Biochem. J. (1979) **184**, 539–546
Printed in Great Britain

Synthesis of *N*-Acetyl-L-aspartate by Rat Brain Mitochondria and its Involvement in Mitochondrial/Cytosolic Carbon Transport

By Tarun B. PATEL* and John B. CLARK
Department of Biochemistry, St. Bartholomew's Hospital Medical College, University of London, Charterhouse Square, London EC1M 6BQ, U.K.

(*Received* 9 *April* 1979)

1. The synthesis and efflux of *N*-acetyl-L-aspartate from brain mitochondria of rats of different ages has been studied. 2. Brain mitochondrial State 3 (+ADP) respiration rate, using 10mM-glutamate and 2.5mM-malate as substrates, increases during the suckling period and reaches approx. 50% of the adult value at 17 days after birth [adult State 3 respiration rate = 160±7ng-atoms of O/min per mg of mitochondrial protein (mean±S.D.; $n = 3$)]. 3. The influence of 5mM-pyruvate or 10mM-DL-3-hydroxybutyrate on aspartate efflux from brain mitochondria from rats of different ages oxidizing glutamate and malate was studied. In all cases the aspartate efflux in State 3 was greater than in State 4, but, whereas the aspartate efflux in State 3 increased as the animals developed, that of State 4 showed only a small increase. However, the rate of aspartate efflux in the presence of pyruvate or 3-hydroxybutyrate as well as glutamate and malate was approx. 60–65% of that in the presence of glutamate and malate alone. 4. An inverse relationship between aspartate efflux and *N*-acetylaspartate efflux was observed with adult rat brain mitochondria oxidizing 10mM-glutamate and 2.5mM-malate in the presence of various pyruvate concentrations (0–5mM). 5. *N*-Acetylaspartate efflux by brain mitochondria of rats of different ages was studied in States 3 and 4, utilizing 5mM-pyruvate or 10mM-DL-3-hydroxybutyrate as acetyl-CoA sources. A similar pattern of increase during development was seen in State 3 for *N*-acetylaspartate efflux as for aspartate efflux (see point 3 above). Also only very small increases in *N*-acetylaspartate efflux occurred during development in State 4. 6. Rat brain mitochondria in the presence of iso-osmotic *N*-acetylaspartate showed some swelling which was markedly increased in the presence of malate. 7. It is concluded that *N*-acetylaspartate may be synthesized and exported from both neonatal and adult rat brain mitochondria. It is proposed that the *N*-acetylaspartate is transported by the dicarboxylic acid translocase and may be an additional mechanism for mitochondrial/cytosolic carbon transport to that of citrate.

The work of Tallan *et al.* (1954, 1955) showed that protein-free extracts of cat brain contained appreciable concentrations (approx. 0.8mg/g wet wt. of brain) of bound aspartate, which was subsequently identified as *N*-acetyl-L-aspartate by ion-exchange chromatography. Similar concentrations were found in rat brain (1mg/g wet wt. of brain), but only 0.01–0.03mg/g wet wt. in other tissues e.g. liver, kidney and muscle. Further regional studies on mouse brain (Tallan, 1957) indicated that *N*-acetyl-L-aspartate was present in all regions with particularly high concentrations in the cerebral grey matter (1.24mg/g wet wt.) and low concentrations in the medulla (0.55mg/g wet wt.). From the point of view of development, rat brain at birth contains only 0.2mg of *N*-acetylaspartate/g wet wt., which increased rapidly to approx. 1.2mg/g wet wt. at weaning (21 days) and then decreased to the adult value (approx. 1mg/g wet wt.) (Tallan, 1957). D'Arcangelo *et al.* (1963) have reported that 85% of the acetylated aspartate is present in the cytosol and Buniatian *et al.* (1965) have demonstrated in brain acetone powders that *N*-acetylaspartate may act as an acetyl-group donor to both glucosamine and choline. By using doubly labelled *N*-acetylaspartate, injected parasagittally into rats, D'Adamo & Yatsu (1966) have demonstrated that the acetyl group of *N*-acetylaspartate is incorporated into brain lipids three times more effectively than acetate alone. Furthermore, in the immature rat brain maximum incorporation of the acetyl moiety of *N*-acetylaspartate into lipids occurs just before and during myelination, i.e. from approx. 8 days after birth (D'Adamo *et al.*, 1968). These

* Present address: Department of Biochemistry, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284, U.S.A.

studies also suggested from the constant $^3H/^{14}C$ ratio in the lipids formed from doubly labelled *N*-acetylaspartate that the mechanism by which the acetyl moiety of *N*-acetylaspartate is incorporated into lipids is similar throughout the developmental period.

In the rat, Benuck & D'Adamo (1968) demonstrated that the enzyme responsible for *N*-acetyl-L-aspartate synthesis, L-aspartate *N*-acetyltransferase (EC 2.3.1.17), was only present in nervous tissue, whereas the enzyme of *N*-acetyl-L-aspartate breakdown (*N*-acetyl-L-aspartate amidohydrolase, EC 3.5.1.15) was widely distributed in liver, kidney, heart and adipose tissue, as well as in the brain. Knizley (1967) suggested that the *N*-acetyl-L-aspartate transferase was located in the particulate fraction of brain homogenates and not in the cytosol as previously reported (Goldstein, 1959; McIntosh & Cooper, 1965). The suggestion that the enzyme was particulate was further investigated by Goldstein (1969), who claimed that the transferase was bound to 'light' microsomal fraction and 'active particulate components' of the crude mitochondrial fraction. The *N*-acetyl-L-aspartate amidohydrolase, however, has a cytosolic location and shows an 8-fold increase in activity from birth to adulthood in rat brain (D'Adamo *et al.*, 1973).

