Regulation of Pyruvate Metabolism via Pyruvate Carboxylase in Rat Brain Mitochondria

By MULCHAND S. PATEL* and SHIRLEY M. TILGHMAN

Departments of Pediatrics and Biochemistry, Temple University School of Medicine and St. Christopher's Hospital for Children, Philadelphia, Pa. 19133, U.S.A.

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1. The fixation of CO_2 by pyruvate carboxylase in isolated rat brain mitochondria was investigated. 2. In the presence of pyruvate, ATP, inorganic phosphate and magnesium, rat brain mitochondria fixed $\text{H}^{14}\text{CO}_3^-$ into tricarboxylic acid-cycle intermediates at a rate of about 250 nmol/30 min per mg of protein. 3. Citrate and malate were the main radioactive products with citrate containing most of the radioactivity fixed. The observed rates of $\text{H}^{14}\text{CO}_3^-$ fixation and citrate formation correlated with the measured activities of pyruvate carboxylase and citrate synthase in the mitochondria. 4. The carboxylation of pyruvate by the mitochondria had an apparent K_m for pyruvate of about 0.5 mM. 5. Pyruvate carboxylation was inhibited by ADP and dinitrophenol. 6. Malate, succinate, fumarate and oxaloacetate inhibited the carboxylation of pyruvate whereas glutamate stimulated it. 7. The results suggest that the metabolism of pyruvate via pyruvate carboxylase in brain mitochondria is regulated, in part, by the intramitochondrial concentrations of pyruvate, oxaloacetate and the ATP:ADP ratio.

The fixation of CO_2 by the brain *in situ* has been well documented (Berl *et al.*, 1962). Although the metabolic role of this process is not well understood, the incorporation of $\text{H}^{14}\text{CO}_3^-$ into aspartate, glutamate and glutamine in the brain is known (Otsuki *et al.*, 1963). Furthermore, the perfusion of ammonia into cat brain enhanced the fixation of bicarbonate and glutamine production (Waelsch *et al.*, 1964). The presence of several CO_2 -fixing enzymes in the brain has been reported (Ochoa & Weisz-Tabori, 1948; Keech & Utter, 1963; Hotta *et al.*, 1963). The subcellular distribution of these enzymes, namely pyruvate carboxylase (EC 6.4.1.1), NADP-isocitrate dehydrogenase (EC 1.1.1.42) and NADP-malate dehydrogenase (EC 1.1.1.40) has been studied in rat brain (Salganicoff & Koeppe, 1968) and an attempt was made to assess the qualitative role of CO_2 -fixing capacity in two anatomically defined compartments.

An important role of the mitochondrial carboxylation of pyruvate for gluconeogenesis and lipogenesis in liver (Lardy *et al.*, 1965) and for lipogenesis in adipose tissue (Patel & Hanson, 1970) has been established. Since glucose is the principal source of energy in the brain, the metabolism of pyruvate derived from glucose via glycolysis plays a key role in energy metabolism. Because rat brain is not a gluconeogenic tissue, pyruvate carboxylase may play a significant

role in transporting acetyl equivalents in the form of citrate across the inner mitochondrial membrane for biosynthetic processes in the cytosol, as well as in the synthesis of dicarboxylic amino acids. In view of this, the fixation of $\text{H}^{14}\text{CO}_3^-$ by pyruvate carboxylase and its regulation in rat brain mitochondria was investigated. A preliminary report of this work has been presented previously (Tilghman & Patel, 1972).

Materials and Methods

Chemicals

$\text{NaH}^{14}\text{CO}_3$ (sp. radioactivity 10–20 mCi/mmol), $[1,5\text{-}^{14}\text{C}]\text{citrate}$ (sp. radioactivity 2–10 mCi/mmol) and $[3\text{-}^{14}\text{C}]\text{sodium pyruvate}$ (sp. radioactivity 1–5 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. L- $[U\text{-}^{14}\text{C}]\text{Malate}$ (sp. radioactivity, 28 mCi/mmol) was obtained from Amersham-Searle Corp., Arlington Heights, Ill., U.S.A. ATP, ADP, NAD^+ , NADH, NADP^+ and acetyl-CoA were purchased from P. L. Biochemicals, Milwaukee, Wis., U.S.A. Avidin and potassium pyruvate were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Malate dehydrogenase (EC 1.1.1.37) and citrate lyase (EC 4.1.3.6) were obtained from Boehringer-Mannheim Corp., New York, N.Y., U.S.A. All other chemicals used were of the highest purity commercially available.

