Purification and Properties of Rat Brain Pyruvate Carboxylase

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Rat brain pyruvate carboxylase was purified 2000-fold and some of its properties and kinetic parameters were investigated. The use of $(\text{NH}_4)_2\text{SO}_4$ gradient solubilization on a Celite column and precipitation with polyethylene glycol permitted purification to an estimated 20% purity. Except for a few subtle kinetic differences this enzyme is indistinguishable from rat liver pyruvate carboxylase.

Although CO_2 fixation in brain tissue was implicated in 1953 (Moldave *et al.*, 1953) and was found earlier in the retina (Crane & Ball, 1951), direct evidence was not available until the early 1960's (Berl *et al.*, 1962). This subject has been reviewed by Cheng (1971).

Since the discovery of pyruvate carboxylase (EC 6.4.1.1) in avian liver (Utter & Keech, 1960), this enzyme has been found in substantial amounts in liver and kidney from several species and is apparently needed for conversion of pyruvate into glucose by glucogenic organs (Utter *et al.*, 1964). Chicken liver pyruvate carboxylase has been studied in great detail (Utter & Scrutton, 1969; Scrutton, 1971; Scrutton & Young, 1972) and purification and kinetic analysis of the rat liver enzyme has been reported (McClure *et al.*, 1971a,b,c; McClure & Lardy, 1971; Seufert *et al.*, 1971). The enzyme has not been found in muscle tissue, but small amounts do occur in brain (Scrutton & Utter, 1968).

Rat brain contains approx. 0.2 unit of enzyme (defined as $1 \mu\text{mol}$ of substrate converted/min at 25°C) per g wet wt. of tissue (Salganicoff & Koeppe, 1968). Assuming a specific activity similar to that of the rat liver enzyme, 25 units/mg at 25°C , each gram of rat brain has approx. 0.02 mg of pyruvate carboxylase (approx. 2 munits/mg of brain protein). Although it is known that this enzyme, like those from liver and kidney, requires acetyl-CoA for activity and is inhibited by avidin (Salganicoff & Koeppe, 1968; Felicioli *et al.*, 1966, 1967), it has not been extensively purified and its properties have not been studied in detail. The small amount of enzyme present, the difficulty in obtaining large amounts of rat brain and the inherent instability of pyruvate carboxylase in dilute solutions complicate the purification process. Herein we report the partial

purification of rat brain carboxylase (to approx. 20% purity) and describe some of its properties.

Experimental Procedures

Materials

The following were purchased from the sources indicated: Tris-ATP, Trizma carbonate, and Ficoll (Sigma Chemical Co., St. Louis, Mo., U.S.A.); polyethylene glycol, average mol. wt. 6×10^3 (Matheson, Coleman and Bell, Kansas City, Mo., U.S.A.); Celite 545 (Curtin Scientific, Tulsa, Okla., U.S.A.) and male albino rats, 150–250 g (Holtzman Co., Madison, Wis., U.S.A.).

Methods

Preparation of reagents. Acetyl-CoA was prepared essentially as described by Simon & Shemin (1953) in 100 mM-Tris base, but by using only 15% excess of acetic anhydride and maintaining the pH at 7–7.5 with small additions of solid Tris base. Ficoll solutions were prepared from the commercial reagent without dialysis. Celite 545 was washed extensively before equilibration with appropriate buffers. Tris bicarbonate was prepared from either commercial Trizma carbonate reagent or by titration of a solution of Tris base to pH 8.0 with solid CO_2 . Adenine nucleotides were determined as described by McClure *et al.* (1971a), pyruvate, in 100 mM-Tris-HCl, pH 8.0, as described by Bucker *et al.* (1969), acetyl-CoA by the method of Srere (1969) and protein by the method of Kalckar (1947). Bicarbonate in stock solutions and buffers was determined manometrically (Van Slyke & Folch, 1940). Endogenous concentrations of bicarbonate in kinetic assay reagents were corrected as described by McClure *et al.* (1971a).