In view of the proposals that *N*-acetylaspartate may function as an acetyl-group donor in cytosolic biosynthetic reactions in the brain and the reported compartmentation between the enzymes of *N*-acetylaspartate metabolism, the experiments reported in the present paper were aimed at elucidating whether (*a*) *N*-acetyl-L-aspartate could be synthesized intramitochondrially and transported out of the mitochondria and (*b*) if this activity changed during brain development consistent with changes in the need for brain cytosolic biosynthetic activities, e.g. lipid formation.

Materials and Methods

ADP, NADH, glutamate–oxaloacetate transaminase (EC 2.6.1.1), malate dehydrogenase (EC 1.1.1.37). lactate dehydrogenase (EC 1.1.1.27) and hexokinase (EC 2.7.1.1) were obtained from Boehringer Corp., London, U.K. Hexokinase was dialysed three times against 50vol. of 50mM-potassium phosphate buffer, pH7.0, and stored at −20°C. This procedure ensured removal of $(NH_4)_2SO_4$ and glycerol from the enzyme. 3-Hydroxybutyrate and *N*-acetyl-L-aspartate were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Koch–Light Laboratories, Colnbrook, Bucks., U.K., supplied pyruvate, which was purified by distillation twice under vacuum. The purified acid was stored at −20°C. Ficoll was obtained from Pharmacia (Uppsala, Sweden) and purified by dialysis against glass-distilled water. Versilube F50 was a gift from Jacobson, Van den Berg and Co., London W3 7RH, U.K., and Bisoflex was from B.P. Chemicals International, London, U.K.

All other reagents used were of the highest quality commercially available and all solutions were made up in double-glass-distilled water.

Animals

Rats of the Wistar strain were used. The birth dates of all litters were carefully recorded after daily inspection and culled to 8–10 pups. Animals of either sex were used up to weaning (21 days), but after this only males were used. Food and water were always available.

Mitochondrial experiments

Rat brain mitochondria were prepared by the method of Clark & Nicklas (1970). Incubations containing 1–3mg of mitochondrial protein were carried out in 1–2.5ml of a medium containing (final concentrations) 100mM-KCl, 75mM-mannitol, 25mM-sucrose, 10mM-phosphate/Tris, 10mM-Tris/HCl and 50μM-EDTA, pH7.4. All incubations were stirred and carried out in a thermostatically controlled (25°C) microincubation chamber. O_2 was blown over the top to prevent anaerobiosis. In addition the duration of the incubation was kept strictly limited to prevent oxygen limitation occurring, the extent of such incubations being calculated from previously determined O_2-uptake data. State 3 (Chance & Williams, 1956) was induced either by the presence of a hexokinase trap [20mM-glucose, 5mM-$MgCl_2$, 1mM-ADP and dialysed hexokinase (approx. 0.1 unit/mg of mitochondrial protein)] or by a large excess of ADP. One unit of hexokinase is the amount of enzyme required to convert 1μmol of substrate/min. Aliquots (0.5–1ml) of the incubation mixture were withdrawn at timed [0→2.5min] intervals and pipetted gently into microcentrifuge tubes (1.5ml) containing 0.5ml of silicone oil [Bisoflex/Versilube F50 (1:4, v/v)] layered on top of 50–100μl of 18% (w/v) $HClO_4$, and centrifuged at 15000*g* for 2min in an Eppendorf microcentrifuge. A known volume of the resulting upper phase was then transferred to 50μl of 18% $HClO_4$, neutralized to pH6.4±0.2 with 3M-K_2CO_3 in 0.5M-triethanolamine, recentrifuged at 15000*g* for 2min to remove precipitated protein and $KClO_4$ and the supernatant taken for metabolite assay.

Metabolite assay

The assay of aspartate was carried out essentially as described by Williamson & Corkey (1969).

Preparation of N-acetyl-L-aspartate amidohydrolase

N-Acetyl-L-aspartate assay required the prep-

aration of the *N*-acetyl-L-aspartate amidohydrolase. This was carried out essentially by the method of Birnbaum (1955) with some modifications (cf. Fleming & Lowry, 1966). Fresh frozen pig kidneys (2.5kg) were thawed, defatted and homogenized in a Waring blender with 2vol. of double-distilled water at 2°C. The homogenate was strained through cheesecloth and centrifuged at 2000*g* for 20min to remove cellular debris. The preparation was chilled to 2°C in an ice-cold bath and brought to pH4.7 by careful addition of 2M-HCl. The resulting thick suspension was immediately centrifuged at 2°C and 10000$g_{av.}$ for 20min. The sediment was discarded and the clear red supernatant was adjusted to pH6.5 with 2M-NaOH. To this was then added 266g of solid $(NH_4)_2SO_4$/litre of solution [final concn. 1.97M-$(NH_4)_2SO_4$], whereupon the pH increased from 6.0 to 6.2. The resulting precipitate, which contained most of the amidohydrolase I (EC 3.5.1.14) activity, was separated by centrifugation at 2000$g_{av.}$ for 15min and the resulting precipitate was dissolved in an equal volume of 0.1M-sodium phosphate buffer, pH6.8, and heated for 10min at 70°C. After centrifugation (2000$g_{av.}$ for 10min) the enzyme was again precipitated from the supernatant by addition of solid $(NH_4)_2SO_4$ to a final concentration of 2.8M. Purification at this stage was about 7-fold (specific activity approx. 0.84 units/mg of protein) and the enzyme remained stable at −20°C for several months. One unit of enzyme is the amount required to convert 1μmol of substrate/min. The preparation contained very little aspartate, but was considerably contaminated by lactate dehydrogenase.