* Present and reprint request address: Department of Medicine, Temple University Hospital, 3401 N. Broad St., Philadelphia, Pa. 19140, U.S.A.

Animals

Male Sprague-Dawley rats weighing about 200g were used in all studies and were fed with Purina Laboratory Chow *ad libitum*.

Isolation of mitochondria from rat brain tissue

Rat brain was obtained immediately after decapitation, and mitochondria were isolated by the method of Clark & Nicklas (1970). All operations were carried out at 4°C. Brains from three rats were pooled, chopped and washed in the isolation medium (0.24M-sucrose, 10mM-Tris, 0.5mM-EDTA, pH 7.4). The tissue was then homogenized slowly in 25 ml of the isolation medium, and centrifuged at 2000g for 3 min. The resulting supernatant was centrifuged again at 12500g for 8 min. The crude mitochondrial pellet was suspended to a final volume of 8 ml in '3% Ficoll medium' [3% (w/v) Ficoll, 0.12M-mannitol, 0.03M-sucrose, 25 μ M-EDTA, pH 7.4] and layered on to 16 ml of '6% Ficoll medium' [6% (w/v) Ficoll, 0.24M-mannitol, 0.06M-sucrose, 50 μ M-EDTA, pH 7.4] and centrifuged for 30 min at 11500g. The pellet was resuspended in 25 ml of isolation medium and centrifuged for 10 min at 12500g. Mitochondria were then suspended in 0.3M-sucrose to a concentration of about 5 mg of protein/ml.

Fixation of $H^{14}CO_3^-$ by mitochondria

Incubations were carried out in tubes (12 cm $\times$ 1.8 cm), which were shaken in a water bath at 37°C. The compositions of the reaction mixtures are described in the legends of the tables and figures. After a temperature-equilibration period of about 5 min, the reaction was started with the addition of mitochondrial suspension. The reaction was terminated after an appropriate time-period by adding perchloric acid to a final concentration of 6% (v/v). The tubes were gassed for 4 min with CO_2 to remove acid labile counts and a portion was counted for radioactivity in 10 ml of Ditol scintillation solution (Herberg, 1960) with a Packard liquid-scintillation spectrometer to determine $H^{14}CO_3^-$ fixed.

Analysis of intermediates

Mitochondrial protein precipitated with $HClO_4$ was removed by centrifugation at 650g for 5 min and a portion of the supernatant was neutralized with 2M- K_2CO_3 . Citrate (Moellering & Gruber, 1966) and malate (Hohorst, 1965) were measured enzymically in the neutralized extracts.

Metabolism of labelled intermediates by mitochondria

Incubations were carried out in 25 ml Erlenmeyer flasks with rubber serum stoppers equipped with

hanging polyethylene cups (Kontes Glass Co., Vine-land, N.J., U.S.A.). The composition of the reaction mixture is described in the legend of Table 3. The reaction was initiated by the addition of the mitochondrial suspension. After the appropriate incubation time 0.4 ml of Hyamine-10X hydroxide was injected into the polyethylene cup through the rubber cap, followed by 0.4 ml of 36% (v/v) $HClO_4$, into the reaction mixture. The flasks were shaken for an additional 40 min to collect $^{14}CO_2$ and then the content of the cup was transferred into 15 ml of toluene containing (per litre) 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene, and the radioactivity was measured.

Separation of labelled intermediates

The labelled intermediates formed were separated by using the high-voltage apparatus (model L24; Shandon Scientific Co., Sewickley, Pa., U.S.A.) as described previously (Patel & Hanson, 1970). A portion of the neutralized sample was spotted on a strip (4 cm $\times$ 61 cm) of Whatman no. 3 MM chromatography paper, which was then run in 2M-acetic acid buffer, adjusted to pH 2.6 with pyridine, for 3.5 h with a current of 10 mA per strip. The strips were dried, cut into sections (4 cm $\times$ 1.2 cm) and counted for radioactivity in 15 ml of toluene scintillation solution. The radioactive spots were identified by comparing their migration with R_F values of known radioactive compounds.