Enzyme assays. These were done as described in the references cited: alcohol dehydrogenase (Bonnichsen, 1969); catalase (Lück, 1969); citrate synthase (Srere, 1969); lactate dehydrogenase (Clark &

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Nicklas, 1970); malate dehydrogenase (*Worthington Enzyme Manual*, 1972); acetyl-CoA deacylase (Srere, 1969); ATPase (adenosine triphosphatase) (Myers & Slater, 1957; Tanaka & Abood, 1964; Goldenberg & Fernandez, 1966). Pyruvate carboxylase activity in crude preparations was monitored by the radioisotope assay (Salganicoff & Koeppe, 1968; slightly modified). Pyruvate carboxylase of the more purified fractions, in which interfering lactate dehydrogenase activity was low, was assayed in a reaction mixture, which contained the following in a final volume of 1 ml: 100 mM-Tris-HCl, pH 8.0; 1 mM-disodium ATP; 5 mM-sodium pyruvate; 30 mM-KHCO₃; 8 mM-MgSO₄; 0.16 mM-acetyl-CoA; 0.16 mM-NADH; and 5 units of malate dehydrogenase. Optimum malate dehydrogenase concentrations were determined as described by McClure (1969). The decrease in E_{340} was monitored at 25°C. One unit of carboxylase is defined as the amount causing a decrease of 1 μ mol of substrate/min at 25°C.

Density-gradient centrifugation. By using the method of Martin & Ames (1961), the average $s_{20,w}$ values for acetyl-CoA deacylase and pyruvate carboxylase were calculated to be 8.0S and 14.8S respectively.

Analysis of kinetic data. The design of the kinetic experiments, analysis of results and nomenclature of kinetic terms are as outlined by Cleland (1967). Lineweaver-Burk plots of the varied substrate or activator were constructed by statistically fitting the data to eqn. (1) by using Fortran computer programs based on the non-linear regression method described by Wilkinson (1961), obtained from either Cleland (1963) or Mushahwar *et al.* (1972). When biphasic kinetics were apparent in the Lineweaver-Burk plots (McClure *et al.*, 1971a; Engel & Ferdinand, 1973) (i.e. abrupt transitions between apparently linear sections) the substrate concentration of each linear region was defined by inspection. All the experiments gave intersecting patterns on double-reciprocal plots. Therefore these data (the entire family of lines for a given experiment) were fitted to eqn. (2):

$$v = \frac{VA}{K_a + A} \quad (1)$$

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \quad (2)$$

$$v = \frac{VAB}{K_{ia}K_b + K_bA + AB} \quad (3)$$

where v is the observed velocity; V is the maximum velocity; A is the variable substrate (or activator) at fixed concentrations of substrate (or activator) B ; K_a and K_{ia} are the Michaelis and inhibition (dissociation of the enzyme-substrate complex) constant for A , respectively; and K_b is the Michaelis constant for B .

Eqn. (2) is the rate equation for the ordered, steady-state addition of two substrates (A, B); eqn. (3) is the rate equation for the equilibrium ordered addition of the first substrate (whose K_a value must be zero). When Mg^{2+} and $MgATP$ were the variable substrates, the K_a for Mg^{2+} approached zero (see the Discussion section); hence these data were fitted to eqn. (3). Computer programs for direct fits for these equations were also obtained from Cleland (1963). When an acyl-CoA derivative was the varied component of a kinetic experiment, the kinetic constants were determined as described by Scrutton & Fung (1972).

Enzyme purification

Mitochondria. Mitochondria were prepared (Clark & Nicklas, 1970) from the cerebral hemispheres (weighing approx. 30 g) of 32 rats, suspended in 20 ml of 0.1 mM-EDTA, freeze-dried and stored evacuated over CaSO₄ at -29°C until used. No loss of pyruvate carboxylase activity was observed over a storage period of several weeks.