Measurement of N-acetyl-L-aspartate

A spectrophotometric assay for the metabolite was developed by extending the aspartate assay of Williamson & Corkey (1969). The principle may be understood from eqns. (1)–(3):

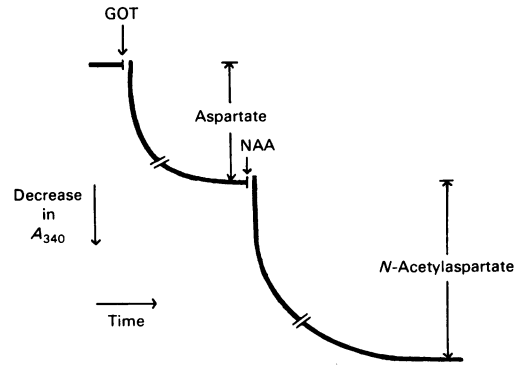

Fig. 1. *Assay for aspartate and N-acetylaspartate*
A typical spectrophotometric trace for the assay of aspartate and *N*-acetyl-L-aspartate in the same sample (see the Materials and Methods section for details). Abbreviations used: GOT, glutamate–oxaloacetate transaminase; NAA, *N*-acetylaspartate amidohydrolase.

$$\textit{N}\text{-Acetyl-L-aspartate} \xrightarrow{\textit{N}\text{-Acetylaspartate amidohydrolase}} \text{aspartate} + \text{acetate} \quad (1)$$

$$\text{Aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{Glutamate–oxaloacetate transaminase}} \text{oxaloacetate} + \text{glutamate} \quad (2)$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{Malate dehydrogenase}} \text{malate} + \text{NAD}^+ \quad (3)$$

Hence only eqn. (1) is additional to the aspartate assay. Since all incubations carried out for acetylaspartate synthesis also generated a relatively high concentration of aspartate it was necessary to first measure the free aspartate and subsequently the aspartate formed from *N*-acetylaspartate by the action of the amidohydrolase. Thus the incubation extract was first incubated with glutamate–oxaloacetate transaminase and malate dehydrogenase for 5min (i.e. eqns. 2 + 3) until all the free aspartate had been converted and measured (see Fig. 1). At this stage, 0.2mg of *N*-acetyl-L-aspartate amidohydrolase, purified from pig kidney was added. The aspartate released by the action of this enzyme on the *N*-acetylaspartate was then measured as a further oxidation of NADH (see Fig. 1).

Certain precautions in this assay had to be carried out when measuring aspartate and *N*-acetylaspartate in samples containing pyruvate since the lactate dehydrogenase contamination of the *N*-acetylaspartate amidohydrolase would convert pyruvate into lactate, thus increasing the NADH oxidation. This was carried out by adding to the incubation extract initially (i.e. before the addition of glutamate–oxaloacetate transaminase) an aliquot [100 units of activity] of commercially available lactate dehydrogenase to convert pyruvate into lactate. Thus the complete reaction mixture for the measurement of both aspartate and *N*-acetylaspartate was (final concentrations): 50mM-triethanolamine/HCl, pH7.4; 10mM-$MgSO_4$; 5mM-EDTA; 10mM-2-oxoglutarate; 20μM-NADH; 5μg (6 units) of malate dehydrogenase. On the addition of 0.1mg (20 units) of glutamate–oxaloacetate transaminase, aspartate

could be measured. Subsequent addition of 0.2mg (0.17 units) of acetylaspartate amidohydrolase yielded a quantitative estimate of acetylaspartate. Fig. 1 indicates a typical spectrophotometric profile for such an assay.

Proteins

These were measured by the method of Gornall *et al.* (1949) with bovine plasma albumin as standard.

Results

In all of the studies reported in the present paper non-synaptic brain mitochondria (see Clark & Nicklas, 1970) were investigated. Furthermore only those with respiratory-control ratios in excess of 4 and undetectable oxidation of NADH were used. In the studies to assess the potential of these mitochondria to synthesize and export *N*-acetylaspartate, both acetyl-CoA and aspartate had to be provided intramitochondrially and since neither of these precursors can be transported into the mitochondria (Dennis, 1976; Dennis *et al.*, 1977) this had to be done indirectly. The acetyl-CoA was provided by allowing the mitochondria to oxidize either pyruvate or 3-hydroxybutyrate in the presence of malate. Glutamate, which is known to enter the mitochondrial matrix by the glutamate–aspartate translocase (Dennis *et al.*, 1976) and to be metabolized in brain mitochondria mainly by transamination (Dennis & Clark, 1977), supplied the intramitochondrial aspartate. Malate was added so that the oxaloacetate availability for transamination was not limiting.