The quantitation of labelled products utilized the specific radioactivities of the labelled substrates and efficiencies of the scintillation counting (50%) and the high-voltage electrophoresis separation (85%). The specific radioactivities (d.p.m./nmol) in the incubation media were as follows: $H^{14}CO_3^-$, 200; [3- ^{14}C]pyruvate, 140; [1,5- ^{14}C]citrate, 110; [U- ^{14}C]malate, 135. The results reported for the fixation of $H^{14}CO_3^-$ by brain mitochondria in Table 2 represent minimum values, for the radioactivity incorporated into [^{14}C]citrate is lost as $^{14}CO_2$ in the conversion of [^{14}C]citrate into succinate in the forward direction of the tricarboxylic acid cycle. The labelling patterns in the products formed from ^{14}C -labelled substrates in Table 3 are more complicated as all the label from each substrate is not lost in the first forward turn of the tricarboxylic acid cycle. Therefore the results are expressed as the conversion of specifically labelled substrate into ^{14}C -labelled products rather than as the concentration of the products.

Assay of mitochondrial enzymes

Mitochondrial suspensions were freeze-dried and resuspended in water. Pyruvate carboxylase was measured by a modification (Ballard & Hanson, 1967) of the $H^{14}CO_3^-$ -fixation method of Utter & Keech (1963). Citrate synthase (EC 4.1.3.7) (Ochoa,

1955a), NAD-malate dehydrogenase (Ochoa, 1955b), NADP-malate dehydrogenase (EC 1.1.1.40) (Ochoa & Weisz-Tabori, 1948) NADP-isocitrate dehydrogenase (EC 1.1.1.42) (Plaut, 1962), aspartate aminotransferase (EC 2.6.1.1) (Karmen *et al.*, 1955) and lactate dehydrogenase (Kornberg, 1955) were measured spectrophotometrically at 37°C. A unit is the amount of enzyme catalysing the formation of 1 μmol of product/min at 37°C.

Measurement of mitochondrial integrity

The P:O ratio and the respiratory control ratio of mitochondrial suspensions were measured by using pyruvate plus malate as substrates with a Clark-type oxygen electrode in a 2ml chamber at 28°C. The reaction mixture used was as described previously (Patel, 1972).

Measurement of protein

Mitochondrial protein was measured as described by Lowry *et al.* (1951) with bovine serum albumin used as a standard.

Results

Rat brain mitochondria isolated by the method of Clark & Nicklas (1970) were found to be functionally intact as judged in several preparations by the P:O ratio (about 2.7) and the respiratory control ratios (about 8.5). Furthermore, in the freeze-dried mitochondrial preparations we found about 0.17 unit of lactate dehydrogenase activity/mg of mitochondrial protein, which suggested that the mitochondria were relatively free of contamination by cytosolic proteins (Clark & Nicklas, 1970).

The cofactor requirements for the fixation of H¹⁴CO₃⁻ by rat brain mitochondria were investigated and the results are shown in Table 1. In the complete system, which contained all the necessary cofactors, the mitochondria fixed H¹⁴CO₃⁻ at a rate of about 269nmol/30min per mg of protein. The omission of pyruvate, MgCl₂ or ATP almost completely abolished the fixation of H¹⁴CO₃⁻, whereas the omission of P_i decreased carboxylation by about 65%. The addition of 10mM-2-oxoglutarate to the system lacking pyruvate had no effect on bicarbonate fixation, indicating that H¹⁴CO₃⁻ was not being incorporated into 2-oxoglutarate by isocitrate dehydrogenase to form isocitrate. The addition of 10 units of avidin, which is known to bind biotin irreversibly, had no effect on pyruvate carboxylation, suggesting an absence of accessible pyruvate carboxylase activity in these preparations.

The optimum conditions for the H¹⁴CO₃⁻ fixation were determined as shown in Figs. 1 and 2. The pH range for maximum fixation of H¹⁴CO₃⁻ was from

Table 1. Cofactor requirements for H¹⁴CO₃⁻ fixation in the presence of pyruvate by rat brain mitochondria

The reaction mixture contained, in a final volume of 1ml: 6.6mM-potassium phosphate, pH7.4; 6.6mM-triethanolamine, pH7.4; 5mM-potassium pyruvate; 4mM-ATP (potassium salt); 10mM-MgCl₂; 20mM-KH¹⁴CO₃ (2μCi); 157mM-sucrose. Where indicated, 10mM-2-oxoglutarate and 1mg of avidin (10 units) were added. The incubation was carried out for 30min at 37°C with about 0.4mg of mitochondrial protein being added to each tube.