(NH₄)₂SO₄ precipitation. The freeze-dried mitochondrial residue was homogenized with 30 ml of a solution containing 50 mM-Tris-HCl (pH 7.4), 0.5 mM-EDTA and 0.5 mM-dithiothreitol (buffer A), the pH of the homogenate was adjusted to 7.2-7.4 with a small amount of solid Tris base, and the homogenate was centrifuged at 10⁵g at 0°C for 30 min. After warming to room temperature (approx. 25°C), the supernatant was adjusted to 33% saturation by addition of solid (NH₄)₂SO₄ (19.6 g/100 ml) over a 30 min period during which the pH was maintained at 7.2-7.4 with solid Tris. Pyruvate carboxylase activity was pelleted by centrifugation at 10⁴g at 20°C for 20 min. Although at this stage rat brain pyruvate carboxylase is not cold-labile, subsequent purification steps were performed at room temperature.

The potential usefulness of the Celite-(NH₄)₂SO₄ gradient solubilization procedure of King (1972) was suggested by the purification scheme of Seufert *et al.* (1971). The 33%-satd.-(NH₄)₂SO₄ pellet was suspended in 1-2 ml of buffer A adjusted to 33% saturation with (NH₄)₂SO₄ (19.6 g/100 ml) (buffer B). To this was added 0.5 ml of wet Celite and the slurry was layered on a column (1 cm × 9 cm) of Celite previously equilibrated with buffer B. Sometimes it was necessary to slurry the top portion of the column to prevent a decrease in flow rate. The column was eluted at 0.33 ml/min with a linear gradient (40 ml) of decreasing (NH₄)₂SO₄ concentration from buffer B to buffer A. An elution profile is shown in Fig. 1.

The fractions containing pyruvate carboxylase activity were pooled, adjusted to at least 33% saturation with solid (NH₄)₂SO₄ (conductivity equal to or greater than that of buffer B) and centrifuged

Fig. 1. $(\text{NH}_4)_2\text{SO}_4$ gradient solubilization of pyruvate carboxylase on a column of Celite 454

Conductivity (○) in mmhos, protein (△) as measured at 280 nm, and enzyme activity (□) expressed as percentage of that of fraction 19 (1 ml fractions were collected) are expressed.

Table 1. Purification of rat brain pyruvate carboxylase

Treatment	Pyruvate carboxylase			Acetyl-CoA deacylase		
	(Units/ml)	(Total units)	(Units/mg of protein)	(Units/ml)	(Total units)	(Units/mg of protein)
Homogenate*	0.04	9.6	0.002	—	—	—
Celite column	0.26	3.9	1.1	0.46	7.1	1.9
Fraction A	0.53	2.7	1.0	0.90	4.5	1.7
10% Polyethylene glycol supernatant	0.36	2.3	—	0.03	0.16	—
10% Polyethylene glycol pellet	0.63	0.63	1.24	1.1	1.1	2.3
18% Polyethylene glycol pellet (fraction B)	1.6	1.6	5.1	0.05	0.05	0.14

* Values for homogenate are based on the activity of one rat brain as determined by the radioisotope assay described and extrapolated to simulate a purification using 32 rat brains.

at 10^5g for 30 min. The pellet was dissolved in 5 ml of buffer A; enzyme at this stage of purification is referred to as fraction A.

Polyethylene glycol treatment. To fraction A was added dropwise 1.25 ml of 50% (w/v) polyethylene glycol in buffer A. This suspension was stirred gently for 30–60 min and centrifuged at 10^5g for 30 min. Then an additional 1.55 ml of the 40% polyethylene glycol in buffer A was added to the supernatant. The suspension was stirred slowly for 20 min and centrifuged for 30 min at 10^5g . The pellet (often invisible) was dissolved in 1 ml of buffer A, this stage of purification being referred to as fraction B. Fraction B was centrifuged at 10^5g for 20 min to remove light-scattering material, which interfered

with the spectrophotometric assay of protein. The supernatant from this centrifugation was stored at 4°C and used for most of the studies reported below.