Fig. 2 shows the O_2-uptake rates when mitochondria derived from brains of rats of different ages were oxidizing glutamate (10mM) in the presence of malate (2.5mM) in State 3 (i.e. plus ADP). These were prepared and showed similar purity and metabolic integrity to those previously described (Land *et al.*, 1977). The mitochondrial respiration rates increased gradually in animals between 5 and 14 days of age and then increased markedly in animals between 14 and 21 days old and in 21 day-old animals was approx. 77% of the adult rate, the latter being 160±6ng-atoms of O/min per mg of mitochondrial protein (mean±S.D., $n = 3$). This pattern of development of O_2 uptake is similar to the developmental profile of rat brain mitochondrial glutamate–oxaloacetate transaminase described by Amores & Bonavita (1965). The oxidation of pyruvate and malate by brain mitochondria from rats of different ages shows a somewhat similar profile to that of the oxidation of glutamate and malate (Land *et al.*, 1977), but that of 3-hydroxybutyrate and malate is different in that it reaches a peak in animals 21 days old and then decreases to approx. 60% of this value in the adult brain (Land *et al.*, 1977).

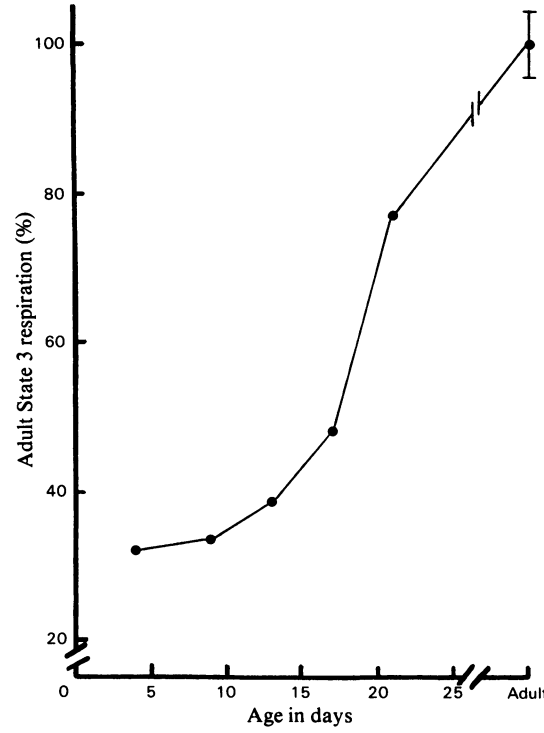

Fig. 2. *Glutamate oxidation by rat brain mitochondria* Mitochondria were isolated and incubated in a medium containing 100mM-K^+ (see the Materials and Methods section). Glutamate (10mM) and malate (2.5mM) were added as substrates and State 3 (Chance & Williams, 1956) was induced by adding 250μM-ADP. The results are plotted as the mean values, expressed as a percentage of the adult value, for at least three experiments, each value of which was derived from a pooled preparation of six animals. Standard deviations were within 7% of the mean values. Adult rat brain mitochondria showed State 3 respiration rates of 160±6ng-atoms of O/min per mg of mitochondrial protein (mean±S.D.; $n = 3$).

When mitochondria were incubated with glutamate, malate and either pyruvate or 3-hydroxybutyrate in either State 3 or State 4 (Chance & Williams, 1956), aspartate and acetylaspartate efflux was observed. Control experiments in the absence of an exogenous intramitochondrial source of acetyl-CoA (i.e. pyruvate or 3-hydroxybutyrate) showed no acetylaspartate efflux; aspartate efflux, however, persisted. Fig. 3 shows the aspartate-efflux rates from brain mitochondria of rats of different ages metabolizing glutamate plus malate in the presence and absence of either pyruvate or 3-hydroxybutyrate in State 3 (+ADP) or State 4 (−ADP). At all ages in either State 3 or State 4 the rate of aspartate efflux in the

