

chondrial aspartate was observed between State 3 and State 4 conditions, and intramitochondrial aspartate was not detectable. However, preliminary experiments with an ammonium electrode indicated an increase in NH₃ production in State 3 conditions, suggesting an increased metabolism of glutamate by glutamate dehydrogenase (J. M. Walsh, unpublished work).

The low rates of aspartate production in the experiments shown in Fig. 5 may be the result of the high concentrations of 2-oxoglutarate present in the incubation mixture, since Braunstein (1973) has shown that purified glutamate-oxaloacetate transaminase is inhibited by 2-oxoglutarate. Fig. 6 indicates the rates of extramitochondrial production of glutamate, 2-oxoglutarate and aspartate from 4-aminobutyrate metabolism under State 3 (Figs. 6a and 6c) and State 4 (Figs. 6b and 6d) conditions when 2-oxoglutarate was being generated intramitochondrially from added pyruvate and malate.

The rate of aspartate production markedly increased in both mitochondrial populations compared with that seen previously (Fig. 5) in the presence of high 2-oxoglutarate concentrations. In fact, in the 'free' brain mitochondria the rate of aspartate

production in State 3 conditions (1.9nmol/min per mg of protein; Fig. 6a) is slightly higher than the rate of glutamate produced (1.6nmol/min per mg of protein), whereas in the synaptic mitochondria (fraction SM₂) (Fig. 6c) aspartate (0.7nmol/min per mg of protein) is produced at about half the rate of glutamate. These differences may be a reflexion of the glutamate metabolism in the two mitochondrial populations, since the rate of glutamate produced under these conditions is a net rate of glutamate production from 4-aminobutyrate aminotransferase activity and glutamate metabolism via glutamate dehydrogenase. A tentative estimate of the flux through the 4-aminobutyrate shunt in both types of mitochondria under State 3 conditions may be made by summing the rates of glutamate and aspartate production (Figs. 6a and 6c), since aspartate arises from part of the glutamate produced via 4-aminobutyrate transamination. These fluxes [3.5 nmol/min per mg of protein for 'free' (fraction M) mitochondria and 2nmol/min per mg of protein for synaptic (fraction SM₂) mitochondria] are, however, underestimates, as no allowance has been made for glutamate deamination. On the basis of these estimates, 54% of the glutamate formed undergoes transamination in

Fig. 6. 4-Aminobutyrate metabolism using intramitochondrially generated 2-oxoglutarate in 'free' (a and b) and synaptic (c and d) rat brain mitochondria

Mitochondrial protein (3–4 mg) was incubated in 2 ml of 100 mM-K⁺ medium (see under 'Methods') together with 5 mM-4-aminobutyrate, 5 mM-pyruvate and 1 mM-malate. State 3 (a and c) and State 4 (b and d) (Chance & Williams, 1956) conditions were maintained as described under 'Methods'. The rates of metabolite production are extramitochondrial plus intramitochondrial values. ●, 2-Oxoglutarate concentration; Δ, glutamate concentration; ■, aspartate concentration.

'free' (fraction M) mitochondria, whereas only 35% is transaminated in the synaptic mitochondria (fraction SM₂).

Experiments similar to those in Figs. 6(a) and 6(c), except that State 4 (i.e. ADP absent) conditions prevail, are shown in Figs. 6(b) and 6(d). Glutamate was produced at a rate of 3.2 nmol/min per mg of protein by the 'free' mitochondria (fraction M) and

3.3 nmol/min per mg of protein by the synaptic (fraction SM₂) mitochondria. These rates are similar to those obtained under State 4 conditions when externally applied 2-oxoglutarate was used (Fig. 5). However, aspartate production was very low under these conditions, being 0.6 nmol/min per mg of protein (fraction M) and 0.3 nmol/min per mg of protein (fraction SM₂), representing 16 and 8% respectively

Fig. 7. Effect of potassium concentration on 4-aminobutyrate metabolism in 'free' (M) mitochondria (a) and synaptic (SM₂) mitochondria (b)

Mitochondrial protein (3–4 mg) was incubated in 2 ml of the 5 or 100 mM-K⁺ medium (see under 'Methods') in the presence of 5 mM-4-aminobutyrate, 2.5 mM-2-oxoglutarate and 1 mM-malate under State 3 conditions (see under 'Methods'). The rates of glutamate production are measured by summing the extra- and intra-mitochondrial concentrations. □, In 100 mM-K⁺ medium; ●, in 5 mM-K⁺ medium.

of the total glutamate production. Further, Figs. 6(b) and 6(d) show that in comparison with the experiments in Fig. 5, the rate of 2-oxoglutarate production no longer increases linearly with time of incubation.

Stimulation of brain mitochondrial respiration by K⁺ ions has been reported by several authors (see Nicklas *et al.*, 1971) and rapid release of 4-aminobutyrate by K⁺ ions has been observed by Machiyama *et al.* (1967). However, no change in 4-aminobutyrate metabolism as measured by glutamate production occurred when the K⁺ concentration was increased from 5 to 100 mM under conditions similar to those in Fig. 2. Also no change in 4-aminobutyrate metabolism was observed when K⁺ ion concentration was similarly increased under State 4 (ADP absent) conditions (cf. Fig. 5). However, under State 3 conditions (cf. Fig. 5), although no change in the rate of glutamate production occurred with 'free' mitochondria (fraction M), an increase in the K⁺ ion concentration from 5 to 100 mM (Fig. 7) with the synaptic mitochondria (fraction SM₂) caused the rates of glutamate production to fall from 2.7 nmol/min per mg of protein in 5 mM-K⁺ to 2.1 nmol/min per mg of protein in 100 mM-K⁺. Previous experiments indicated that the rates of aspartate (see Fig. 5) production under these conditions could not account for the differences in the rates of glutamate production.