It usually required 10–12 h to obtain fraction B from the freeze-dried mitochondria. Data for a typical purification are presented in Table 1. The specific activity of pyruvate carboxylase in the fractions B from 15 purifications ranged from 2.7 to 7.8 units/mg of protein, the average being 4.6 units/mg of protein. From 0.6 to 1.9 (average 1.2) units of pyruvate carboxylase activity was obtained in the fraction B. This is an average yield of 42% (from freeze-dried mitochondria), a 2000-fold purification from rat brain and a fourfold purification from the Celite column eluate (fraction A).

Results and Discussion

General properties

Contaminating enzymes. No ATPase or citrate synthase was detected in fraction A, whereas lactate dehydrogenase and malate dehydrogenase activities equal to 0.3 and 2.5%, respectively, of pyruvate carboxylase activity were detected. In contrast, the acetyl-CoA deacylase activity of fraction A was often fivefold that of pyruvate carboxylase. Remarkably, polyethylene glycol fractionation removed 99% of this serious contaminant (Table 1). On average, acetyl-CoA deacylase activity of fraction B was only 3.5% of that of pyruvate carboxylase. Moreover, the deacylase activity was less stable during storage than was pyruvate carboxylase.

Disc gel electrophoresis of a fraction B preparation having a specific activity of 5.0 on 4% polyacrylamide gel at pH 9.5 (*Chemical Formulation for Disc Electrophoresis*, 1965) gave at least four protein bands. Assuming a maximal specific activity similar to that of rat liver (25 units/mg) (McClure *et al.*, 1971a) the rat brain pyruvate carboxylase preparation reported here is approx. 20% pure.

Stability. During storage at 4°C of fraction B with a protein concentration of 0.32 mg/ml, the loss of enzyme activity followed first-order kinetics with a half-life of approx. 7 days and first-order rate constant of 0.004 h^{-1} . Protein concentrations of the fraction B preparations ranged from 0.11 to 0.39 mg/ml, averaging 0.24 mg/ml. When protein concentrations are lowered to less than 0.1 mg/ml, stability on storage is greatly decreased. Like the rat liver enzyme (McClure *et al.*, 1971a), but in contrast with that from chicken liver and sheep kidney (Ling & Keech, 1966), rat brain pyruvate carboxylase is not cold-labile.

pH optimum. Rat brain pyruvate carboxylase has a pH optimum of 8.0–8.5, similar to that reported for the enzyme from rat liver (McClure *et al.*, 1971a; Seufert *et al.*, 1971).

Molecular weight. The $s_{20,w}^0$ of 14.8S (see under 'Methods') for rat brain pyruvate carboxylase, determined by density gradient centrifugation, is close to the value of 14.5S reported for the rat liver enzyme by using the same technique (McClure *et al.*, 1971a) and similar to those obtained by analytical ultracentrifugation of the rat (McClure *et al.*, 1971a) and chicken (Scrutton & Utter, 1965) liver enzymes. Except for the enzyme isolated from *Pseudomonas citronellolis* (Scrutton & Young, 1972), all pyruvate carboxylases described to date have molecular weights of slightly over 500 000, including that from rat brain.

Inhibition

One unit (i.e. the amount that will bind $1\mu\text{g}$ of biotin; approx. 0.4 mg/unit) of avidin completely inhibited 0.02 unit of brain pyruvate carboxylase.

Prior incubation of the avidin with $25\mu\text{g}$ of biotin completely prevented this inhibition. The observation that avidin does not inhibit CO_2 fixation in intact brain mitochondria (Patel & Tilghman, 1973) suggests that the enzyme is located on the inner membrane, as is true for the rat liver enzyme (McClure *et al.*, 1971a). Confirming the report of Patel *et al.* (1973), we found phenylpyruvate to be a non-competitive inhibitor of rat brain pyruvate carboxylase (Fig. 2). Like the rat liver enzyme (McClure & Lardy, 1971), rat brain pyruvate carboxylase is not inhibited by up to 25 mM-SO_4^{2-} ; at acetyl-CoA concentrations between 6 and $190\mu\text{M}$, chicken liver carboxylase is (Scrutton & Fung, 1972).