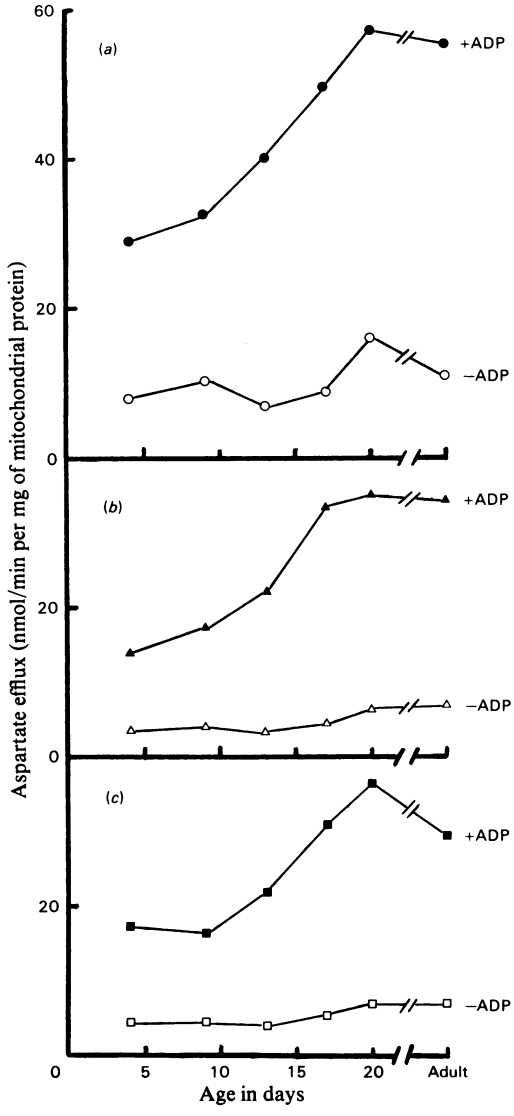

Fig. 3. *Development of aspartate efflux from rat brain mitochondria*

Mitochondria from the brains of rats of different ages were incubated in a medium containing 100mM-K^+ (see the Materials and Methods section) and 2.5mM-malate, and the additional compounds indicated below. Symbols: ●, 10mM-glutamate, State 3; ○, 10mM-glutamate, State 4; ▲, 10mM-glutamate+5mM-pyruvate, State 3; △, 10mM-glutamate+5mM-pyruvate, State 4; ■, 10mM-glutamate+10mM-DL-3-hydroxybutyrate, State 3; □, 10mM-glutamate+10mM-DL-3-hydroxybutyrate, State 4. State 3 was induced by a hexokinase trap and mitochondria were separated from the medium (see the Materials and Methods section). The 'mitochondria-free' supernatant was assayed for aspartate. Each value is a mean of at least two different experiments in each of which the metabolite was measured at a minimum of three time points. Standard deviations were within 7% of the mean values and the rates of aspartate efflux are expressed as nmol/min per mg of mitochondrial protein.

presence of glutamate and malate alone (Fig. 3*a*) was higher than when either pyruvate (Fig. 3*b*) or 3-hydroxybutyrate (Fig. 3*c*) were present in addition. Thus in State 3 the rate of aspartate efflux from mitochondria oxidizing glutamate and malate alone

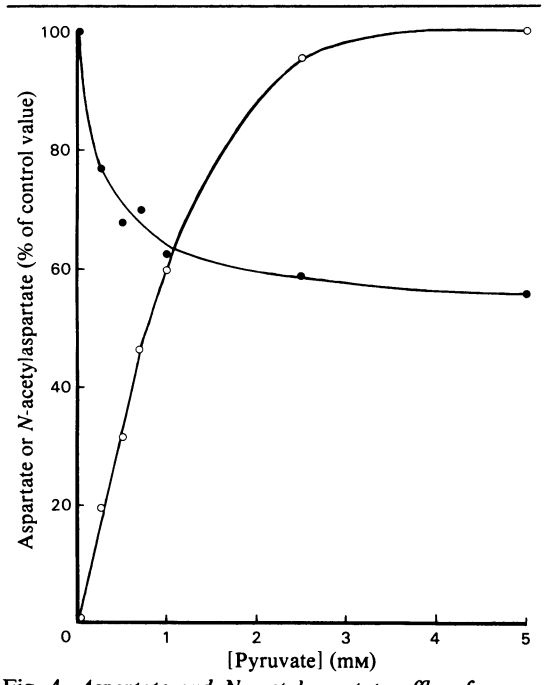

Fig. 4. *Aspartate and N-acetylaspartate efflux from rat brain mitochondria*

Adult rat brain mitochondria were incubated under State 3 conditions (see the Materials and Methods section) in the presence of 2.5mM-malate, 10mM-glutamate and various concentrations of pyruvate (0–5mM). Aspartate (●) and *N*-acetylaspartate (○) were assayed at several time points in the 'mitochondria-free' incubation medium (see the Materials and Methods section). Zero-time samples were taken immediately after the instigation of State 3 in the presence of all components. The results are expressed as a percentage of the control values where the control rate of aspartate efflux (in the absence of added pyruvate) was 54±3nmol/min per mg of mitochondrial protein (mean±S.D.; $n=3$) and for *N*-acetylaspartate efflux (in the presence of 5mM-pyruvate) was 8.6±0.6nmol/min per mg of mitochondrial protein (mean±S.D.; $n=3$). Each value is a mean of at least two distinct experiments in each of which the metabolite was measured at a minimum of three time points to calculate the rate. Standard deviations were within 7% of the mean values.

increased approx. 2-fold between 5 and 21 days of age. The increased efflux rate at 21 days was then maintained into adulthood. The approximate doubling of the aspartate efflux rate over this developmental period was also observed with mitochondria oxidizing glutamate plus malate and additionally pyruvate (Fig. 3*b*) or 3-hydroxybutyrate (Fig. 3*c*) although the actual rates were approx. 60–65% of the aspartate efflux rates in the presence of glutamate and malate alone.

In State 4 the aspartate efflux from brain mitochondria was considerably lower than that in State 3 at all ages of rats with any combination of substrates used. This presumably reflects the energy dependence of the glutamate–aspartate translocase (Dennis *et al.*, 1976). However, whereas in State 3 there was an approximate doubling of the aspartate efflux rate between 5 and 21 days, with all substrates tested in State 4 only, very small insignificant changes were observed during the development of the animal from 5 days to adulthood. However, when actual rates of aspartate efflux were observed the rates in the presence of glutamate, malate and additionally pyruvate or 3-hydroxybutyrate were only 60–65% of those in the presence of glutamate and malate alone, similar to the difference observed in State 3.