Conditions	Pyruvate carboxylation (nmol of H ¹⁴ CO ₃ ⁻ fixed/mg of protein)
Complete system	269
-Phosphate buffer	88
-Pyruvate	1
-MgCl ₂	13
-ATP	6
-Pyruvate	3
+2-oxoglutarate	
+Avidin	264

Fig. 1. Effect of pH, phosphate, potassium bicarbonate and MgCl₂ on the fixation of H¹⁴CO₃⁻ by rat brain mitochondria

Unless otherwise indicated, the reaction mixture is described in Table 1. When the effect of pH was studied, potassium phosphate buffer and triethanolamine buffer of indicated pH were added. Incubations were carried out at 37°C for 30min.

Fig. 2. Effect of pyruvate, ATP and mitochondrial protein on the fixation of $H^{14}CO_3^-$ by rat brain mitochondria

Unless otherwise indicated, the reaction mixture is described in Table 1. When 8mM- or 12mM-ATP was used, additional 20mM- $MgCl_2$ was also added to the reaction mixture. Incubations were carried out at 37°C for 30 min.

7.4 to 8.0, and 7.4 was used in all studies. The optimum concentrations of substrates and cofactors were 6.6mM-potassium phosphate, 20mM- $KHCO_3$, 10mM- $MgCl_2$, 5mM-potassium pyruvate and 4mM-ATP. The rate of pyruvate carboxylation had an apparent K_m for pyruvate of about 0.5mM. The fixation of $H^{14}CO_3^-$ was linear with protein up to at least 0.75mg, and in most studies about 0.4mg of protein was used.

The dependence of the fixation of $H^{14}CO_3^-$ by brain mitochondria on ATP was further investigated. ADP concentrations of 1.25mM and 5.0mM in the presence of 4mM-ATP resulted in about 25% and 60% inhibition of pyruvate carboxylation respectively, whereas the addition of 2,4-dinitrophenol at concentrations of 25 μ M to 100 μ M produced inhibitions of over 60%, owing to its uncoupling effect on oxidative phosphorylation (Balázs *et al.*, 1964), which would raise the concentration of intramitochondrial ADP. As noted in Table 1, the removal of P_i from the incubation medium also decreased the fixation of $H^{14}CO_3^-$,

Fig. 3. Effect of the tricarboxylic acid-cycle intermediates, glutamate and aspartate on the fixation of $H^{14}CO_3^-$ by rat brain mitochondria

The reaction mixture is described in Table 1. Various intermediates were added to the final concentrations indicated: $\circ$, malate; $\bullet$, fumarate; Δ , succinate; $\blacktriangle$, oxaloacetate; $\square$, aspartate; $\blacksquare$, glutamate. Incubations were carried out at 37°C for 30 min.

presumably owing to the absence of the rephosphorylation of ADP to ATP. These results suggest that the carboxylation of pyruvate is regulated, in part, by the concentrations of intramitochondrial adenine nucleotide and are consistent with similar observations in rat liver (Lardy *et al.*, 1965) and adipose tissue mitochondria (Patel & Hanson, 1970).

The effects of intermediates of the citric acid cycle on $H^{14}CO_3^-$ fixation in the presence of pyruvate were examined (Fig. 3). No effect on $H^{14}CO_3^-$ fixation was observed with citrate and isocitrate. However, the addition of any of the dicarboxylic acid intermediates of the cycle decreased the carboxylation of pyruvate and the inhibition was concentration-dependent. Succinate, fumarate and oxaloacetate at 5mM decreased pyruvate carboxylation by 70%, 70% and 45% respectively. Malate was the most potent inhibitor, exhibiting 90% inhibition at 5mM concen-

Table 2. *Formation of organic acids from pyruvate and labelled $H^{14}CO_3^-$ in the absence and the presence of glutamate or 2-oxoglutarate by rat brain mitochondria*

The reaction mixture is described in Table 1. Glutamate or 2-oxoglutarate was added to give the final concentrations indicated. The incubations were carried out for 30 min at 37°C. The products were measured enzymically and also separated by high-voltage electrophoresis and calculated as described in the Materials and Methods section. Values are the means $\pm$ S.E.M. for four experiments.