Activation

Rat liver (Scrutton & White, 1972) and sheep kidney (Ashman *et al.*, 1972) pyruvate carboxylases both catalyse a slight acetyl-CoA-independent carboxylation. We have observed the same phenomenon with the rat brain enzyme, although this carboxylation varied considerably from preparation to preparation and was only a very small fraction of the acetyl-CoA-dependent carboxylation (less than 5%).

For the activation of rat brain pyruvate carboxylase by acetyl-CoA, we determined a Hill coefficient of 2.0, close to the values of 2.0 (McClure *et al.*, 1971a) and 2.1 (Scrutton, 1971) obtained with the rat liver enzyme, but somewhat lower than the 2.9 reported (Utter & Scrutton, 1969) for the avian liver enzyme. The K_a for activation of avian liver pyruvate carboxylase by acetyl-CoA is $2\text{--}3\mu\text{M}$, compared with $20\text{--}25\mu\text{M}$ for the rat liver enzyme (Scrutton, 1971; McClure *et al.*, 1971a) and $17\mu\text{M}$ reported here for the rat brain carboxylase.

Additional kinetics

The kinetics presented here for rat brain pyruvate carboxylase are modelled after those described by McClure *et al.* (1971a) for the rat liver enzyme. Kinetic plots are presented in detail by Mahan (1974).

K^+ ions. The various kinetic constants are presented in Table 2. Univalent-cation activation of rat liver pyruvate carboxylase has been studied extensively by McClure *et al.* (1971a). The values reported here (Table 2) are in general agreement with those reported by these authors. Although all pyruvate carboxylases are activated by univalent cations (K^+ is the best) only the chicken liver carboxylase is activated by Tris (Scrutton, 1971). Because most of the early work with the chicken liver enzyme was done with Tris buffer, the univalent-cation activation of this enzyme was not immediately recognized (Scrutton, 1971).

As noted by others (McClure *et al.*, 1971a; Scrutton *et al.*, 1965), when pyruvate is the varied substrate in kinetic experiments with several pyruvate carboxy-

Fig. 3. Effect of MgATP on the activation of pyruvate carboxylase by K^+

(a) Reciprocal velocities are plotted as a function of the KCl concentration at the fixed concentrations (mM) of MgATP indicated. (b) Reciprocal velocities are plotted as a function of the MgATP concentration at the fixed concentrations (mM) of KCl indicated. Inset, secondary plot of slopes (■) and intercepts (□) of (b). The 1.0 ml reaction mixtures (pH 8.0) contained (in μmol): pyruvate, 4.1; MgATP, as indicated; HCO_3^- , 28; MgSO_4 , 6.0 above the indicated [MgATP]; KCl, as indicated; acetyl-CoA, 0.13; Tris-HCl, 100; NADH, 0.16; and 6 units of malate dehydrogenase.

lases, the double-reciprocal plots are biphasic. We have made similar observations with the rat brain carboxylase. The data presented in Table 2 follow the previous convention in which pyruvate I refers to the low concentration range of this substrate and pyruvate II refers to the high concentration range (McClure *et al.*, 1971a).

With the brain enzyme we also noted that when KCl was the varied activator at fixed concentrations of MgATP biphasic double-reciprocal plots were obtained (Fig. 3). The convention used in Table 2

is that KCl I and KCl II refer to the low and high concentration ranges respectively.

To date no completely adequate explanations have been offered for the biphasic kinetics (i.e. the abrupt transitions in the Lineweaver-Burk plots). However, it may be noted that with brain pyruvate carboxylase, with one exception, the KCl-MgATP biphasic kinetics presented in Table 2 and Fig. 3, all of the biphasic kinetics were characterized by a constant apparent K_a with changes in K_{Ia} and/or K_{Ix} (where $x = \text{K}^+$ or Mg^{2+}).

Lineweaver-Burk plots of KCl at fixed concentrations of acetyl-CoA were linear and fit eqn. (1). However, secondary plots of slopes and intercepts were parabolic, as previously reported in experiments with the rat (McClure *et al.*, 1971a) and chicken (Ling & Keech, 1966) liver enzymes. The kinetic constants shown in Table 2 were determined as described by McClure *et al.* (1971a).