Fig. 4 shows aspartate and *N*-acetylaspartate efflux rates from adult rat brain mitochondria incubated in State 3 conditions with glutamate, malate and various concentrations of pyruvate. Maximum aspartate efflux was observed when mitochondria were incubated in the absence of added pyruvate [54±3.5nmol/min per mg of mitochondrial protein (mean±S.D., n = 3)]. With increasing pyruvate concentrations in the incubation medium the rate of aspartate efflux decreased until at 5mM-pyruvate the efflux rate was only 53% of the value in the absence of pyruvate. An increase in the pyruvate concentration above 5mM did not cause a further decrease in the rate of aspartate efflux. At the same time as the rate of aspartate efflux decreased, the rate of *N*-acetylaspartate efflux increased, reaching a maximum plateau of 8.6±0.6nmol/min per mg of mitochondrial protein (mean±S.D.; $n = 3$) at 5mM-pyruvate and above.

By using the conditions derived from Fig. 4 for optimal *N*-acetylaspartate production, i.e. 10mM-glutamate, 2.5mM-malate and 5mM-pyruvate (or 10mM-DL-3-hydroxybutyrate), acetylaspartate synthesis and efflux was studied in both State 3 and State 4 utilizing mitochondria isolated from brains of rats of different ages (Fig. 5). At all stages of development the rate of *N*-acetylaspartate synthesis and efflux by rat brain mitochondria was greater in State 3 than in State 4. Also, whereas the rate of acetylaspartate efflux in State 3 showed a marked increase with age whether pyruvate or 3-hydroxybutyrate was the source of acetyl-CoA, no such

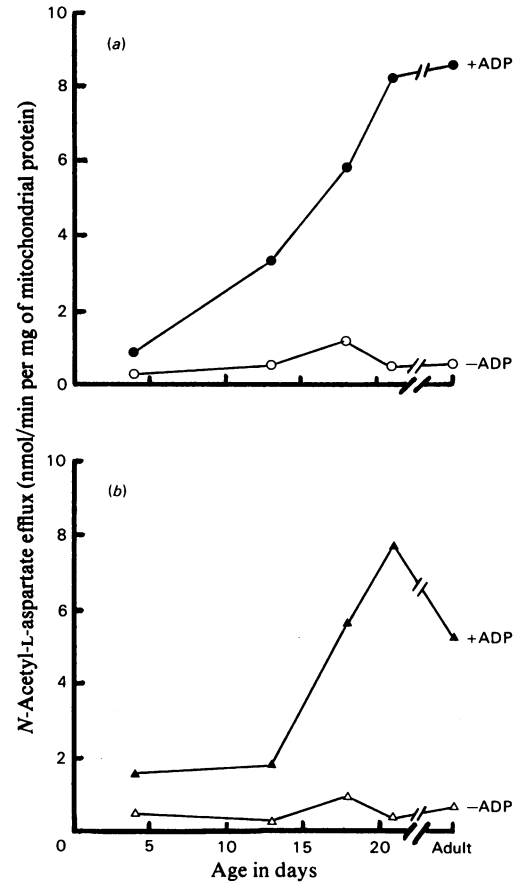

Fig. 5. *Development of N-acetylaspartate efflux from rat brain mitochondria*

Mitochondria incubation conditions were similar to those described in the legend to Fig. 3. Substrates used were 10mM-glutamate+2.5mM-malate and either (*a*) 5mM-pyruvate (State 3, ●; State 4, ○) or (*b*) 10mM-DL-3-hydroxybutyrate (State 3, ▲; State 4, △). Rates are expressed as nmol/min per mg of mitochondrial protein and each value is a mean of at least two distinct experiments in each of which the metabolite was measured at a minimum of three time points. Standard deviations were within 8% of the mean values.

increases were apparent in State 4, where the rate of efflux remained relatively low throughout all the periods of development studied. In State 3, utilizing either pyruvate or 3-hydroxybutyrate as acetyl-CoA sources, the rate of *N*-acetylaspartate efflux increased some 6–7-fold (Fig. 5) in animals between 4 and 21 days old. This high rate of *N*-acetylaspartate efflux seen in brain mitochondria from 21 day-old rats was maintained into adulthood when pyruvate was the

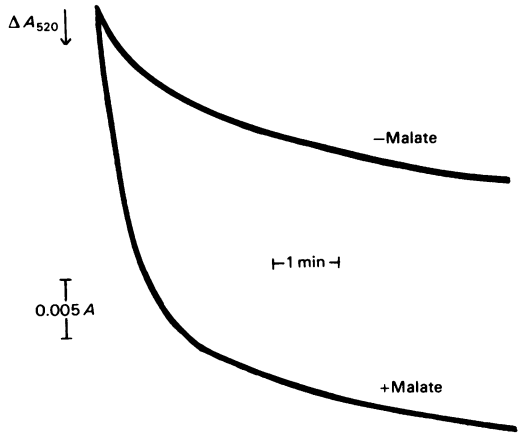

Fig. 6. *Swelling of rat brain mitochondria in iso-osmotic N-acetyl-L-aspartate*

Adult rat brain mitochondria (0.5mg of protein) were suspended in a medium containing 125mM-ammonium *N*-acetylaspartate, 20mM-Tris/HCl, 4mM-KH_2PO_4, 2mM-$MgCl_2$, 2mM-EDTA, 1 μg of rotenone and 1 μg of Antimycin A (final volume = 1 ml; pH 7.4). Changes in light scattering were followed at 520nm as outlined in the Materials and Methods section, in the presence or absence of 2.5mM-malate.

source of acetyl-CoA, but decreased by about 25% with 3-hydroxybutyrate as the source of intramitochondrial acetyl-CoA.