Product measured (nmol/mg of protein)	Addition ...	None	5mm-Glutamate	2mm-2-Oxoglutarate
$H^{14}CO_3^-$ fixed		251 $\pm$ 10	317 $\pm$ 6	308 $\pm$ 8
Total citrate formed		209 $\pm$ 8	—	—
^{14}C incorporated into citrate		149 $\pm$ 13	157 $\pm$ 19	124 $\pm$ 10
Total malate formed		7 $\pm$ 1	—	—
^{14}C incorporated into malate		10 $\pm$ 2	8 $\pm$ 1	25 $\pm$ 1
^{14}C incorporated into aspartate		>1	102 $\pm$ 9	>1

tration. In contrast, the addition of glutamate enhanced pyruvate carboxylation by about 20%. The enhancement reached a maximum at 2mm-glutamate, and did not change with further increases in glutamate concentration (Fig. 3). The products of pyruvate carboxylation by brain mitochondria were mainly citrate and a very small amount of malate, as measured enzymically as well as by high-voltage electrophoresis (Table 2). The increase in $H^{14}CO_3^-$ fixation in the presence of 5mm-glutamate was due to the formation of labelled aspartate with no changes in the amounts of [^{14}C]citrate and [^{14}C]malate formed (Table 2). The addition of 5mm-aspartate to the incubation medium had, however, no appreciable effect on pyruvate carboxylation (Fig. 3). About 20% increase in $H^{14}CO_3^-$ fixation was observed in the presence of 2mm-2-oxoglutarate (Table 2). However, in the absence of pyruvate no appreciable fixation of $H^{14}CO_3^-$ was observed upon the addition of 10mm-2-oxoglutarate (Table 1). In the presence of 2-oxoglutarate the formation of [^{14}C]citrate was not affected but about a three-fold increase in [^{14}C]malate was noted (Table 2). A similar phenomenon has been previously observed with rat kidney mitochondria (Mehlman, 1968).

The metabolism of [$3-^{14}C$]pyruvate, [$1,5-^{14}C$]citrate and [$U-^{14}C$]malate by the isolated rat brain mitochondria was investigated. As seen in Table 3, 35 and 20 nmol of labelled pyruvate/30 min per mg of protein were converted into labelled CO_2 and malate respectively, whereas 563 nmol appeared in the labelled citrate. The high citrate:malate ratio agrees well with results reported for the products of the fixation of $H^{14}CO_3^-$ in Table 2. The addition of malate decreased the formation of $^{14}CO_2$ but produced about a twofold increase in [^{14}C]malate and [^{14}C]citrate. As shown in Fig. 3, the addition of malate to rat brain mitochondria decreased the fixation of $H^{14}CO_3^-$

by pyruvate carboxylase. A marked increase in the formation of [^{14}C]citrate is attributed to the increase in pyruvate metabolism via pyruvate dehydrogenase due to increased availability of oxaloacetate. Rat brain mitochondria oxidized exogenous [$1,5-^{14}C$]citrate to $^{14}CO_2$ at a rate of about 20 nmol/30 min per mg of protein (Table 3). The addition of 5mm-pyruvate had no effect on $^{14}CO_2$ formation from labelled citrate, but the addition of 1mm- and 5mm-malate enhanced the production of $^{14}CO_2$ to 94 and 169 nmol/30 min per mg of protein respectively. The enhancement of $^{14}CO_2$ formation by malate is attributed to increased transport of citrate across the inner mitochondrial membrane (Chappell & Haerhoff, 1967). Uniformly labelled malate was metabolized by brain mitochondria to [^{14}C]citrate and $^{14}CO_2$ at a rate of about 389 and 139 nmol/30 min per mg of protein, respectively. The addition of 5mm-pyruvate produced over a twofold increase in both [^{14}C]citrate and $^{14}CO_2$ formation as the availability of acetyl-CoA from pyruvate enhanced the metabolism of labelled oxaloacetate formed from [$U-^{14}C$]malate.