Mg^{2+} ions. Although the MgATP chelate is the

actual substrate for the enzyme, pyruvate carboxylase requires an additional amount of Mg^{2+} for total activation (Scrutton & Young, 1972). The apparent kinetic parameters for Mg^{2+} activation of the brain pyruvate carboxylase are presented in Table 2. The concentration of free Mg^{2+} ion was calculated as outlined by Blair (1969), by using the formation constants $7 \times 10^4 M^{-1}$ for MgATP and $15 M^{-1}$ for Na^+ and K^+ complexes of ATP (O'Sullivan & Perrin,

Fig. 4. Effect of HCO_3^- on the activation of pyruvate carboxylase by Mg^{2+}

(a) Reciprocal velocities as a function of the free Mg^{2+} concentration at fixed concentrations (mM) of $KHCO_3$. (b) Reciprocal velocities as a function of the $KHCO_3$ concentrations at fixed concentrations (mM) of free Mg^{2+} . (c) Secondary plot of slopes (■) and intercepts (□) of (b). Total K^+ was maintained at 28.3 by varying KCl. The 1.0 ml reaction mixtures (pH 8.0) contained (in μ mol): pyruvate, 4.6; MgATP, 0.8; $KHCO_3$, as indicated; acetyl-CoA, 0.12; $MgSO_4$, as indicated; triethanolamine-HCl, 100; NADH, 0.16; and 5.4 units of malate dehydrogenase.

Fig. 5. Initial-velocity pattern for pyruvate carboxylase with MgATP and pyruvate as a variable substrate

(a) Reciprocal velocities are plotted as a function of MgATP concentrations as the fixed concentrations of pyruvate indicated. (b) Secondary plot of intercepts of (a). (c) Secondary plot of slopes of (a). The 1.0 ml reaction mixture (pH 8) contained (in μ mol): pyruvate, as indicated; MgATP, as indicated; MgSO₄, 6.0 above the indicated [MgATP]; acetyl-CoA, 0.12; KHCO₃, 30; triethanolamine-HCl, 70; NADH, 0.16; and 6.0 units of malate dehydrogenase.

1964). Because these constants were determined in triethanolamine buffers, triethanolamine was substituted for Tris in the Mg²⁺ experiments. McClure *et al.* (1971a) used 70 mm-triethanolamine-HCl in some of their experiments. Therefore to compare the rat brain carboxylase with that from rat liver, we also used 70 mm rather than 100 mm-triethanolamine in some of our Mg²⁺ studies.

When Mg²⁺ was varied at fixed concentrations of MgATP, slope replots of the double-reciprocal plots passed through the origin. Similar observations have been made with the rat (McClure *et al.*, 1971a) and pig (Warren & Tipton, 1974) liver enzymes. As discussed by both groups of authors these results indicate that the K_a of Mg²⁺ approaches zero at infinite concentrations of MgATP and the data may be fitted to eqn. (3). Also these data suggest that free Mg²⁺ binds to the enzyme at equilibrium, followed by the binding of MgATP (Warren & Tipton, 1974).

When Mg²⁺ was varied at fixed concentrations of HCO₃⁻ biphasic double-reciprocal plots were obtained (Fig. 4). Biphasic kinetics with these substrates have not been reported previously. The biphasic kinetic constants observed when pyruvate was varied at fixed concentrations of Mg²⁺ are shown in Table 2, where MgSO₄ I and MgSO₄ II refer to the low and high range of Mg²⁺ concentrations respectively.