The swelling of mitochondria in iso-osmotic solutions of ammonium salts of permeant and non-permeant anions has been used as a method of investigating the transport of anions across the mitochondrial membrane (Chappell, 1968). Fig. 6 shows such an experiment in which the swelling of adult rat brain mitochondria in iso-osmotic ammonium acetylaspartate has been monitored. It is clear that only a small amount of swelling occurs in the presence of ammonium acetylaspartate alone, but that this is greatly enhanced in the presence of malate. Previous studies have shown that no swelling of brain mitochondria occurs in the presence of iso-osmotic ammonium aspartate (Dennis *et al.*, 1976).

Discussion

The experiments reported in the present paper indicate that rat brain mitochondria provided with a source of intramitochondrial acetyl-CoA (derived from either pyruvate or 3-hydroxybutyrate) and aspartate (from glutamate transamination) synthesize and export *N*-acetyl-L-aspartate. Control experiments without an intramitochondrial source of acetyl-CoA (Fig. 4) or aspartate showed no *N*-acetylaspartate efflux. Furthermore as the concentration of pyruvate and hence intramitochondrial acetyl-CoA increased, so did the rate of *N*-acetylaspartate efflux, whereas that of aspartate efflux decreased (Fig. 4). Several groups (Haslam & Krebs, 1963; Balázs, 1965; Nicklas *et al.*, 1971; Dennis & Clark, 1978) have reported decreased aspartate production from glutamate transamination in the presence of a source of acetyl-CoA. These authors have attributed the phenomenon to a competition between the glutamate–oxaloacetate transaminase and citrate synthase for available oxaloacetate, causing a decreased aspartate production on the introduction of more acetyl-CoA. It would appear from the results reported in the present paper that the situation is more complex, in that part of the decreased aspartate production may also be accounted for by the conversion of aspartate into *N*-acetylaspartate. From the data of Fig. 4 it is apparent that *N*-acetylaspartate production could account for about one-third of the decrease in aspartate production at saturating pyruvate concentrations.

The efflux (which in these experiments represents a composite rate of both synthesis and efflux *per se*) of both aspartate and *N*-acetylaspartate from mitochondria of rats of different ages show similar developmental profiles in that, with either pyruvate or 3-hydroxybutyrate as sources of acetyl-CoA, the rate of efflux is much faster in State 3 (+ADP) than in State 4 (−ADP) (Figs. 3 and 5). Indeed with both metabolites in State 4 there is virtually no change with development, whereas in State 3 the rates increase severalfold when the brains of animals 5 days or 21 days old are compared (Figs. 3 and 5). This apparent dependence on the mitochondrial energy state is probably a consequence of the involvement of the glutamate–aspartate translocase, which has been shown to be electrogenic and hence under coupled conditions unidirectional (glutamate inwards) and therefore strongly influenced by the mitochondrial energy state (LaNoue & Tischler, 1974; LaNoue *et al.*, 1974*a*,*b*; Dennis *et al.*, 1977).

The *N*-acetylaspartate-efflux rates (Fig. 5) seen in State 3 show a developmental pattern in the neonatal and suckling rat brain (5–21 days) that is similar to that of glutamate-oxidation rates (Fig. 2), aspartate-efflux rates in State 3 with glutamate and malate alone as substrates (Fig. 3) and with glutamate–oxaloacetate transaminase activities, as reported by Amores & Bonavita (1965). This suggests that during this period both pyruvate and 3-hydroxybutyrate are producing sufficient intramitochondrial acetyl-CoA for *N*-acetylaspartate synthesis despite the fact that the two enzyme systems involved, pyruvate dehydrogenase and 3-hydroxybutyrate dehydrogenase, show different developmental patterns (Land *et al.*, 1977). In the former case, the pyruvate dehydrogenase activity at 21 days is approx. 50% of

the adult, whereas that of 3-hydroxybutyrate dehydrogenase is almost 300% of that of the adult (Land *et al.*, 1977). The latter case may explain the decrease in *N*-acetylaspartate production seen in the adult mitochondria with 3-hydroxybutyrate as substrate, compared with when pyruvate is providing intramitochondrial acetyl-CoA (Fig. 5).

Thus in the suckling and adult rat brain, with the possible exception of the case in the adult when the brain is dependent on 3-hydroxybutyrate for acetyl-CoA production i.e. long-term starvation, *N*-acetylaspartate production is regulated by the availability of aspartate, which because of the nature of the glutamate–aspartate translocase, will be higher in State 3 than in State 4. Indeed, although mitochondrial acetyl-CoA concentrations are higher in State 4 than in State 3 (Nicklas *et al.*, 1971), the aspartate production in State 4 seems inadequate to allow significant *N*-acetylaspartate efflux.