The activities of several enzymes involved in pyruvate carboxylation and in the metabolism of the products in the tricarboxylic acid cycle were measured in the freeze-dried rat brain mitochondria (Table 4). Pyruvate carboxylase activity was about 66 munits/mg of mitochondrial protein, sufficient to account for the rate of pyruvate carboxylation observed. This activity of pyruvate carboxylase is considerably higher than has been previously reported (Salganicoff & Koeppe, 1968). Among three important enzymes that utilize oxaloacetate in rat brain mitochondria, citrate synthase activity was about 313 munits per mg of protein, which is lower than that reported by Balázs (1965). About 2.2 units of NAD-malate dehydrogenase and 1.6 units of aspartate

Table 3. *Metabolism of labelled intermediates of the tricarboxylic acid cycle by rat brain mitochondria*

The reaction mixture contained, in a final volume of 2 ml: 6.6 mM-potassium phosphate, pH 7.4; 6.6 mM-triethanolamine, pH 7.4; 4 mM-ATP; 10 mM-MgCl₂; 20 mM-potassium bicarbonate; 157 mM-sucrose. The concentrations of the labelled intermediates were 5 mM in all cases, plus any additions as indicated. The incubations were carried out for 30 min at 37°C with about 1 mg of mitochondrial protein per incubation. The collection of ¹⁴CO₂ and the measurement of labelled products were carried out as described in the Materials and Methods section.

Labelled substrate (mM)	Addition (mM)	Incorporation of labelled substrate (nmol/mg of protein)		
		Into ¹⁴ CO ₂	Into [¹⁴ C]malate	Into [¹⁴ C]citrate
[3- ¹⁴ C]Pyruvate (5)	None	35	20	563
	L-Malate (5)	9	37	1324
[1,5- ¹⁴ C]Citrate (5)	None	20	—	—
	Pyruvate (5)	26	—	—
	L-Malate (1)	94	—	—
	L-Malate (5)	169	—	—
[U- ¹⁴ C]Malate (5)	None	139	—	389
	Pyruvate (5)	359	—	882

Table 4. *Activity of several enzymes in rat brain mitochondria*

Enzyme activities in the isolated brain mitochondria were assayed as described in the Materials and Methods section. Activities are expressed as nmol/min per mg of mitochondrial protein at 37°C. Values are the means ± S.E.M. for six experiments.

Enzymes	Activity (munits/mg of protein)
Pyruvate carboxylase	66 ± 2
Citrate synthase	313 ± 12
NAD-malate dehydrogenase	2235 ± 96
Aspartate aminotransferase	1573 ± 12
NADP-malate dehydrogenase	44 ± 3
NADP-isocitrate dehydrogenase	59 ± 2

aminotransferase were found. The glutamate-induced stimulation of pyruvate carboxylation as seen in Table 2 can now be explained in view of the high activity of aspartate aminotransferase in the mitochondria. The activities of the two other CO₂-fixing enzymes, NADP-malate dehydrogenase and NADP-isocitrate dehydrogenase, were 44 munits and 59 munits/mg of protein respectively. The presence of the former enzyme in rat brain mitochondria is in agreement with a previous report (Salganicoff & Koeppel, 1968). However, we found a much higher activity of this enzyme.

Discussion

The presence of several CO₂-fixing enzymes, namely pyruvate carboxylase (Keech & Utter, 1963;

Felicioli *et al.*, 1967), NADP-malate dehydrogenase (Ochoa & Weisz-Tabori, 1948) and NADP-isocitrate dehydrogenase (Hotta *et al.*, 1963) in the brain has been demonstrated and in a more recent study the subcellular distribution of these enzymes has been reported (Salganicoff & Koeppel, 1968). In the present study the role of pyruvate carboxylase in CO₂ fixation by brain mitochondria was investigated for two reasons: (1) under physiological conditions the equilibria of both NADP-malate dehydrogenase and NADP-isocitrate dehydrogenase favour decarboxylation (M. S. Patel, unpublished work) and (2) the exclusive localization of pyruvate carboxylase in the mitochondria (Salganicoff & Koeppel, 1968) may play an important role in pyruvate metabolism in these organelles. As shown in Table 1, in the presence of pyruvate, ATP and MgCl₂, brain mitochondria fixed about 250 nmol of H¹⁴CO₃⁻/30 min per mg of protein. A negligible amount of H¹⁴CO₃⁻ was fixed by the mitochondria when 2-oxoglutarate replaced pyruvate in the incubation medium, suggesting that a reversal of NADP-dependent isocitrate dehydrogenase reaction did not play a significant role in rat brain mitochondrial carboxylation under the experimental conditions used. Furthermore, the very small amount of [¹⁴C]malate formed from pyruvate by rat brain mitochondria (Table 2) would tend to eliminate NADP-malate dehydrogenase as a possible route for H¹⁴CO₃⁻ fixation. Supporting this is the requirement for ATP, as no such requirement is known for this enzyme.