One explanation might be that the increase in K⁺ ion concentration stimulated the deamination pathway of glutamate metabolism in the synaptic (fraction SM₂) mitochondrial population.

General Discussion

4-Aminobutyrate metabolism may theoretically be affected by a number of parameters: notable amongst these must be the availability of 2-oxoglutarate for transamination and the redox state of the mitochondria particularly with respect to the succinate semialdehyde dehydrogenase enzyme. The studies reported here are further complicated, since 4-aminobutyrate metabolism as measured by glutamate accumulation is subject to any variations in glutamate metabolism itself that may occur under the conditions used. From the data of Fig. 1 and of other workers (Brand & Chappell, 1974) it appears that access of 4-aminobutyrate to the mitochondrial matrix would not normally present a control point. However, carrier-mediated transport systems for 4-aminobutyrate with *K_m* values in the region 10⁻⁸–10⁻⁶ M have been reported in brain slices (Bond, 1973) and may be important in the overall control of brain 4-aminobutyrate metabolism. The dependence of 4-aminobutyrate metabolism on the presence of

2-oxoglutarate (Fig. 5) and its stimulation in the presence of malate (malonate) (Fig. 2) suggest that a potential control point may exist at the level of the brain 2-oxoglutarate-malate mitochondrial translocase system. The sensitivity of 4-aminobutyrate metabolism to 2-oxoglutarate is also shown in the experiments of Fig. 6, where 2-oxoglutarate was generated intramitochondrially from pyruvate and malate and not added externally as in Fig. 5. This leads to substantial rates of aspartate production in both mitochondrial populations, suggesting that, in the presence of high external 2-oxoglutarate (Fig. 5), glutamate metabolism by the glutamate-oxaloacetate transaminase might be drastically inhibited. Recent studies have indicated that the glutamate-oxaloacetate transaminase of both the 'free' (M) and synaptic (SM₂) mitochondrial populations is competitively inhibited by 2-oxoglutarate (K_i of 2-oxoglutarate for 'free' mitochondria, 1.45 mM and for synaptic mitochondria 1.1 mM; Dennis, 1976). It should also be noted from the data of Fig. 6 that under State 4 conditions (Chance & Williams, 1956) the rate of 2-oxoglutarate production decreased with time, whereas under State 3 conditions it remained linear. The increased redox and phosphate potential characteristic of State 4 conditions tends to decrease the flux through the citric acid cycle which might be responsible for this decrease in 2-oxoglutarate availability. All these observations suggest that the intramitochondrial 2-oxoglutarate concentration might play an important role in controlling 4-aminobutyrate metabolism.

It is also evident from the data of Fig. 4 that the redox state of the mitochondrial NAD will play an important role in the control of the 4-aminobutyrate-shunt activity. It is noteworthy that the apparent K_i of NADH for this system (Fig. 4) is of the same order as the concentration of NADH found in 'free' brain mitochondria (fraction M) in State 4 conditions in the presence of rotenone (Clark & Nicklas, 1970). There were, however, only very minor differences in the rate of 4-aminobutyrate metabolism under State 4 conditions between the two populations of brain mitochondria in the presence of externally added 2-oxoglutarate (Fig. 5). Under State 3 conditions, however, the rates of glutamate produced paralleled the enzyme activities of the two mitochondrial populations, the 'free' mitochondria (fraction M) having the potential to metabolize 4-aminobutyrate approximately 1.5 times as fast as the synaptic mitochondria (fraction SM₂) (Table 1). The fact that the State 3 rates of glutamate production are actually lower than those of State 4 (Fig. 5) may be a reflexion of the fact that more of the glutamate produced from 4-aminobutyrate is being metabolized under State 3 conditions than under State 4 conditions. This may be due to the high NADH/NAD⁺ ratio under State 4 conditions, inhibiting the glutamate dehydrogenase

activity as was suggested by the presence of increased NH₃ concentrations in the medium during the State 3 incubations, despite the fact that both mitochondrial populations possess glutamate-oxaloacetate transaminase activities at least an order of magnitude higher than those of glutamate dehydrogenase (Lai *et al.*, 1975).

Increasing the potassium concentration from 5 mM to 100 mM caused little alteration in the rate of glutamate production from 'free' brain mitochondria (fraction M) under State 3 conditions (Fig. 7a). This observation is consistent with the observations of Machiyama *et al.* (1970) on brain slices, who noted that, although on increasing the K⁺ ion concentration from 6 to 66 mM the flux through the citric acid cycle increased by some 2.5-fold, the 4-aminobutyrate shunt was little affected. However, with the synaptic mitochondria (fraction SM₂), less glutamate appears to be metabolized in the presence of 5 mM-K⁺ than in the presence of 100 mM-K⁺, suggesting that the glutamate dehydrogenase pathway is stimulated by high concentrations of K⁺. This effect may have important physiological implications in view of the suggestion that in addition to the release of 4-aminobutyrate on depolarization of the pre-synaptic membrane, a continuous release of 4-aminobutyrate also occurs independently of the state of polarization of the membrane (Tapia, 1974). Therefore the synaptic brain mitochondria (fraction SM₂), derived from the pre-synaptic nerve terminals, would require not only a continual 4-aminobutyrate-metabolizing system, but also a system to cope with the discontinuous release of 4-aminobutyrate acting in a neurotransmitter role. Such a system might also be expected to be responsive to changes in K⁺ ion concentration. It is tempting to suggest therefore that the glutamate dehydrogenase and its potassium-sensitivity observed in the experiments of Fig. 7 of the synaptic mitochondria might fulfil such a role.

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