The nature of the translocase system whereby *N*-acetylaspartate is transported from its site of synthesis in the mitochondrial matrix to the cytosol is unknown. The evidence of the iso-osmotic swelling experiments of Fig. 6 suggest that the mechanism is malate-dependent. *N*-Acetylaspartate at physiological pH values will have a double negative charge, whereas aspartate itself has a single negative charge. *N*-Acetylaspartate is therefore unlikely to fit into the electrogenic mechanism proposed for the glutamate–aspartate translocase (LaNoue & Tischler, 1974; LaNoue *et al.*, 1974*a*,*b*). The malate-dependence of the swelling experiments and its double negative charge suggest that *N*-acetylaspartate is more likely to be transported on the dicarboxylic acid translocase (see Meijer & Van Dam, 1974).

In summary the experiments reported in the present paper indicate that acetylaspartate may be synthesized in brain mitochondria of both neonatal and adult rats and may be transported across the mitochondrial membrane, probably by the dicarboxylic acid translocase. Since the enzyme *N*-acetyl-L-aspartate amidohydrolase is present in the brain cytosol (D'Adamo *et al.*, 1973), this suggests that *N*-acetylaspartate may act as a carbon transport molecule in an analogous way to citrate and provide cytosolic carbon from intramitochondrial sources. This would be in accord with the suggestion by D'Adamo & Yatsu (1966) that *N*-acetylaspartate may provide carbon for lipogenesis.

T. B. P. is grateful to the S.R.C. for a studentship. We thank Professor E. M. Crook for his continued support and encouragement.

References

Amores, G. & Bonavita, V. (1965) *Life Sci.* **4**, 2417–2424
Balázs, R. (1965) *Biochem. J.* **95**, 497–508
Benuck, M. & D'Adamo, A. F., Jr. (1968) *Biochim. Biophys. Acta* **152**, 611–618
Birnbaum, S. M. (1955) *Methods Enzymol.* **2**, 115–119
Buniatian, H. Ch., Hovhannisian, V. S. & Aprikian, G. V. (1965) *J. Neurochem.* **12**, 695–703
Chance, B. & Williams, G. R. (1956) *Adv. Enzymol Relat. Areas Mol. Biol.* **17**, 65–134
Chappell, J. B. (1968) *Br. Med. Bull.* **24**, 150–157
Clark, J. B. & Nicklas, W. J. (1970) *J. Biol. Chem.* **245**, 4724–4731
D'Adamo, A. F., Jr. & Yatsu, F. M. (1966) *J. Neurochem.* **13**, 961–965
D'Adamo, A. F., Jr., Gidez, L. I. & Yatsu, F. M. (1968) *Exp. Brain Res.* **5**, 267–273
D'Adamo, A. F., Jr., Smith, J. C. & Weiler, C. (1973) *J. Neurochem.* **20**, 1275–1278
D'Arcangelo, P., Lino, A. & Brancati, A. (1963) *Bull. Soc. Ital. Biol. Sper.* **39**, 967
Dennis, S. G. C. (1976) Ph.D. Thesis, University of London
Dennis, S. G. C. & Clark, J. B. (1977) *Biochem. J.* **168**, 521–527
Dennis, S. G. C. & Clark, J. B. (1978) *Biochem. J.* **172**, 155–162
Dennis, S. G. C., Land, J. M. & Clark, J. B. (1976) *Biochem. J.* **156**, 323–331
Dennis, S. G. C., Lai, J. C. K. & Clark, J. B. (1977) *Biochem. J.* **164**, 727–736
Fleming, M. C. & Lowry, O. H. (1966) *J. Neurochem.* **13**, 779–783
Goldstein, F. B. (1959) *J. Biol. Chem.* **234**, 2702–2705
Goldstein, F. B. (1969) *J. Biol. Chem.* **244**, 4257–4260
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
Haslam, R. J. & Krebs, H. A. (1963) *Biochem. J.* **88**, 566–578
Knizley, H., Jr. (1967) *J. Biol. Chem.* **242**, 4619–4622
Land, J. M., Booth, R. F. G., Berger, R. & Clark, J. B. (1977) *Biochem. J.* **164**, 339–348
LaNoue, K. F. & Tischler, M. E. (1974) *J. Biol. Chem.* **249**, 7522–7528
LaNoue, K. F., Bryla, J. & Bassett, D. J. P. (1974*a*) *J. Biol. Chem.* **249**, 7514–7521
LaNoue, K. F., Meijer, A. J. & Brouwer, A. (1974*b*) *Arch. Biochem. Biophys.* **161**, 544–550
McIntosh, J. C. & Cooper, J. R. (1965) *J. Neurochem.* **12**, 825–835
Meijer, A. J. & Van Dam, K. (1974) *Biochim. Biophys. Acta* **346**, 213–244
Nicklas, W. J., Clark, J. B. & Williamson, J. R. (1971) *Biochem. J.* **123**, 83–95
Tallan, H. H. (1957) *J. Biol. Chem.* **224**, 41–45
Tallan, H. H., Moore, S. & Stein, W. H. (1954) *J. Biol. Chem.* **211**, 927–939
Tallan, H. H., Moore, S. & Stein, W. H. (1955) *J. Biol. Chem.* **219**, 257–264
Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol.* **13**, 434–512