In the present study indirect evidence is presented to show that pyruvate carboxylation by rat brain mitochondria may be controlled by the concentration of pyruvate and also by the intramitochondrial ATP:ADP ratio. The apparent *K_m* of pyruvate for carboxylation by the mitochondria was about 0.5 mM

(Fig. 2), so small alterations in pyruvate concentration could elicit large changes in this process in the brain. The carboxylation of pyruvate in the brain *in vivo* would be supported by the observed high ATP:ADP ratio (approx 8.9) in the rapidly frozen mouse brain *in situ* (Goldberg *et al.*, 1966).

An important factor controlling pyruvate metabolism that was not examined in the present study is the concentration of acetyl-CoA in the brain mitochondria. Acetyl-CoA is an obligatory cofactor for pyruvate carboxylase activity in crude mitochondrial extracts (Felicoli *et al.*, 1967) and for the partially purified rat brain enzyme (M. C. Scrutton & S. M. Tilghman, unpublished work). Since pyruvate is also metabolized via pyruvate dehydrogenase (EC 1.2.4.1), the presence of pyruvate in the incubation medium was probably sufficient for adequate acetyl-CoA production to activate this enzyme in the present experiments. It is possible that the increment in $\text{H}^{14}\text{CO}_3^-$ fixation with increasing pyruvate concentrations (Fig. 2) is due, in part, to an increase in the intramitochondrial acetyl-CoA concentration.

The regulatory roles of the intermediates of the tricarboxylic acid cycle are difficult to assess in these studies, as concentrations above the physiological one were used. The absence of any observed effect on $\text{H}^{14}\text{CO}_3^-$ fixation by citrate or isocitrate is probably due to the impermeability of the intramitochondrial membrane. The transport of tricarboxylic acids across the inner mitochondrial membrane of rat liver (Chappell & Haarhoff, 1967) and adipose tissue (Patel *et al.*, 1971) has been shown to require malate, and similar requirements for malate in brain mitochondria have been observed in this laboratory (M. S. Patel, unpublished work; see also Table 3). In contrast to tricarboxylic acids, dicarboxylic acids decreased the fixation of $\text{H}^{14}\text{CO}_3^-$ by rat brain mitochondria to varying degrees depending upon their concentrations (Fig. 3). The source of the inhibition of pyruvate carboxylation in the presence of these dicarboxylic acids, including oxaloacetate, cannot be determined in studies with intact mitochondria. However, in experiments with freeze-dried rat brain mitochondria, 1 mM-oxaloacetate inhibited pyruvate carboxylase by approx. 85% at saturating acetyl-CoA concentrations (M. S. Patel, unpublished work). With a partially purified rat brain-enzyme preparation, it was observed that concentrations of L-malate as low as 0.5 mM were effective inhibitors of pyruvate carboxylase at various concentrations of acetyl-CoA. All other dicarboxylic acids had no inhibitory effect on the enzyme (M. C. Scrutton & S. M. Tilghman, unpublished work). The addition of glutamate to the brain mitochondria enhanced pyruvate carboxylation by removing oxaloacetate to form aspartate. Since there was no change in the formation of citrate and malate (Table 3) the formation of aspartate could lead to a decrease in the oxaloacetate pool, which in

turn was compensated for by an increase in CO_2 fixation.

Pyruvate carboxylase may play an important role in the translocation of the acetyl moiety for lipogenesis in the developing rat brain as well as for the synthesis of acetylcholine. The precursor required for these biosynthetic processes in the cytosol is acetyl-CoA, which is produced from pyruvate in the mitochondria. Since acetyl-CoA does not readily cross the inner membrane of the mitochondria, several alternative pathways for the transport of the acetyl moiety have been suggested (Spencer & Lowenstein, 1962; Tuček, 1967). From experiments with brain slices with labelled substrates it is inferred that citrate formed from acetyl-CoA and oxaloacetate in the mitochondria carries the acetyl moiety across the barrier of the inner mitochondrial membrane (Mann *et al.*, 1938; Browning & Schulman, 1968; Sollenberg & Sörbo, 1970). The accumulation of citrate by brain mitochondria metabolizing pyruvate as shown in Tables 2 and 3 is consistent with such a thesis.

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