Substrates. The double-reciprocal plots obtained when pyruvate was varied at fixed concentrations of MgATP were biphasic, and, as observed previously with the rat liver enzyme (McClure *et al.*, 1971b), with MgATP as the varied substrate the lines of the double-reciprocal plots in the low pyruvate range are almost parallel, whereas in the high-concentration range of pyruvate they intersect (Fig. 5). The kinetic constants for these data and those for the HCO₃⁻-MgATP relationships (Fig. 6) are shown in Table 2. In contrast with the rat liver enzyme (McClure *et al.*,

Fig. 6. Initial-velocity patterns for pyruvate carboxylase with MgATP and KHCO_3 as variable substrates

(a) Reciprocal velocities are plotted as a function of the MgATP concentration at fixed concentrations of KHCO_3 (mM) indicated. (b) Reciprocal velocities are plotted as a function of the KHCO_3 concentrations at fixed concentrations of MgATP (mM) indicated. Insets, secondary plots of slopes (■) and intercepts (□) of the corresponding primary plot. The total K^+ concentration was maintained at 30mM by varying KCl. The 1.0ml reaction mixtures (pH 8.0) contained in (μmol): pyruvate, 5.5; MgATP, as indicated; KHCO_3 , as indicated; MgSO_4 , 6.0 above the indicated [MgATP]; acetyl-CoA, 0.12; triethanolamine-HCl, 70; NADH, 0.16; and 6 units of malate dehydrogenase.

1971b) there is no substrate inhibition by MgATP at low pyruvate concentrations.

Identity and metabolic significance

At present we must conclude that either rat brain and rat liver pyruvate carboxylase are identical or that only subtle differences distinguish these enzymes. The large Michaelis constant for phosphoenolpyruvate of rat brain phosphoenolpyruvate carboxy-

kinase (Cheng & Cheng, 1972) suggests that this enzyme has a decarboxylation function. The soluble isoenzyme of the 'malic' enzyme of bovine brain has a carboxylation rate that approaches one-fifth of the decarboxylation velocity (Frenkel, 1972). Assuming a similar situation in rat brain, the carboxylation capability of this enzyme would be less than 0.1 unit/g of brain (Salganicoff & Koeppe, 1968) at 25mM-pyruvate (Frenkel, 1972). Probably as much as 0.2 μmol of CO_2 fixed into oxaloacetate/min per g is needed for

the synthesis of glutamine *de novo* by rat brain during acute NH_4^+ toxicity (Hawkins *et al.*, 1973). If one assumed a tricarboxylic acid-cycle turnover in rat brain of $0.5\text{--}1\ \mu\text{mol/min per g}$ (Hawkins *et al.*, 1973; Waelsch *et al.*, 1964; Cremer, 1970), it may be calculated from the labelling data of Waelsch *et al.* (1964) with cat brain that CO_2 fixation into oxaloacetate may proceed at a rate as high as $1\ \mu\text{mol/min per g}$. This is not a net rate of synthesis of oxaloacetate from pyruvate, since recycling may occur via phosphoenolpyruvate carboxykinase and pyruvate kinase. Data reported here, and previously (Salganicoff & Koeppe, 1968), of approx. 0.2 unit of pyruvate carboxylase/g of brain at 25°C are in reasonably good agreement with the values of 0.5 (Scrutton & Utter, 1968) and 0.9 at 37°C (Ballard *et al.*, 1970) reported by others. The claim of much higher pyruvate carboxylase activity in brain mitochondria (Patel & Tilghman, 1973) probably needs further documentation.

Kinetic constants presented here for brain pyruvate carboxylase suggest that carboxylation by this enzyme may proceed in brain at a rate which represents a considerable portion of the theoretical maximum. The K_a for pyruvate of 0.1 mM approximates to the reported pyruvate concentration of brain (Hawkins *et al.*, 1973), and the K_a of 0.02 M for activation by acetyl-CoA is somewhat larger than the reported (Heinrich *et al.*, 1973) $6\ \mu\text{M}$ concentration of acetyl-CoA in brain. However, it is quite likely that the intramitochondrial concentrations of these components exceed those of the whole tissue. Thus functional pyruvate carboxylase activity in brain of $0.2\text{--}0.5\ \mu\text{mol/min per g}$ at 37°C seems likely and would account for most of the CO_2 fixation into oxaloacetate.

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